

UPSIDE

Deliverable D4.1

Grant Agreement No.	101070931
Start date of Project	1 September 2022
Duration of the Project	48 months
Deliverable name	D 4.1
Partner Leader	UF
Dissemination Level	PU

<i>Status</i>	Final
<i>Version</i>	V1.2
<i>Submission Date</i>	04-03-2024

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UPSIDE project has received funding from the European Union's Horizon Europe EIC-PATHFINDER programme under grant agreement No 101070931.





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History of Changes

Version	Change made	Date
V1.0	First draft	25-02-2024
V1.1	Feedback internal review	28-02-2024
V1.2	Final version	29-02-2024

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Abbreviations

AP:	anterior-posterior
BSA:	bovine serum albumin
CMOS:	Complementary Metal Oxide Semiconductor
DV:	dorso-ventral
EBI:	Epidural Brain Interface
eFUS:	epidural Focused Ultrasound
FUS:	Focused Ultrasound
H&E:	Hematoxylin & Eosin
mfb:	medial forebrain bundle
ML:	medio-lateral
PCB:	printed circuit board
PFA:	Paraformaldehyde
WP:	Work Package

Executive Summary

Clinical depression is associated with dysfunctional neural networks that regulate mood, emotion and reward-orientated behaviours. Neuromodulation is a therapeutic strategy aiming to correct the activity of the pathological pathways that underpin the key symptoms. The current project aims to produce an implantable epidural brain interface (EBI) with low-intensity focused ultrasound and cortical recording capability that can regulate neural activity in a spatial and temporal defined fashion via focused acoustic pressure waves. The EBI will combine the CMOS Integrated focused ultrasound stimulation chip (henceforth referred to as “eFUS”) and the CMOS integrated ion gated transistor neural recording arrays (henceforth referred to as “eREC”).

The objectives leading into D4.1 were to procure and test the commercially available FUS on diverse media to verify its technical characteristics (e.g. focal length). The media used were temperature sensitive film, an agar-based tissue phantom, post-mortem 4% PFA fixed brains, or in unfixed ex vivo brains. Furthermore, the preliminary studies were to identify appropriate stimulation parameters – in terms of intensity and targeting - that could serve as the basis for future deliverables. Targeting in the brain of freshly sacrificed animals was confirmed with a histological staining. An additional important commitment (and component of D4.1) was to design together with our consortium partners a prototype for the EBI (including both eFUS and eREC), and generate the appropriate consequent surgical method and protocol for the implantation of the EBI on the rodent’s head/ skull. The surgical protocol needed to take into account both physical constraints on the rat’s head, such as the skull’s thickness or its anterior-posterior or lateral dimensions, as well all the physical characteristics of the EBI. Throughout the protocol refinement consideration had to be given to animal welfare, particularly in view of optimizing craniotomy size and EBI fixation options. At this stage, the two components of the implantable EBI, the eREC array and the eFUS chip, along with their PCBs and connections, are developed separately but in consultation across the consortium members. On these grounds, the prototype EBI for which we have developed the implantation protocol could evolve over time.

The document describes the effort leading up to the completion of Deliverable D4.1, work carried out in the context of WP4 Tasks 4.1. Deliverable D4.1 concerns i.) the implantation protocol for the (EBI) on the rodent’s head/ skull and ii.) targeting the focused ultrasound (FUS) in a rat brain using a commercially available transducer.

Using the commercial transducer, we performed the planned in vitro and ex vivo tests. With the use of a power amplifier the maximum focal pressure was sufficient to cause a lesion in an explanted fixed rat brain. It was not possible to form a lesion in the mfb in unfixed tissue with the same parameters, instead a larger area of the tissue was heated. While further tests will be conducted and the temperature of the brain at the time of stimulation has to be taken into account as well as shorter stimulation times, at this point the commercial transducer does not seem to be the ideal tool to target the mfb in vivo. The large necessary craniotomy and focal spot size seem to make it difficult to target deep structures reliably and accurately. Therefore the Task 4.2 will be achieved by looking at brain slice cultures of the mfb and shallower targets using histological methods and electrophysiological recordings. As soon as the first generation of the chip is ready, the mfb can be targeted and the parameters confirmed.

In the second part of Task 4.1 an implantation protocol was established for the eFUS chip and eREC electrode on their ownm, as well as the integrated EBI. Since the design and development is an ongoing

process, especially for the used PCB boards, exact craniotomy sizes and PCB dimensions might change in the future. The general method, as well as the size constrictions for both the craniotomies and PCBs have been successfully tested. With the use of two layered PCB boards, sufficient pressure can be applied to assure the FUS Chip is in constant contact with the brain surface. We will need to confirm whether the angle of the chip can be kept perpendicular to the target during fixation. The gap between the 7 mm craniotomy and 5 mm chip would allow for additional electrodes surrounding the chip although for the recording of potential biomarkers the extension of the electrode onto the prefrontal cortex is essential.

1. Introduction

Clinical depression is associated with dysfunctional neural networks that regulate mood, emotion and reward-orientated behaviours. Neuromodulation is a therapeutic strategy aiming to correct the activity of the pathological pathways that underpin the key symptoms. The current project aims to produce an implantable epidural brain interface (EBI) with low-intensity focused ultrasound and cortical recording capability that can regulate neural activity in a spatial and temporal defined fashion via focused acoustic pressure waves. The EBI will combine the CMOS Integrated focused ultrasound stimulation chip (henceforth referred to as “eFUS”) and the CMOS integrated ion gated transistor neural recording arrays (henceforth referred to as “eREC”).

The objectives leading into D4.1 were to procure and test the commercially available FUS on diverse media to verify its technical characteristics (e.g. focal length). The media used were temperature sensitive film, an agar-based tissue phantom, post-mortem 4% PFA fixed brains, or in unfixed ex vivo brains. Furthermore, the preliminary studies were to identify appropriate stimulation parameters – in terms of intensity and targeting - that could serve as the basis for future deliverables. Targeting in the brain of freshly sacrificed animals was confirmed with a histological staining. An additional important commitment (and component of D4.1) was to design together with our consortium partners a prototype for the EBI (including both eFUS and eREC), and generate the appropriate consequent surgical method and protocol for the implantation of the EBI on the rodent’s head/ skull.

