

Preclinical Oncolysis Project

Overview

The goal of this preclinical oncolysis study was to ascertain if focused ultrasound at relatively low intensities can have a lysing effect on glioblastoma (GBM) cells while also not harming healthy cells. To facilitate this study, experimental setups were created to treat 3D cultures of cells and *in vivo* mouse flanks. In a cohort of 38 mice, a single treatment of low intensity ultrasound corresponded to a dramatic decrease in the growth of glioblastoma tumors! Likewise, *in vitro* studies suggested that low intensity ultrasound treatment of tumors has a dependence on the frequency of the applied ultrasound, and that optimizing this parameter may enable more effective treatment. Presented here is a brief overview of the studies that were performed along with the results of these initial feasibility studies. These initial results only explore a subset of available treatment parameters, however, Openwater's open source therapeutic ultrasound platform will allow researchers to explore treatment parameters over a wide range of treatment parameters as never before. With this new freedom, the research community will be able to investigate the mechanisms of ultrasound oncolysis, optimize parameters, and enable clinical studies.

The preclinical oncolysis project consisted of two phases. The initial phase used focused ultrasound at various frequencies and parameters (burst length, duty cycle, pressure, and total time) to treat *in vitro* 3D cultures of glioblastoma cells (and later healthy) to determine if their viability changed following the treatment. The final phase of the study was to use the same focused ultrasound transducers on the flanks of an *in vivo* mouse GBM model.

The manuscript is organized as follows. Section 1 provides a description of the Openwater preclinical ultrasound system used in the study. Section 2 is a summary of the sample and treatment parameters for the treat *in vitro* tumor spheroids, *in vitro* cell-laden hydrogel domes, and *in vivo* mouse glioma tumors implanted in the flanks of NSG mice. The results for these experiments are summarized in Section 3. An appendix is also included, documenting the results of individual *in vitro* experiments performed during the course of the study.

I. Preclinical Ocolysis System Description

Openwater preclinical ultrasound system overview

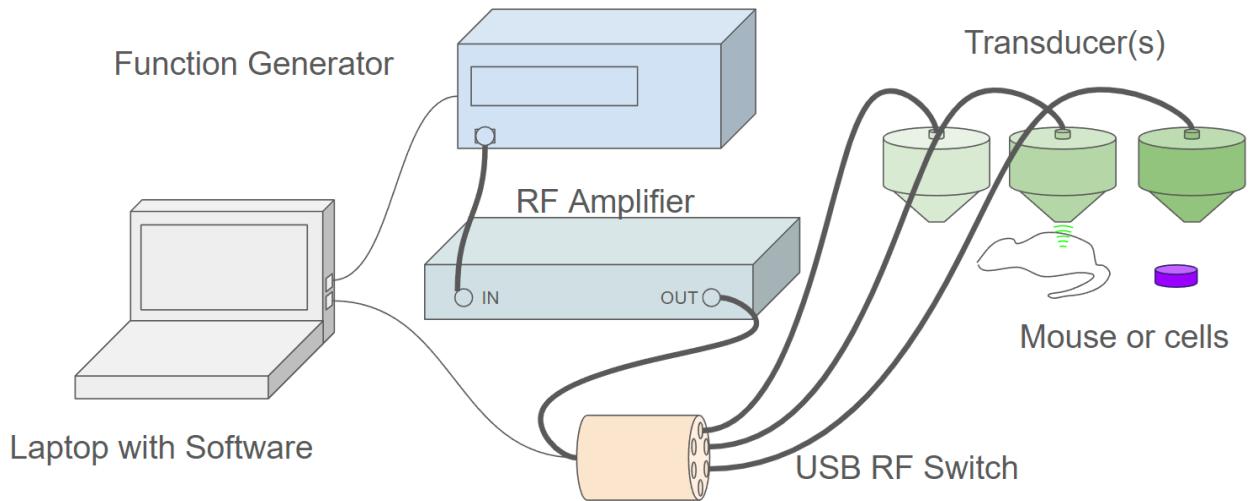
Common to all studies (*in vitro* and *in vivo*) were the transducers. Most of the transducers were custom spherically focused transducers with radiiuses of curvature of 60mm and diameters of

60mm for all frequencies (70kHz, 100kHz, 150kHz, 200kHz, 300kHz, 500kHz, and 1000kHz) fabricated by Benthowave, except the F#1 670 kHz transducer which was an off the shelf transducer manufactured by Precision Acoustics. The actual focus of each transducer varied and was measured in a water tank with a calibrated hydrophone attached to a motor-controlled 3-dimensional stage to map the acoustic field and determine the precise pressure to driving voltage relationship.

For operation in the experiments, all transducers were fitted with custom 3D printed coupling cones which enabled the ultrasound energy to be delivered without having to be immersed in a water bath. The individual transducers were all acoustically characterized, with their respective coupling cones, in a water tank. Each transducer was coupled to a specific coupling cone that enabled the “best” focus to be achieved. This was necessary because if you change the coupling cone, the acoustic field of the transducer also changes, so it is imperative to do this characterization for each transducer/coupling cone combination.

For each experiment, the acoustic outputs of the transducers were calibrated such that the maximum pressure that could be applied to the sample (in vitro or in vivo) was targeted and reported. For some of the transducers (predominantly the lower frequency ones), this meant that the pressure applied on the side of the target closest to the transducer received the most energy.

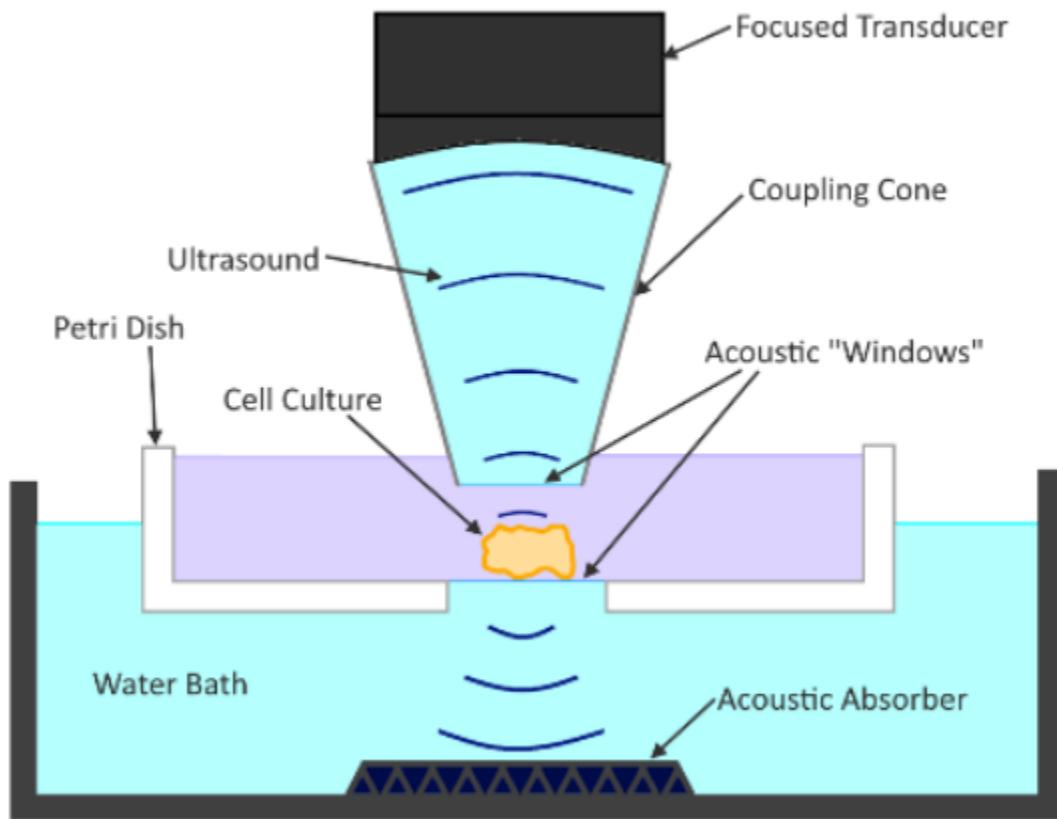
The general schematic of the electronic equipment and components is shown in the figure below which is the same for all of the in vitro and in vivo experiments.



In Vitro experimental setups

The in vitro experimental setups evolved throughout the study and details regarding the specific designs are described below and can also be found in the two instruction manuals.

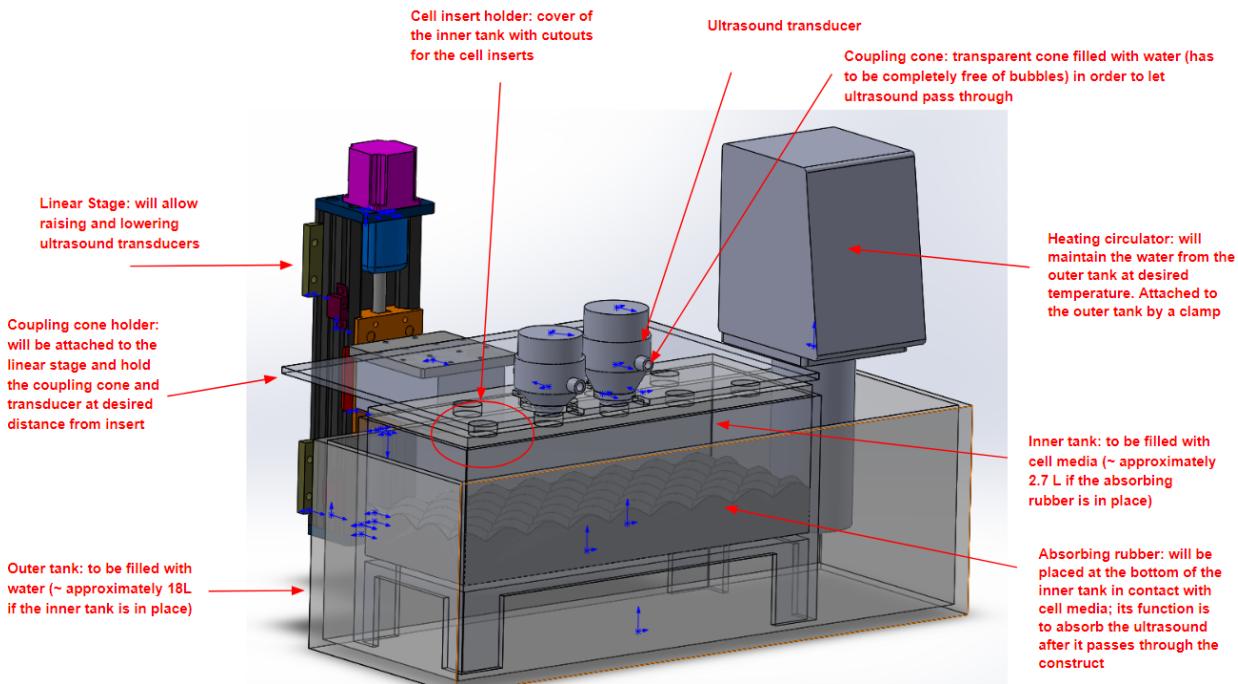
The primary difference between the two in vitro setups was the use of a common cell media bath vs. individual containers for each of the transducers. Otherwise, the concept remained the same which was applying focused ultrasound to the cellular constructs while trying to mitigate standing waves. The following figure is a generic schematic of the setup.



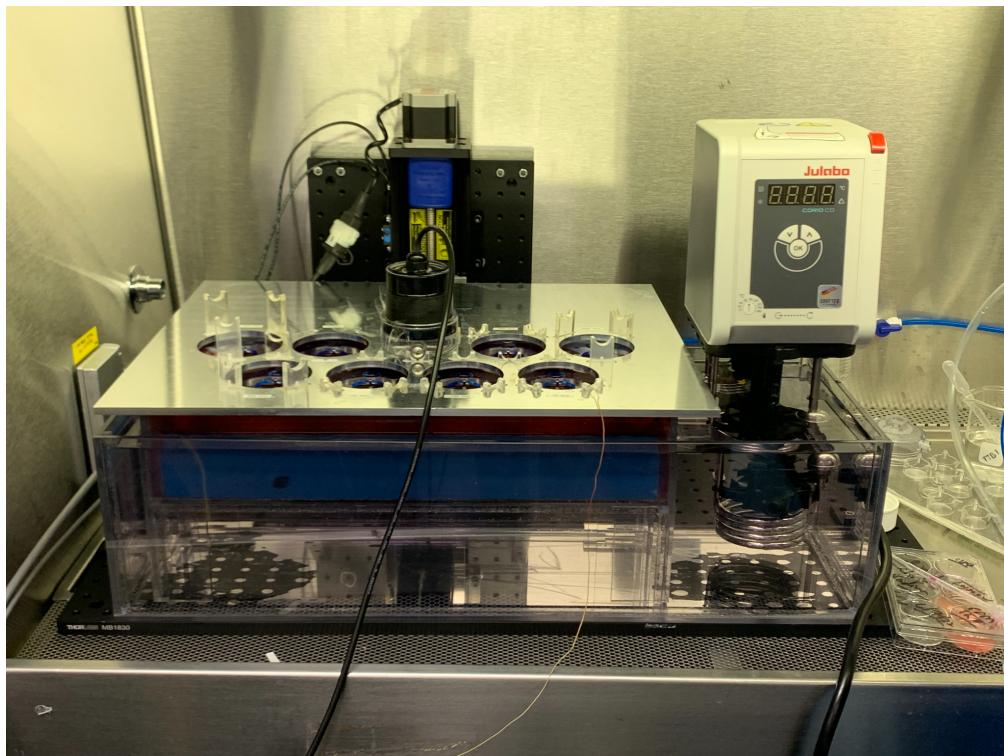
Common to all of the in vitro experiments, the 3D cell constructs were placed in the center of a 6-well plate insert that had an acoustically transparent bottom. The specific inserts that were used were [Greiner Bio-one Thincert for 6 well plate](#) (shown below). The inserts have a porous acoustically transparent membrane on the bottom which is and also allows cell media to permeate both sides.



The first experimental setup, shown in the figure below, consisted of an external temperature-controlled water bath, that had an internal tank that was filled with cell media. The transducers each had a position in the setup such that they could be lowered into place with a motorized linear stage. The coupling cones on each transducer were brought into contact with the cell media where the 3D cultures were located. Due to the different coupling cone lengths specific positions for each transducer were necessary to make sure their acoustic fields covered the sample appropriately.



Photographs of the above schematic are shown below. The clear plastic “arms” around each transducer hole are specifically sized such that the acoustic focus is when the treatment is underway.



Photos below is the inner tank cover where the inserts are placed with the cell samples

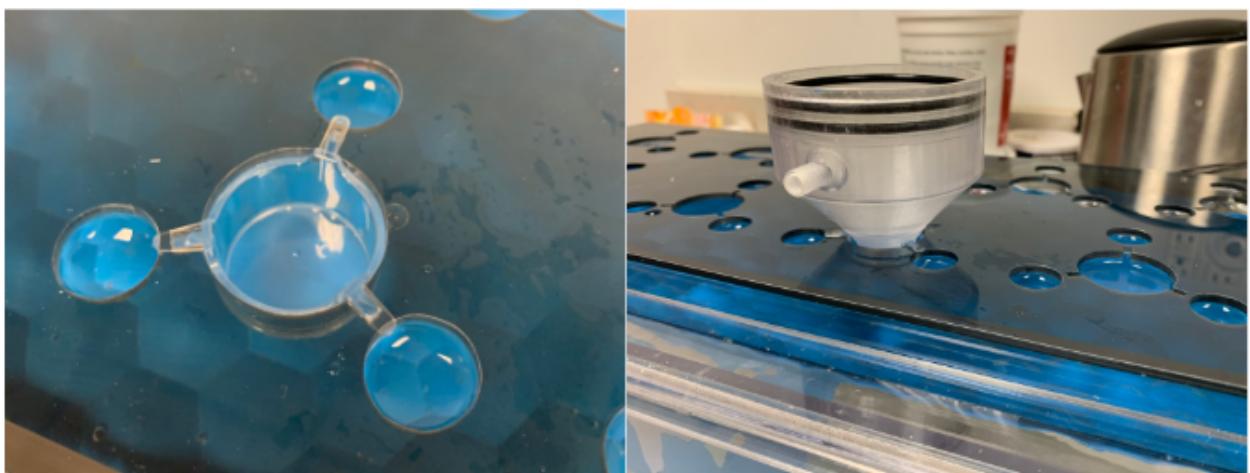
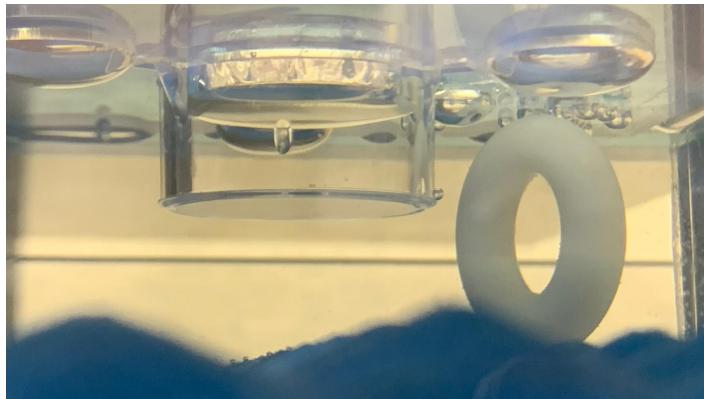
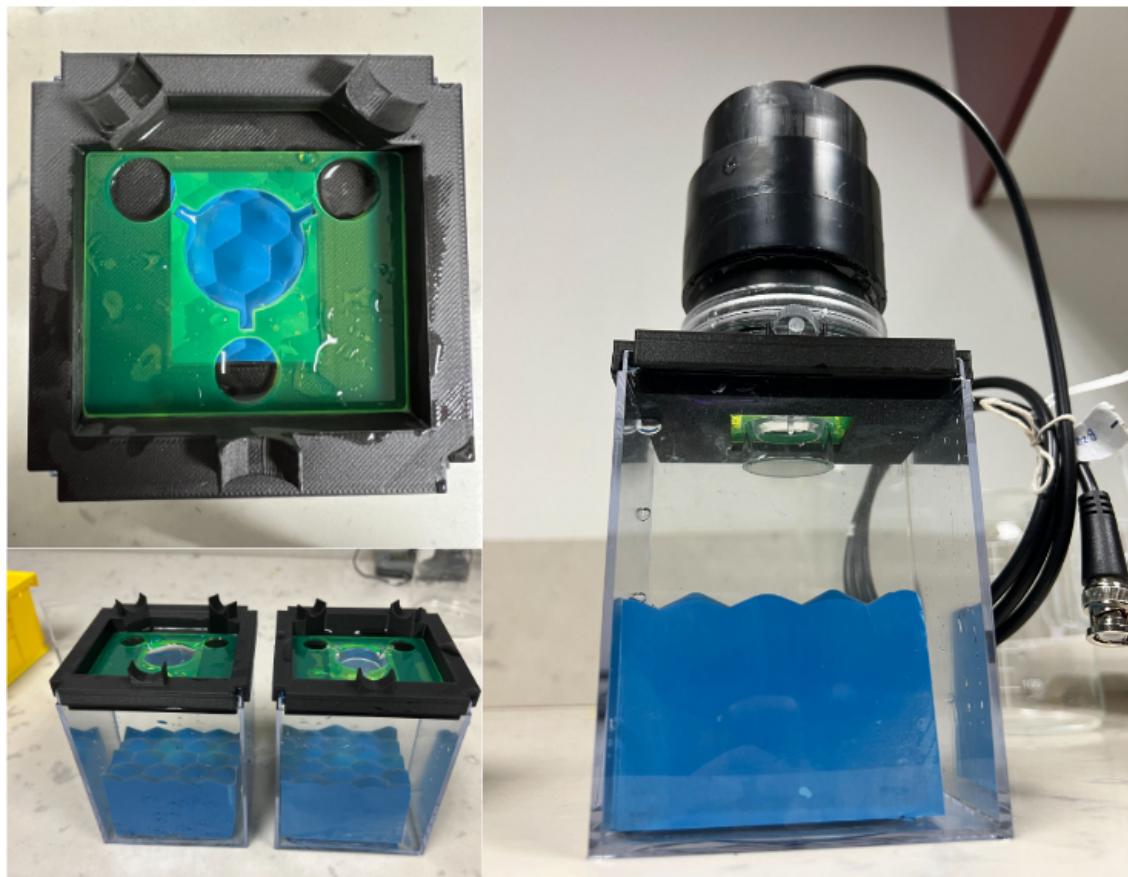


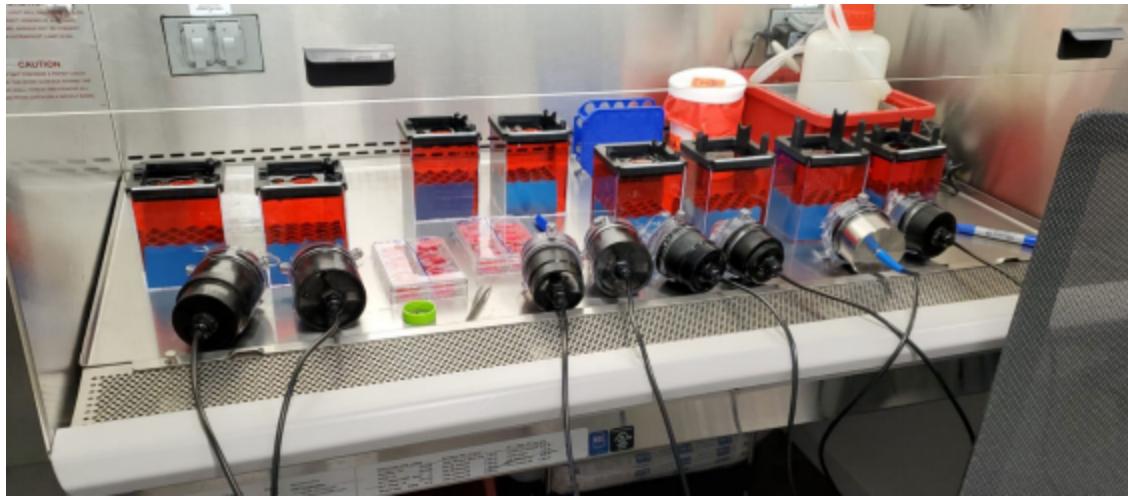
Photo of an insert in the tank with the coupling cone inside of it in contact with the liquid.



Swallowing several experimental studies with cells it was discovered that the large common tank with the shared cell media caused many contamination issues. It was also cumbersome to operate and difficult to ensure that there were no air bubbles between the coupling cones and the samples. Therefore a redesign of the setup was done where each transducer had its own, individual water tank. This eliminated many of the contamination issues and also made it much easier to remove bubbles that were in the acoustic path.

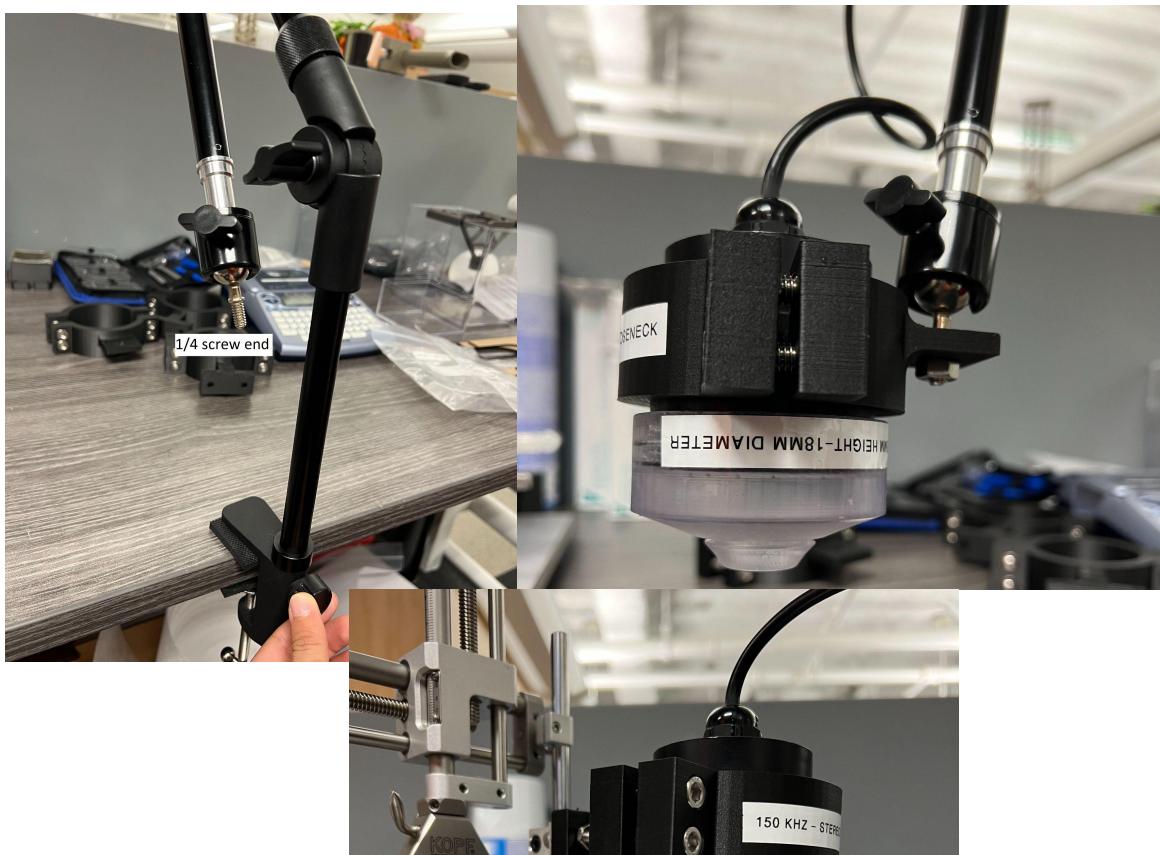
Pictures of the newer setup that was used in the later part of Phase 1.1 through Phase 2 of the studies are shown below.





In Vivo experimental setup

The in vivo setup consisted of the same transducers as the in vitro, but with different coupling cones as the desired acoustic field was different for these experiments. The transducers were characterized with the new coupling cones such that the desired (maximum) pressure was located 2mm from the face of the coupling cone exit plane. This enabled the transducer to be placed directly over the tumor to apply treatment. In order to perform the treatment the mice were sedated and the ultrasound transducer was mounted to a boom arm, or a stereotactic frame. The coupling cones were attached and filled and plastic wrap was used to seal the coupling fluid inside. Between the coupling cone plastic wrap and the tumor being treated a small amount of ultrasound gel was used to facilitate the final coupling of the energy to the target.

