Project Acronym:

BRAINSONIC (ENTERPRISES/0223/Sub-Call1/0057)

MRI-guided Focused Ultrasound robotic system for brain tumors.

Deliverable number: 5.3

Title: Evaluation of the therapeutic system and protocol in a mouse model of GBM

Prepared by:

Nikolas Evripidou (CUT)
Christakis Damianou (CUT)

Date: 23/12/2025

Funded by the European Union NextGenerationEU

Cyprus tomorrow RECOVERY AND RESILIENCE PLAN

Republic of Cyprus

RESEARCH & INNOVATION FOUNDATION

The BRAINSONIC project is funded by the Recovery and Resilience Facility of the NextGenerationEU instrument, through the Research and Innovation Foundation (RIF) of Cyprus.

Table of Contents

Executive summary

The study evaluated a custom-designed 3 degrees-of-freedom robotic system integrated with focused ultrasound (FUS) technology for precise and controlled brain tumor ablation in a Glioblastoma (GBM) mouse model. Ten adult male C57BL/6 mice were used in this study. Five mice served as healthy controls, and five mice were GBM models generated by stereotactic injection of GL261 cells. Two weeks after tumor induction, the animals were assigned to two groups: an experimental *Tumor* group consisting of the GBM-bearing mice that received FUS treatment over the tumor site, and a *Control* group consisting of healthy mice that received identical FUS to provide a procedural reference and ensure consistency.

Anaesthesia was carefully administered, and the mice were positioned for treatment on a 3D-printed platform ensuring optimal acoustic coupling. FUS was delivered using a 2.75 MHz spherically focused transducer mounted on a robotic arm allowing precise targeting within the mouse brain. Sonications were performed with standardized parameters (60 W acoustic output for 30 s) while continuous physiological monitoring ensured animal welfare.

Following treatment, mice were observed for adverse effects, then euthanized, and their brains were extracted for histological evaluation with hematoxylin and eosin (H&E) staining. Histology confirmed preserved brain architecture in unsonicated regions and effective, localized tumor ablation of FUS-treated GBM tissue characterized by coagulative necrosis, neuronal loss, and vascular disruption in the experimental (*Tumor*) group.

Materials and Methods

Ethical Approval

All experimental procedures involving mice were performed at the premises of the Cyprus Institute of Neurology and Genetics (CING) (Nicosia, Cyprus) under the study license CY/EXP/PR.L02/2024 granted by the Veterinary Services of Cyprus (Ministry of Agriculture, Rural Development and Environment, Nicosia, Cyprus), and in full compliance with all applicable guidelines and protocols.

Animals and Tumor Model

C57BL/6 mice were chosen because they constitute one of the most commonly used inbred strains in biomedical research. Ten male inbred adult (aged more than 6 weeks) C57BL/6 mice were acquired from the controlled facilities of the CING (Cyprus). Water and food were provided to the mice ad libitum.

Orthotopic GL261 glioma models were established (n=5) by stereotactically injecting GL261 GBM cells into the brains of C57BL/6 mice. These cells, sourced from DSMZ GmbH (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and kept at 37°C in a 5% CO2 incubator until reaching 80–90% confluence. Before injection, the cells were washed and carefully prepared as a single-cell suspension at a concentration of 1×10^6 cells in 3 μL. Each mouse received an injection 2 mm lateral to the sagittal suture and 3 mm deep into the brain. The mice were then monitored over a 2-week period to allow tumors to develop prior to receiving FUS therapy. This entire procedure is outlined in **Figure 1**.

FUS system

The fabricated 3 degrees-of-freedom (DOF) robotic system, integrating a single-element spherically focused ultrasonic transducer, was employed to deliver FUS treatment to mice.

The custom-built transducer—manufactured in-house using a specialized piezoceramic element (PiezoHannas, Wuhan, Hubei, China)—operates at a central frequency of 2.75 MHz and features a 50 mm aperture diameter with a radius of curvature of 65 mm, enabling precise targeting of murine brain tumors. The transducer is electrically connected and tuned to a dedicated RF amplifier (AG1016, T&C Power Conversion Inc., Rochester, NY, USA) that supplies the controlled power required for operation.