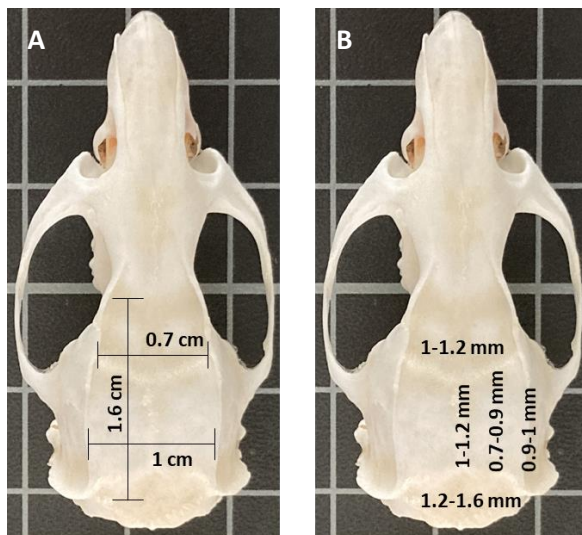


Figure 1: Dimensions of a rat skull. **A** Available space for the placement of a PCB board and implants. **B** Estimation of the skull thickness in different areas.

The surgical protocol needed to take into account both physical constraints of the rat’s head, such as the skull’s thickness or its anterior-posterior or lateral dimensions, as well all the physical characteristics of the EBI. The skull dimensions and thickness can be seen in fig. 1. Multiple measurements were taken from several male and female rats to obtain the maximum size for the PCB board to fit all rats. Since the skull thickness varies, special attention had to be paid to ensure the chip is in constant contact with the brain, when the PCB board is fixed on top of the skull. The current solution is to wire bond the 5x5 mm chip to a 7x7 mm PCB which will also be inserted into the craniotomy and provide the needed thickness (see also fig. 7).

Throughout the protocol refinement consideration had to be given to animal welfare, particularly in view of optimizing craniotomy size and EBI fixation options. At this stage, the two components of the implantable EBI, the eREC array and the eFUS chip, along with their PCBs and connections, are developed separately but in consultation across the consortium members. On these grounds, the prototype EBI for which we have developed the implantation protocol could evolve over time.

The document describes the various approaches we have taken during period M6-M18 to design with our colleagues the EBI, and to optimize the implantation/ fixation protocol of the EBI on the rodent's head/ skull given both the physical constraints on the head and keeping the animals welfare at the forefront of our thinking. Furthermore, we describe emerging stimulation parameters following our early use of the commercially available FUS in both in vitro and ex vivo. It must be noted that the characteristics of the commercial FUS transducer, and the effects we tried to achieve during this period (high-intensity FUS), are significantly different from those that we will aim to achieve with the eFUS chip (low-intensity FUS). The use of the commercial transducer is informative, but the directly relevant data will be generated with the eFUS chip once it is transferred to Freiburg.

2. Content paragraphs

Activity in WP4 started in M6 with task 4.1. The sub-tasks focused on purchasing and developing necessary accessories for the in vitro and in vivo use of the commercially available ultrasound transducer device (4.1.1); and ii.) starting to use the device to work out parameters for lesioning and stimulation (4.1.2); iii.) discussions with our partners concerning the design of the eFUS/ eREC device based on the physical constraints imposed by the dimensions of the rodent skull and brain anatomy (4.1.3); and iii.) working out surgical protocols for the epidural implantation of the eFUS/ eREC device, including the required craniotomy and methods for the fixation of the device for future chronic studies (4.1.4).

2.1. Ex vivo studies

2.1.1. Set up

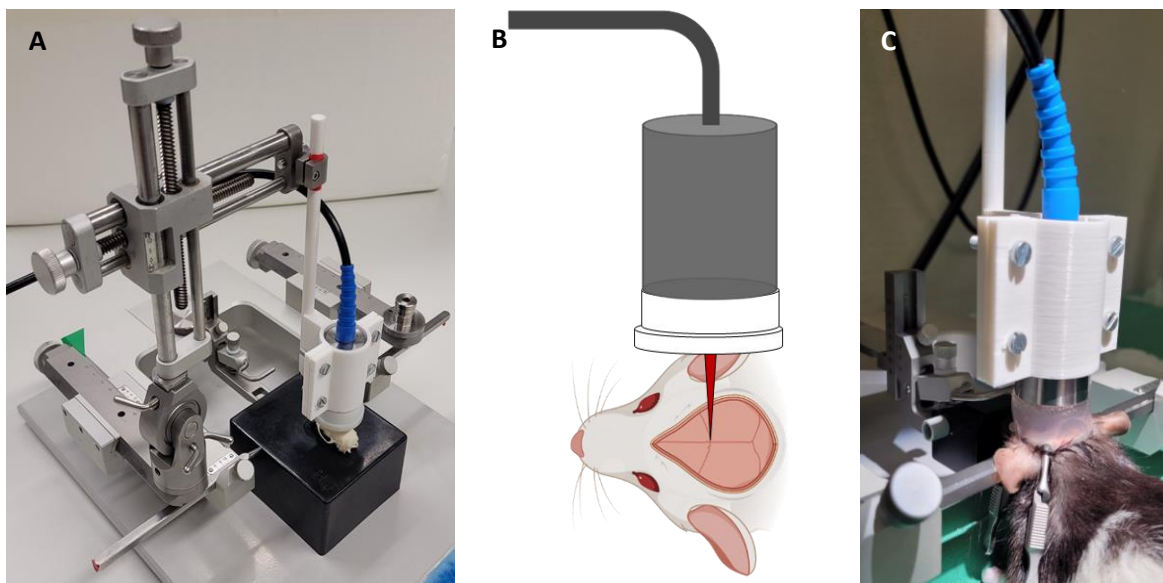


Figure 2 Set up of the commercial FUS transducer. Accessories built to adapt the transducer to the stereotactic frame (A, holding device) and to permit the targeting of the transducer's focal spot (B, targeting cone). The transducer is coupled to the brain using an ultrasound gel pad to allow the undeviated propagation of the ultrasound into the brain (C).

To complete tasks 4.1 and 4.2 before the first generation of the eFUS chip was available, a commercially available FUS transducer (5 MHz, 19 mm focal length) by Precision Acoustics was delivered to Freiburg from the TU Delft. The fixation of the transducer head during surgery was achieved through the design of a 3D printed holding device, which could be integrated in the stereotactic frame (fig. 2 A). The targeting of the reference points on the rat skull was achieved by a similarly 3 D printed targeting cone (fig. 2 B). In order to see the tip of the cone with the microscope even from a steep angle during surgery, it is 4 cm in length. The targeting cone is temporarily attached to the transducer head to manoeuvre the middle of the transducer, where the focal spot is formed, to the reference point bregma and measure the corresponding coordinates. Based on these, the target coordinates can be calculated for the transducer. The depth of the dura, once the craniotomy was performed, can be measured as well, to calculate the dorso-ventral coordinate.

During the characterisation of the transducer and first in vitro tests degassed, deionized water was used as a coupling medium. During surgery in ex vivo and upcoming in vivo tests sterile ultrasound gel and a sterile, cut to size 10 mm thick ultrasound gel pad are used (fig. 2 C).