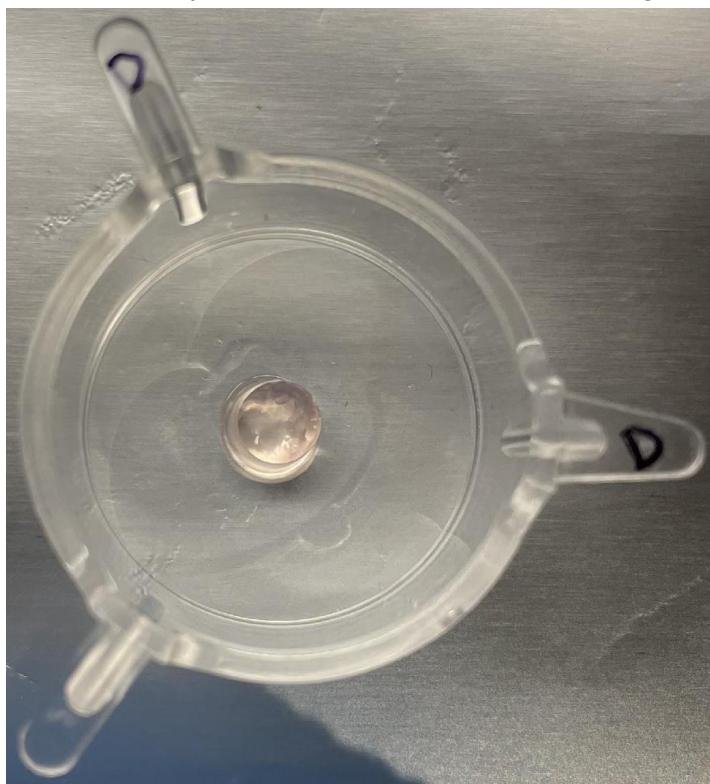


II. Description of experiments

In Vitro experiments

Phase 1.1 - In Vitro setup validation and biological component optimization

This initial phase of the in vitro study was to optimize the biological components of the experiments (3D cell cultures to have brain-like rheological properties and optimal concentrations of cells or spheroids) and to narrow the ultrasound parameters to ones that would most likely cause an effect. The hydrogels that were used in the first phase of the study were Gelatin-Methacryloyl (GelMA) Hydrogels attached to the bottom of the insert using Fibronectin. To determine the viability of the cells after treatment, live-dead staining was used and pictures of the fluorescent cells were taken with a confocal microscope. The pictures were taken in the geometric center of the construct within the first 1mm from the top (the side ultrasound entered). This ensured that the cells that were counted received the full target pressure of the ultrasound treatment. A custom ImageJ script was used to determine the percent viability of the cells from the confocal images.



Phase 1.2 - Optimizing Ultrasound Parameters and testing of GBM spheroids and Healthy Cells

Initially, these in vitro experiments were done with all frequencies of the transducers. However, due to early success in our parameter search space with two of the cell lines, future studies focused on only using 3 frequencies of transducers which enabled more cell lines to be investigated. The ultrasound parameters kept constant in this phase of the study were burst length (40ms), duty-cycle (10%), and total time (120 seconds). The % MI was varied (25% to 150%) to see if there was a lysis threshold based on the peak negative pressure at a given frequency. The determination of the cell viability was the same as in Phase 1.1, where the cells were dyed using a live/dead assay and imaged using a confocal microscope.

Phase 2 - In Vitro Cell-laden Hydrogel Domes with optimized parameters and flow cytometry

There were challenges associated with using laden hydrogels with respect to the throughput time and analysis of the cell lysing caused by ultrasound. The hydrogels required confocal microscopy of the cells to determine if they had lysed, and therefore it was extremely time-consuming and somewhat subjective. Although the results and analysis were useful, it was determined that a more quantitative approach of analysis would be better suited.

Therefore, a new method of 3D cell encapsulation was introduced in Phase 2 of the study. This method relied on putting cells into a different type of hydrogel (VitroGel) in a dome-like configuration that allowed for dissociation after treatment. Once the cells were dissociated from the hydrogel construct they were passed through flow cytometry to determine viability.



This methodology worked well for quantitative flow cytometry analysis but was still challenging to use in a high throughput scenario. It required a minimum of two people on treatment day to move the domes to the insert in a timely enough fashion to get through triplicates of every testing parameter for every frequency in under 8 hours.

Further improvements to the in vitro setup design would help facilitate higher throughput analysis. Enabling many cells to be treated inside 96 well plates or similar would be very advantageous. This is recommended for future studies as there are many parameters that should be investigated.

In Vivo experiments

Phase 3 - In Vivo - Mouse Flank

Using the same basic setup that was used for the in vitro experiments, an in vivo experimental plan was created. The in vivo experiments utilized NSG mice for both control and experimental arms. In the experimental arms, GBM (PDM140 and GL261) cells were injected into the flank of the mice. Once the tumors reached a volume of approximately 100-200mm³ ultrasound treatment was applied.

During the first set of experiments, where dosing of the tumor cells was being determined and experimental setup was performed, 230kHz ultrasound was used on tumors with different coupling mediums (gel and water). These results showed that fluid-filled coupling cones worked better but they also showed that the treatments had the potential to slow the growth of the tumors.

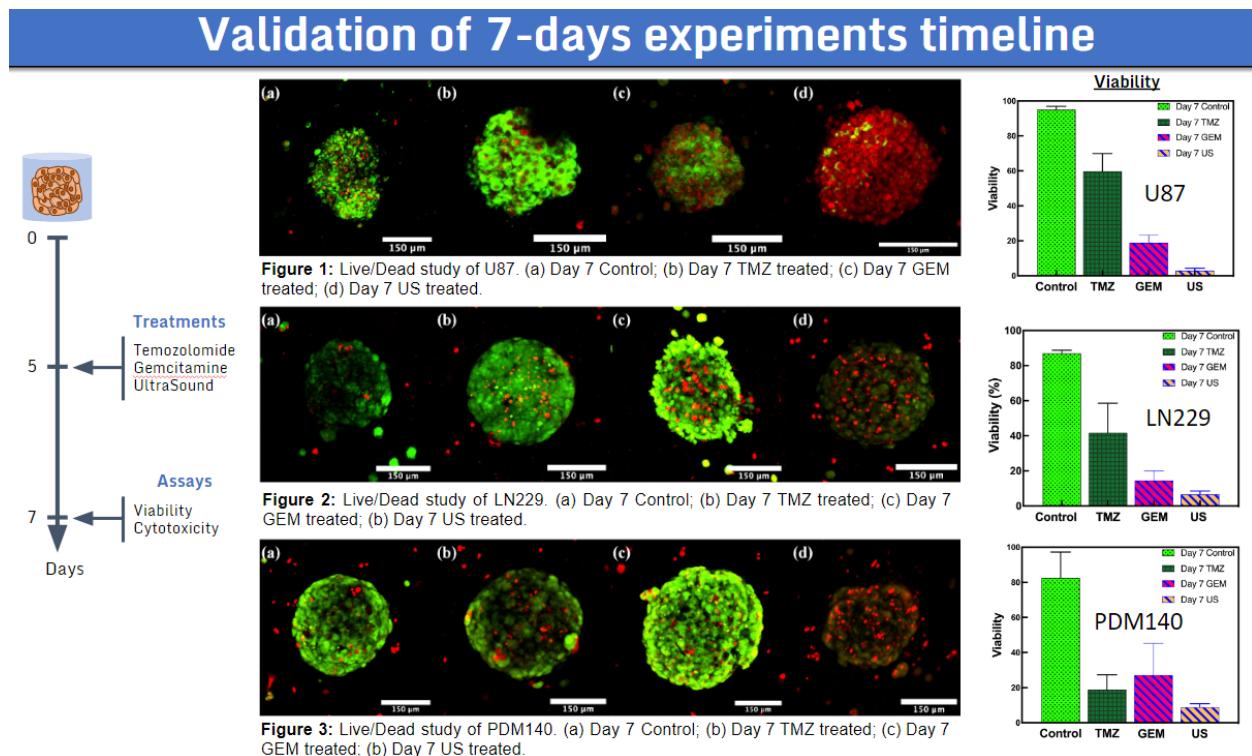
The final and most comprehensive round of in vivo experiments was performed on 38 NSG mice injected with GL261 GBM tumor cells. There were 30 mice in three treatment groups (100kHz, 150kHz, and 230kHz), and 8 control mice (no ultrasound). The ultrasound parameters that were used were 40ms burst duration, 10% duty cycle, 100% MI, and 120 second treatment time. From the first day of treatment the flank tumors were measured using calipers and their sizes were recorded. These measurements were made until one of the following 3 criteria were met at which case the animals were sacrificed and their tissue was harvested for histological analysis. If the tumors grew to exceed 2cm³, if the tumor broke the skin, or at 28 days. After sacrificing, the tissue was preserved per the protocol of Charles River for their third party analysis of the samples.

III. Results

Phase 1.1: In Vitro setup validation and cellular construct optimization

Cellular constructs were fabricated and rheologically analyzed such to that a formulation could be made that mimics the biological properties of brain tissue. Once the hydrogel formulation was determined, the different GBM cell lines that were going to be used in the study needed to be optimized for ideal spheroid size based upon the seeding. The details of the hydrogel formulation and cell seeding strategy for spheroid formation are not presented in this white

paper since they are the intellectual property of Openwater's collaborators. After optimizing the cellular constructs, the first in vitro experiments were performed in the first in vitro setup with the 300kHz transducer and three different cell lines (U87, PDM140, and LN229). The parameters used for these studies were 40ms burst length, 10% duty-cycle, 100% MI, and 120-second treatment time. These experiments were performed with controls receiving no ultrasound, and with Temozolomide and Gemcitamine (Chemotherapeutic drugs), for comparison. The initial study showed that focused ultrasound at these levels could lyse the GBM spheroids as shown in the following figure. It was also shown that a 7-day read out was sufficient to monitor the cell lysing.



Additional studies were performed with the first in vitro setup and all of the transducers during this stage as well. Preliminary results were promising but repeatability was very difficult as there were many contamination issues which led to a redesign of the in vitro setup. After redesigning the experimental setup, many more experiments were able to be run without contamination issues.

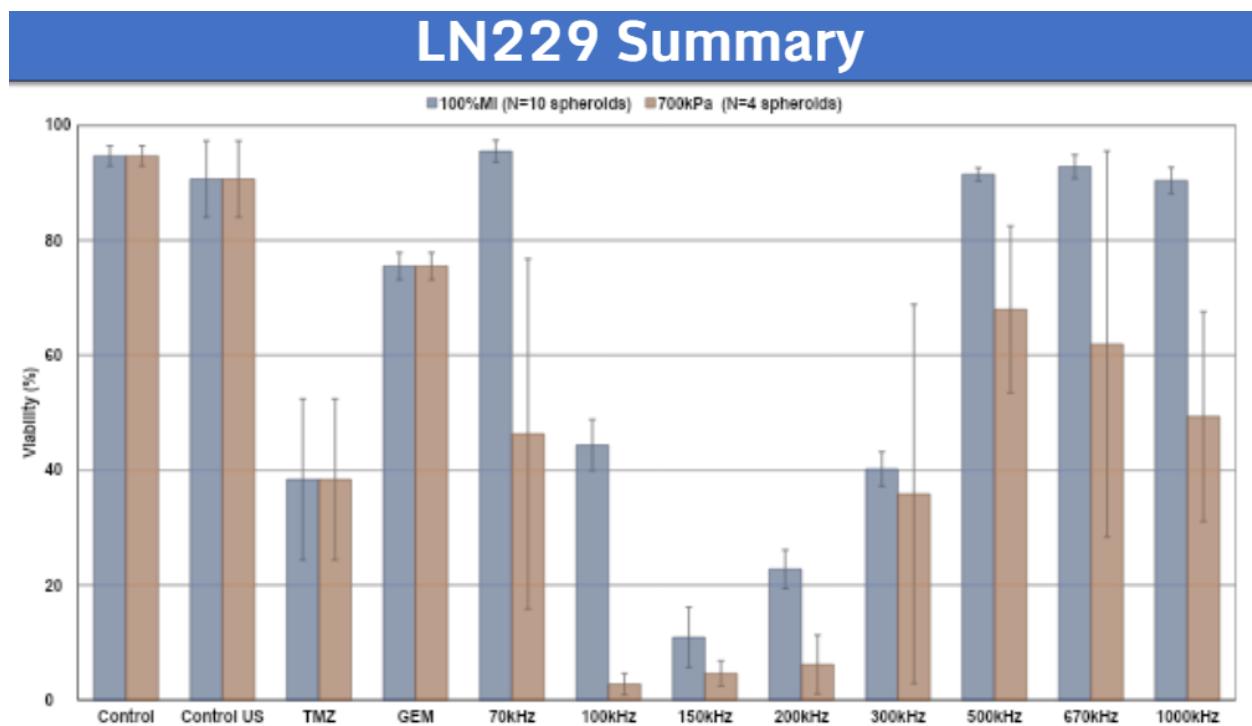
Phase 1.2 Optimizing Ultrasound Parameters and testing of GBM spheroids and Healthy Cells

Since the goal was to optimize an effect without inducing cavitation, a mechanical index of 1.9 was used as a maximum pressure. Mechanical index is the peak-negative pressure (PNP) in MPa divided by the squared root of the frequency in MHz. It was unclear if the effect we were trying to induce (lysing GBM cells) was governed by pressure alone or if there was a frequency

component to the effects. Therefore experiments were performed at both constant pressure (same for all frequencies), and constant MI. Based upon the limited experiments that were done on two cell types, constant MI appeared to show more differentiation between the frequencies used so further studies used the same MI and not the same PNP.

Another ultrasound parameter that was investigated was the burst length, while keeping the MI (100%), duty-cycle (10%) and total time (120 seconds) constant. These experiments showed that having a longer burst length of 40ms with other parameters fixed caused more lysis of the GBM cells than a burst length of 20ms. Further in vitro experiments (Phase 1.2 and beyond) used constant MI and burst lengths of 40ms unless otherwise noted.

Figure showing LN229 cell viability plot comparing constant pressure to 100% MI with 40ms burst duration



The above plot shows one of the experiments that was done comparing 100% MI to 700kPa. 100% MI represents a different pressure level depending on the frequency. From 70kHz to 1MHz, the pressure at 100% MI varied from approximately 500kPa to 1.9MPa respectively.

Parameter Optimization Conclusion

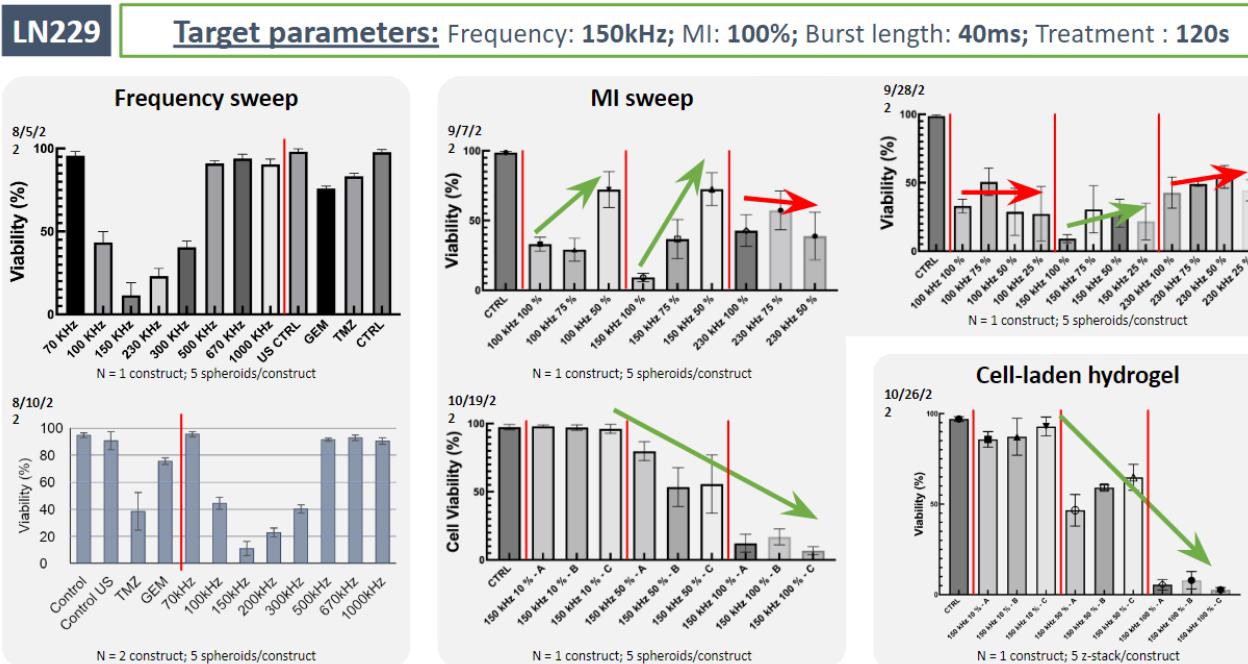
With the results of the prior experiments in this phase, a fixed set of treatment parameters was decided upon, and further experiments use these parameters unless otherwise specified:

Parameter	Value	Units
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Burst Length	40	ms
Duty Cycle	10	%
Duration	120	seconds

In addition to determining the optimal parameters, the frequency range of investigation was reduced based upon results showing more of an effect to viability at frequencies ranging from 100kHz to 230kHz. Therefore, many of the later studies did not use any of the higher frequency transducers, and only used 100kHz, 150kHz, and 230kHz transducers.

Once the ultrasound parameters were optimized for inducing an effect on the viability of the cells, the %MI was used to determine if there was a thresholding effect based on the pressure. 100% MI is the highest pressure one can use and still be under the diagnostic ultrasound limits for pressure. Viability was expected to increase as the %MI decreased.

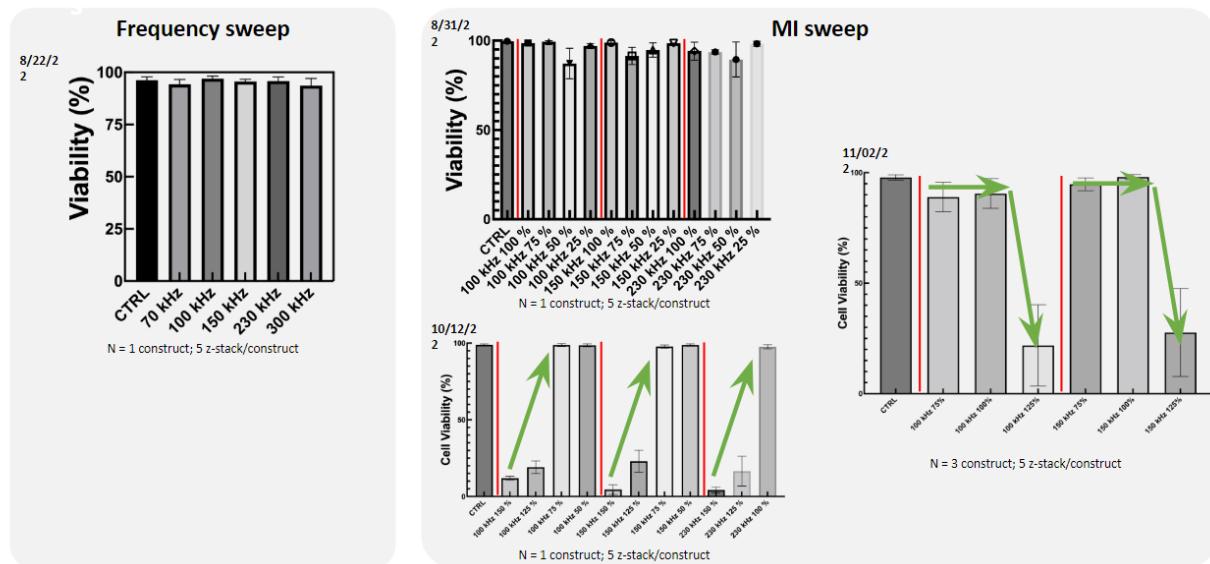


The above figure shows an example of experiments done with LN229 spheroids at different frequencies and different %MIs. Some of these experiments demonstrated a thresholding effect where a lower %MI had a higher viability.

In addition to spheroid laden hydrogels of GBM cell lines, healthy cell-laden hydrogels were also treated of pericytes and human brain endothelial cells (HBMECs) to see if they had resistance to the same levels of ultrasound that caused lysis in the GBM lines. evidence that at levels less than 100% MI less lysis occurred in these cells.

Pericyte

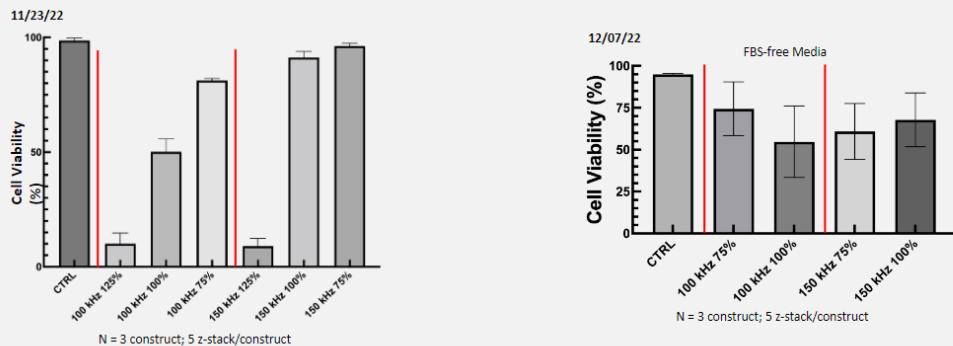
Resistance to 100kHz and 150kHz at 100%MI



The above figure shows an example of experiments done with healthy Pericyte cells. Here is shown that the Pericyte cells were resistant to ultrasound at 100%MI and less. The viability did decrease once the MI exceeded 100%.

Primary HBMECs

MI sweep

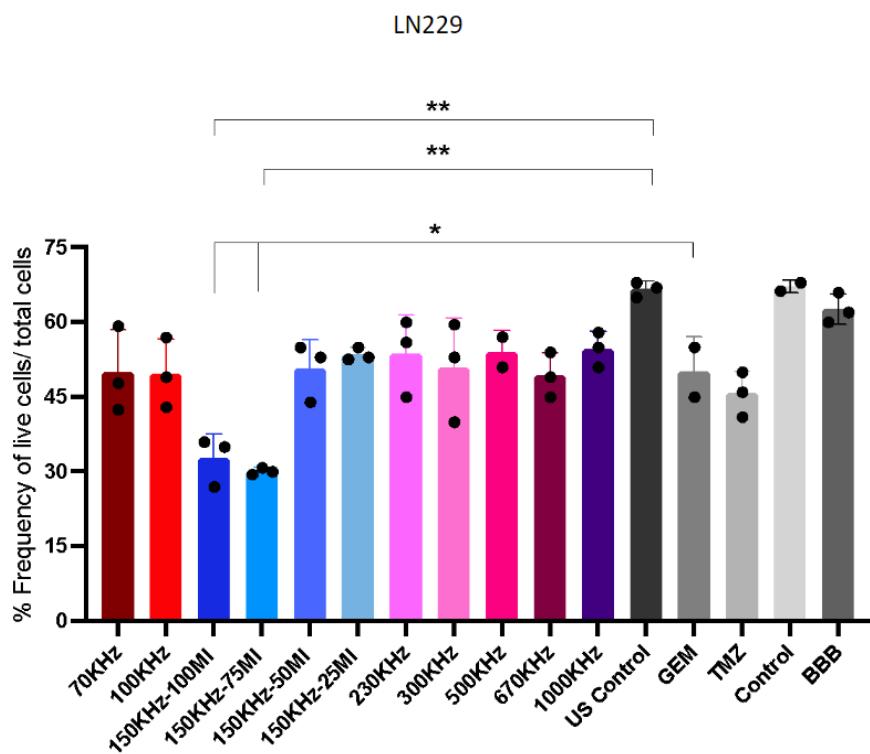


The above figure shows an example of experiments done with healthy Human Brain Microvascular Endothelial Cells (HBMECs). Similar to the healthy Pericyte cells, HBMEC cells were resistant to ultrasound at 100%MI and less. The viability did decrease once the MI exceeded 100%.

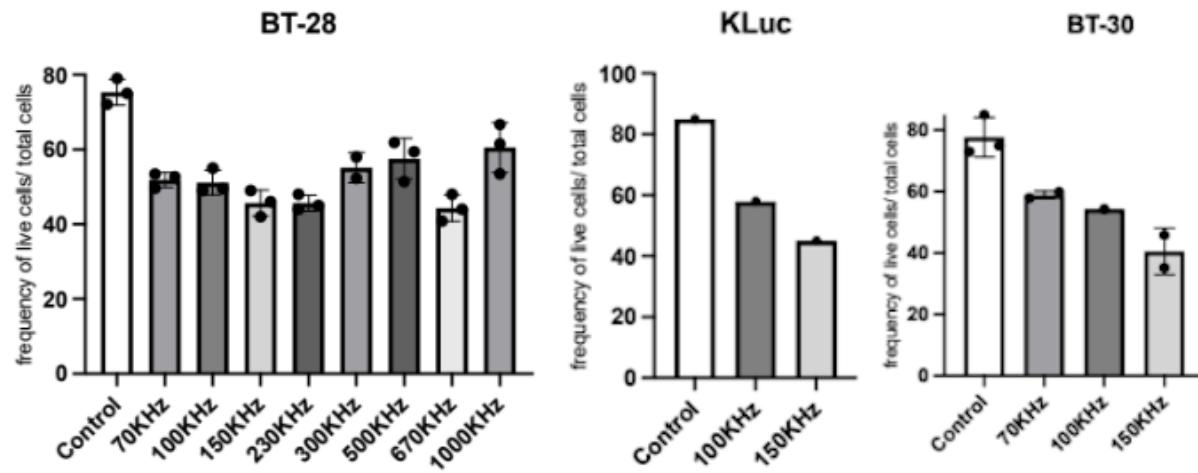
Phase 2: In Vitro Cell-laden Hydrogel Domes with optimized parameters and flow cytometry

Much of the phase 2 study consisted of optimizing the VitroGel for encapsulation and dissociation of the cells so that they were able to be passed through the flow cytometer for analysis. Once the methodology was worked out, four different cell lines were investigated with this method. Frequency sweeps and %MI were varied to identify if there was a resonance and thresholding effect. Below are the results of select experiments with this method of cellular encapsulation and analysis.

LN229 cell results - not labeled for precise ultrasound parameters but should be 40ms burst duration, 10% duty cycle, 120 second total time. The %MI should be 100% in those not labeled.



In the above figure, the 150kHz ultrasound treatment at 100% and 75% MI showed statistically significant differences to the ultrasound control arm.