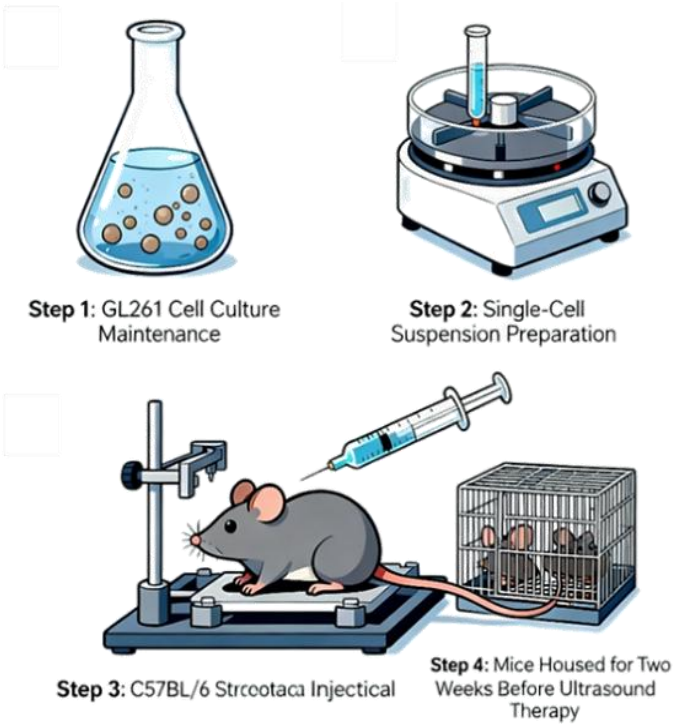

***Figure 1**: Procedure for GL261 cell preparation, injection, and tumor development in C57BL/6 mice.*

A dedicated treatment planning, control, and monitoring software platform was developed and interfaced with the hardware, providing automated and synchronized control of robotic motion and programmable FUS sonication protocols. Accordingly, the robotic system allows three-axis motion control (X, Y, and Z) through PC-controlled linear stages, facilitating accurate positioning of the ultrasound focus within the mouse brain. This integrated approach ensures precision and reproducibility in therapeutic ultrasound delivery, supporting systematic preclinical investigations of FUS effects in GBM models.

FUS ablation in GBM mouse models

Ten C57BL/6 mice were used to assess the effects of FUS on healthy and GBM brain tissue. Five mice served as GBM models following stereotactic injection of GL261 cells, while the remaining five were healthy controls. The animals were divided into two subgroups:

1) Group A (n=5): Experimental group consisting of GBM-bearing mice treated with FUS to assess the effects of FUS therapy on tumor.
   Referred to as *Tumor group*.
2) Group B (n=5): *Control* group consisting of healthy mice treated with identical protocol to provide a reference for normal tissue under the same experimental conditions.

The mice appointed to each group were assigned with specific ID tags. Specifically, mice in the "Tumor group" were given the tag "TUMOR" followed by a number from 1 to 5 (TUMOR1, TUMOR2, TUMOR3, TUMOR4 and TUMOR5), while the mice of the "Control group" were assigned the "CTR" tag followed by the corresponding numbers (CTR1, CTR2, CTR3, CTR4, and CTR5).

Experiments were initiated 2 weeks (14 days) after tumor cell implantation. Before execution of any experimental procedure, mice were anaesthetised by the principal investigator and veterinarian (DVM Kyriakos Spanoudes, VET-EX MACHINA) using Avertin (Sigma-Aldrich, USA). Anaesthesia was delivered by injection. Each mouse received a weight-based dose (15 μL/g). Following anaesthesia, any hair over the tumor site and surrounding areas was shaved. Anaesthetised mice were then individually positioned on the base of the robotic system for intervention. **Figure 2** shows a photo of the experimental set-up.