2.1.2. Transducer characterization

The manufacturer precision acoustics provided an initial calibration of the transducer (see table 1).

Table 1 Transducer calibration by the manufacturer precision acoustics with a sinusoidal burst signal and 18 Vpp amplitude.

peak frequency [MHz]	focal spot diameter [mm]	focal spot length [mm]	focal peak [mm]	focal intensity (W/cm ²)	max input voltage [Vpp]
5.24	0.44 (-3 dB)	4.38 (-3 dB)	20.3	4705 (-6dB)	186
	0.6 (-6dB)	5.98 (-6dB)			

The length of the acoustic path as well as the peak focal pressure were measured again with a purchased 0.2 mm needle hydrophone (see fig. 3A, B). The focal peak was on average located at a distance of 19.3 mm (input voltage 10 Vpp) from the transducer surface with a lower limit of about 18.7 mm and an upper limit of 20.4 mm at about 3 dB below the peak amplitude (see fig. 3 C). This is smaller than the provided focal peak of 20.3 mm and focal spot length of 4.38 mm indicated in the calibration sheet (see table 1). A possible explanation for these differences could be, that any misalignment of the focal spot with the hydrophone can lead to lower measurements or the occurrence of focal shortening when the transducer is driven for a longer time.

To initially confirm if the reliable targeting of the medial forebrain bundle (mfb) in the rat brain was possible with this transducer and to validate the measured focal spot dimensions, we first aim to create a lesion. Therefore a temperature increase to about 60 °C was necessary. For this purpose an amplifier was purchased, since tests and a simulation by the TU delft (see fig. 3F) showed that the drive power from the available wavelength generator alone was not sufficient to achieve this. It was able to amplify the input power (max. 1Vpp) by the factor 143 leading to a maximum power output of 143 Vpp. In case of temporary power oscillations, the maximum input used was 960 mVpp with an output of 137 Vpp. Since the hydrophone and the oscilloscope were limited in their maximum measured pressure and power respectively, the output of the amplifier and the acoustic pressure were extrapolated assuming a linear correlation (see fig. 3D, E).

This would lead to a maximum focal pressure of about 8 MPa at 137 Vpp, which was according to the simulation not enough to create a lesion after a short application times (see fig.3 F), but potentially after a longer time.

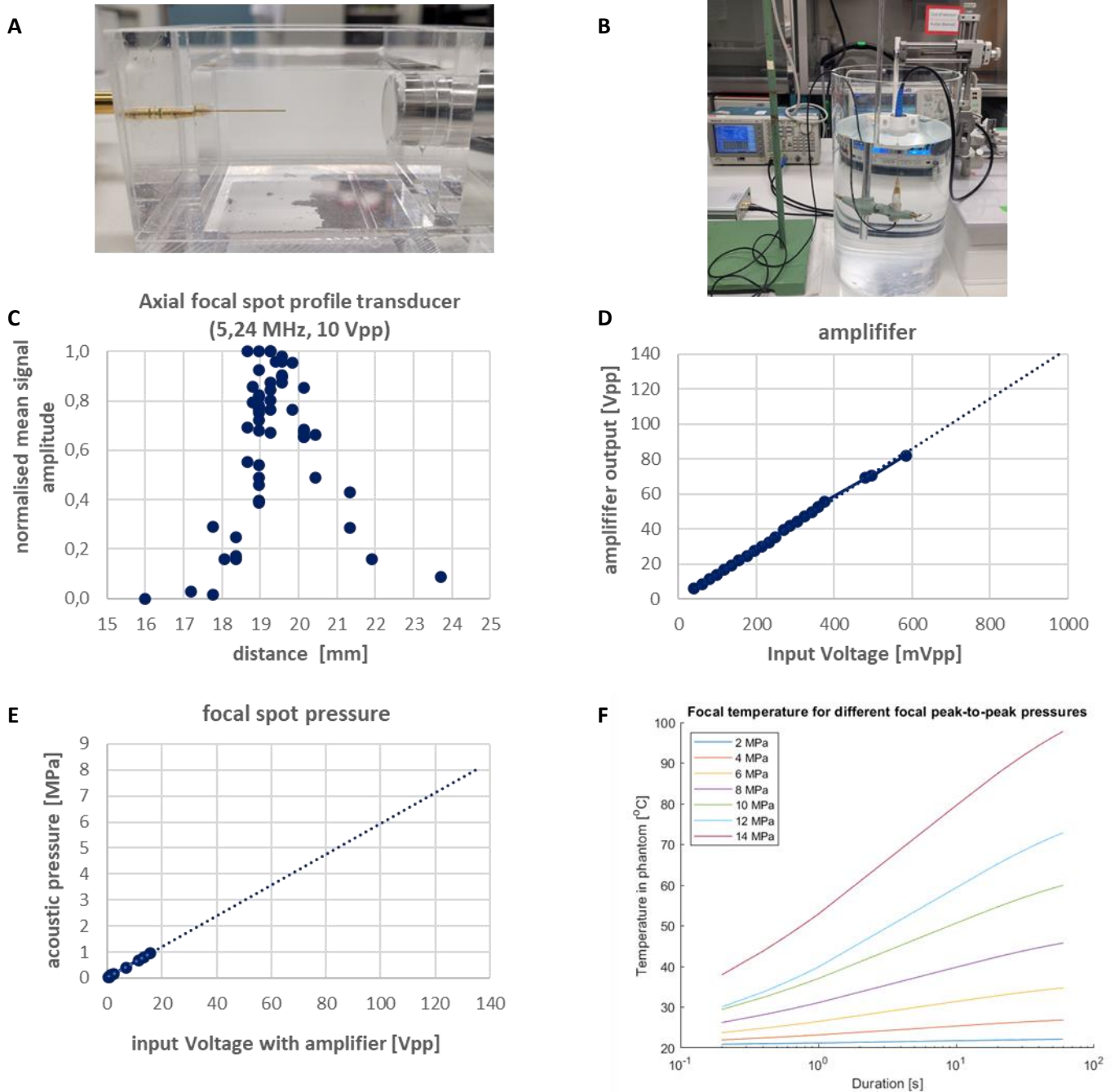


Figure 3 Characterisation of the commercially available transducer with a 0.2 mm needle hydrophone. **A** Measuring of the focal pressure in a small water tank, build for an easier alignment of the hydrophone with the focal spot. **B** Measuring of the focal pressure in a larger water tank, used for a more targeted steerability of the transducer mounted in the stereotactic frame. **C** Axial focal spot profile of the transducer measured in pulsed burst mode with about 4% duty cycle at 5.24 MHz frequency and 10 Vpp amplitude. **D** Power output of the amplifier at different inputs (5.24MHz frequency). **E** Measured focal spot pressure at different input powers. **F** Simulation of expected focal temperature in brain tissue at different focal pressures and stimulation durations (by TU Delft).