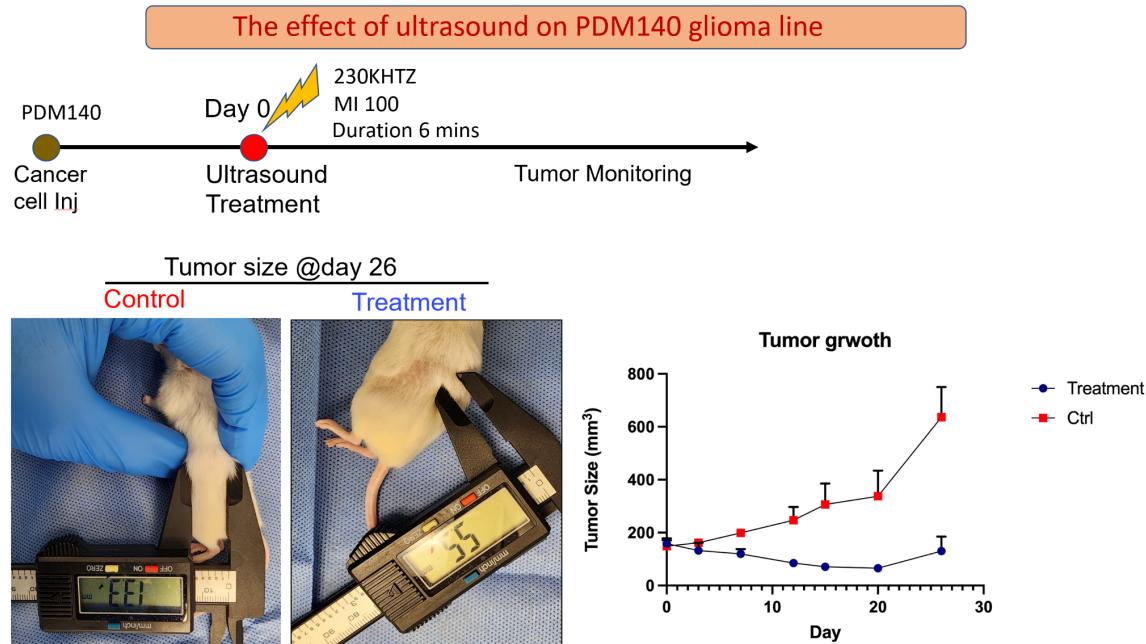
The above figure shows that 150kHz ultrasound treatment showed the lowest frequency of live cells to total cells than any of the other ultrasound frequencies or control for all three cells lines (BT-28, KLUC, and BT-30).

Phase 3: In Vivo - GBM tumor in NSG mouse flank

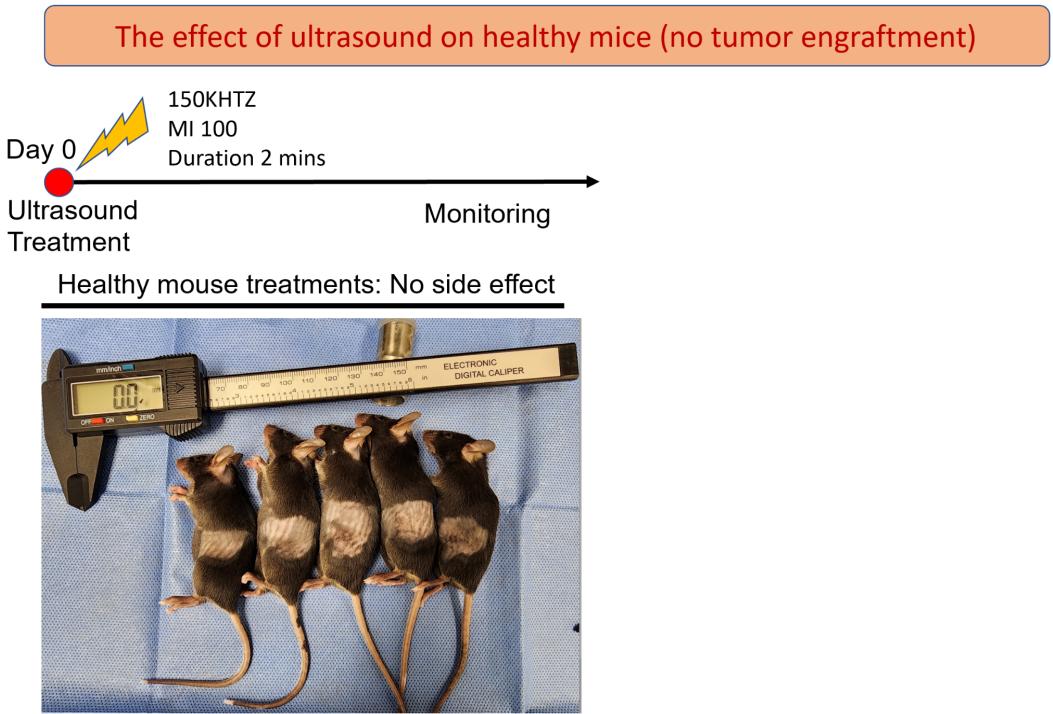
The data from the first in vivo treatment of mice with tumors is presented below. The first experiment was performed as an initial proof of concept but also to validate the setup after optimizing the tumor growth in the flanks of the mice. Because of this, the experimental procedure was still being worked out and the study was only done with a single frequency of transducer with one set of ultrasound parameters. The mice that were treated, we treated for an unknown duration, however it was estimated that they were treated with for approximately 6 minutes each. In addition to the unknown time, the treatment pressure is also unknown as the coupling to the engrafted tumors was not at a controlled distance from the output of the cone. Because of these limitations was difficult to draw any definitive conclusions based on the pressure or time. However, it was clear that the treated tumors reduced in size and had stunted growth compared to the control mice.

The results of the first in vivo treatment are shown in the figure below. The tumor volumes were calculated from caliper measurements.

in vivo treatment results of mice @ 230kHz, MI 100% for 6 minutes

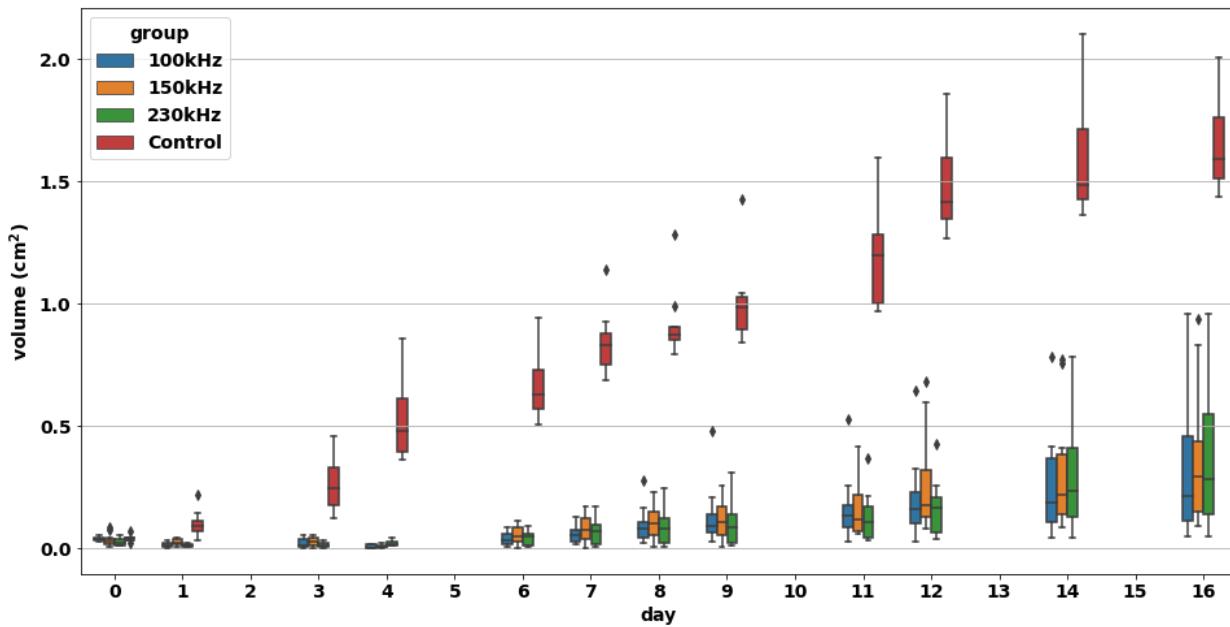


Picture of healthy mice treated with ultrasound at 150kHz, MI 100% for 2 minutes is shown below. No side effects were identified using visual inspection or monitoring of their health.



Tumor volume measurements following ultrasound treatment are shown in the figure below.

These measurements come from the in vivo study with 38 NSG mice injected with GL261 GBM tumor cells. There were 30 mice in the three treatment groups (100kHz, 150kHz, and 230kHz), and 8 control mice (no ultrasound).



Histopathology Results (summary tables and conclusions):

The histopathological results that are found below come from the in vivo study with 38 NSG mice injected with GL261 GBM tumor cells. There were 30 mice in the three treatment groups (100kHz, 150kHz, and 230kHz), and 8 control mice (no ultrasound). At the end of the study all animals were sacrificed and their tumor volumes were preserved according to the protocol provided by Charles River. 5 samples from each of the experimental arms were randomly selected for analysis and delivered to Charles River for their expert histological analysis. The following table summarizes which animals were selected for analysis from each of the experimental arms.

Text Table 1
Experimental Design

Animal Numbers Assigned to Various Treatment Groups													
Control		100 kHz				150 kHz				230 kHz			
4		17				2				26			
9		18				8				30			
19		20				11				32			
31		21				12				34			
33		22				13				38			

Below are the results from the histological analysis of the study performed by Charles River. For each experimental arm, 5 mice were analyzed.

Table 1 - Microscopic Findings

TISSUE/DIAGNOSIS	Control					100 kHz					150 kHz					230 kHz									
	4	9	19	31	33	INC	17	18	20	21	22	INC	2	8	11	12	13	INC	26	30	32	34	38	INC	
Tumor																									
Infiltration, mononuclear cell	2	2	3	3	3	5 / 5	2	3	4	2	1	5 / 5	4	3	3	3	4	5 / 5	4	3	3	1	3	5 / 5	
Infiltration, neutrophil	2	2	3	2	2	5 / 5	2	2	2	2	1	5 / 5	3	3	1	2	3	5 / 5	4	2	2	2	4	2 / 5	
Necrosis, central	-	4	2	3	-	3 / 5	4	4	4	2	-	4 / 5	3	-	-	1	-	2 / 5	1	4	4	2	4	5 / 5	
Necrosis, peripheral	2	-	-	-	-	1 / 5	-	-	-	-	4	1 / 5	-	-	-	-	-	0 / 5	-	-	-	-	0 / 5		
Hemorrhage	1	-	-	-	-	1 / 5	-	-	-	-	2	1 / 5	-	-	-	-	-	0 / 5	-	-	-	-	0 / 5		
Infiltration, muscular	-	2	3	2	3	4 / 5	-	-	-	5	3	2 / 5	-	-	2	-	-	1 / 5	1	-	-	-	-	1 / 5	
Skeletal muscle present in section	N	Y	Y	Y	Y	4 / 5	N	Y	Y	Y	Y	4 / 5	N	N	Y	N	N	1 / 5	Y	N	N	N	N	1 / 5	
Well circumscribed						0 / 5	P	P	P		3 / 5	P	P		P	P	4 / 5		P	P	2 / 5		P	P	
Borders not present in section	P					1 / 5					0 / 5		P			1 / 5		P	P		2 / 5				
Skin present in section						0 / 5	P				1 / 5	P	P	P		3 / 5				P	1 / 5				
Subcutis present in section		P		P	2 / 5	P	P	P		3 / 5	P	P	P	P	P	4 / 5	P		P	P	3 / 5				
Accumulation, amorphous material						0 / 5					0 / 5					3	3	2 / 5	2	2			3	3 / 5	

- = finding not present; 1 = grade 1 (minimal); 2 = grade 2 (mild); 3 = grade 3 (moderate); 4 = grade 4 (marked); 5 = grade 5 (severe)

P = finding present, not graded

Y = Yes; N = No

Histology Conclusions (copied directly from the report of Charles River):

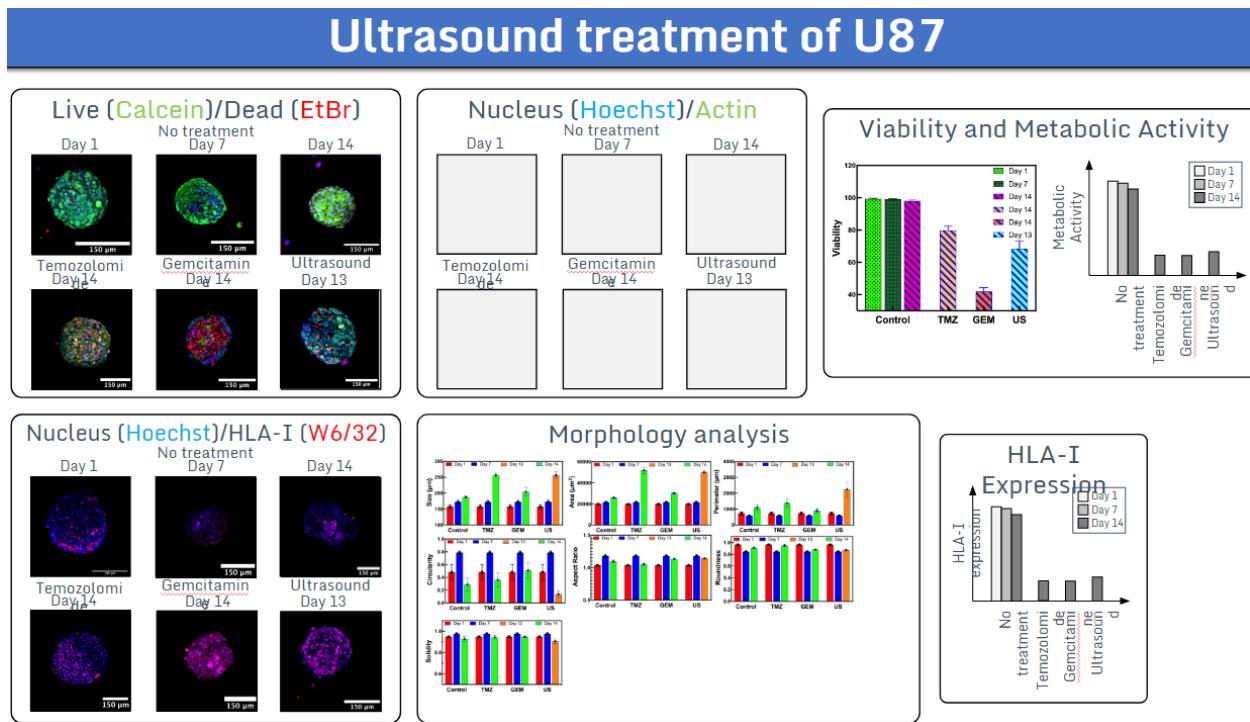
Necrosis was present in tumors from all experimental groups but was less common in tumors from the 150 kHz group. Tumor cell infiltration into adjacent skeletal muscle was noted in the majority of specimens in which skeletal muscle was present in the section. Inflammatory cell infiltration was present around the periphery of all tumors but was notably sparse around one small tumor (animal 34) in the 230 kHz group. Accumulations of unidentified amorphous material observed within 2/5 and 3/5 tumors from the 150 kHz and 230 kHz groups, respectively, were microscopically consistent with treatment-related injury to the tumor cells.

IV. Appendix (all progress report data from the clinical collaborators)

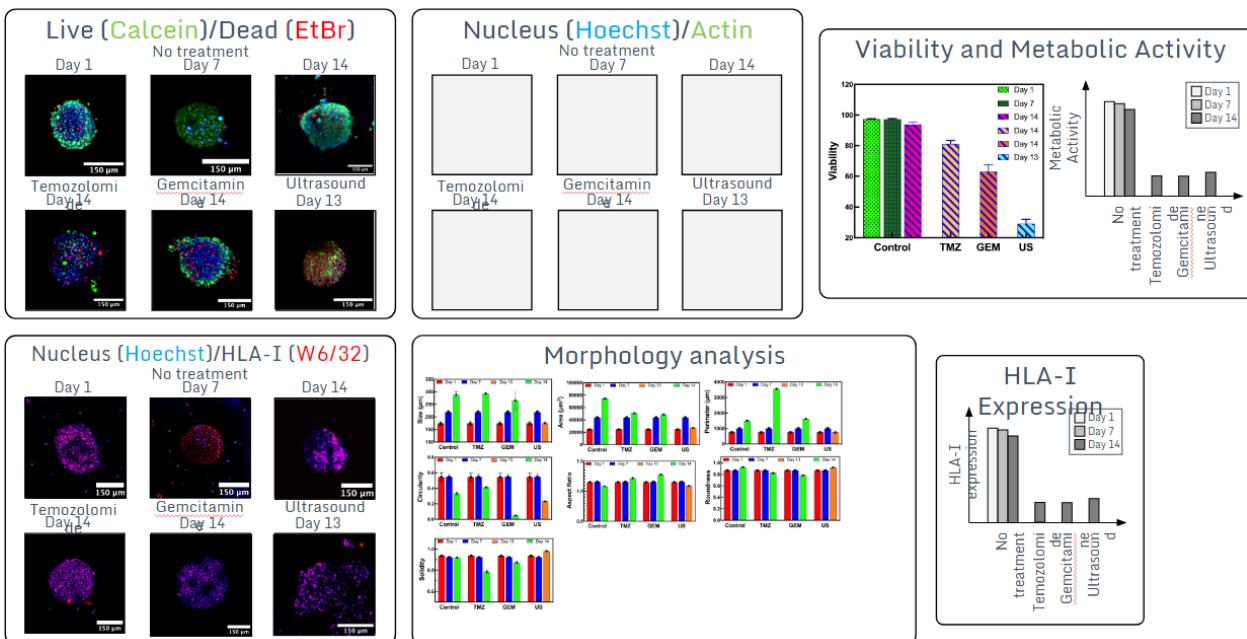
All data presented above is also here in the appendix. This section compiles all of the results provided to Openwater by its collaborators who ran the oncolysis studies. It is organized by date, in the order that these results were presented to Openwater and separated into the various phases of the study.

Phase 1.1 - In Vitro setup validation and biological component optimization

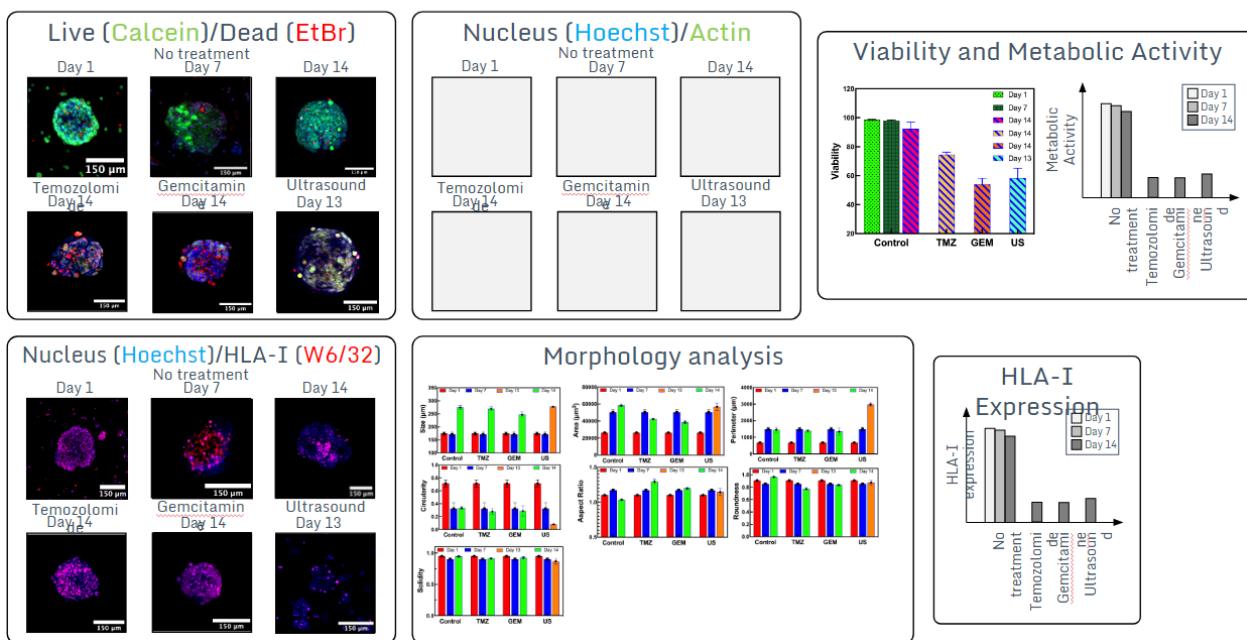
June 29, 2022 - Ultrasound treatment assays of U87, LN229 (ultrasound parameters unclear)



Ultrasound treatment of LN229



Ultrasound treatment of PDM140



July 13, 2022 - 7 day experiment timeline validation

Validation of 7-days experiments timeline

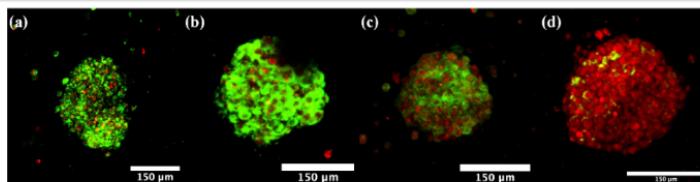
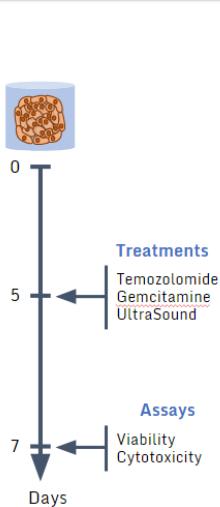


Figure 1: Live/Dead study of U87. (a) Day 7 Control; (b) Day 7 TMZ treated; (c) Day 7 GEM treated; (d) Day 7 US treated.

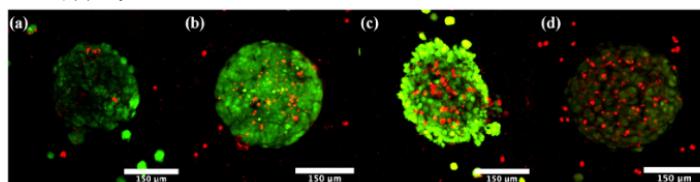
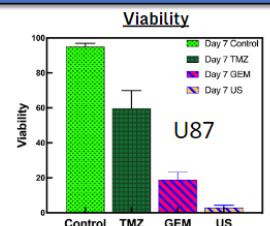


Figure 2: Live/Dead study of LN229. (a) Day 7 Control; (b) Day 7 TMZ treated; (c) Day 7 GEM treated; (d) Day 7 US treated.

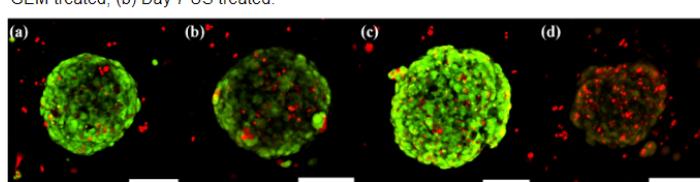
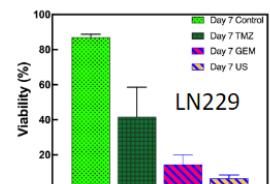
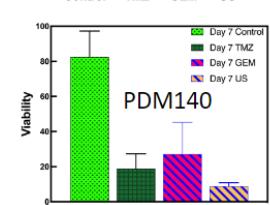
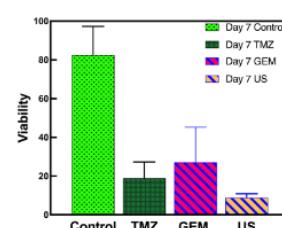
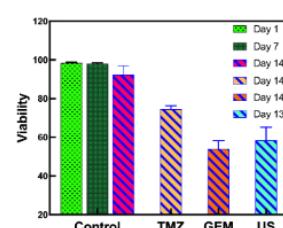
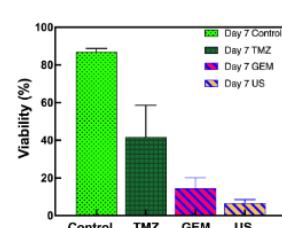
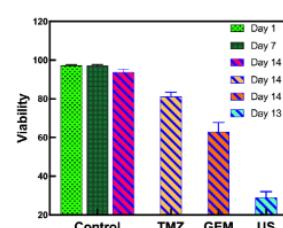
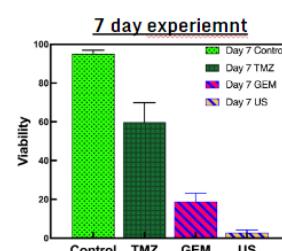
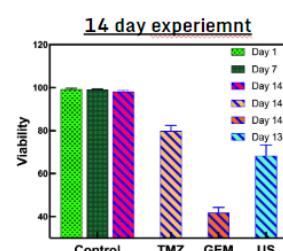
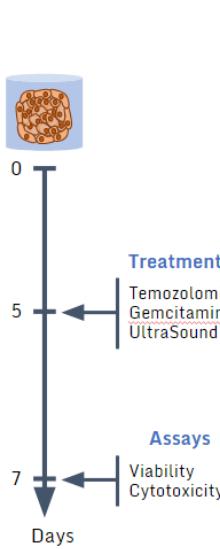


Figure 3: Live/Dead study of PDM140. (a) Day 7 Control; (b) Day 7 TMZ treated; (c) Day 7 GEM treated; (d) Day 7 US treated.