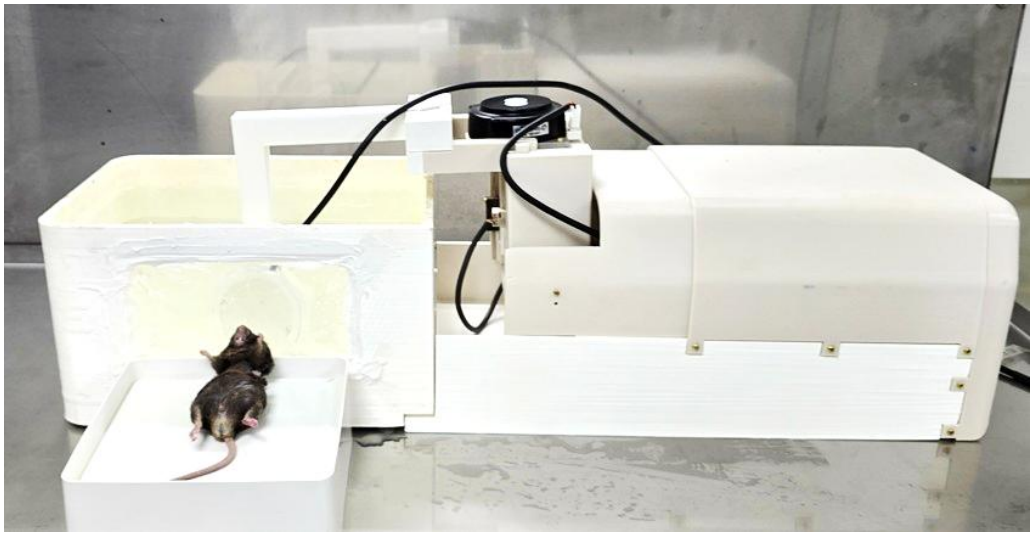

***Figure 2**: Photo of the experimental setting for FUS ablation in mice.*

The water container of the robotic system was filled with degassed/deionised water to create an environment for efficient transmission of ultrasound. A 3D-printed (Raise3D E2, Raise3D, Shanghai, China) Polylactic Acid (PLA) platform having a height of 4 cm was employed to raise the mouse to the level of the acoustic window of the robotic system, thus allowing contact of the tumor site with the thin membrane film covering the acoustic window. A layer of ultrasound gel (Quick-Eco Gel, AB Medica Group S.A., Barcelona, Spain) was applied on the tumor to achieve efficient acoustic coupling. This configuration ensured efficient delivery of acoustic energy from the ultrasonic transducer to the targeted area in the mouse brain. **Figure 3** shows a schematic illustration of the acoustic coupling arrangement —including the water-filled path, sealing membrane, and ultrasonic gel—as well as FUS targeting in a GBM model.

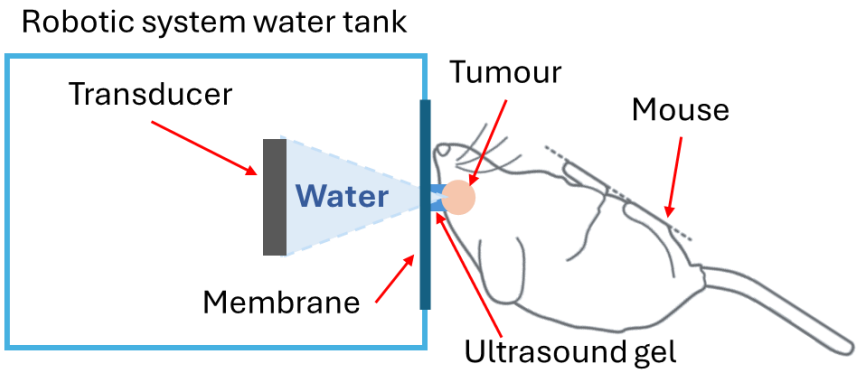

***Figure 3**: Schematic illustration of acoustic coupling and FUS targeting in a GBM model.*

After appropriate positioning on the robotic system, all mice were treated with FUS using the same sonication protocol. Specifically, a single sonication was delivered at an electric power of 200 W—which, given the 30 % efficiency of the transducer, corresponds to an output acoustic power of 60 W—for a duration of 30 s. Sonications were performed using a fixed 60 mm transducer-to-membrane distance, with the focus centered on the tumor site. During the experimental procedure, the breathing and motion patterns of the mice were monitored to evaluate any FUS-induced acute effects. If there were any indications of pain or irregular breathing, the procedure was halted to ensure the welfare and safety of animals.