2.1.3. Targeting the mfb ex-vivo

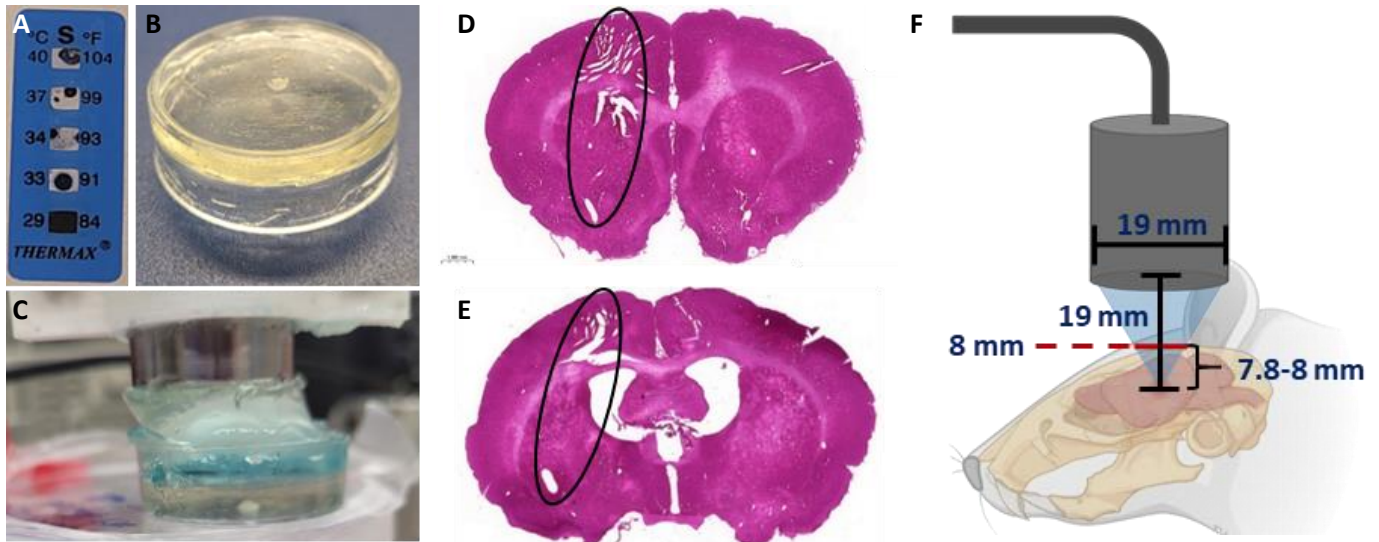


Figure 4 In vitro and ex vivo test of lesioning parameters. **A** Temperature indicator from 29°C to 40°C showed colour change after 5-20 s of stimulation with 7,3 MPa. **B,C** Stimulation of BSA agar for 30 s caused the petri dish to melt but no white colour change of the agar (indicating coagulation at 70°C). **D,E** Successful untargeted lesioning at multiple depths in a perfused explanted rat brain. **F** Based on the transducer characterization (18.7-20.4 mm focal length) a craniotomy of about 8 mm is necessary to stimulate the mfb at a depth of 7.8-8 mm.

To test the possible temperature increase with the commercial transducer, initially temperature indicators from 29 to 40°C were used. Depending on the distance and angle of the transducer, as well as the duration of the stimulation different sized discolorations were visible indicating heating to the respective temperature (see fig. 4 A). This showed that 5-20 s at 7.3 MPa (860 Vpp input) were sufficient to cause a temperature increase to 40°C, depending on the distance of the transducer. When the same parameters were tested on agarose with bovine serum albumin (BSA) for 30 s, the plastic of the petri dish melted, although the agar itself didn't show visible changes (fig. 4B,C; BSA coagulates and turns white around 70°C), most likely because the thermal properties of the agar and the plastic were different. To ensure the maximum temperature increase was sufficient for lesioning, the maximal parameters were used in an explanted rat brain fixated with 4% paraformaldehyde (fig. 4 D, E): 30 s (maximum according to manufacturer) sinusoidal, continuous wave with 137 Vpp amplitude and 5.24 MHz frequency. Since outside of a stereotactic frame the focal spot cannot be targeted to a certain brain structure the beam was focused onto cortical areas of the brain with decreasing distance of the transducer to cause several lesions. As the results in figure 4 D and E show, the creation of a lesion was successful. In the cortical area, potentially due to repeated heating of the tissue, star like lesions formed, unlike in deeper structures where one distinct lesion can be seen.

To aim the transducer at a specific target within the brain, it had to remain within the skull to be fixed in the stereotactic frame. Therefore a large craniotomy (min. 8 mm) was performed in a dead rat and the transducer was coupled to the brain with an ultrasound gel pad as seen in fig. 2C. Several areas of the mfb (marked in fig. 5 A,B) were targeted. Unlike in a perfused brain the targeted right hemisphere didn't show a hole, but anterior to the target a discoloration after staining with haematoxylin and eosin (H&E) was visible. This was initially attributed to an inconsistency in staining due to the use of unfixated tissue.