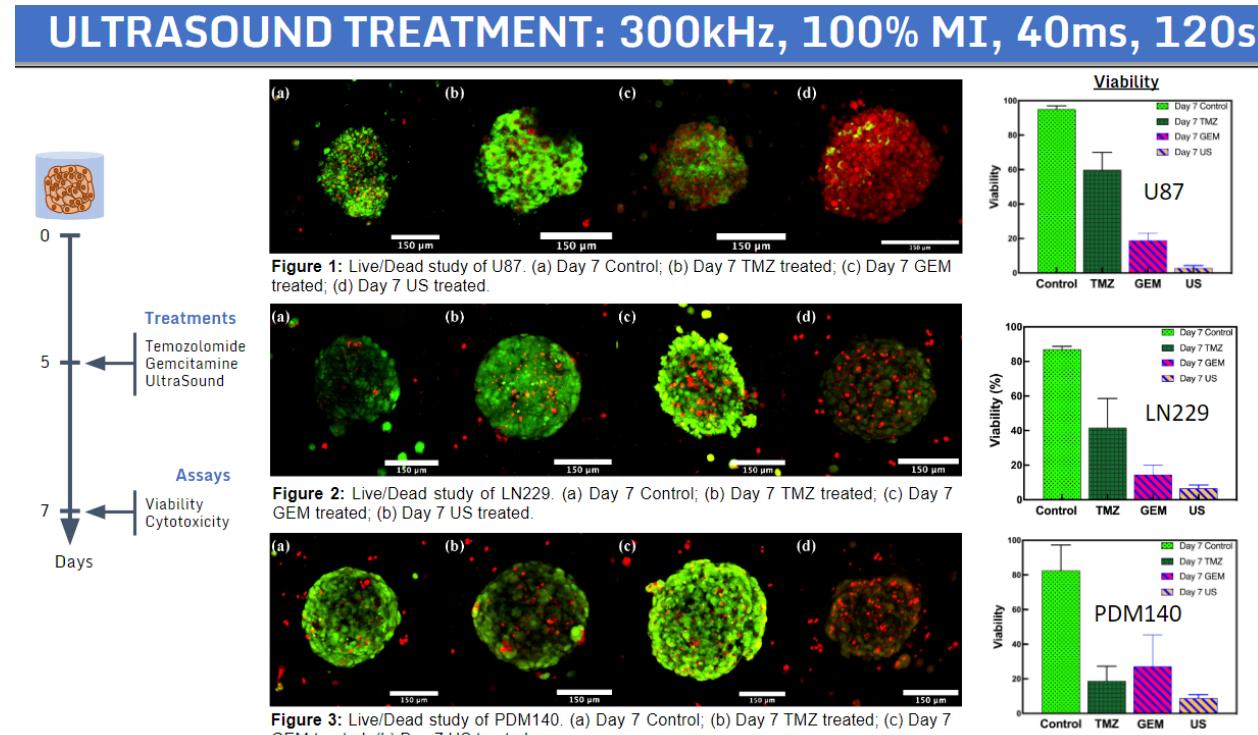
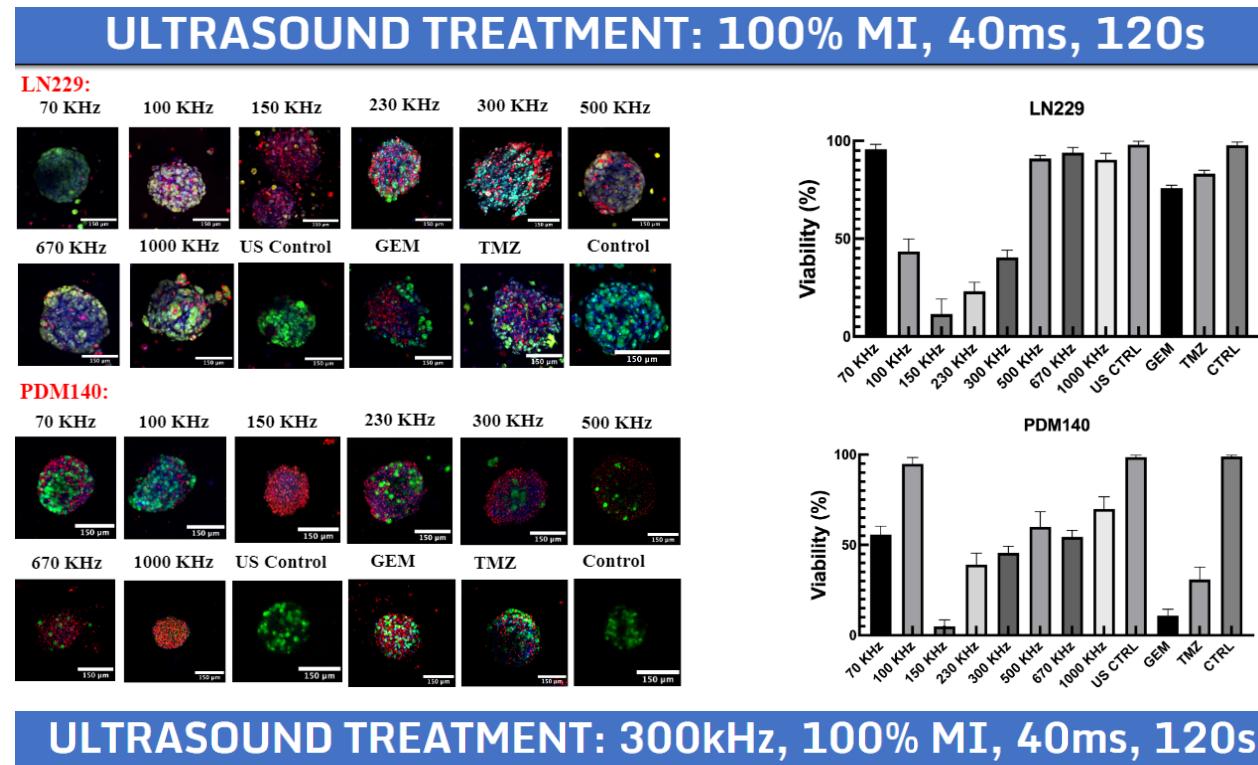


Validation of 7-days experiments timeline



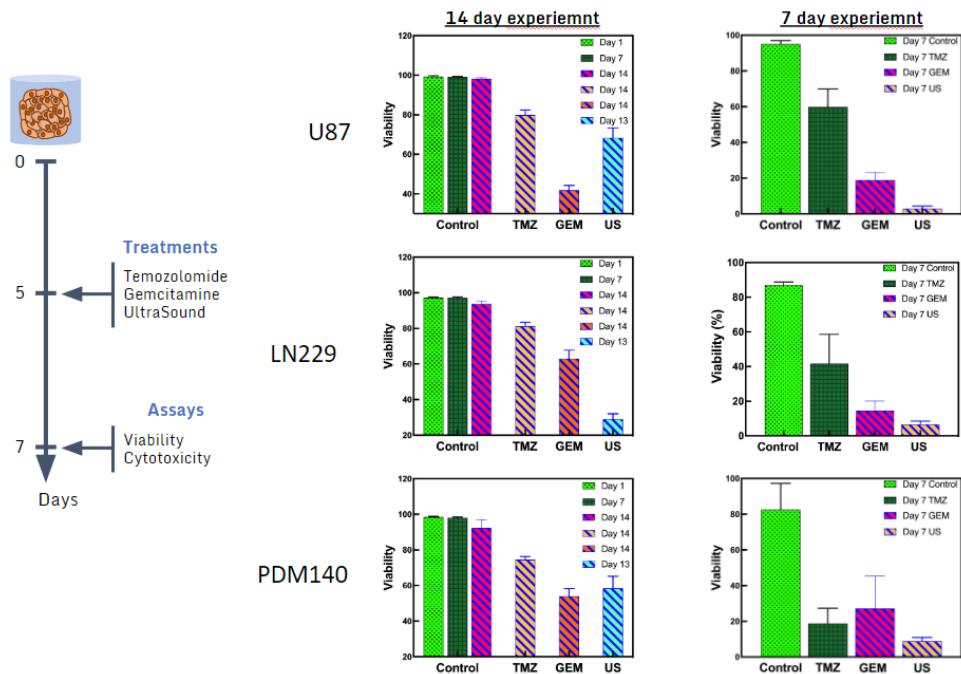
August 5, 2022 - in vitro frequency sweeps. (new single container treatment to mitigate contamination issues)

MI = Mechanical Index, 100% MI is when MI = 1.9, 40ms burst length, 10% duty-cycle, 120 second total treatment time

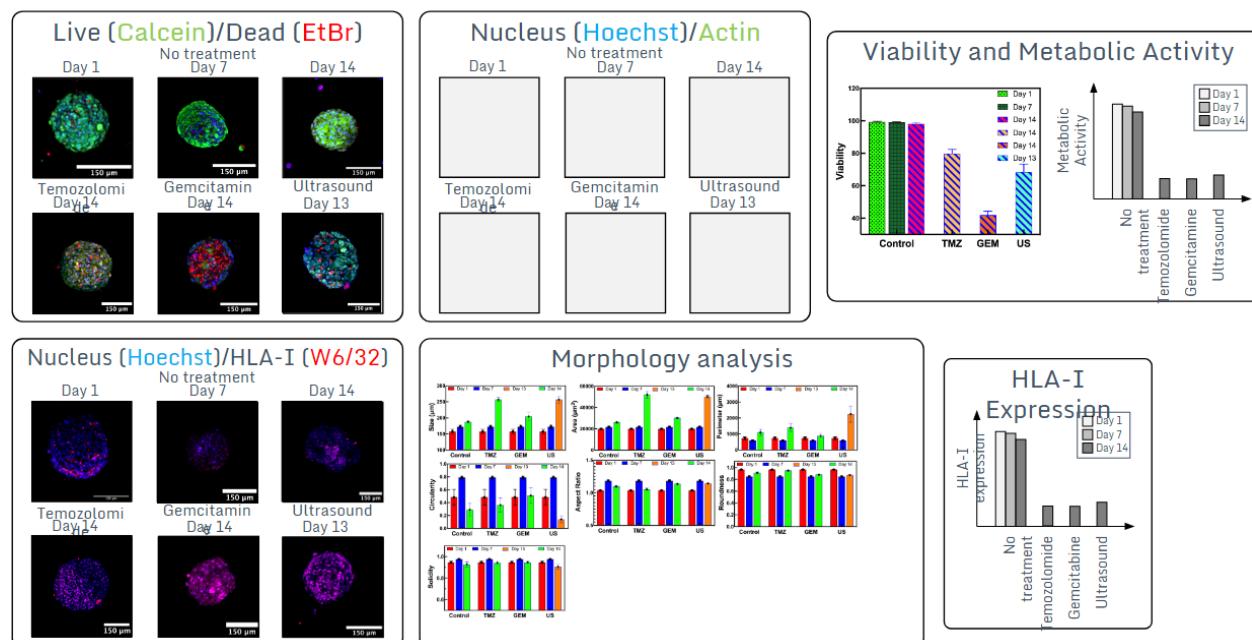


Same pressure, different burst length, same duty-cycle, same total ON time

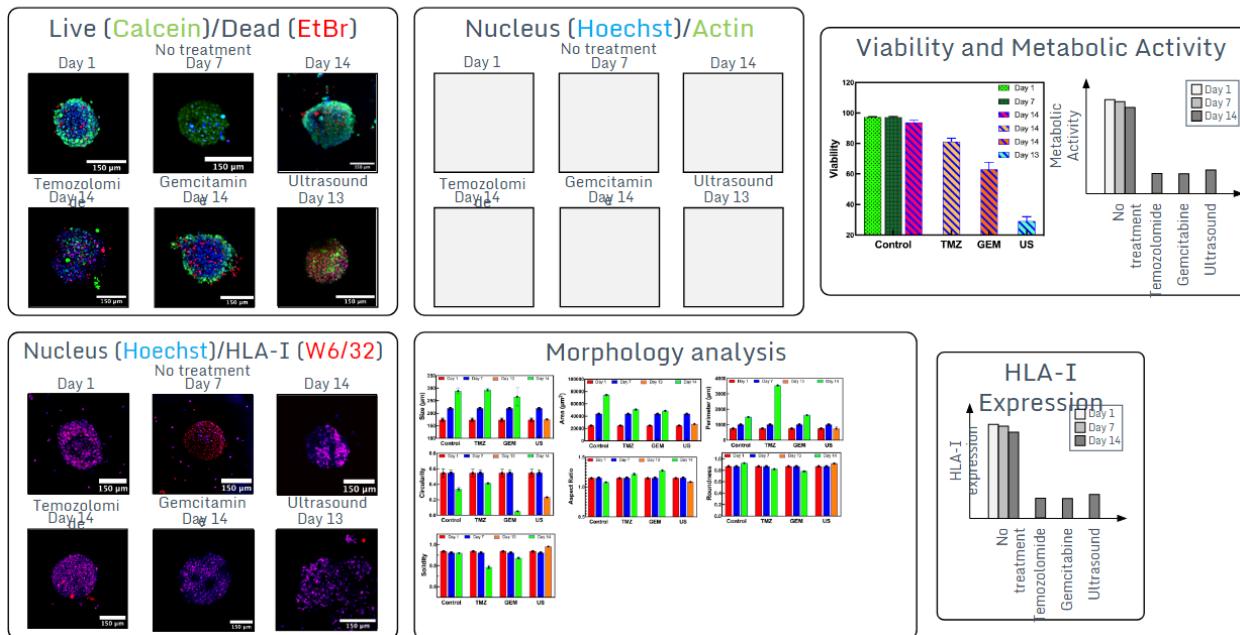
ULTRASOUND TREATMENT: 100% MI, 20ms vs 40ms, 120s



U87 ULTRASOUND TREATMENT: 100% MI, 20ms, 120s

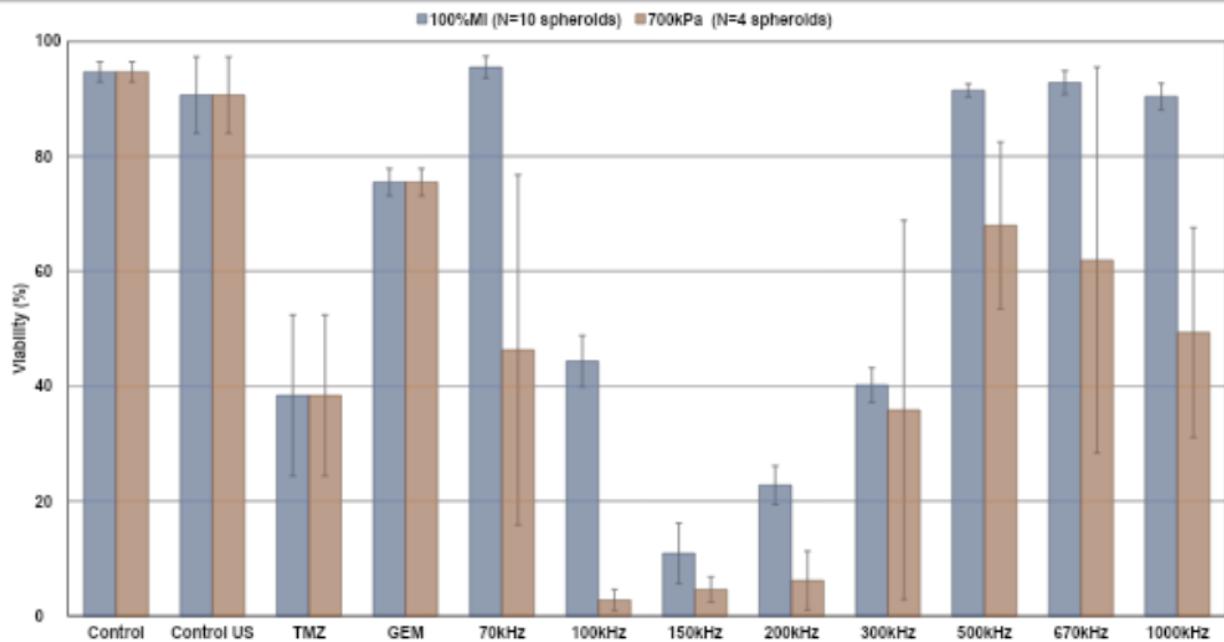


LN229 ULTRASOUND TREATMENT: 100% MI, 20ms, 120s

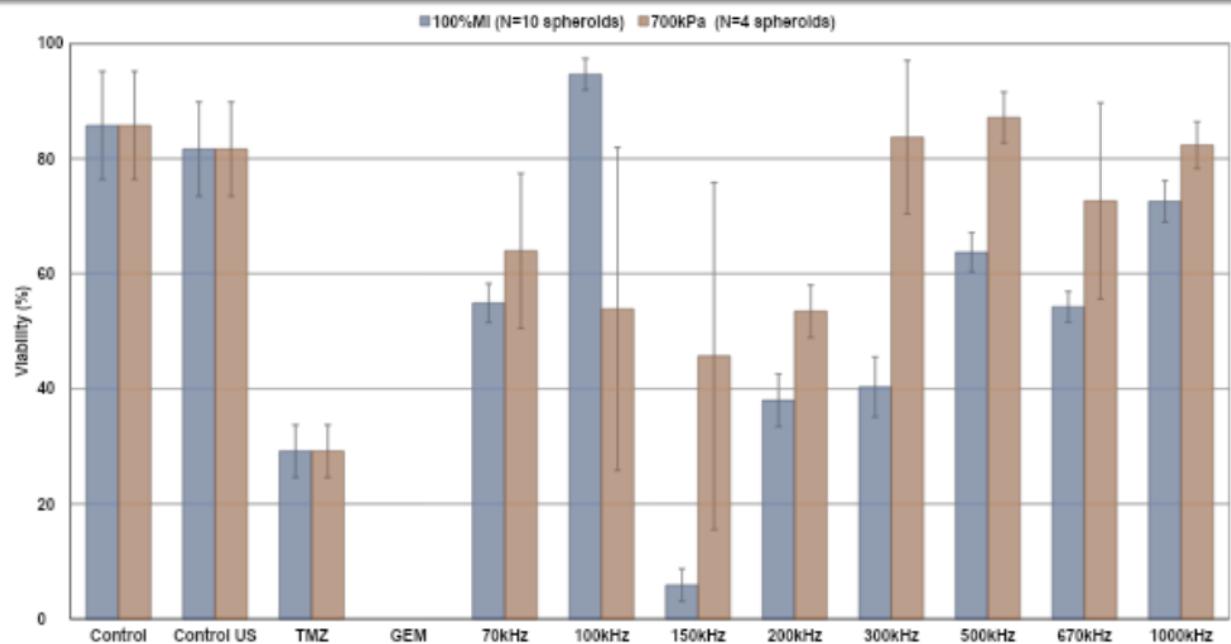


August 10, 2022 - frequency sweep summary graphs with constant pressure and constant MI

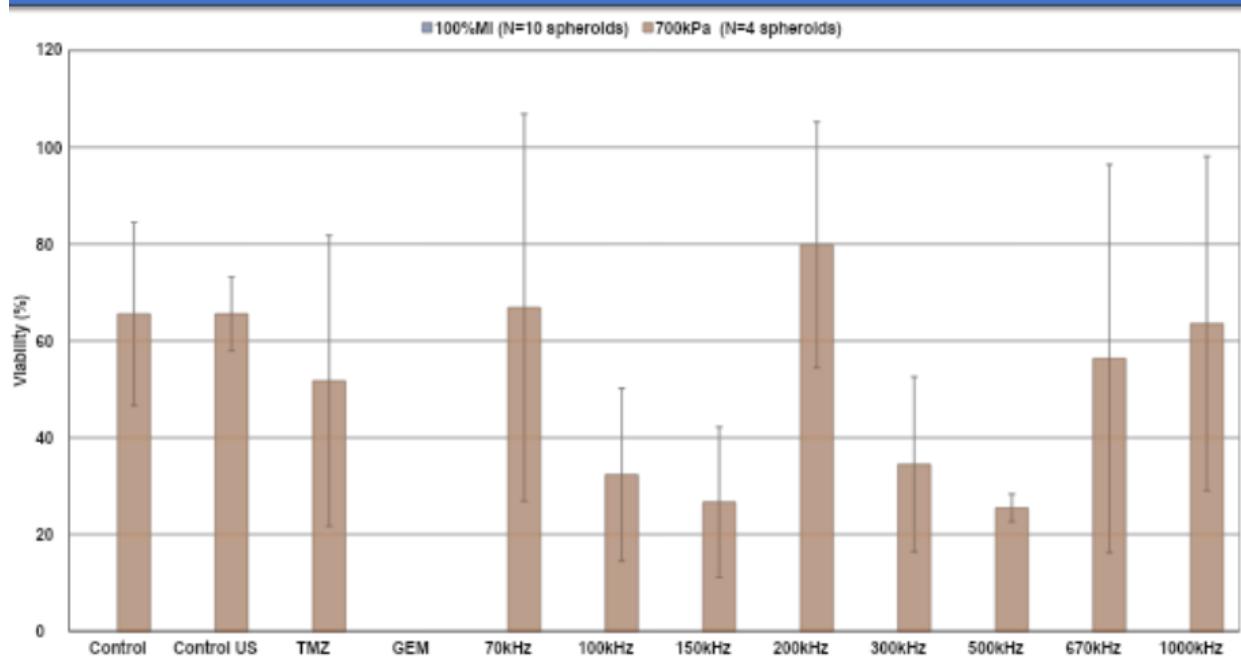
LN229 Summary



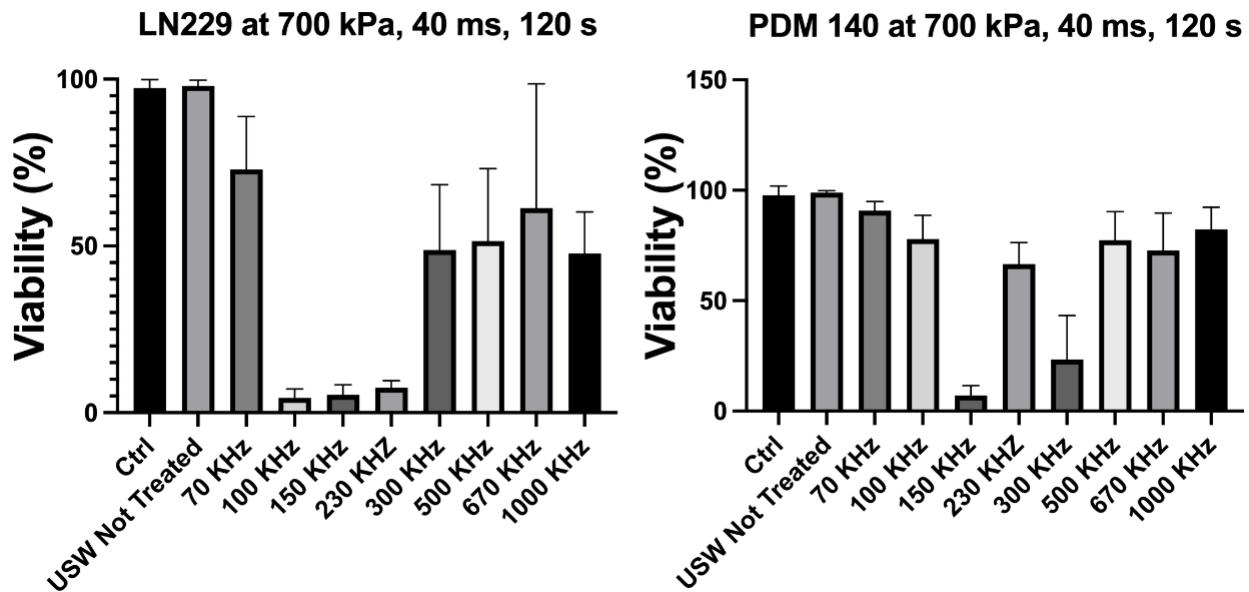
PDM140 Summary



U87 Summary



August 17, 2022 - Frequency sweeps of two cell types (LN229 and PDM140) - constant pressure



Parameter Optimization Conclusion

With the results of the prior experiments in this phase, a fixed set of treatment parameters was decided upon, and further experiments use these parameters unless otherwise specified:

Parameter	Value	Units
Burst Length	40	ms
Duty Cycle	10	%
Duration	120	seconds

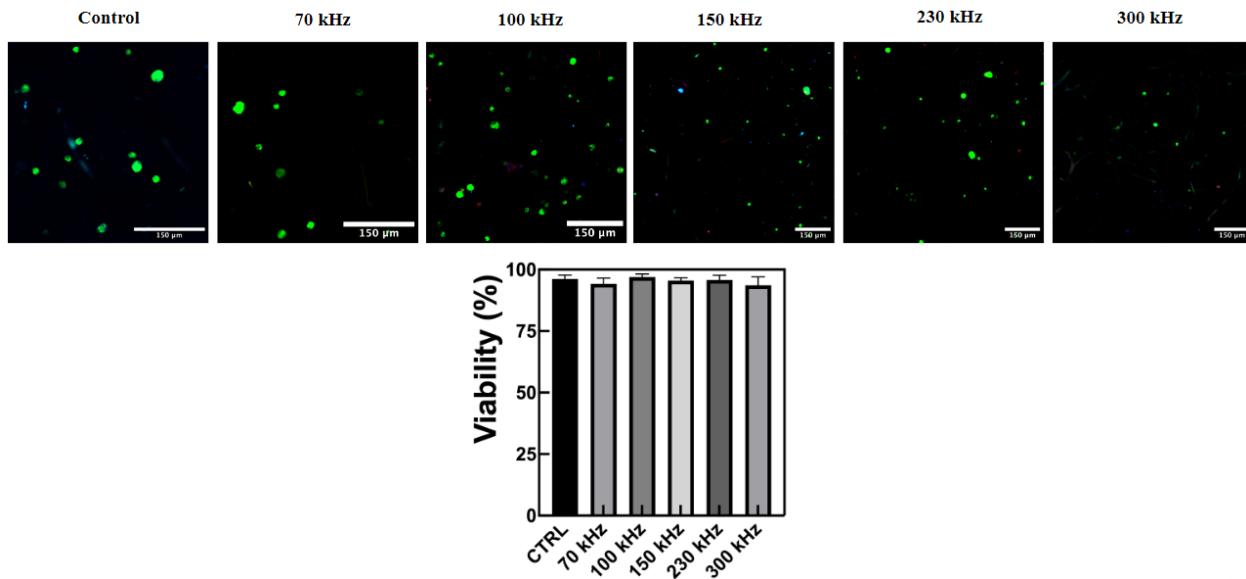
Phase 1.2 In Vitro Spheroids with Optimized Parameters

All experiments in this phase use the optimized parameters unless specified

August 22, 2022 - Frequency sweeps of two cell types (Pericytes and U87)

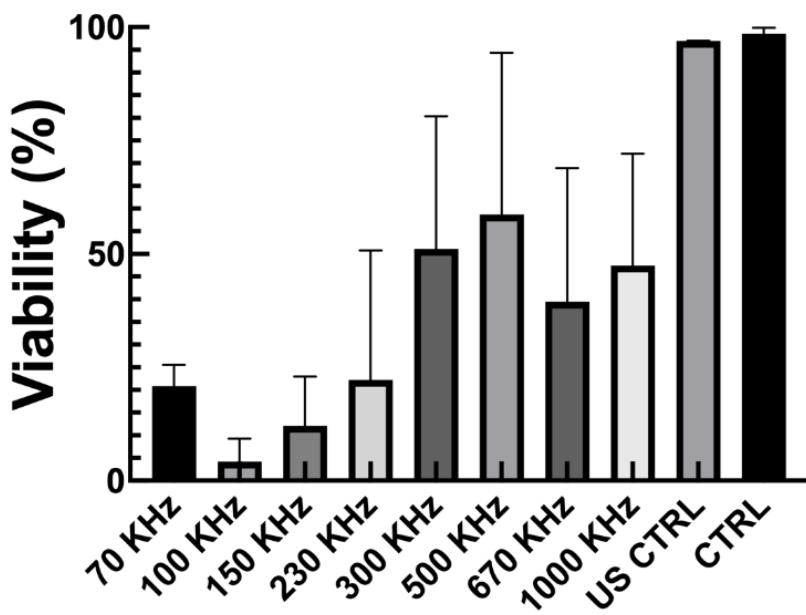
ULTRASOUND TREATMENT: 100% MI, 40ms, 120s

