

Preclinical oncolysis project progress reports

Overview

This document compiles all of the results provided to Openwater by its collaborators that ran the oncolysis studies. It is organized by date, in the order that these results were presented to Openwater. Openwater provided the collaborators with its custom *in vitro* and *in vivo* systems, all non-biological equipment, and training necessary to run the studies. A brief summary of the experiments follows, but for more details please refer to the document “Overview of preclinical oncolysis experiments and results” as it contains more complete explanations of the system and experiments.

In Vitro experiments were organized into the following phases:

Phase 1.1 - In Vitro setup validation and biological component optimization.

This 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 varied ultrasound parameters were the burst length, duty-cycle, total time, and peak negative pressure. The Mechanical Index (MI) was also used as a parameter, which is defined as the frequency divided by the peak-negative pressure. When using %MI as a parameter, all frequencies of the transducers received different pressures.

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.

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.

In Vivo experiments were all performed on NSG mice with tumor cells injected into their flanks.

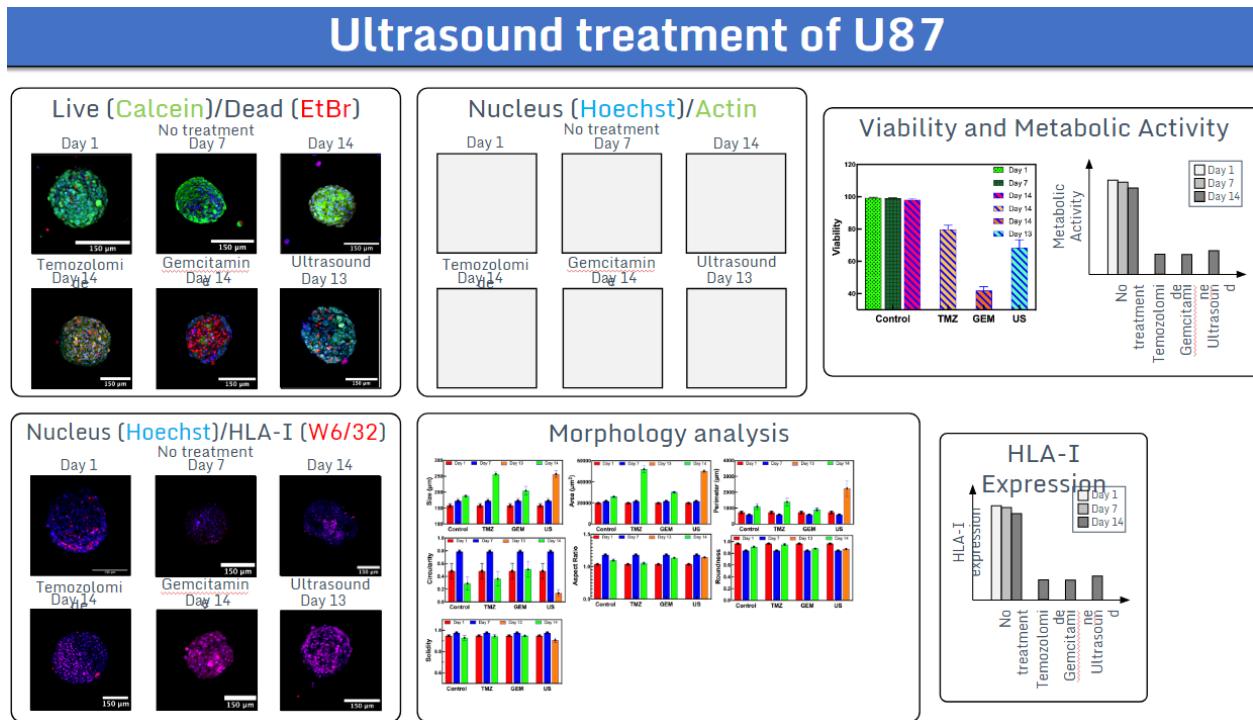
Phase 3 - In Vivo - Mouse Flank

The same basic components used for the in vitro experiments were used for the in vivo experiments. They shared all electronic equipment and transducers, and only the fixturing holding the transducers and the coupling cones changed. 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 at 100kHz, 150kHz, or 230kHz.. 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 analyses of the samples.