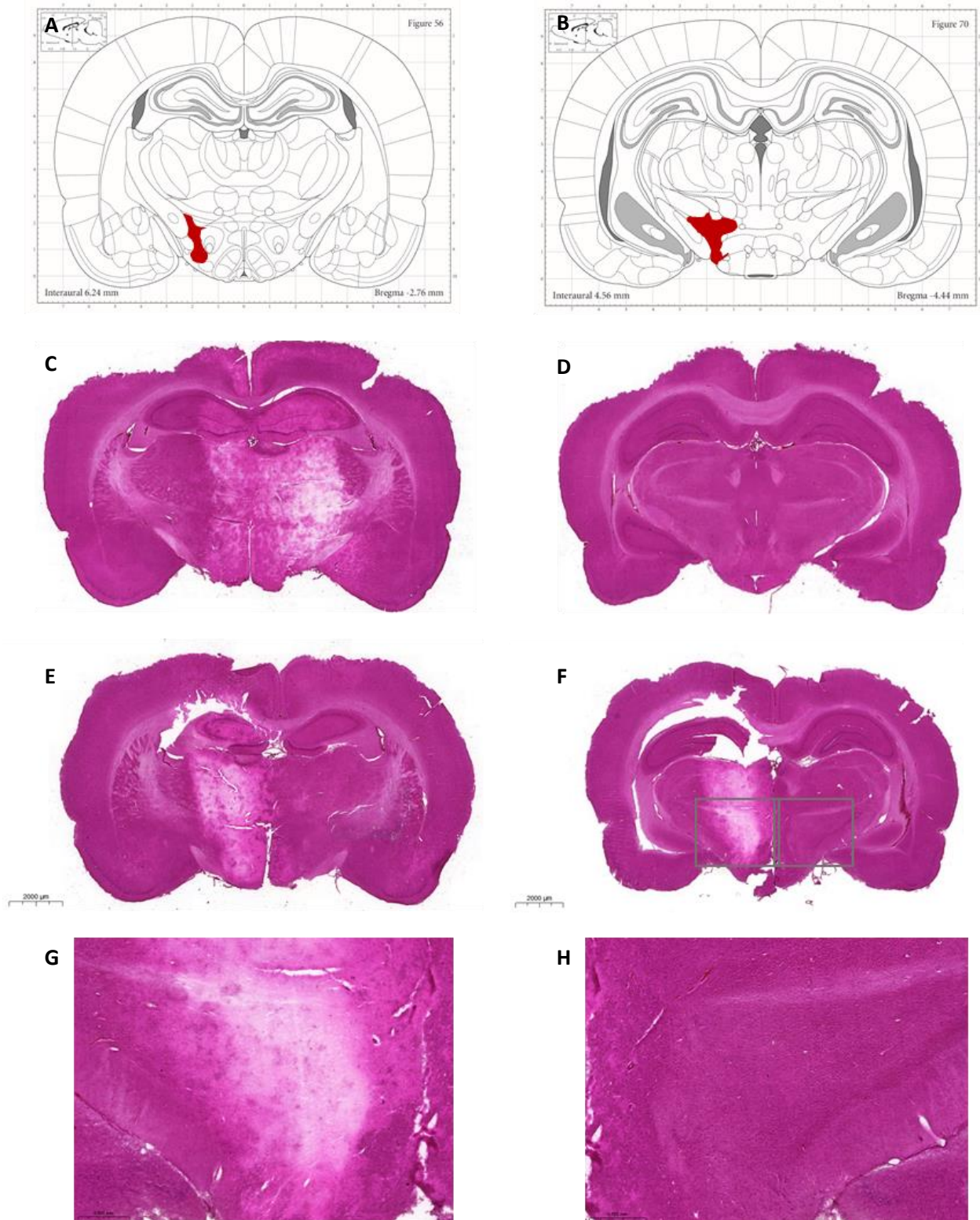


Figure 5 Targeting test with commercial transducer. C-H H&E stained 40 μ m coronal brain slices. Tissue was subjected to HIFU after the animal was killed and afterwards postfixed with 4%PFA for staining. **A,B** Rat brain atlas pictures of the targeted regions, the mfb is indicated in red. **C, D** The area shown in B was targeted on the right hemisphere in a dead animal. **C** Anterior to the target a large discoloration is visible around the midline and right mfb. **D** No visible changes in the targeted right mfb. **E, F** In an additional test, regions shown in A and B were targeted on the left hemisphere. The discoloration and ruptures in the tissue around the targeted areas of the left mfb were confirmed as effects of the tissue heating. **G, H** Magnification (scale bar 500 μ m) of the mfb in figure F, marked in grey, left hemisphere (G), right hemisphere (H).

But in a repeated test (fig. 5 G, H) no lesion could be detected and the same discoloration seemed to be more targeted. Additionally the tissue around the mfb became very brittle and often ripped during cutting. This led to the assumption that the discoloration, as well as the compromised tissue integrity is the effect of the tissue heating due to the transducer stimulation. This applied to a large area also above the actual target.

While further tests will be conducted and the temperature of the brain at the time of stimulation has to be taken into account, at this point the commercial transducer does not seem to be the ideal tool to target the mfb in vivo. The large necessary craniotomy and focal spot size seem to make it difficult to target deep structures reliably and accurately. Therefore the Task 4.2 will be achieved by looking at brain slice cultures of the mfb and shallower targets (e.g. prefrontal cortex) using histological methods and electrophysiological recordings. As soon as the first generation of the chip is ready, the mfb can be targeted and the parameters confirmed.

2.2. Implantation protocol

Aim of this procedure:

- a) Chronic implantation of the in the UPSIDE project developed eFUS chip for the stimulation of the medial forebrain bundle in rats.
- b) Chronic implantation of the in the UPSIDE project developed eREC electrode for the recording of ECoG signals in rats.
- c) Implantation of the integrated epidural brain interface (EBI; eFUS + eREC) for stimulation of the medial forebrain bundle and eCOG recoding from the prefrontal cortex in rats. (preliminary)

Before starting: all instruments must be sterilized

Duration: 2-3 h

Species: rat

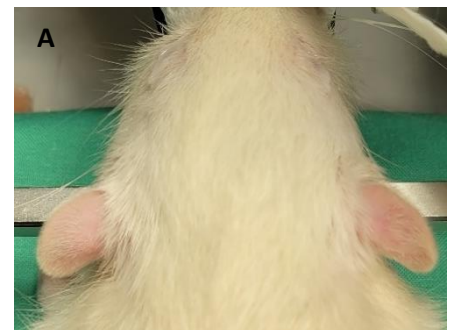
For this project: Sprague Dawley (controls)

Flinders sensitive line (depression model)

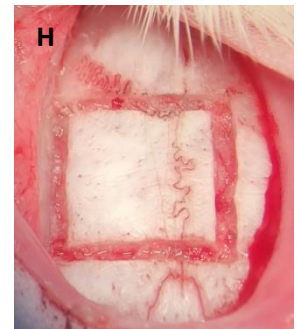
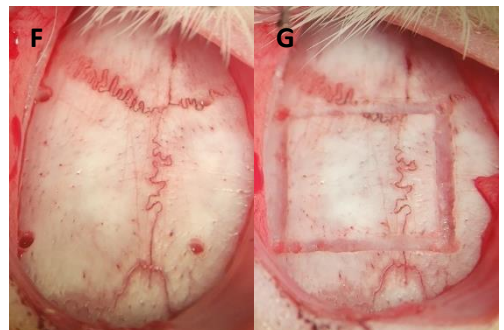
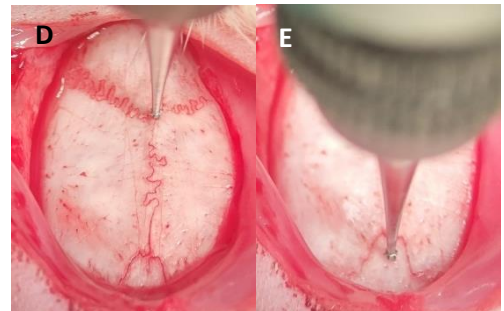
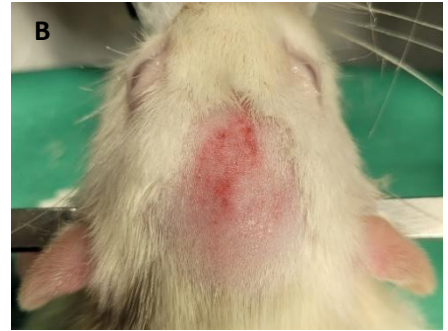
Age of animals: around 12 weeks, when the skull is fully-grown