Pericytes



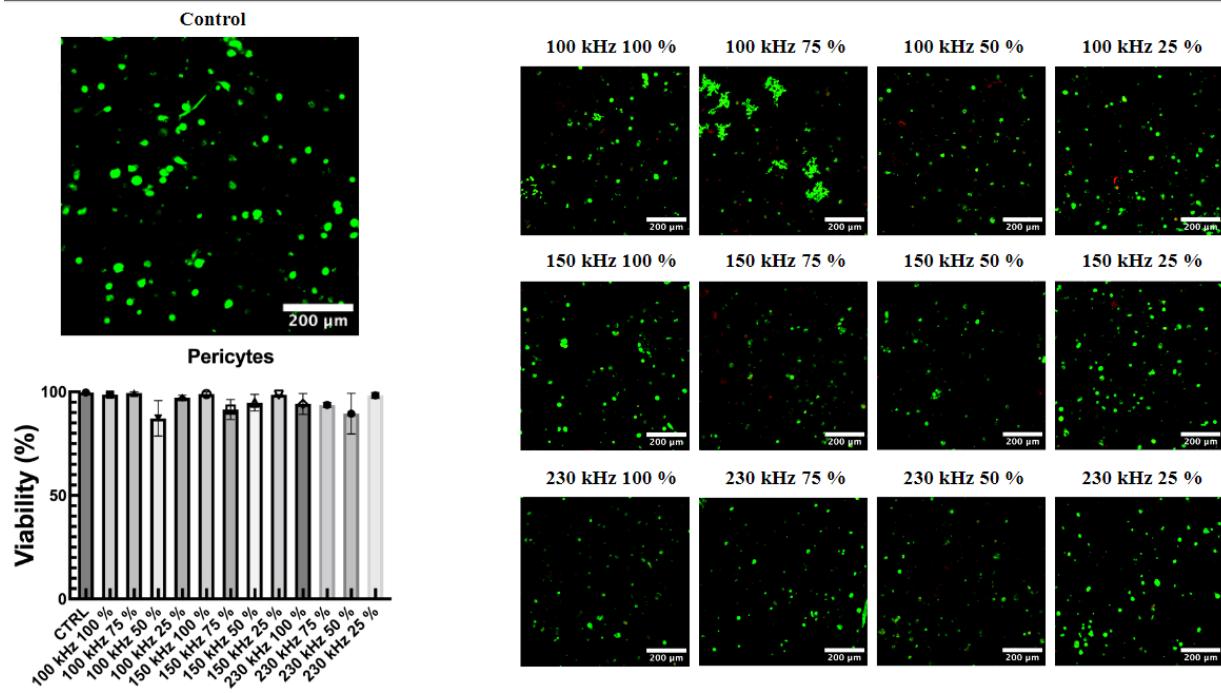
ULTRASOUND TREATMENT: 100% MI, 40ms, 120s

U87



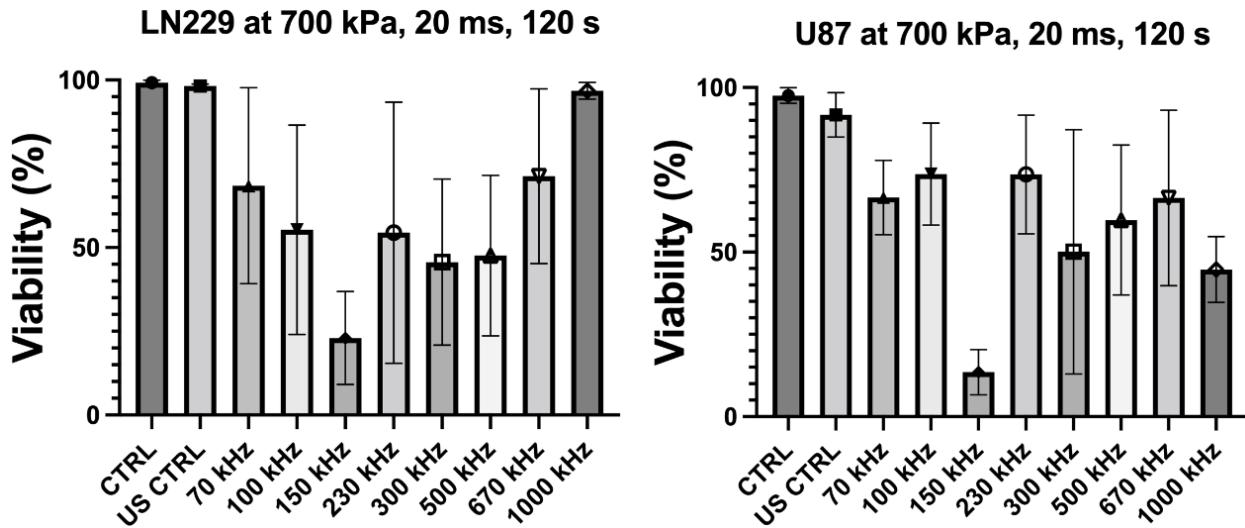
August 31, 2022 - Pericyte frequency and MI sweeps from 100 kHz to 230kHz

ULTRASOUND TREATMENT: Pericytes (200,000 cells/sample)



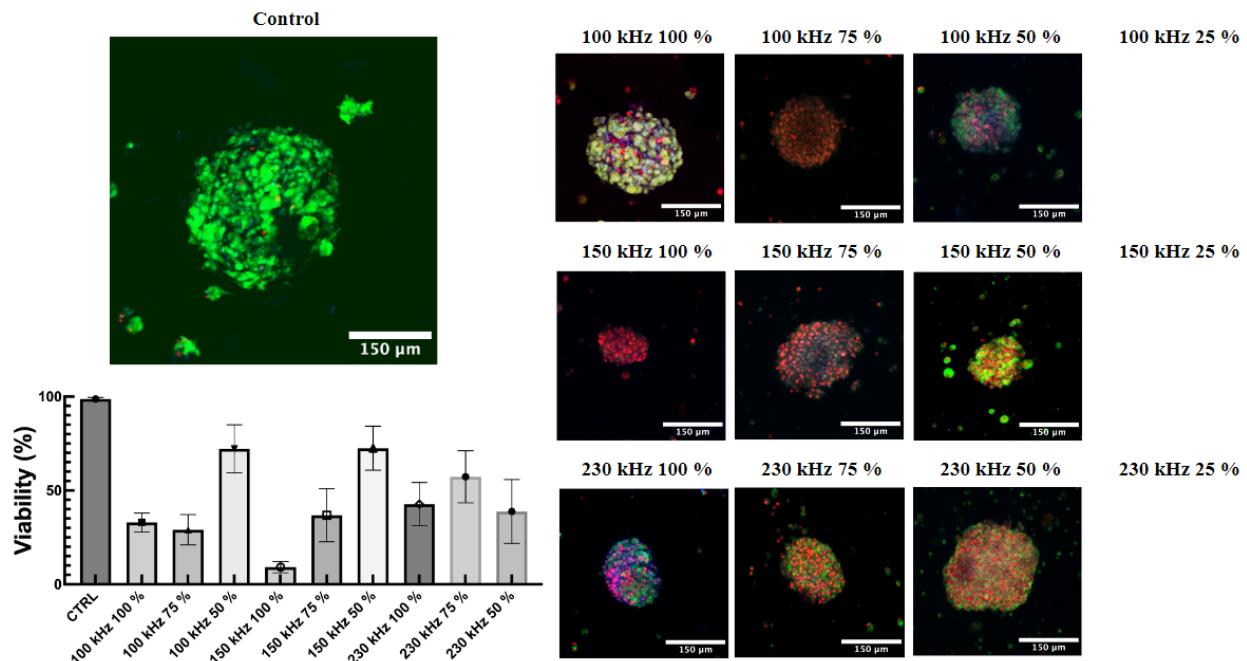
Frequency sweeps of two cell types (LN229 and PDM140) - constant pressure

ULTRASOUND TREATMENT: 700 kPa, 20ms, 120s

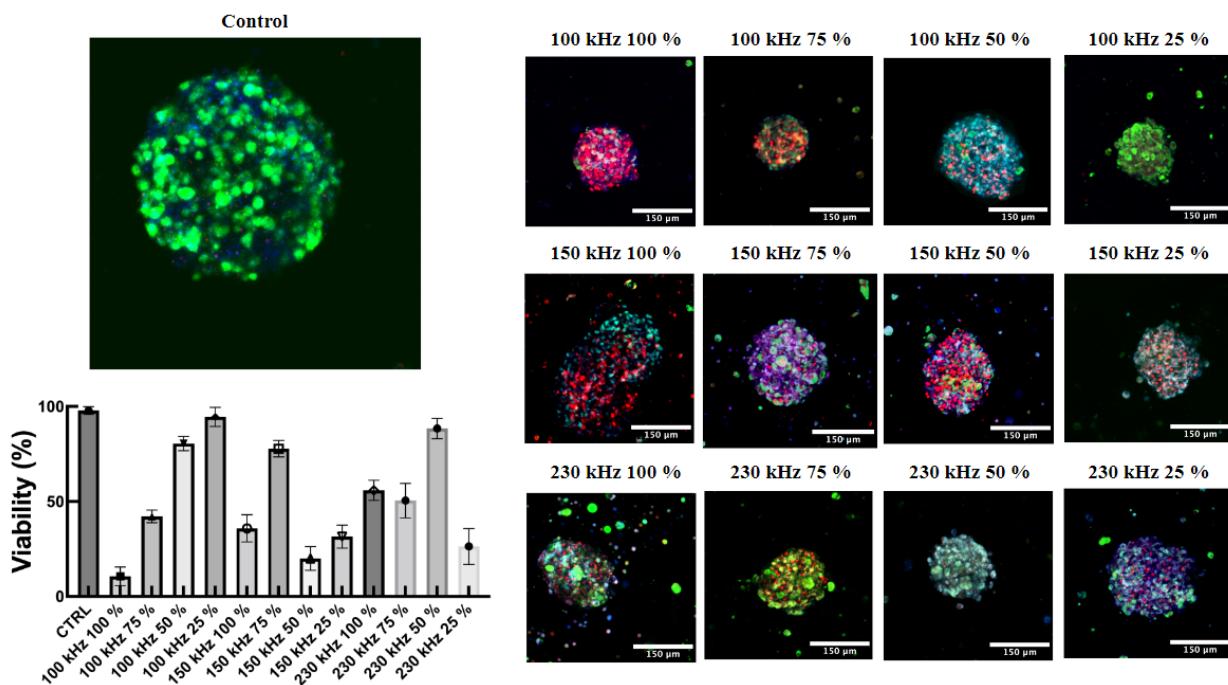


September 7, 2022 - Cell spheroid ultrasound treatment 100kHz - 230kHz
(variable MI)

ULTRASOUND TREATMENT: LN229

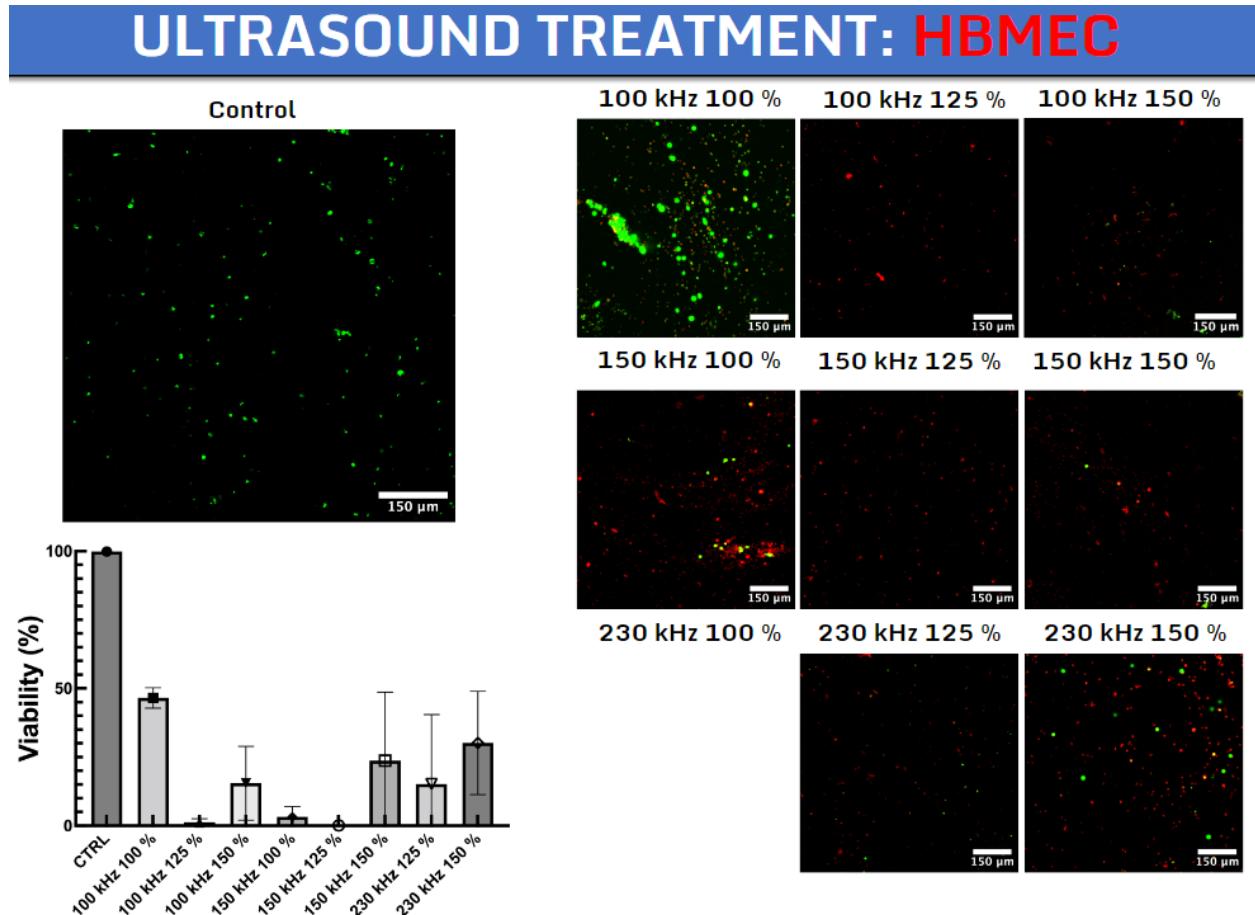


ULTRASOUND TREATMENT: U87

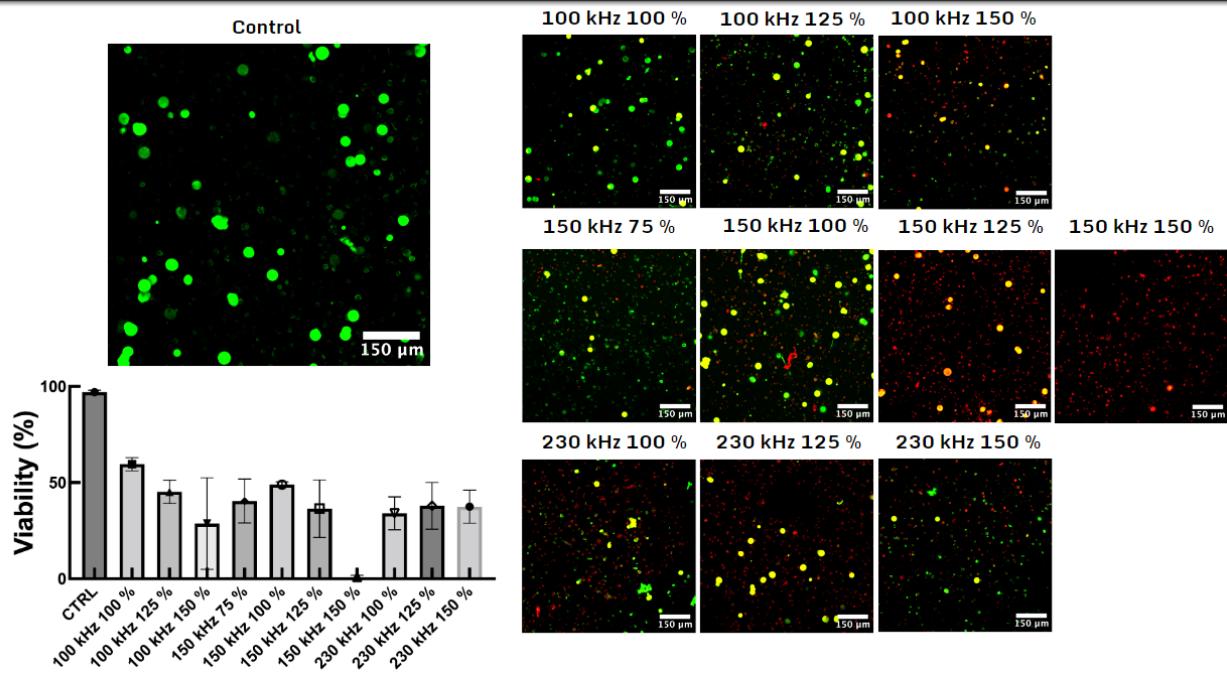


September 14, 2022 - no useful data

September 21, 2022 - Cell spheroid ultrasound treatment 100kHz - 230kHz (variable MI)

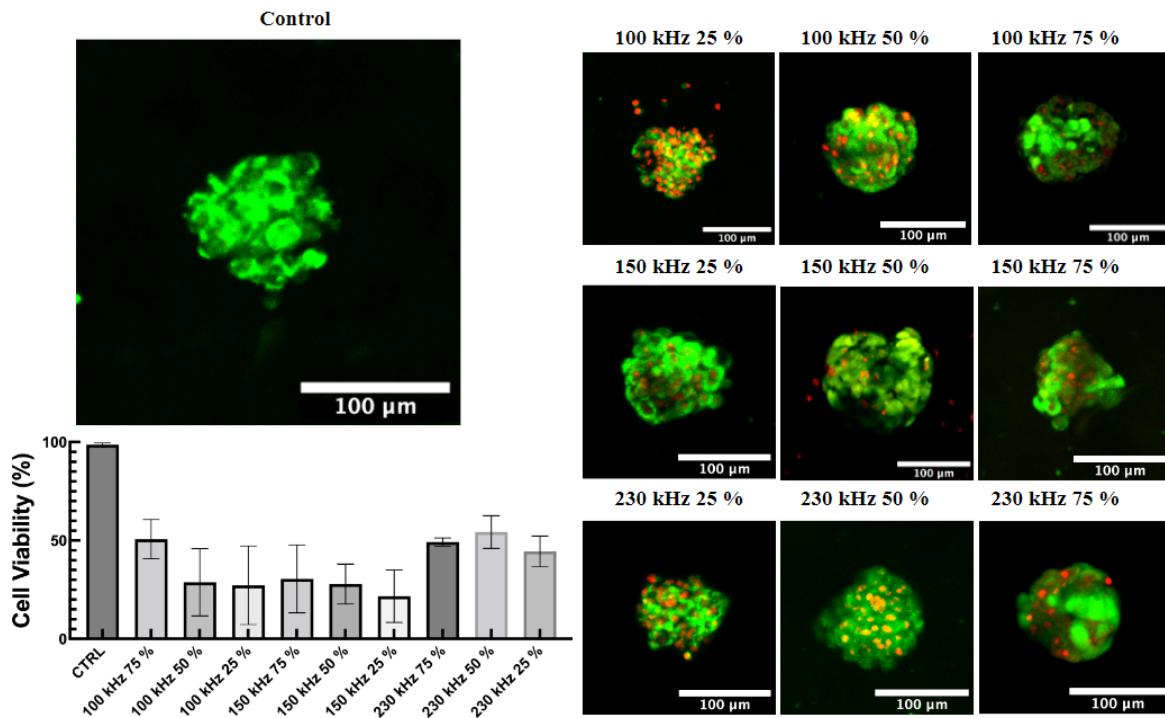


ULTRASOUND TREATMENT: HDF

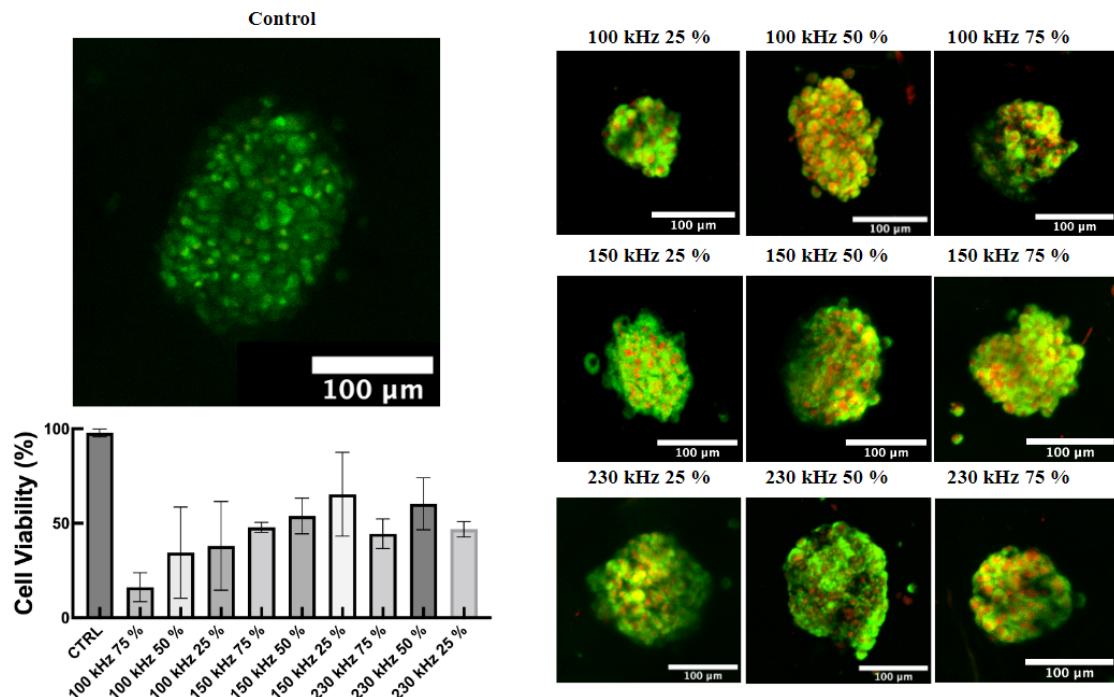


September 28, 2022 - Cell spheroid ultrasound treatment 100kHz - 230kHz (variable MI)

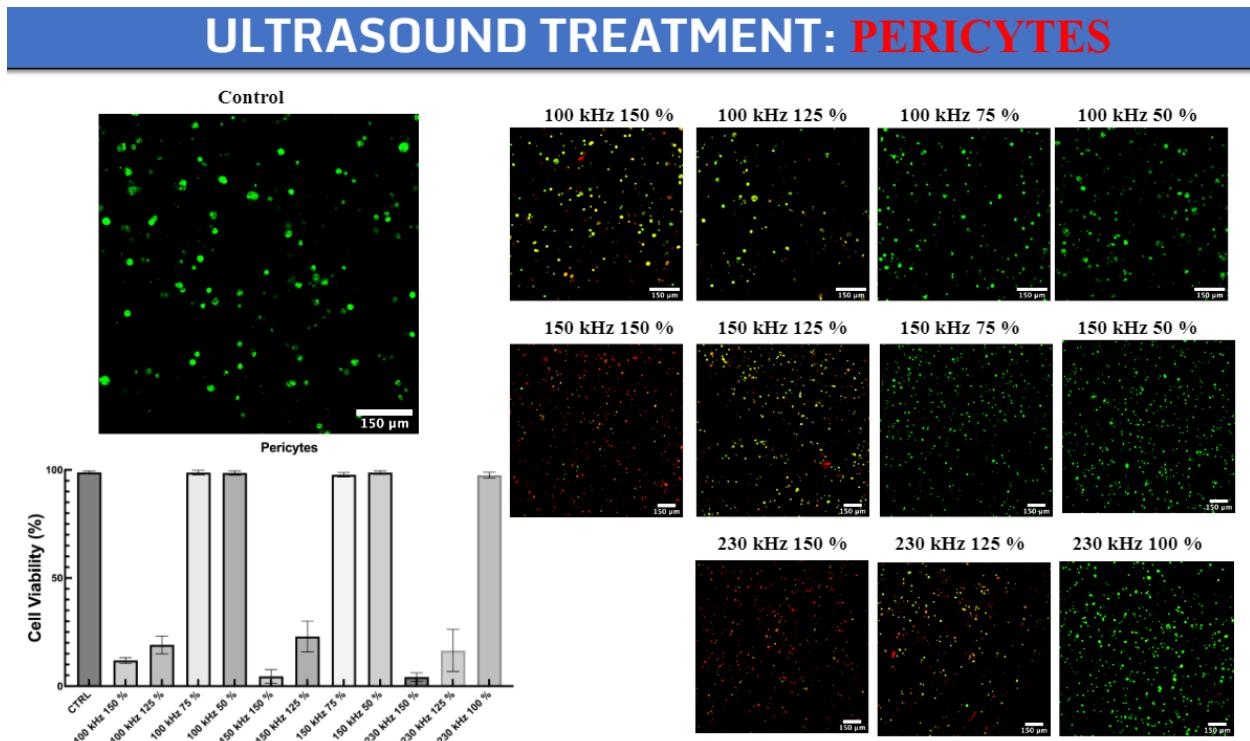
ULTRASOUND TREATMENT: LN229



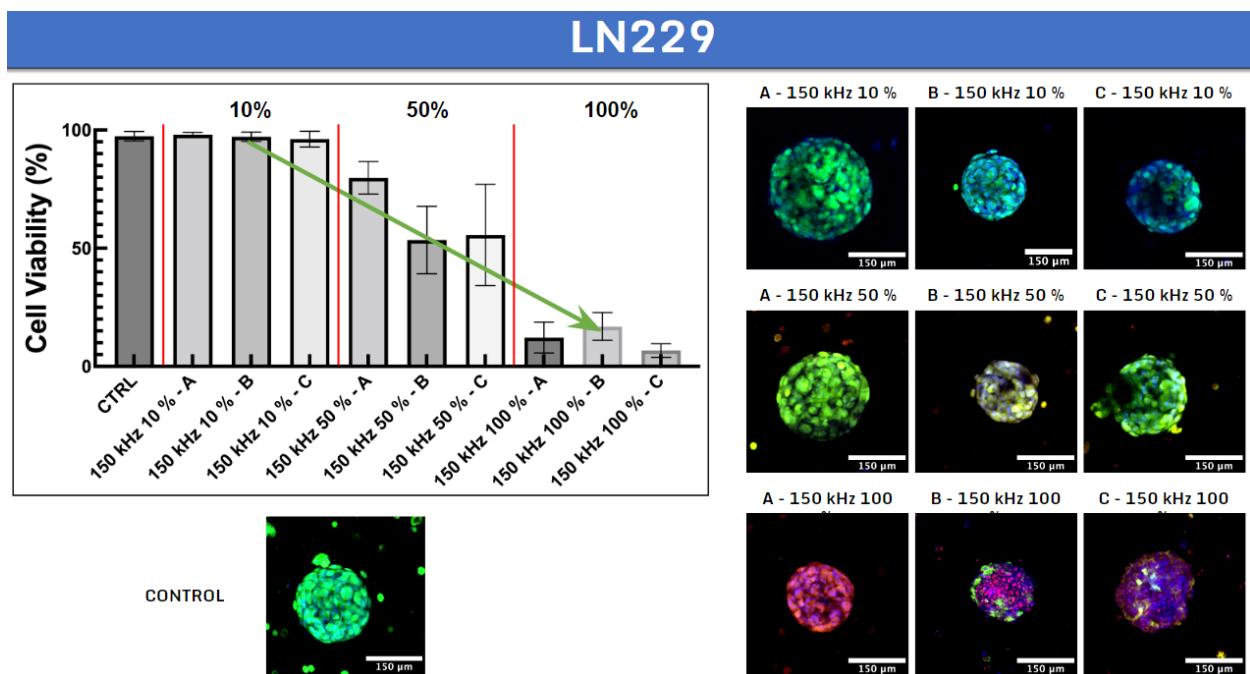
ULTRASOUND TREATMENT: U87



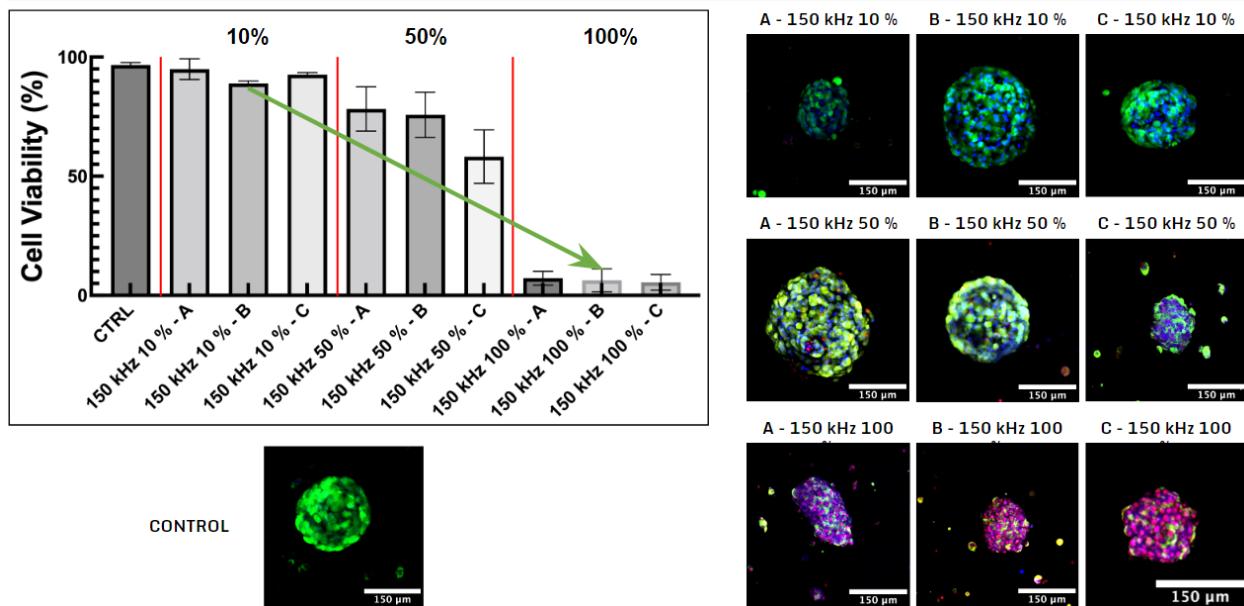
October 12, 2022 - ultrasound treatment slides of pericytes at 50-150% MI