After the end of the treatment, the sonicated part was grossly examined to check for any evidence of coagulative lesions on the skin surface. Mice were then checked for normal breathing and were placed in their cages to recover from anaesthesia. Once full recovery was verified, the animals were humanely euthanized, and their brains were surgically extracted (**Figure 4**) for comprehensive histological analysis.

This workflow facilitated evaluation of the procedural safety and cellular-level effects induced by FUS in the tumor and surrounding brain tissue.

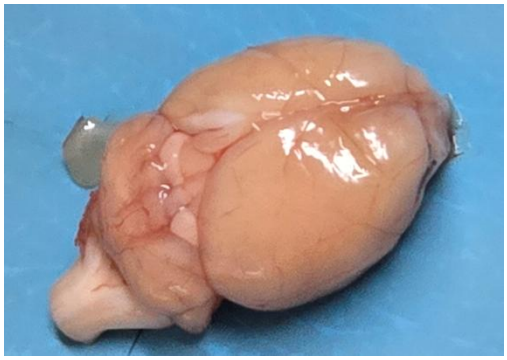

***Figure 4**: Mouse brain surgically removed following euthanasia.*

The overall stepwise timeline of the experimental protocol—from induction of anaesthesia to histological analysis—is illustrated in **Figure 5**.

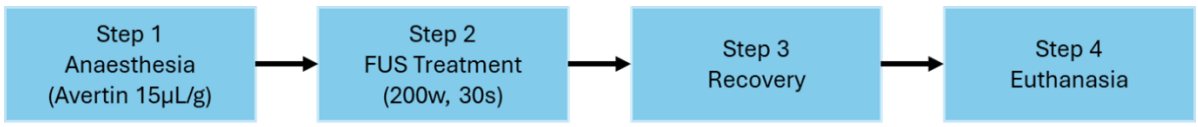

***Figure 5**: Experimental protocol timeline.*

Results

FUS ablation was successfully performed in all mice. In unsonicated regions, brain tissue preserved its typical architecture with no observable damage. Histology images revealed densely packed, healthy neurons within a well-organized neuropil, with no signs of necrosis, hemorrhage, or inflammatory infiltrates. The vascular network remained intact, consistent with normal brain morphology. These findings indicate that regions not exposed to FUS maintained normal histology, supporting the localization of effects to the targeted region. Representative images are shown in **Figure 6** and **Figure 7**.