Protocol:

1. Weight animal prior to surgery.
2. Place the animal in an induction box and anesthetize with 4 % isoflurane and 1-2 l/min oxygen.
3. Transfer the animal to the stereotactic frame, place snout in mask and reduce the Isoflurane flow gradually over time until the maintenance dose of 1.5-2.5% (depending on sex, age and individual animal) is reached. The depth of narcosis should be regularly checked with toe pinch and eye reflexes.
4. Monitor pulse and oxygenation with a pulsoxymeter throughout surgery; maintain a constant body temperature by placing the animal on a heating mat.



5. Administer Caprofen (4 mg/kg bodyweight) subcutaneously.
6. Fixate the head in the frame by inserting the earbars into the ear canal (fig. 6 A).
7. Apply eye ointment to avoid eyes from drying out (no eye lids).
8. Shave the incision area on the head (fig. 6 B).
9. Disinfect the skin with Povidon-Jod solution or Octenisept[®] and apply 2% Lidocaine gel to the skin.
10. Make a 2-3 cm long incision along the midline.
11. Using a scalpel scrape the periosteum from the skull to expose the bone. Make sure the bone sutures are visible and retract the skin till the lateral bone ridges of the parietal bone are visible. Manage bleeding with saline and dry skull surface with cotton swaps.
12. Place clips to hold incision open if necessary (fig. 6 C).
13. Measure coordinates of bregma (fig. 6 D) and lambda (fig. 6 E) with a frame mounted drill using a small drill head.
14. Level the head by adjusting the mask height until the dorso-ventral (DV) coordinates of both reference points are the same.
15. Measure the anterior-posterior (AP) length of the midline. As long as AP is between 8 -9 mm usually no adjustment of coordinates is necessary. When the head is very large (9+ mm) or very small (8- mm) consider adjusting the AP coordinates of the target, by multiplying with the AP correction factor (AP length/9 mm).
16. Using bregma as a reference, calculate the target coordinates.
17. Depending on the desired craniotomy size, calculate the four corners of the square craniotomy with the target in the middle.
18. Drill through the skull in the four corners without penetrating the dura (fig. 6 F).
19. Connect the four corners with the frame-mounted drill by lowering the drill only lightly onto the skull and slowly moving it along the AP or medio-lateral (ML) axis (be careful since skull height differs). Deepen groove as wanted (fig. 6 G).
20. Switch to a free hand drill to finish the craniotomy, stop drilling when the bone piece is easily movable with a forceps and “floats” on the dura (fig. 6 H).
Be extra careful of the depth of narcosis during craniotomy drilling, since prolonged drilling could cause the animal to be more awake, adjust if necessary.



- Try to drill at a steep angle for higher precision, the larger the drill head and the shallower the angle the larger the craniotomy will be independent of calculated size.
 - Be extra careful above the midline, since the bone is quite thick but the superior sagittal sinus runs right underneath causing large bleedings when pricked.
 - The skull is thinnest in the middle of the bone plate between the midline and the lateral ridge of the parietal bone, drill carefully.
21. Try to clean away bone debris and place a drop of saline on top of the craniotomy.
 22. Remove the bone piece carefully and slowly under the saline, by slowly peeling it to one side (fig. 6 I).
 - Try to start at the side furthest away of the midline to avoid ripping the dura above the superior sagittal sinus.
 - If possible you can try to help to detach the dura by scraping the back of the bone with a forceps.
 23. Clean the craniotomy as well as possible, stop any bleeding (fig. 6 J).
 - Put mild pressure with a cotton swap to the source of the bleeding till it stops.
 - Otherwise products with haemostatic properties (e.g. gelfoam®) can be used.

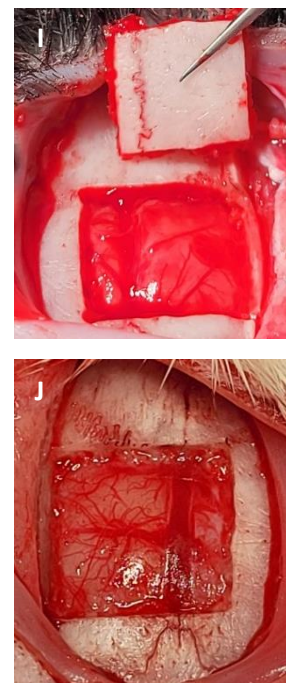


Figure 6 Stereotactic rodent surgery: 7x7 mm craniotomy. A Rat head is fixated in a stereotactic frame. B Incision area is shaved. C Periosteum is removed and bone sutures exposed. D Bregma coordinates are measured. E Lamda coordinates are measured. F Holes are drilled in corners of craniotomy. G Holes are connected with frame fixed drill. H Craniotomy is drilled with free hand drill till dura is exposed. I Bone piece is carefully removed. J Bleeding is stopped and craniotomy cleaned.

a) Chip implantation

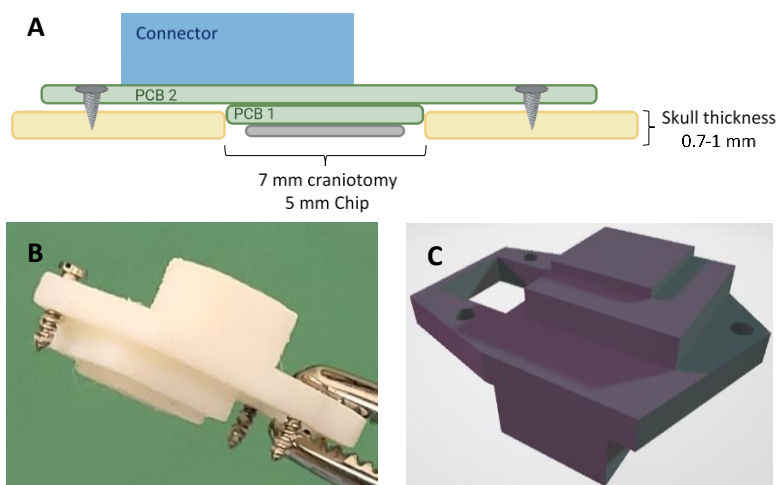


Figure 7 Prototype of eFUS chip PCB board. A Design concept: since the chip is only 0.5 mm thick, the contact with the brain would not be ensured with a skull thickness of min 0.7 mm if the PCB board is placed on the skull. Therefore a 2nd PCB is layered between the actual PCB and the chip, to create more depth. This PCB and the respective craniotomy have to be 7x7 mm wide to ensure enough space for wire bonding. B A 3D printed dummy of the design with screws (diameter 0.85 mm, length 3.85mm) C 3D design from the bottom.