October 19, 2022 - 150kHz spheroid treatments with variable MI (10%-100%)

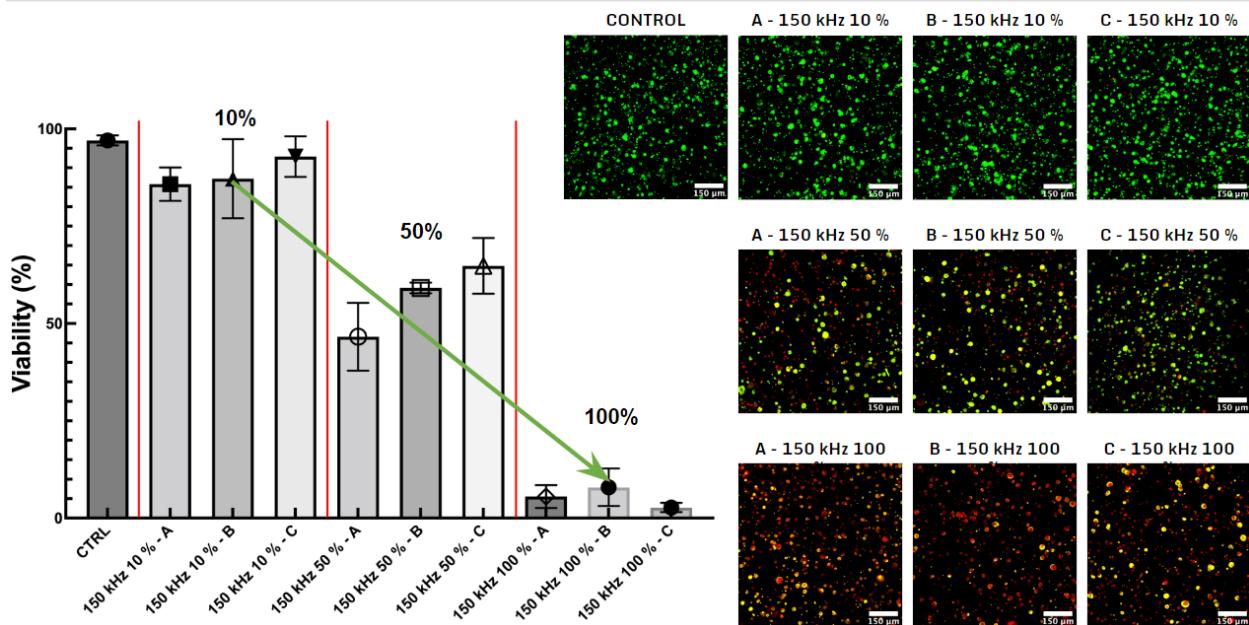


PDM140

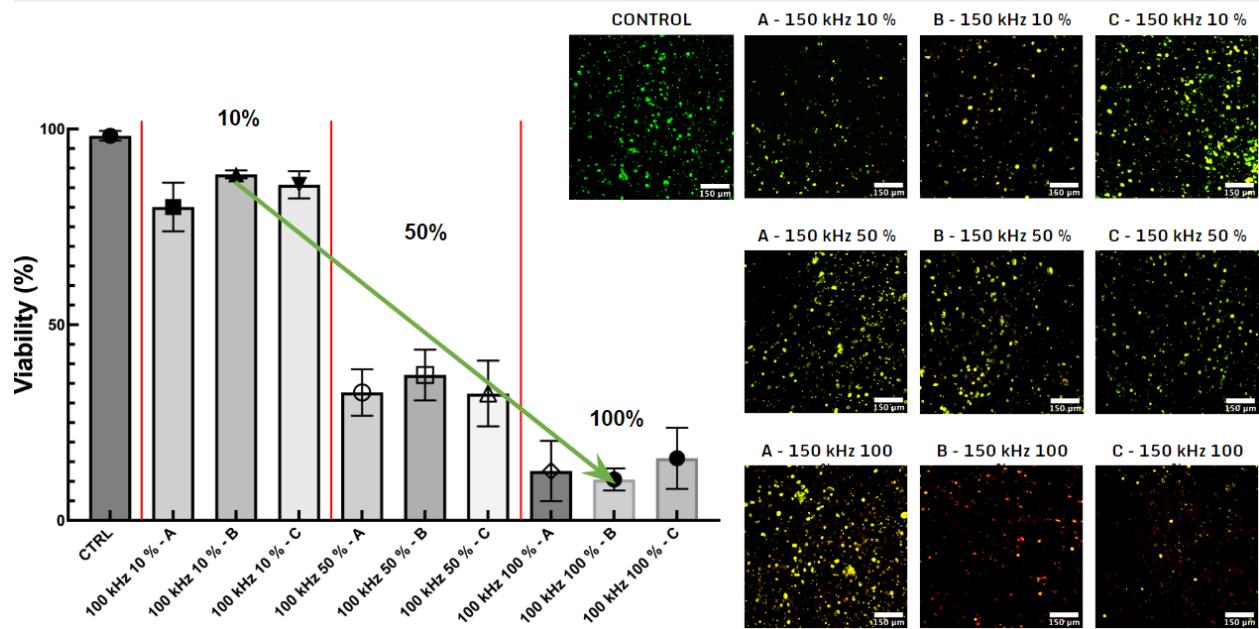


October 26, 2022 - 150kHz cell-laden hydrogel treated with variable MI (10%-100%)

LN229 (cell-laden hydrogel)

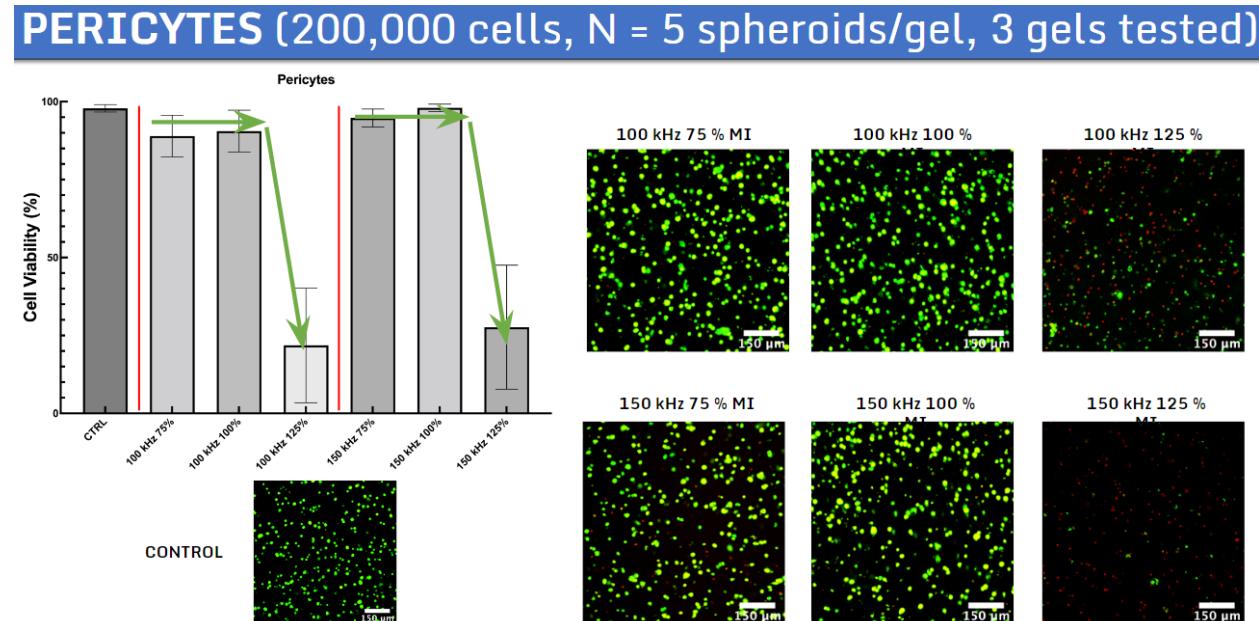


U87 (cell-laden hydrogel)

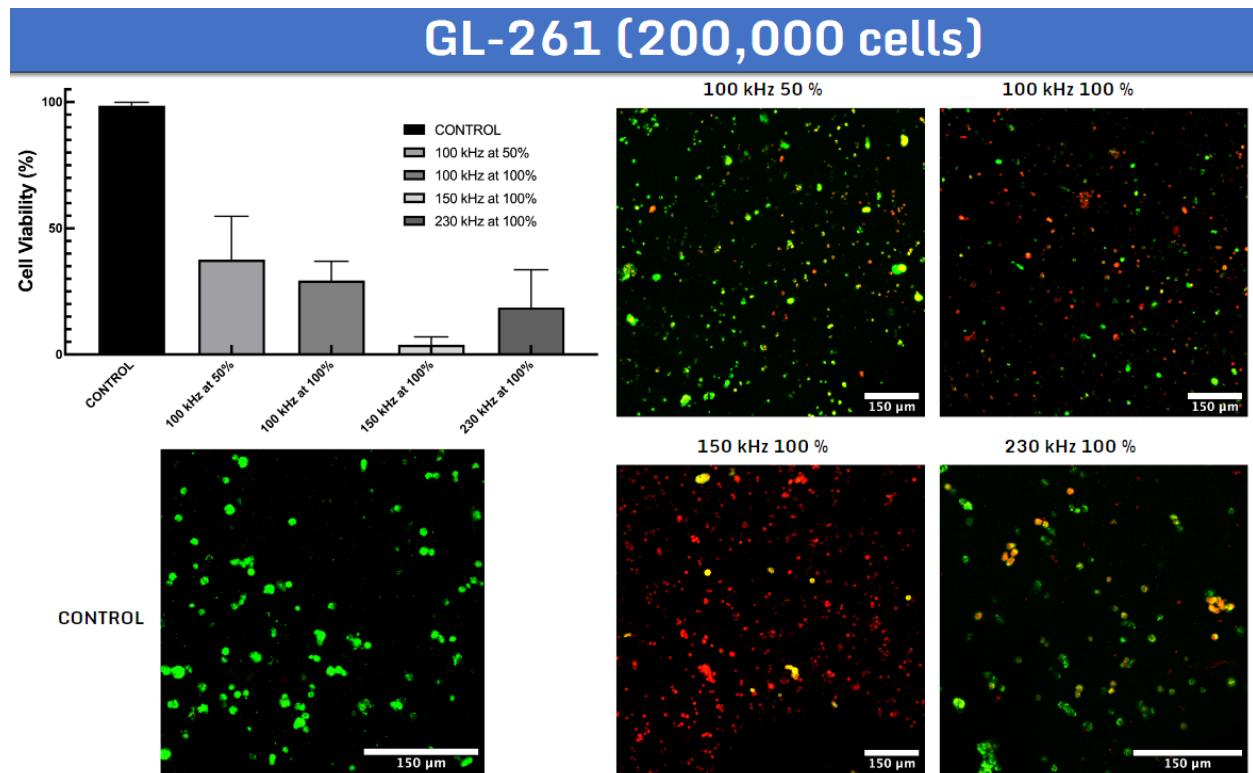


November 2, 2022 - 150kHz cell-laden hydrogel treated with variable MI (10%-100%)

Note: it is unclear why it says 5 spheroids per gel as they are cell-laden gels and not spheroids in the pictures

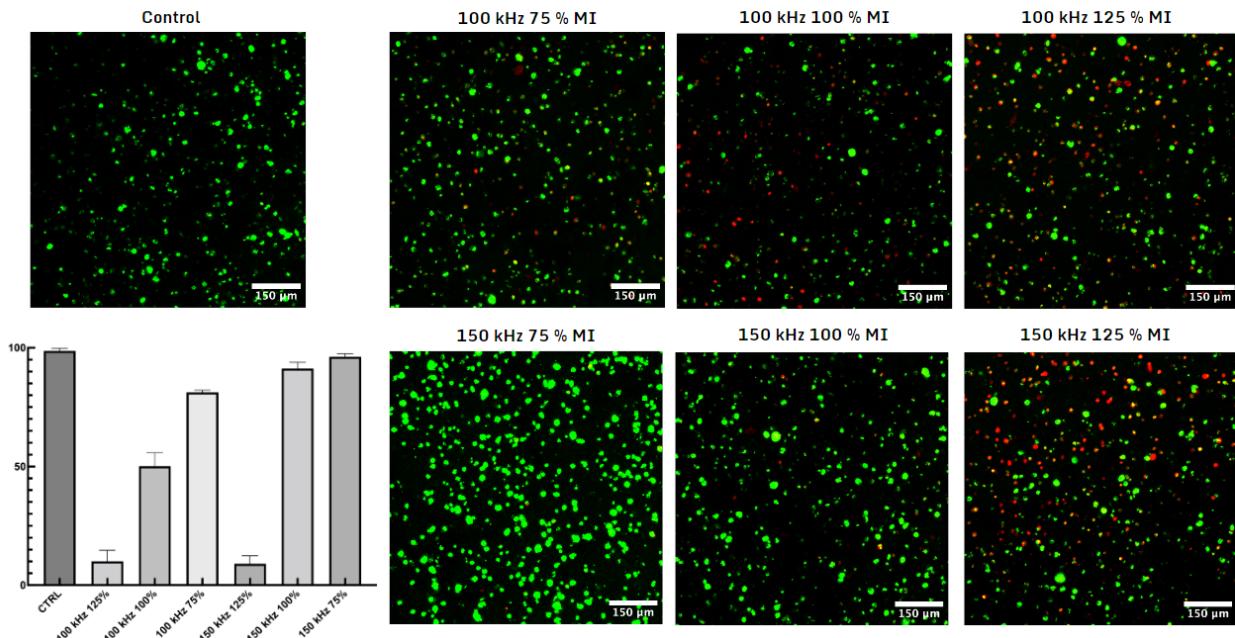


November 9, 2022 - 100kHz, 150kHz, and 230kHz US treatments of GL261 cell-laden hydrogel treated with variable MI (50% and 100%)

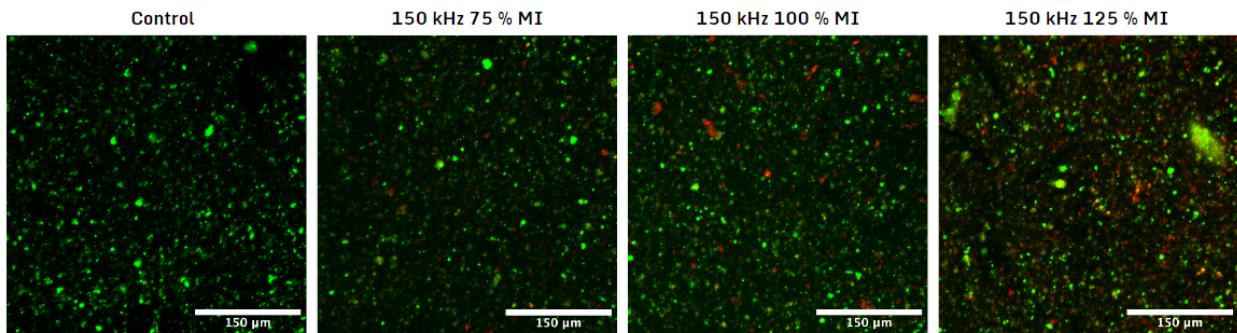


November 23, 2022 - 100kHz and 150kHz US treatments of HBMECs and mouse brain cell-laden hydrogel treated with variable MI (75%, 100%, and 125%)

HBMECs (200,000 cells per hydrogel)

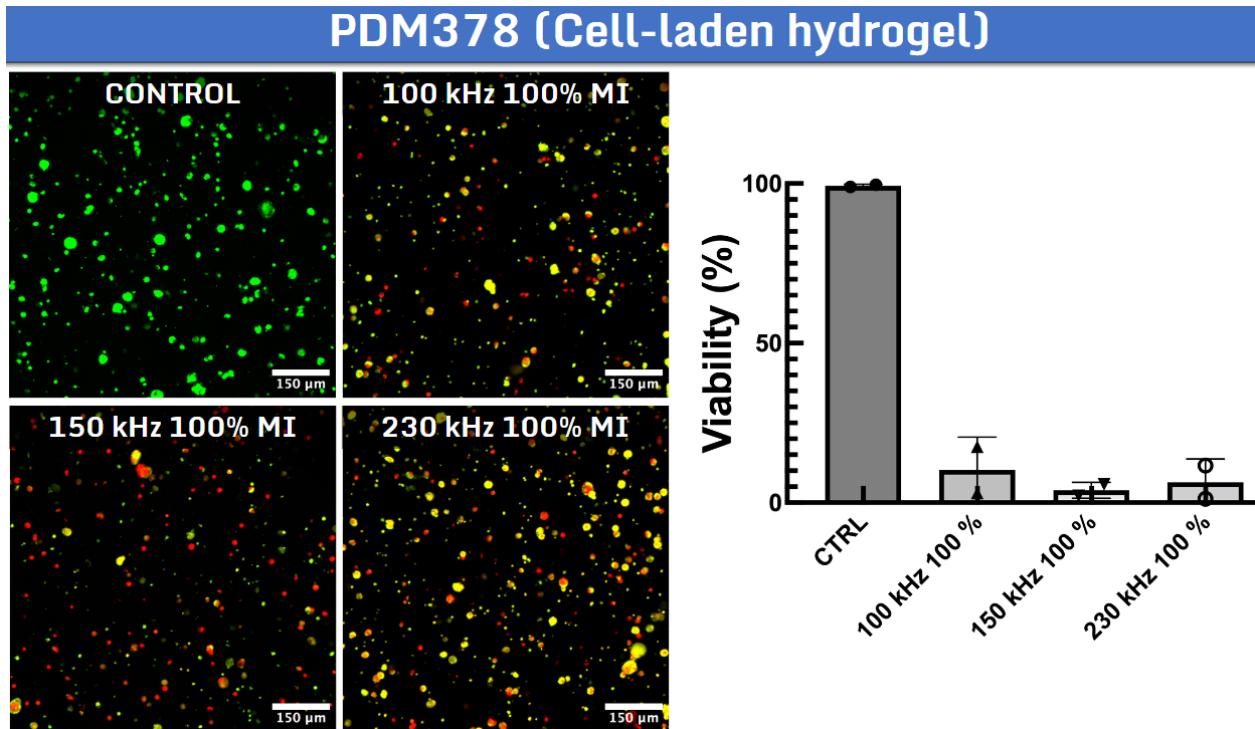


Mouse Brain Cells (400,000 cells per hydrogel)



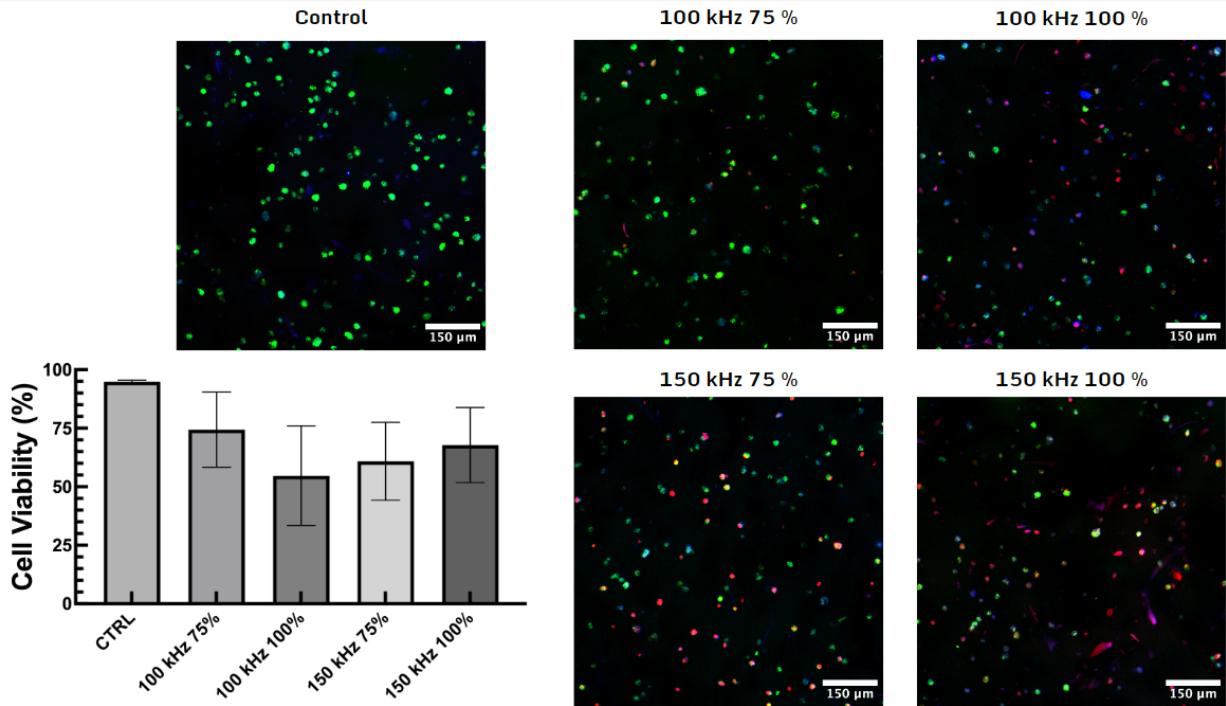
Quantification of viability was not possible due to background noise

November 30, 2022 - 100kHz, 150kHz, and 230kHz US treatments of PDM378 cell-laden hydrogels treated at 100% MI



December 7, 2022 - 100kHz and 150kHz US treatments of HBMEC cell-laden hydrogels treated with variable MI (75% and 100%)

HBMECs in FBS-free media (Cell-laden hydrogel)

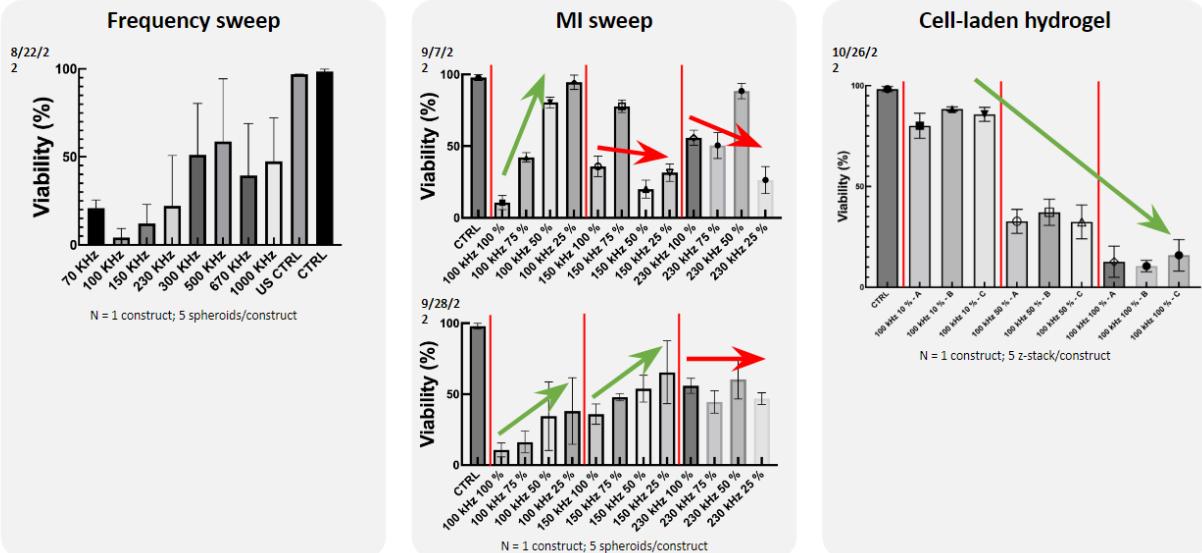


December 21, 2022 - Summary slides of in vitro treatments of both tumor and non-tumor cells