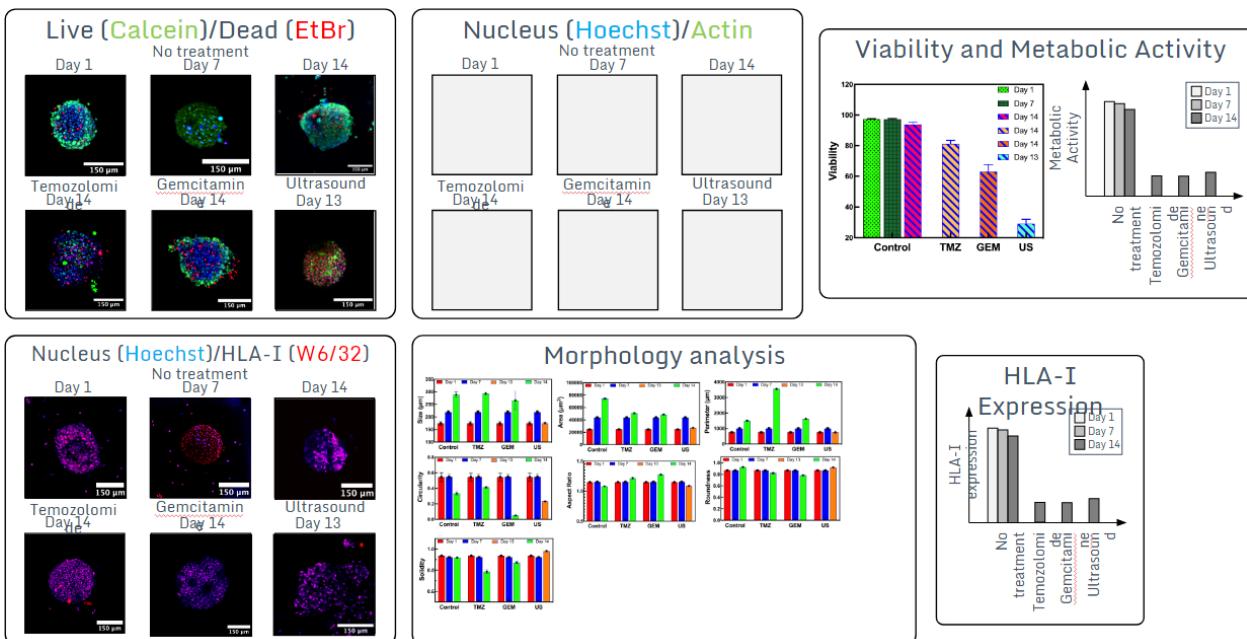
Data from Progress Reports:

Phase 1.1 - In Vitro setup validation and biological component optimization.

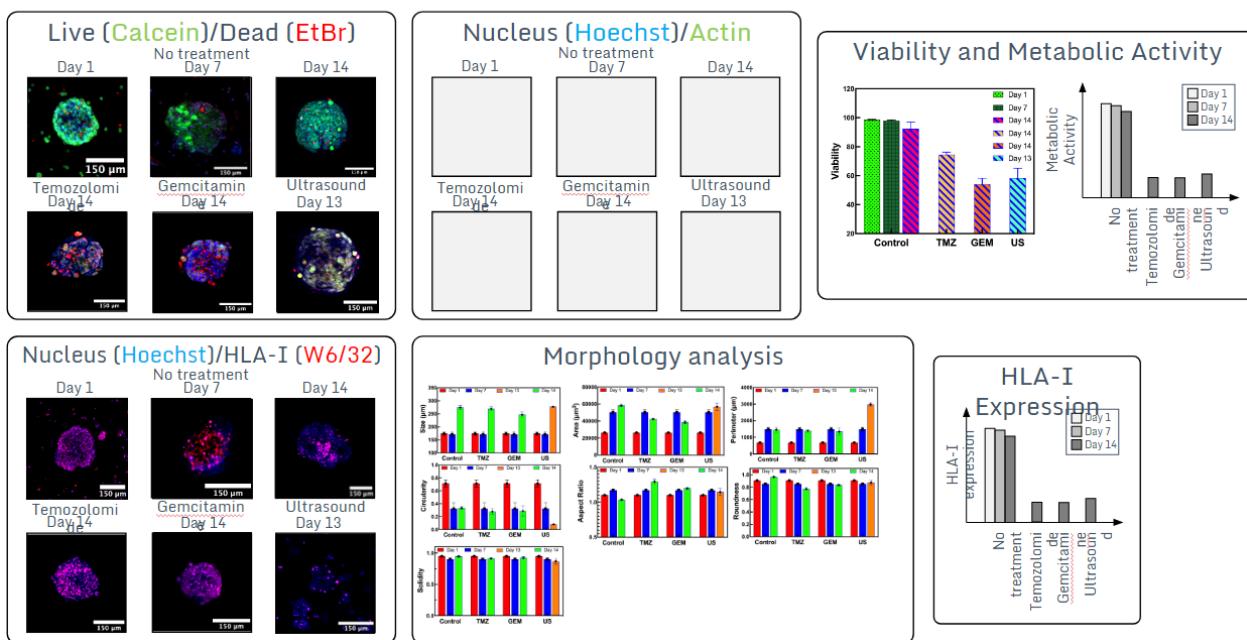
June 29, 2022 - Ultrasound treatment assays of U87, LN229 (ultrasound parameters unclear)