Calculate the coordinates for a 6.6 x 6.6 mm craniotomy around the left mfb (coordinates: AP -2.8/ML +1.8/DV -8.0):

- AP: +0.5/-6.1
- ML: +5.1/-1.5

- This should result in a 7x7 mm craniotomy depending on drill head size. A high precision in craniotomy size should help the exact positioning of the chip and therefore targeting.
24. Place the chip with the PCB board in the craniotomy, mark the position of the screw holes with a pen on the skull or position the drill head in the screw hole (fig. 8 A, B).
 25. Remove the PCB, drill the holes for the screws, clean and dry the skull surface (fig. 8 C).
 26. Make sure dura is as free of bone debris and blood as possible and clean the chip surface carefully with 70% Ethanol.
 27. Place a drop of saline in the craniotomy and place chip inside.
 28. Place screws in pre-burred holes and slowly screw them in until they are anchored in the bone and put slight pressure onto the chip (fig. 8 D, E).
 - Do not drill them in as far as possible, but rather until the PCB seems level and stable. The curvature of the skull will otherwise cause the PCB and potentially the chip to be at an angle, if one side is forced to be flush with the bone.
 29. Seal the PCB to the skull by covering the sides with dental cement.

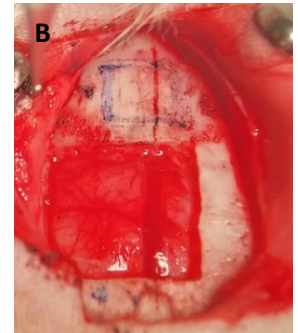
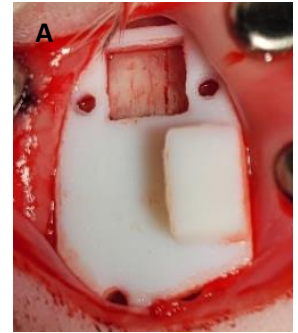
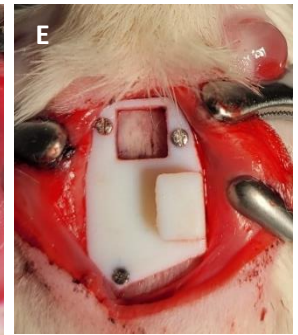
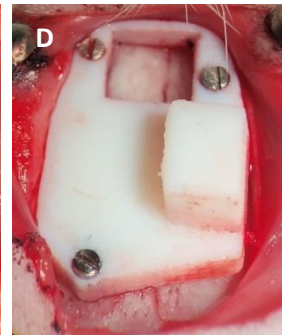
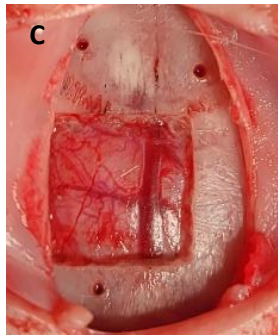


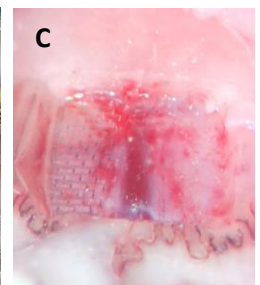
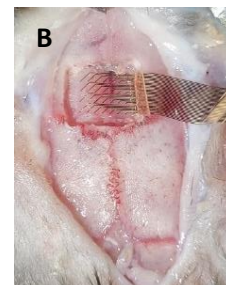
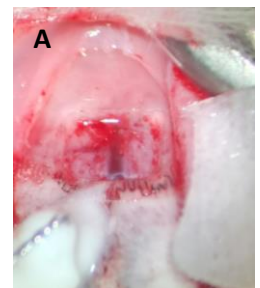
Figure 8 Implantation of the eFUS chip with the head mounted PCB board. A Placement of PCB in craniotomy to mark screw holes. B Marked screw holes and window. C Drilled screw holes for PCB. D,E PCB is screwed in the skull. Screws are adjusted so PCB is level.



b) Electrode implantation

Calculate the craniotomy slightly larger than the actual probe to assist proper placement; e.g. drill a 3.5x 3 mm craniotomy for 64 electrode probe of 3.2 x 2.5 mm (potentially this can be adjusted to as small a craniotomy as possible, depending on the electrode shape, size and position; fig. 9 A).

24. Place the removed bone piece in sterile saline until reimplantation.
25. Drill two holes for the ground (in the bone) and reference electrode (touching the dura) above the cerebellum.
26. Screw in the screws with the attached connectors.
27. Wet a small piece of tissue or Parafilm® with saline and place the electrode on top. Ensure it is flat.
28. Place tissue and electrode next to the craniotomy.
29. Slide the probe slowly onto the brain, if necessary try to direct or correct the position by very carefully pulling with a forceps (fig. 9 B, C).



- Make sure all electrodes lie flat and have contact with the brain.
 - Try to position the probe as straight as possible
 - The probe should not be positioned above a large blood vessel, as this would disturb the recording.
 - If necessary, remove any excess saline with a cotton swap or a sponge.
30. Ensure the recording is working by shortly connection the electrode to the headpiece (fig. 9 D).
- Be careful to ground yourself as not to cause any damage.
 - Be careful not to move the probe during this or to reposition it.
 - Place a small piece of Parafilm® underneath the headstage to avoid any moisture damaging it.
31. Place the removed bone piece carefully back on top of the probe without moving it (fig. 9 E, F).
32. Fill gaps between the bone piece and the skull with either bone wax or a separately mixed paste of Paladur® dental cement (fig. 9 G).
33. Cover all four sides with light curable dental cement. Stop recording and cover craniotomy with thin layer of Paladur® (fig. 9 H).
34. Place PCB in 3D printed head stage and fixate it with dental cement securely to the skull (fig. 9 I).