1.2. In vitro evaluation of ultrasonic therapy efficacy

U87

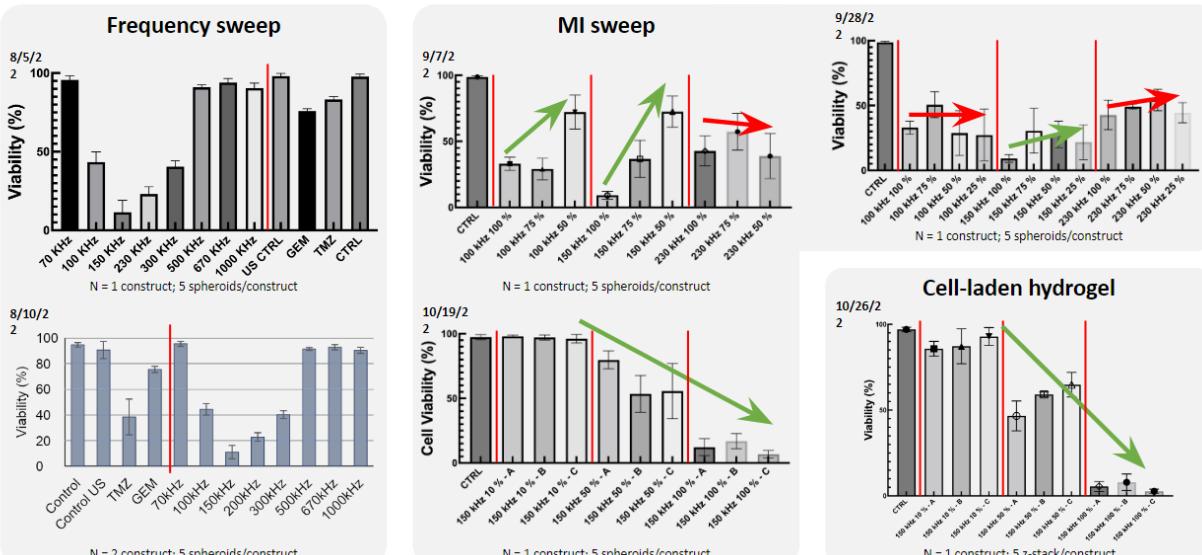
Target parameters: Frequency: 100kHz; MI: 100%; Burst length: 40ms; Treatment : 120s



1.2. In vitro evaluation of ultrasonic therapy efficacy

LN229

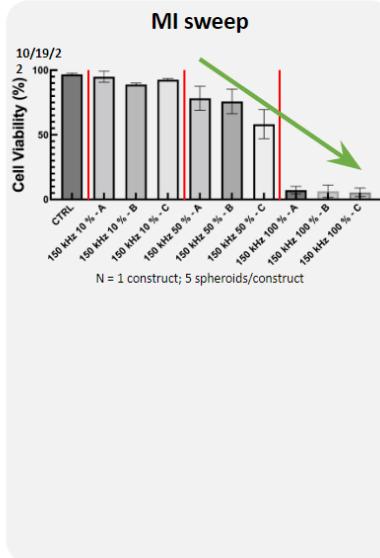
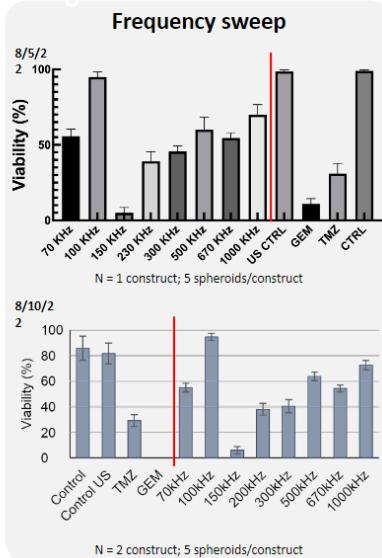
Target parameters: Frequency: 150kHz; MI: 100%; Burst length: 40ms; Treatment : 120s



1.2. *In vitro* evaluation of ultrasonic therapy efficacy

PDM14

Target parameters: Frequency: 150kHz; MI: 100%; Burst length: 40ms; Treatment : 120s

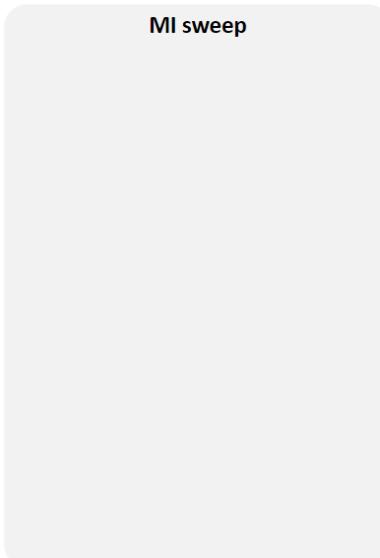
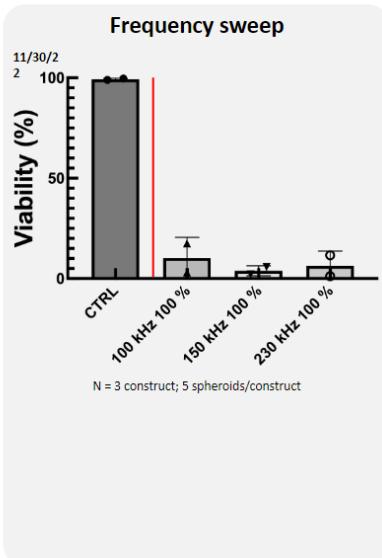


Cell-laden hydrogel

1.2. *In vitro* evaluation of ultrasonic therapy efficacy

PDM378

Target parameters: Frequency: 150kHz; MI: 100%; Burst length: 40ms; Treatment : 120s

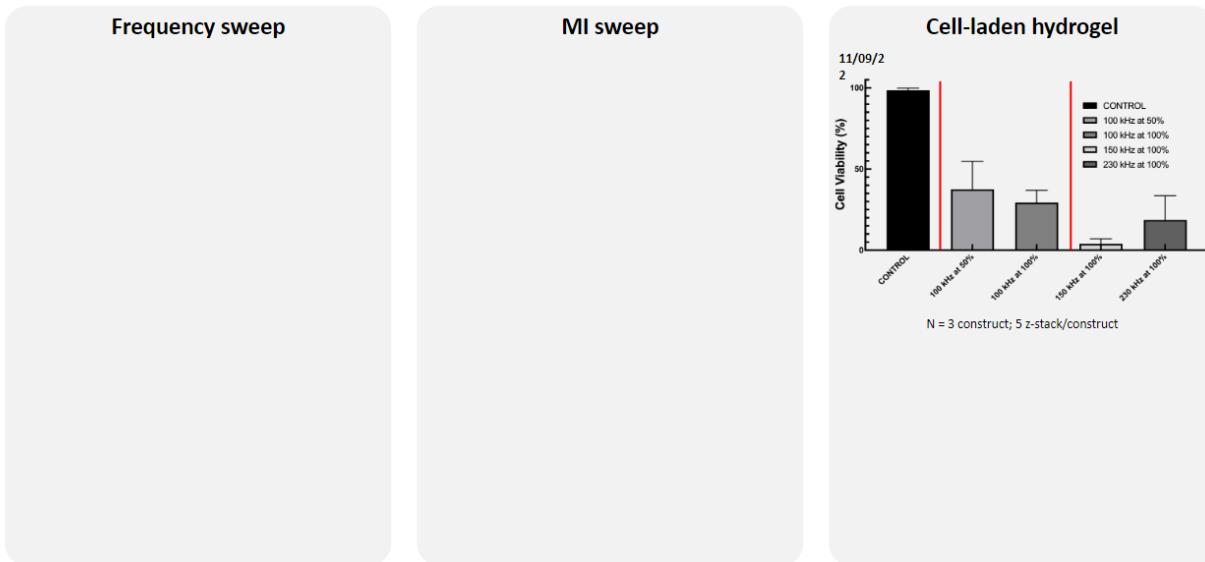


Cell-laden hydrogel

1.2. *In vitro* evaluation of ultrasonic therapy efficacy

GL261

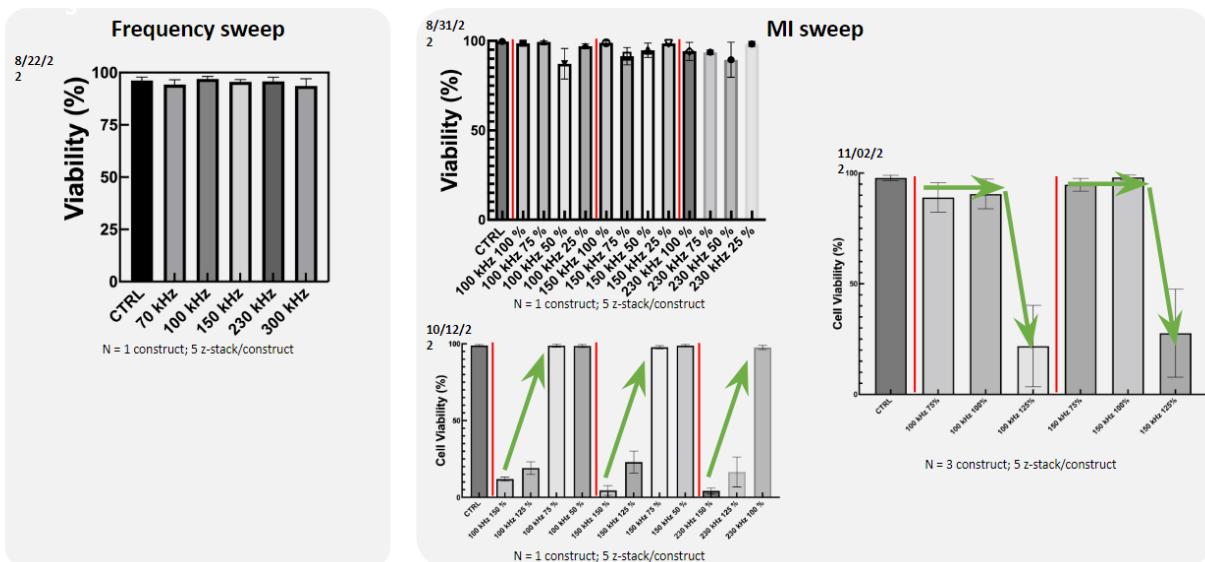
Target parameters: Frequency: **150kHz**; MI: **100%**; Burst length: **40ms**; Treatment : **120s**



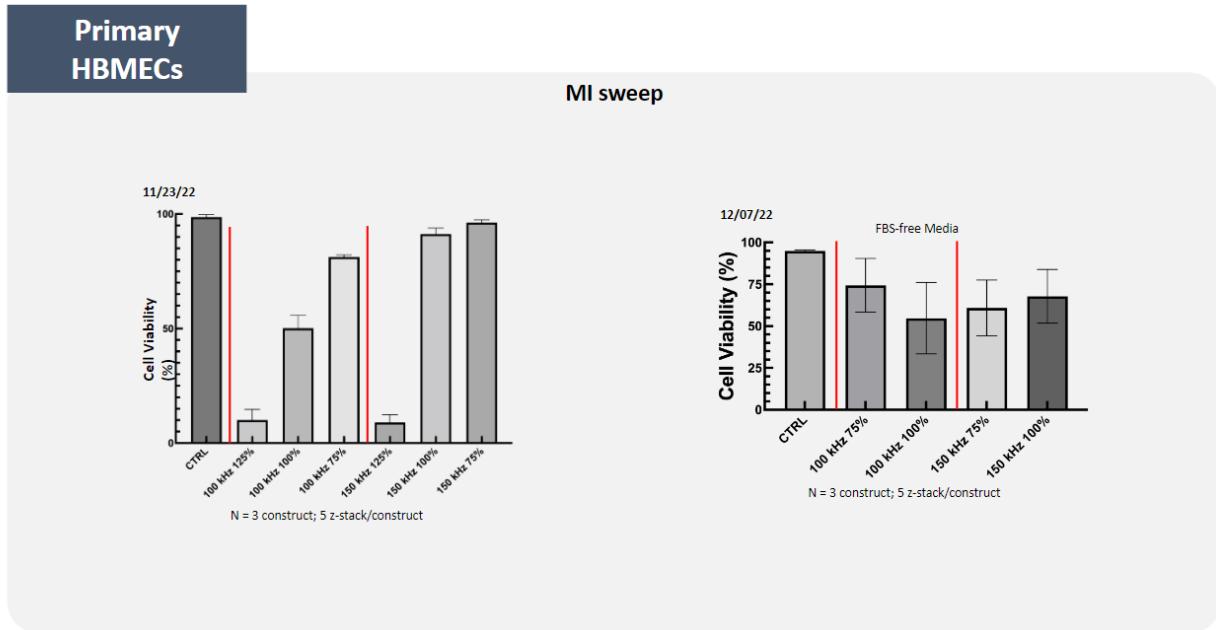
2.2. *In vitro* evaluation of ultrasonic therapy safety

Pericyte

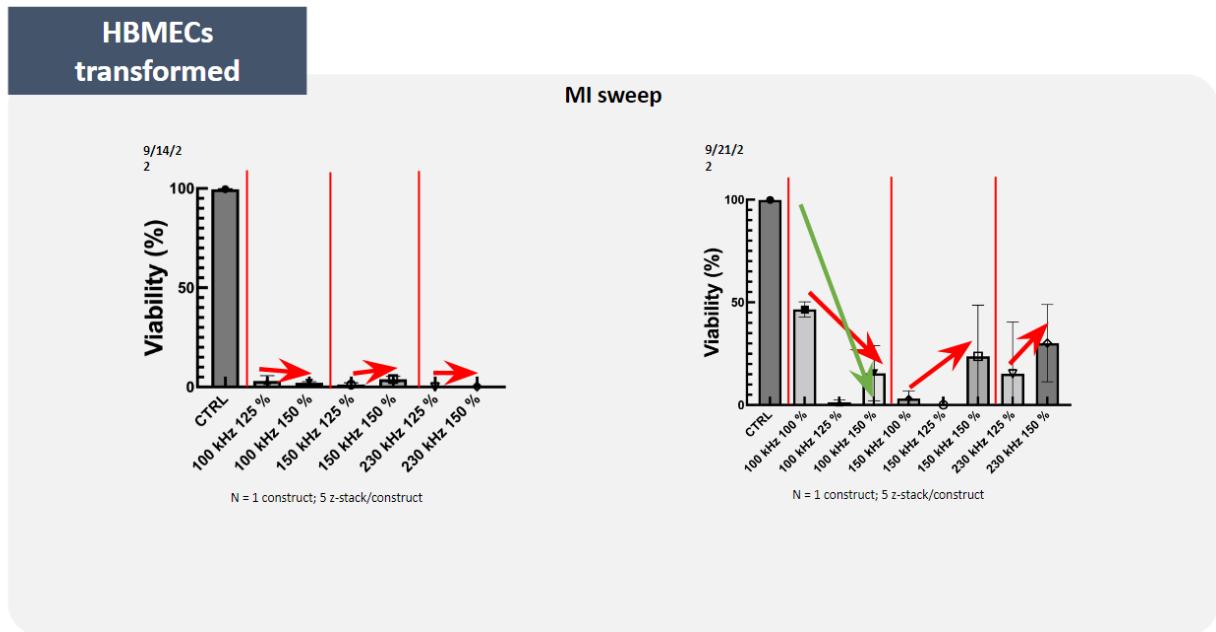
Resistance to 100kHz and 150kHz at 100%MI



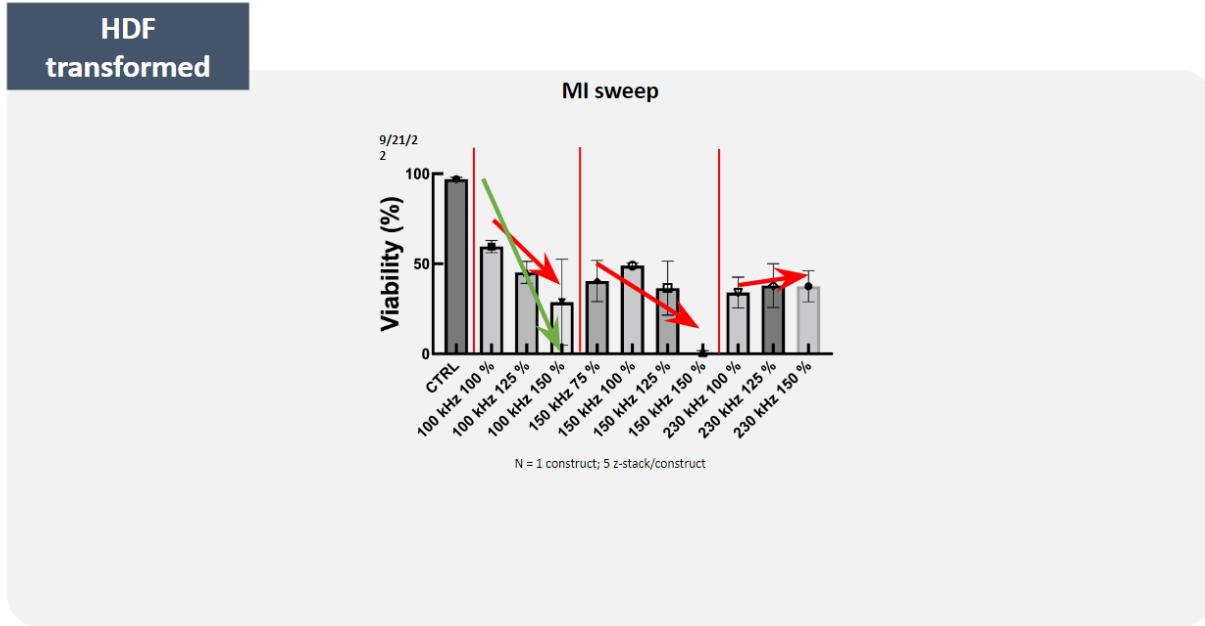
2.2. *In vitro* evaluation of ultrasonic therapy safety



2.2. *In vitro* evaluation of ultrasonic therapy safety



2.2. *In vitro* evaluation of ultrasonic therapy safety



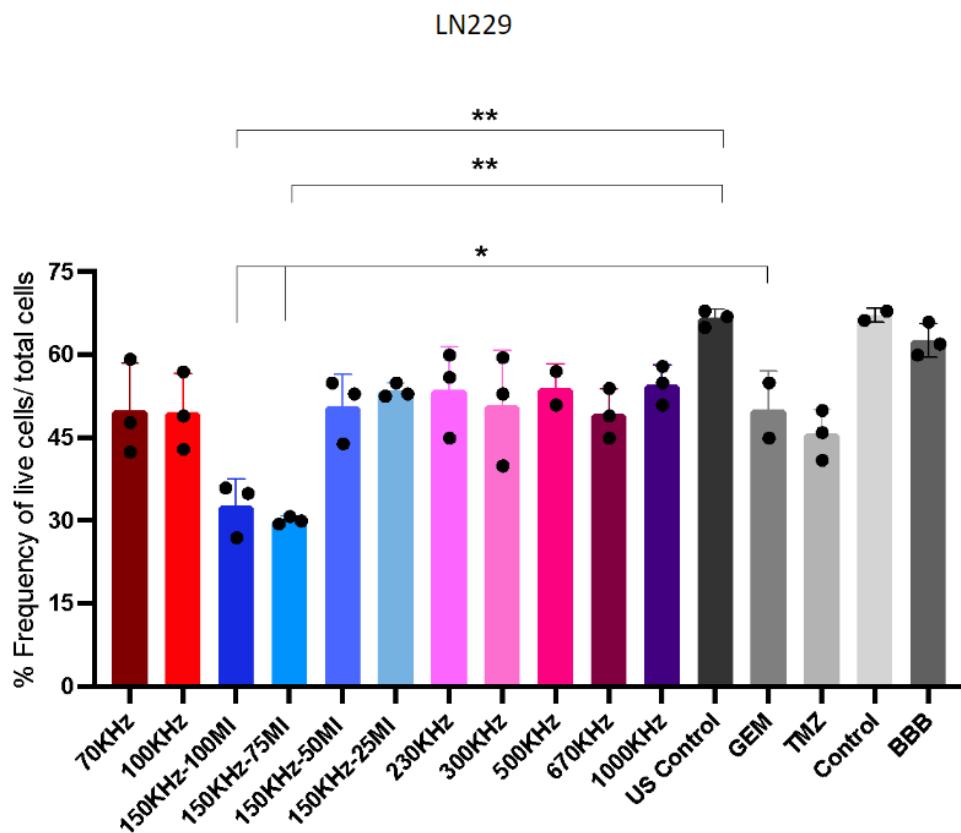
Summary of in vitro experiments done in Phase 1.1 and 1.2

Cell	Frequency (kHz)	MI (%)	Burst Length (ms)	Treatment time (s)	Replicate	Viability values for each spheroids			Avg	SD
						1	2	3		
Pericytes	70	100	40	120	1					
		150			2					
		125			3					
		100	40	120	4	99	98	99		0.7
		75			5	100	100	99		0.5
		50			6	79	86	96		8.5
	100	25			7	96	99	97		1.4
		150			8					
		125			9					
		100	40	120	10	98	100	99		0.9
		75			11	93	96	86		4.8
	150	50			12	96	98	90		4.0
		25			13	99	99	99		0.1
		150			14					
		125			15					
HBMECs	230	100	40	120	16	98	89	96		5.0
		75			17	95	94	92		1.2
		50			18	98	79	92		9.8
		25			19	100	97	99		1.4
		300	100	40	20					
		150			21	17	28	1	15	13.4
	100	125			22	1	3	0	1	1.3
		100	40	120	23	47	50	43	47	3.7
		75			24					
		50			25	0	0	0	0	0.0
		25			26	7	1	2	3	3.7
	150	150			27					
		125			28	31	11	49	30	18.8
		100			29	0	44	1	15	25.3
		75			30					
		50			31					
HDF	230	25			32					
		150			33	38	46	2	29	23.8
		125			34	49	48	38	45	6.1
		100	40	120	35	63	57	58	60	3.4
		75			36					
		50			37	1	0	2	1	0.8
	100	25			38	44	46	19	36	14.9
		150			39	50	50	47	49	1.4
		125			40	46	27	48	40	11.4
		100			41					
		75			42					
	150	50			43					
		25			44					
		150			45	46	29	37	37	8.6
		125			46	40	25	49	38	12.2
		100			47	44	27	31	34	8.6
	230	75			48					
		50			49					
		25			50					

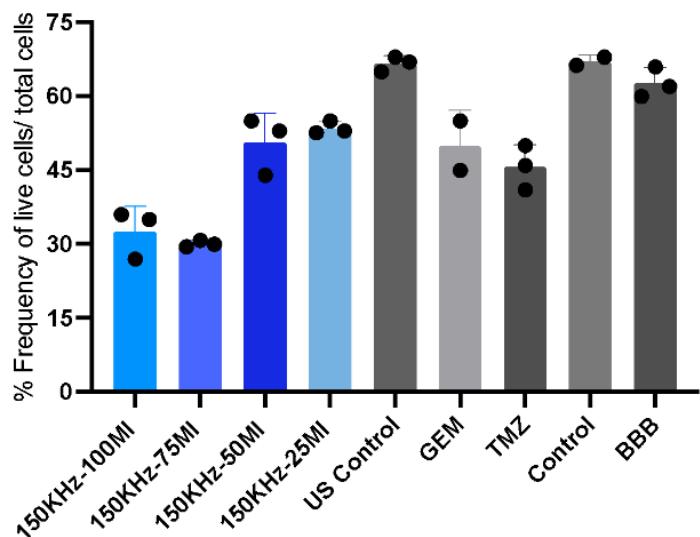
Phase 2: In Vitro - Hydrogel Domes

Feb 15, 2023 in vitro dome preparation protocol development. Protocol development went through May 15, 2023

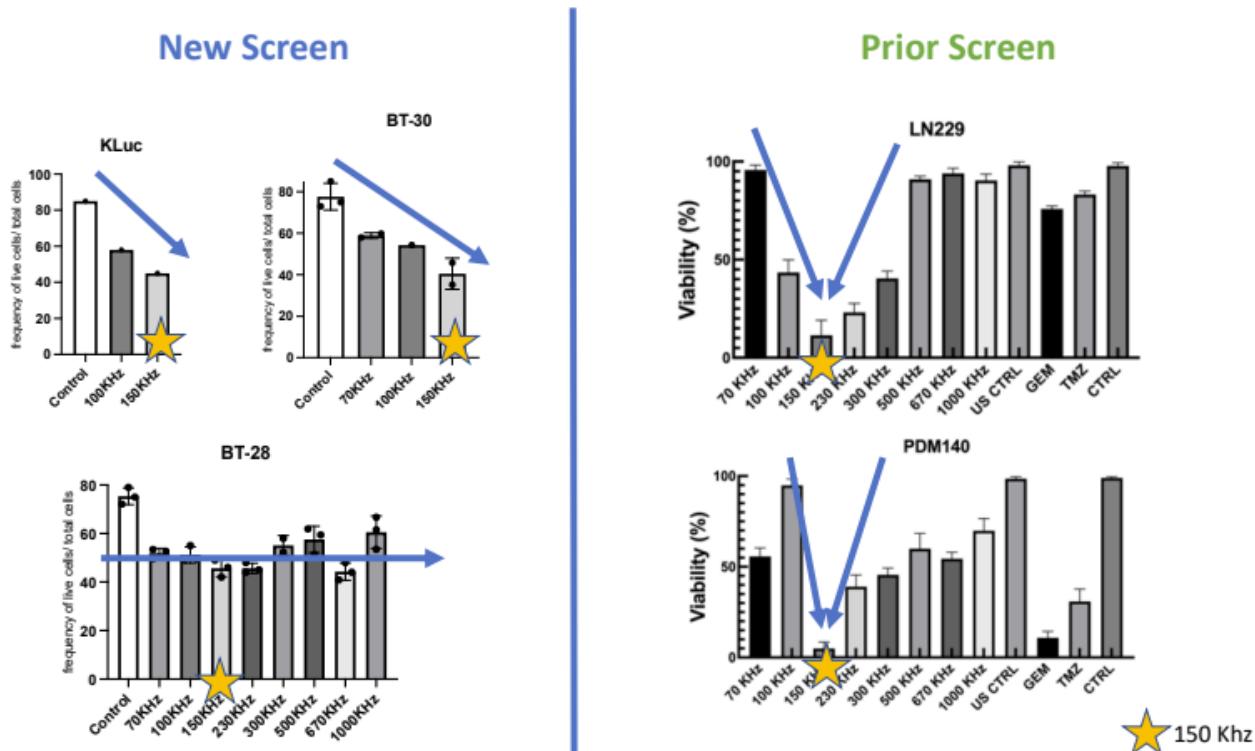
May 15, 2023 - LN229 cell results - not labeled for precise ultrasound parameters but should be 40ms burst duration, 10% duty cycle, 120 seconds total time.