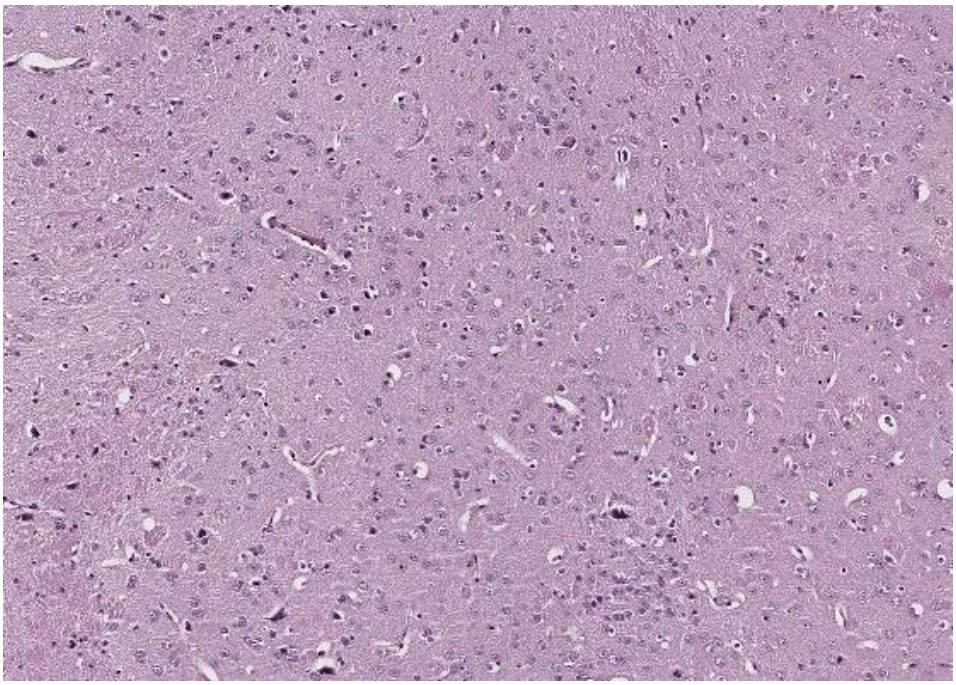

***Figure 6**: Indicative histology image from unsonicated brain tissue (1).*

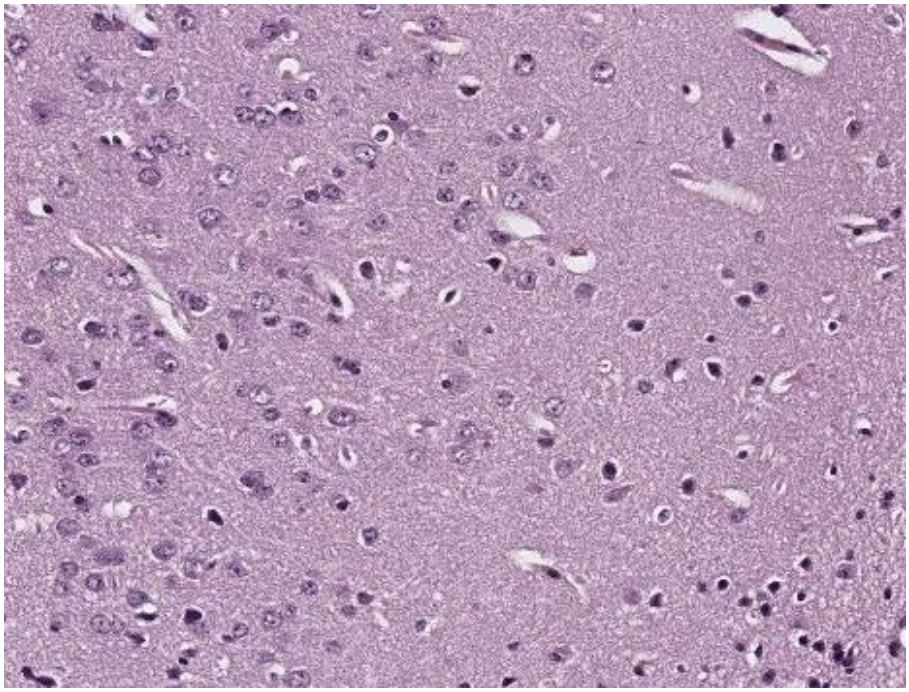

***Figure** 7: Indicative histology image from unsonicated brain tissue (2).*

Conversely, sonicated GBM tumors exhibited significant histopathological changes indicating effective ablation. The sonicated regions showed coagulative necrosis, characterized by eosinophilic staining, loss of nuclear features, and marked neuronal dropout. The ablated areas exhibited tissue rarefaction consistent with thermal injury. Surrounding tumor regions showed increased cellularity and scattered inflammatory cells, reflecting a localized response to FUS exposure. Mild vascular irregularities, including the presence of perivascular spaces, were also noted. Figures 8, 9, and 10 present representative histological sections.