Ultrasound treatment of LN229



Ultrasound treatment of PDM140



July 13, 2022 - 7 day experiment timeline validation

Validation of 7-days experiments timeline

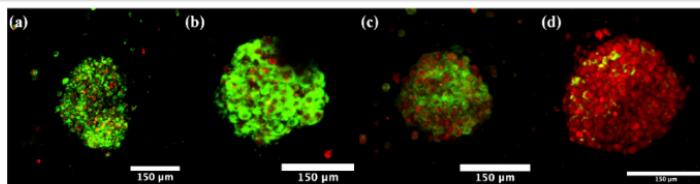
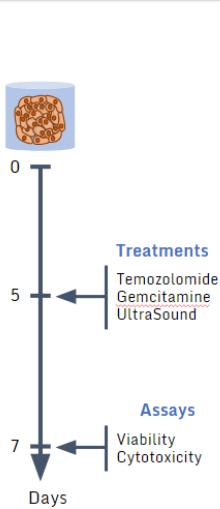


Figure 1: Live/Dead study of U87. (a) Day 7 Control; (b) Day 7 TMZ treated; (c) Day 7 GEM treated; (d) Day 7 US treated.

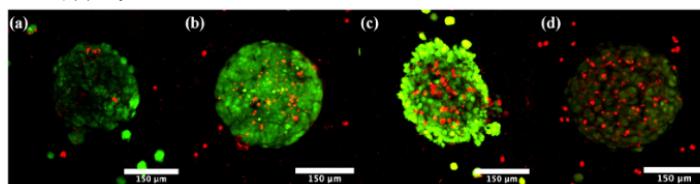
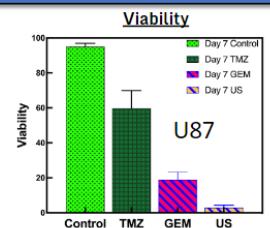


Figure 2: Live/Dead study of LN229. (a) Day 7 Control; (b) Day 7 TMZ treated; (c) Day 7 GEM treated; (d) Day 7 US treated.

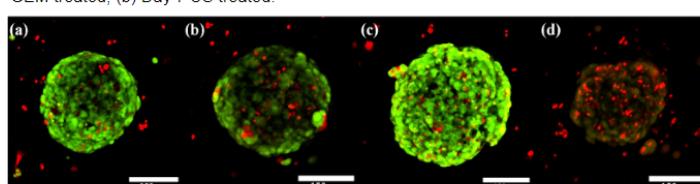
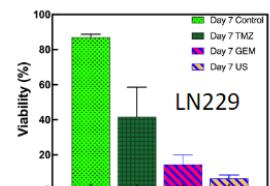
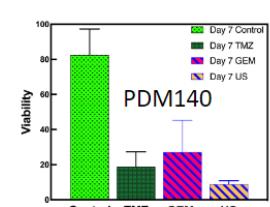
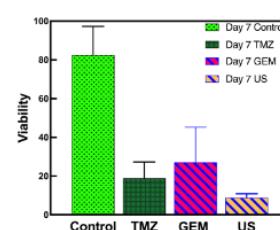
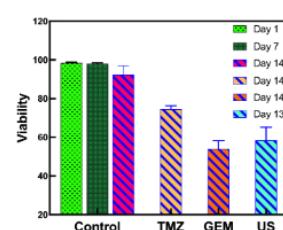
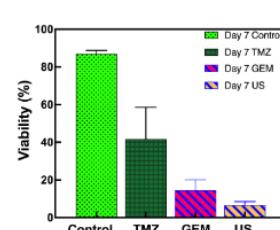
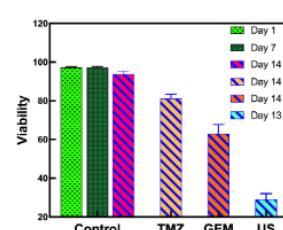
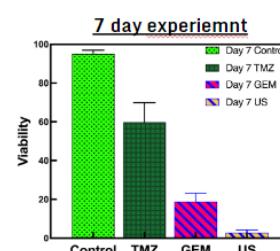
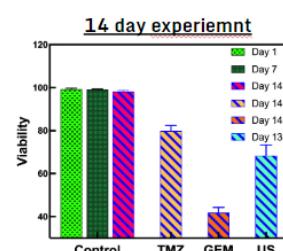
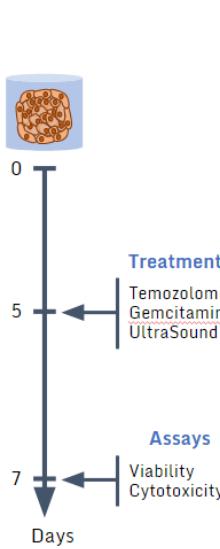