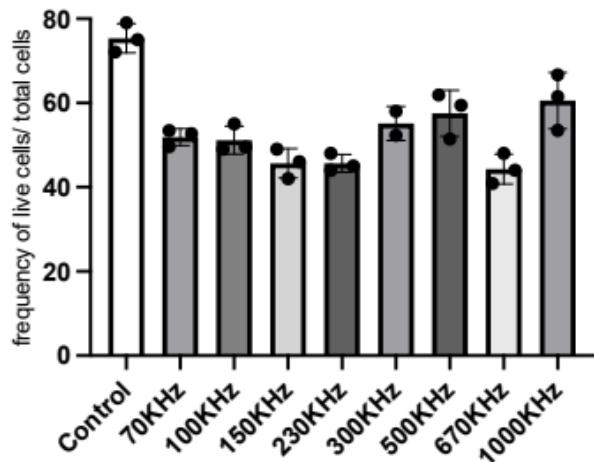
LN229



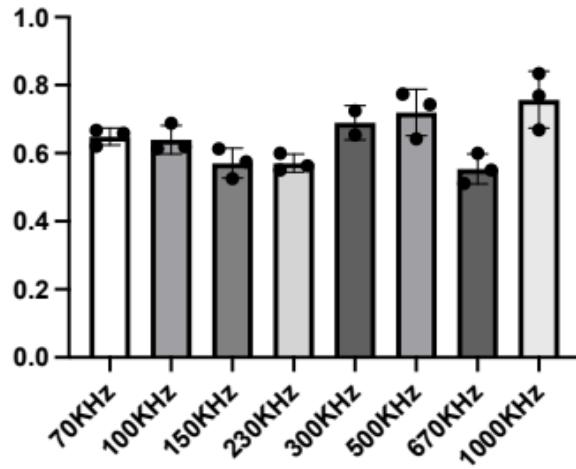
Apr 26, 2023 and May 3, 2023 -



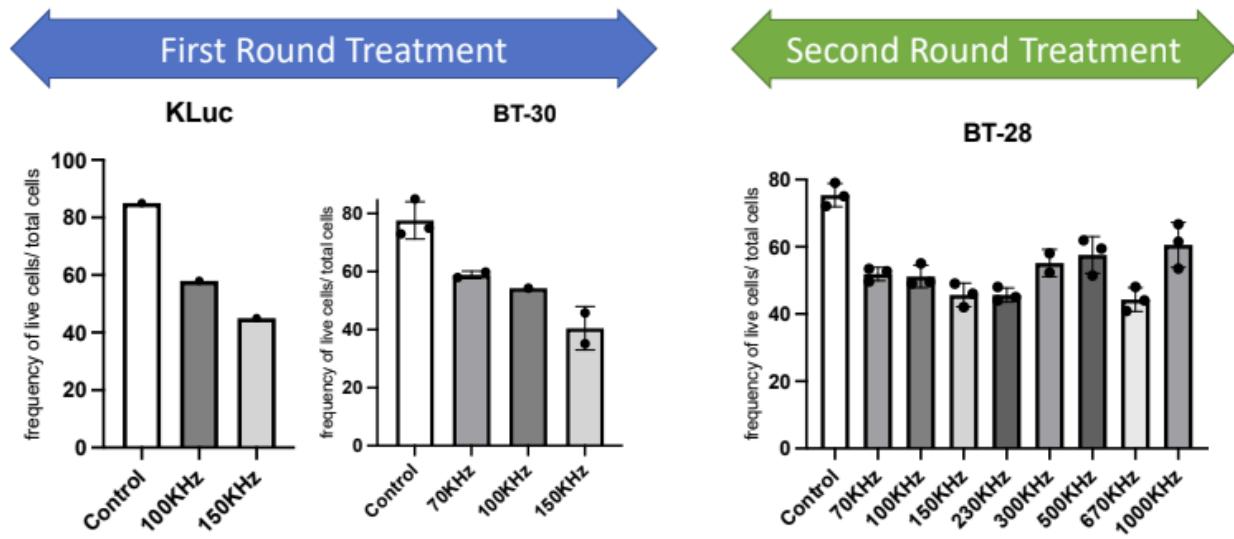
BT-28



BT28-second experiment (normalized)



Ultrasonic Treatment: Results

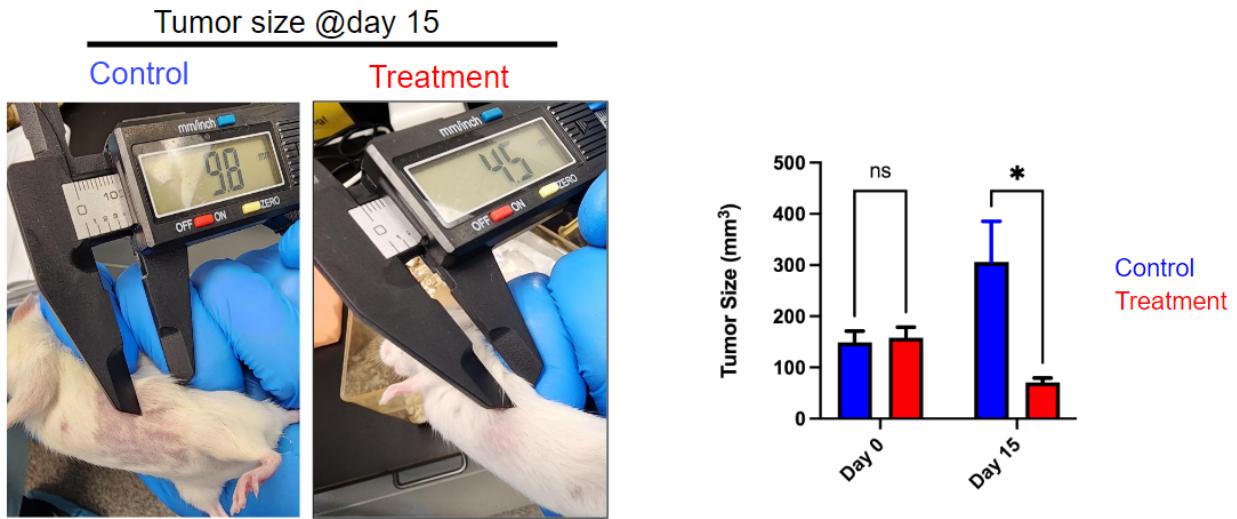


Phase 3: In Vivo - Mouse Flank

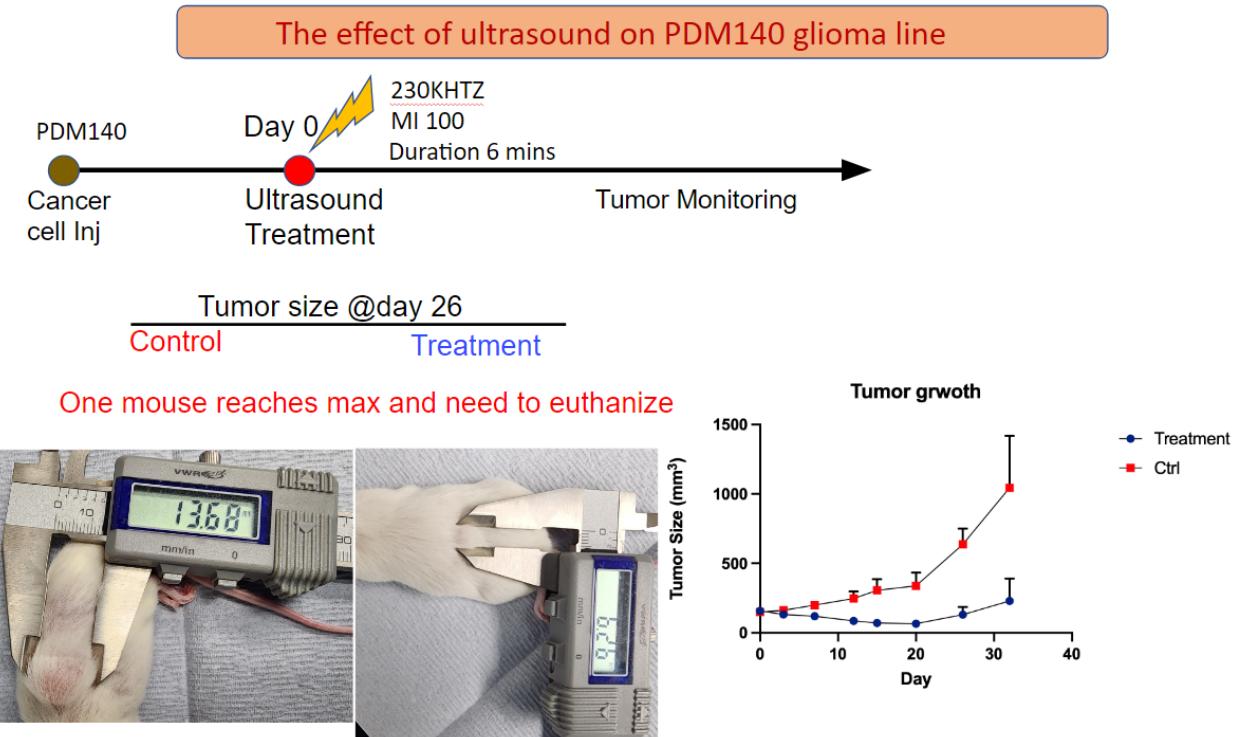
Mar 16, 2023 - in vitro dome preparation protocol development - in vivo treatment result (1 mouse?)

The US treatment used is unclear. No frequency was reported, nor other parameters including total time. **Treatment was not done according to a specific protocol.**

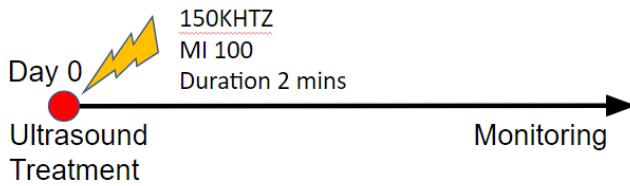
In Vivo tumor growth after a single treatment



Mar 22, 2023 in vivo treatment results of 1 mouse @ 230kHz, MI 100% for 6 minutes, and some mice @ 150kHz



The effect of ultrasound on healthy mice (no tumor engraftment)



Healthy mouse treatments: No side effect

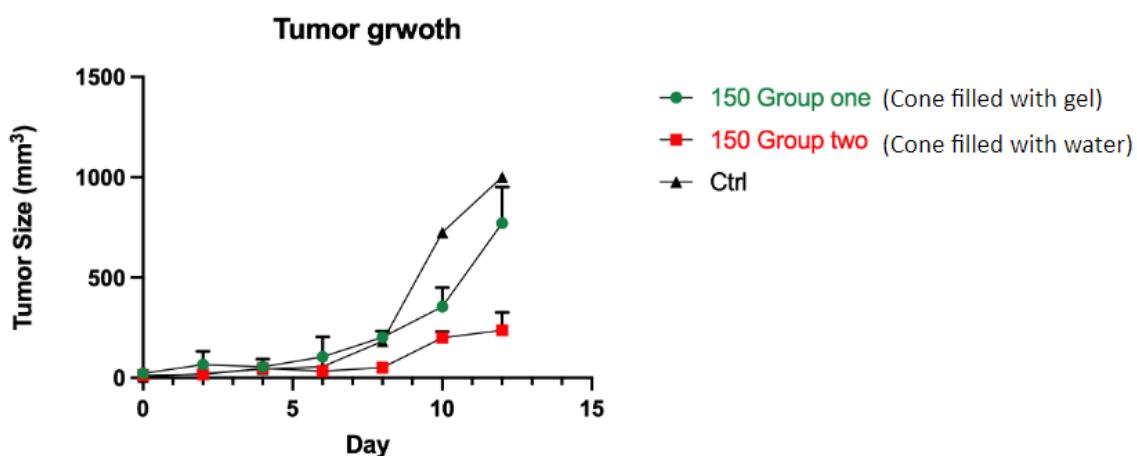


Healthy mouse treatments: No side effect, Vet tech Check

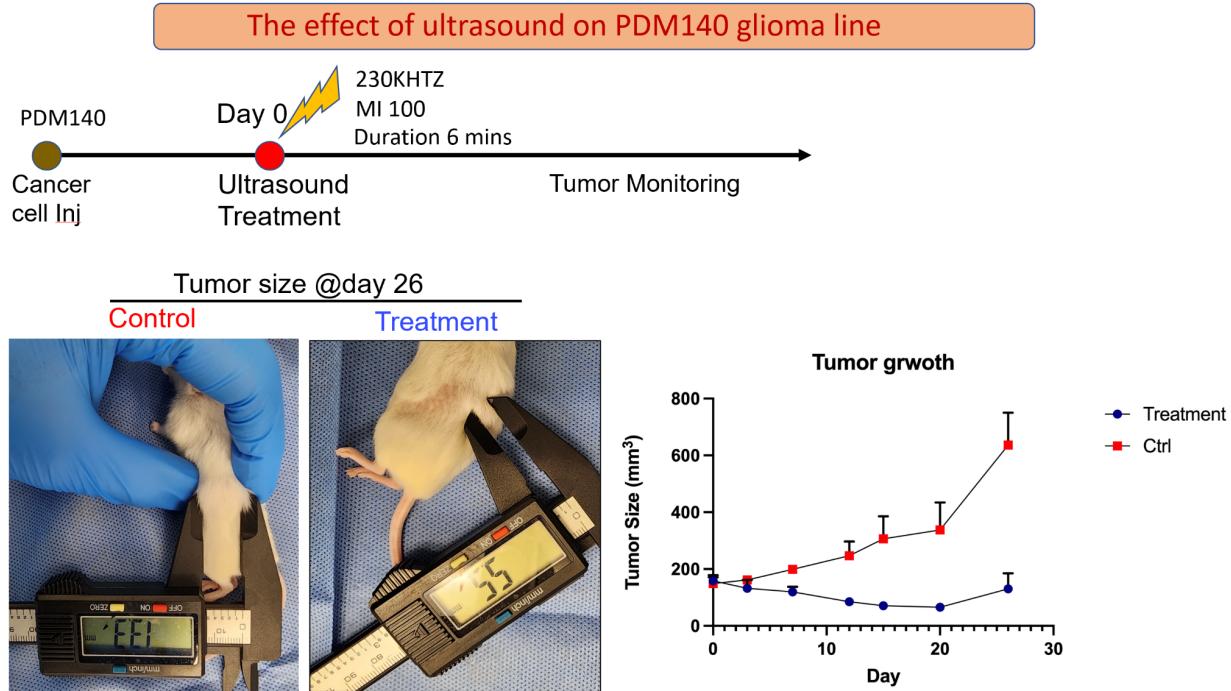


The cone filled with gel had a lot of air in it so it likely did not transmit the ultrasound completely

In vivo tumor growth (B16 OVA) after a single treatment



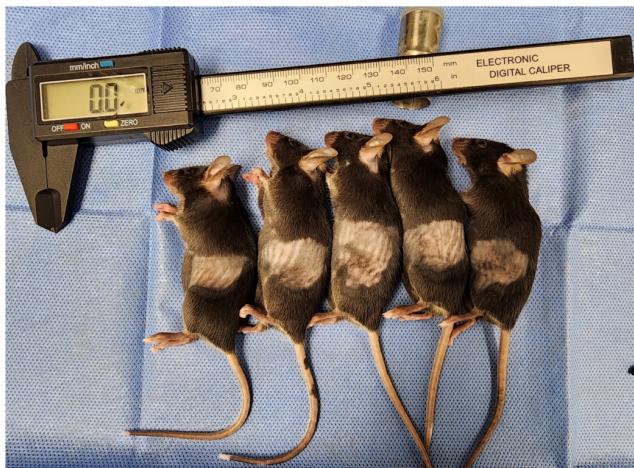
Mar 28, 2023 in vivo treatment results of 1 mouse @ 230kHz, MI 100% for 6 minutes, and some mice @ 150kHz, MI 100%, and 2 minutes.



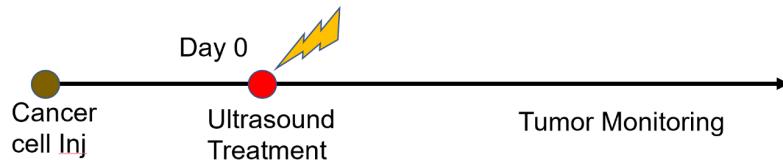
The effect of ultrasound on healthy mice (no tumor engraftment)



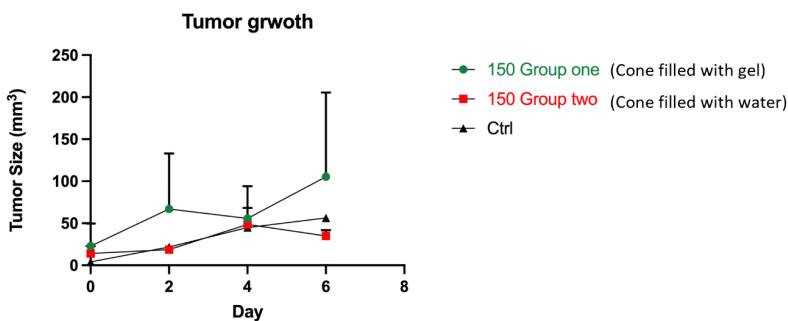
Healthy mouse treatments: No side effect



In vivo tumor growth (B16 OVA) after a single treatment

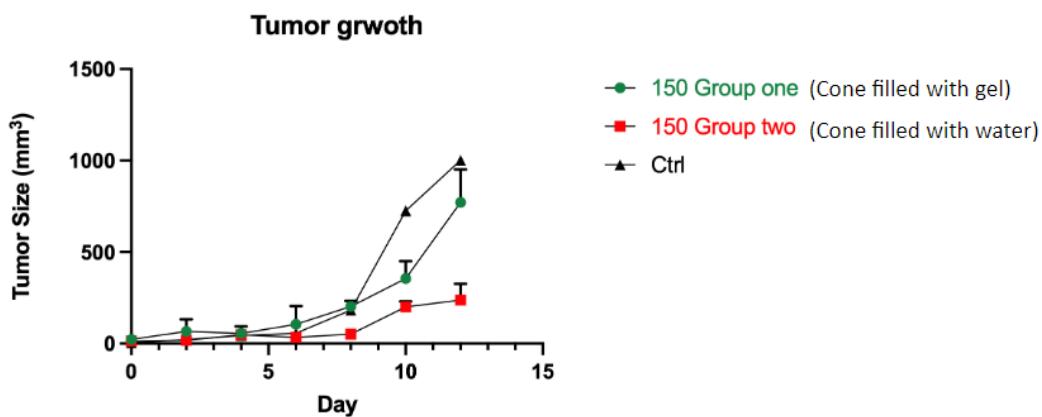


One mouse from group 150 one has issue



Apr 5, 2023 - Further analysis of in vivo 150kHz mouse group.

In vivo tumor growth (B16 OVA) after a single treatment



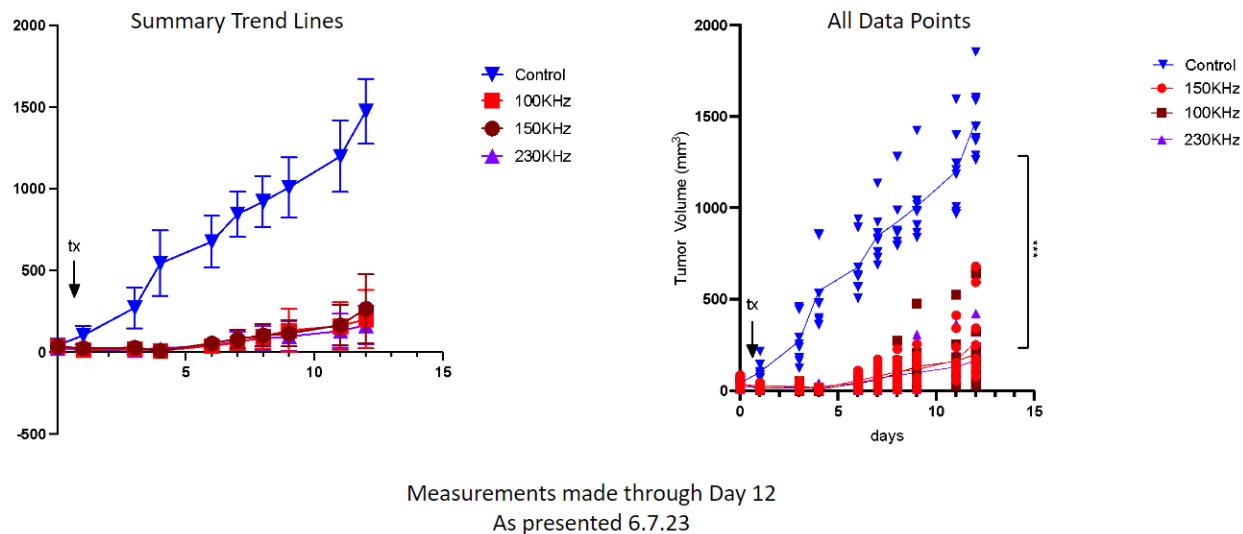
Apr 19, 2023 - no data - just planning meeting slides

Jun 7, 2023 - in vivo flank tumor growth with some details of the experiment

Flank Tumor Growth Measurements (GL261)

Injection Date: 5/17 (N=38 mice; N=10 in each treatment arm, N=8 control)

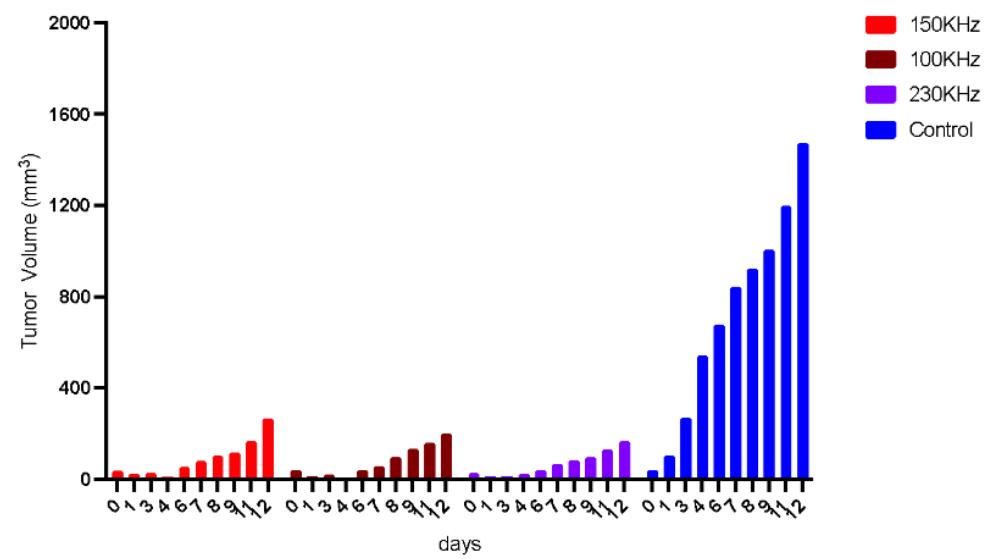
Treatment Date: 5/25



Flank Tumor Growth Measurements (GL261)

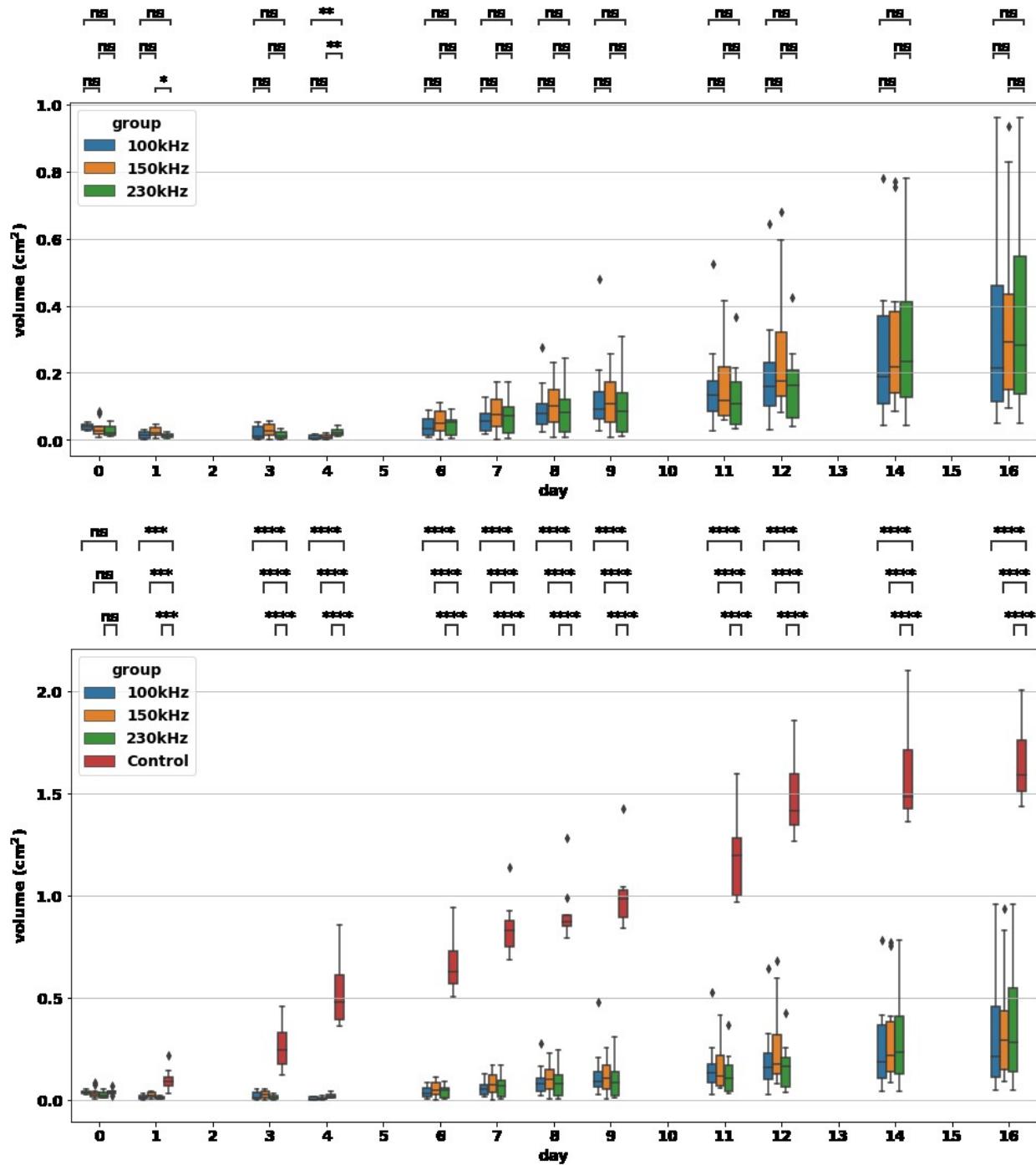
Injection Date: 5/17 (N=38 mice; N=10 in each treatment arm, N=8 control)

Treatment Date: 5/25



Measurements made through Day 12
As presented 6.7.23

Jun 16, 2023 - Results from the in vivo study through day 16 (post US treatment)



Histopathology Results (summary tables and conclusions):