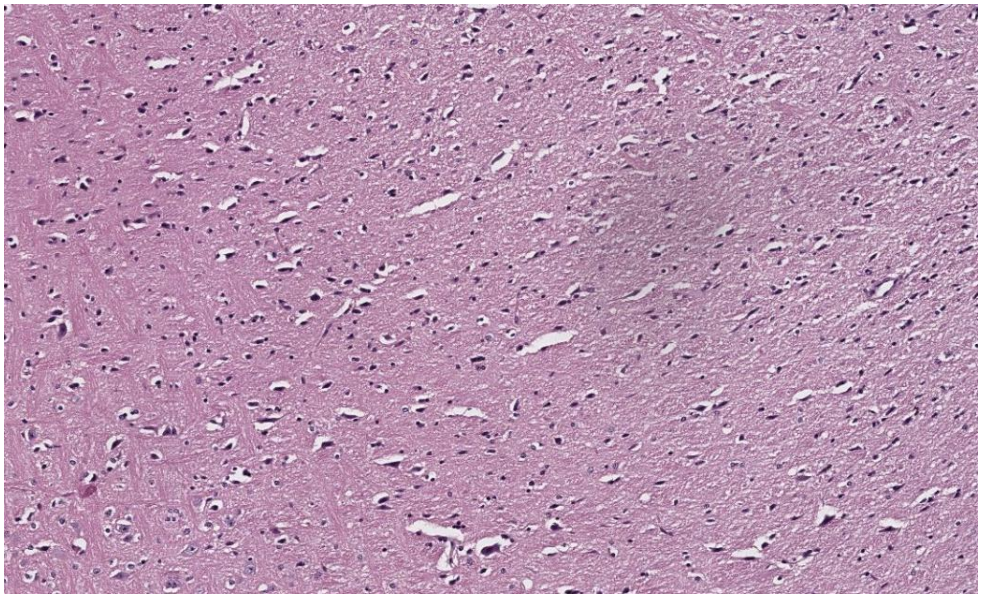

***Figure 8**: Indicative Tumor group histology image (1).*

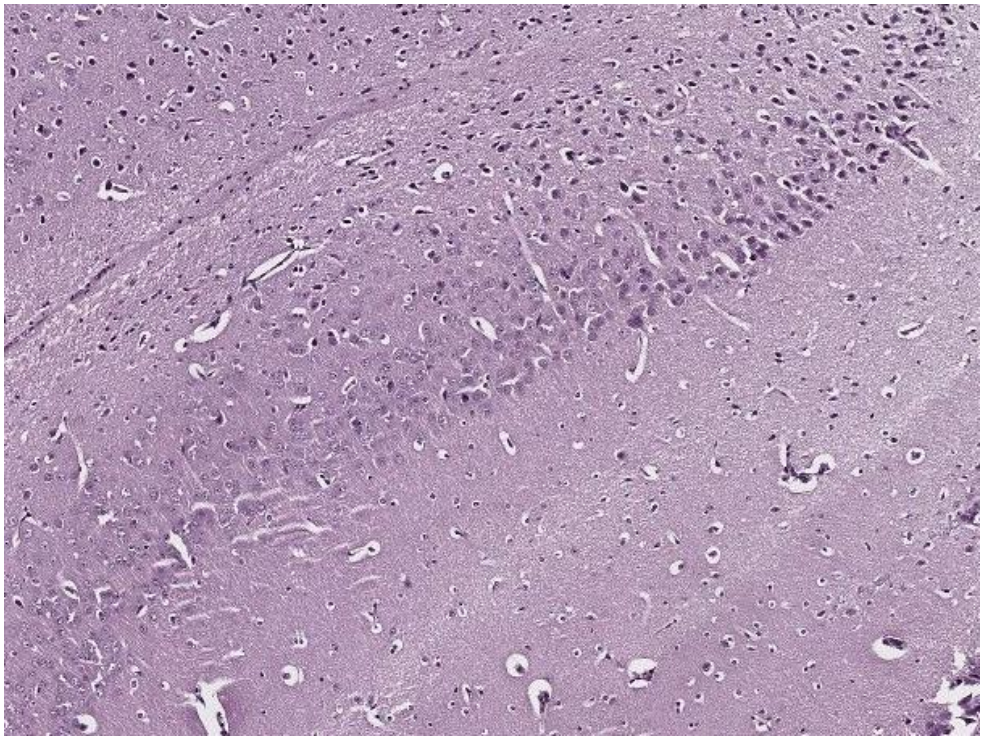

***Figure 9**: Indicative Tumor group histology image (2).*

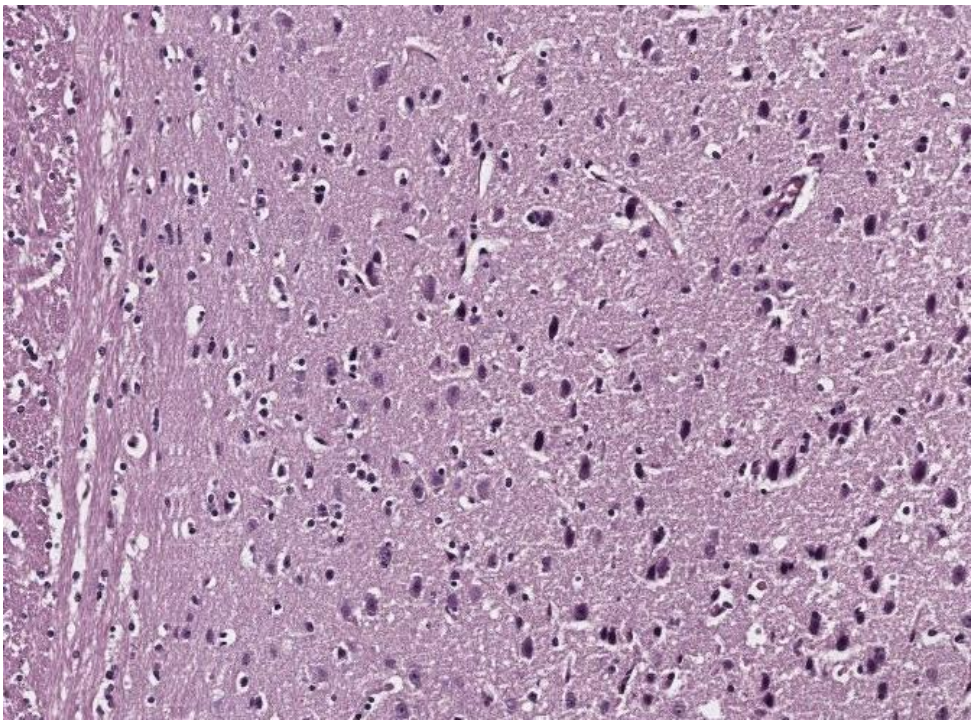

***Figure 10**: Indicative Tumor group histology image (3).*

Discussion

In this study, adult male C57BL/6 mice were employed to investigate the histological effects of FUS treatment on GBM mouse models using a novel robotic FUS delivery system. Consistent with established preclinical models, mice were divided into tumor-bearing and control groups. FUS was administered as a single sonication session based on predefined intensity and duration parameters consistently applied across all subjects. The protocol follows previous murine studies reporting localized cytotoxic effects within brain tumors while sparing non-malignant tissue.

Histopathological evaluation revealed that tumor-bearing mice experienced distinct tissue responses characterized by pronounced coagulative necrosis within the tumor mass, accompanied by neuronal dropout, and inflammatory changes. These findings underscore the acute tissue-damaging effects of FUS on GBM cells, consistent with prior reports demonstrating localized thermal and mechanical tumor ablation in orthotopic GBM models. Importantly, adjacent unsonicated brain tissue retained normal histological features, reflecting expected morphology outside the targeted zone. No acute complications were noted during or immediately after the procedure, suggesting good procedural tolerability of the applied FUS parameters in this model.

Although the current study did not evaluate longitudinal tumor size reduction or long-term survival, histological outcomes indicate localized tumor disruption (within the targeted region) following a single FUS ablation session. In this context, study limitations include the focus on acute histological evaluation. Future studies may incorporate longer-term follow-up to assess tumor growth dynamics and overall treatment impact. Future studies may examine approaches to improve ablation coverage.

In conclusion, this study demonstrates that robotic FUS technology can effectively induce selective tumor ablation in a preclinical GBM model, producing cytotoxic effects within the tumor while sparing off-target tissue. These findings support the further development and future *in-vivo* translation of the proposed therapeutic approach and validate the BRAINSONIC technology as a powerful platform for experimental neuro-oncology research.