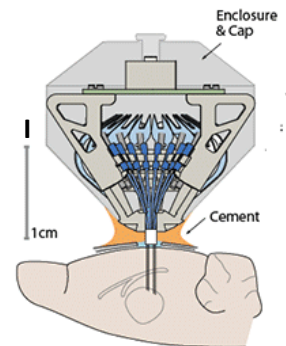
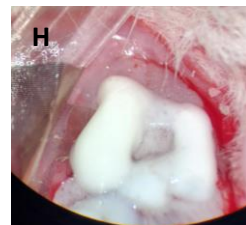
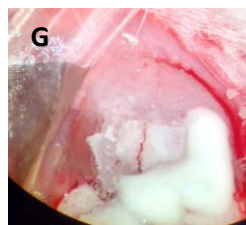
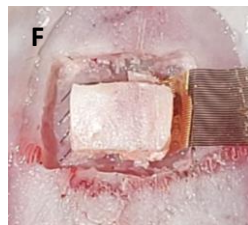
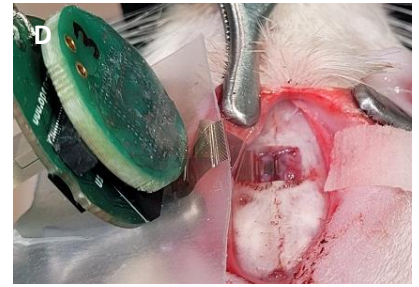


Figure 9 eREC electrode implantation. **A** Drilling of a 3.5 x 3 mm craniotomy. **B** Placement of 32 electrode probe on one hemisphere. **C** Placement of 64 electrode probe on one hemisphere. **D** Test recording before closing of the craniotomy. **E, F** Bone piece is placed back on top of the electrode. **G** Gaps are filled with bone wax. **H** Craniotomy is covered with dental cement. **I** Potential design for 3D printed headstage, adapted from <https://open-ephys.github.io/flexDrive/>

c) epidural brain interface (EBI) implantaion

Drill the corner holes for a square craniotomy for the chip as well as the top corners of a rectangular craniotomy for the eREC probe.

Since the chip is placed on top of the mfb and the craniotomy for the eREC extends above the prefrontal cortex this would result in an L shaped craniotomy (see fig. 10).

24. Drill the small craniotomy for the eREC probe first. Remove the bone piece and store it in sterile saline until reimplantation.

25. Drill the large craniotomy and remove the bone.
26. Drill any holes for anchor and reference screws.
27. Clean the craniotomy and the device.
28. Place the device by placing the probe on top of a wet tissue in the front of the craniotomy and slowly slide it backwards until the chip rests in the large craniotomy and the electrode rests flat on the brain.
29. If necessary, correct the position and replace the bone piece above the eREC probe.
30. Fill in the gaps and dental cement the piece with a thin layer.
31. Screw the PCB board to the skull and secure the device with dental cement.

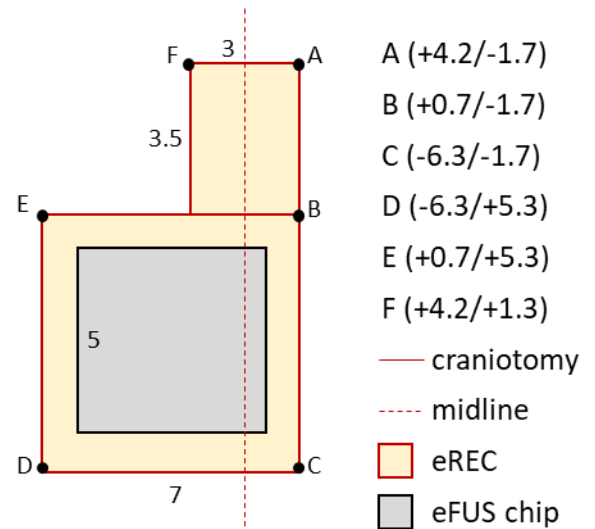


Figure 10 Dimensions and coordinates of craniotomy for EBI (epidural brain interface). Potential space on the craniotomy for the placement of eREC probes is marked in yellow.

Post-surgical care

- Clean away any blood etc. and dampen skin with saline.
- -Inject 0.05 mg/kg KGW Buprenorphine subcutaneously.
- Close the skin around the plug/headstage with a suture (fig.11).
- Disinfect the suture and clean with saline.
- Stop isoflurane flow and remove ear bars but leave animal at the oxygen for several minutes.
- After the animal has recovered from narcosis it can be transferred to a fresh cage. The animal should be kept single housed in a high cage without bars till the end of the experiment to prevent damage to the implant by other rats or the animal hurting itself on cage bars.
- In the next days the animal can receive wet food, which should be changed daily.
- Depending on the time of surgery ends the animal will receive another injection of Buprenorphine on the same day. The next day the animal receives three injections of Buprenorphine in an interval of 6 h. During the night 0.005 mg/ml Buprenorphine + 0.5% Glucose will be added to the drinking water, wet food is replaced with dry food. Once a day Caproven will be injected as long as deemed necessary. The health of the animal, including their body weight will be monitored daily for at least seven days to control proper recovery.



Figure 11 Skin is closed around connector with a suture.

3. Conclusions

After the establishment of the commercial transducer, it was possible to perform the planned in vitro and ex vivo tests. With the use of a power amplifier the maximum focal pressure was sufficient to cause a lesion in an explanted fixed rat brain. It was not possible to form a lesion in the mfb in unfixed tissue with the same parameters, instead a larger area of the tissue was heated. While further tests will be conducted and the temperature of the brain at the time of stimulation has to be taken into account as well as shorter stimulation times, at this point the commercial transducer does not seem to be the ideal tool to target the mfb in vivo. The large necessary craniotomy and focal spot size seem to make it difficult to target deep structures reliably and accurately. Therefore the Task 4.2 will be achieved by looking at brain slice cultures of the mfb and shallower targets using histological methods and electrophysiological recordings. As soon as the first generation of the chip is ready, the mfb can be targeted and the parameters confirmed.

In the second part of Task 4.1 an implantation protocol was established for the eFUS chip and eREC electrode on their own as well as the integrated EBI device. Since its design and development is an ongoing process, especially for the used PCB boards, exact craniotomy sizes and PCB dimensions might change in the future. But the general method, as well as the size constrictions for both the craniotomies and PCBs have been successfully tested. With the use of two layered PCB boards, sufficient pressure can be applied to assure the FUS chip is in constant contact with the brain surface. If the angle of the chip can be kept perpendicular to the target during fixation will have to be confirmed. The gap between the 7 mm craniotomy and 5 mm chip would allow for additional electrodes surrounding the chip although for the recording of potential biomarkers the extension of the electrode onto the prefrontal cortex is essential. While a craniotomy size of 40 % of the skull surface (max 60 mm²; total skull surface ca. 150 mm²) is very large, larger openings have been used in the past in rodents and with a sterile surgical procedure and proper pain management this should not be a problem for the wellbeing of the rat.