Figure 3: Live/Dead study of PDM140. (a) Day 7 Control; (b) Day 7 TMZ treated; (c) Day 7 GEM treated; (d) Day 7 US treated.

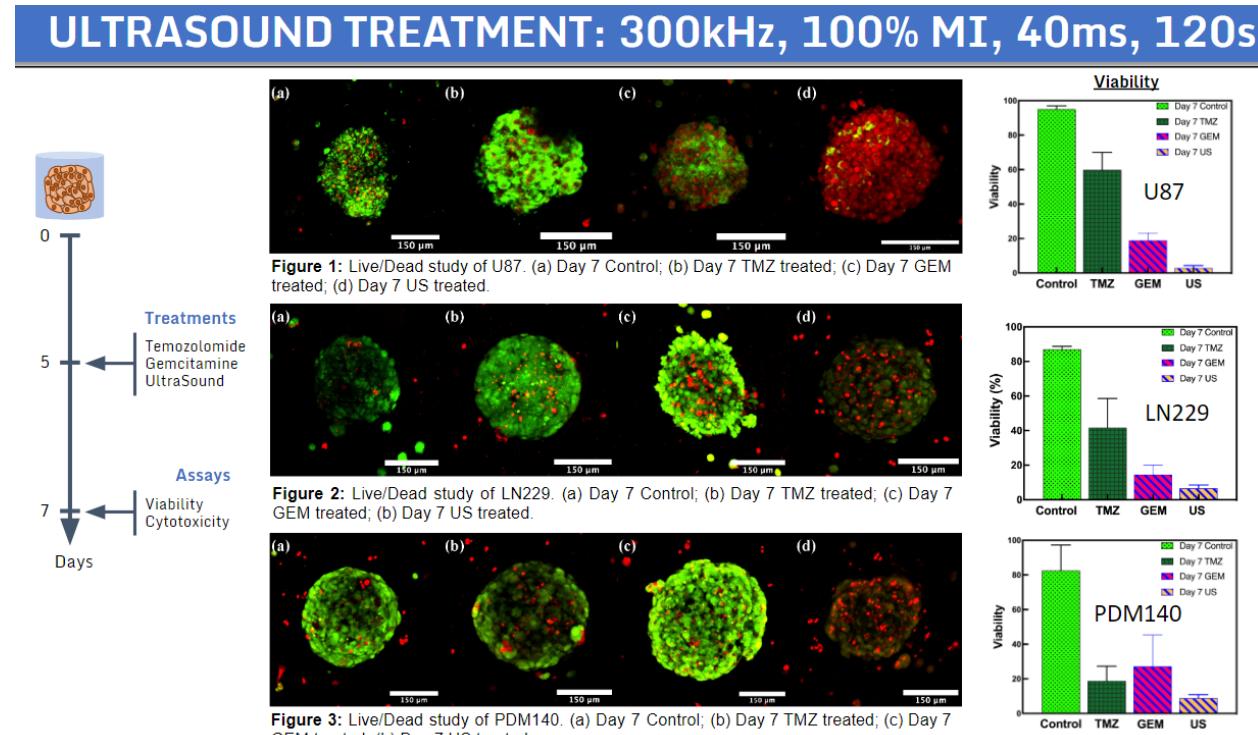
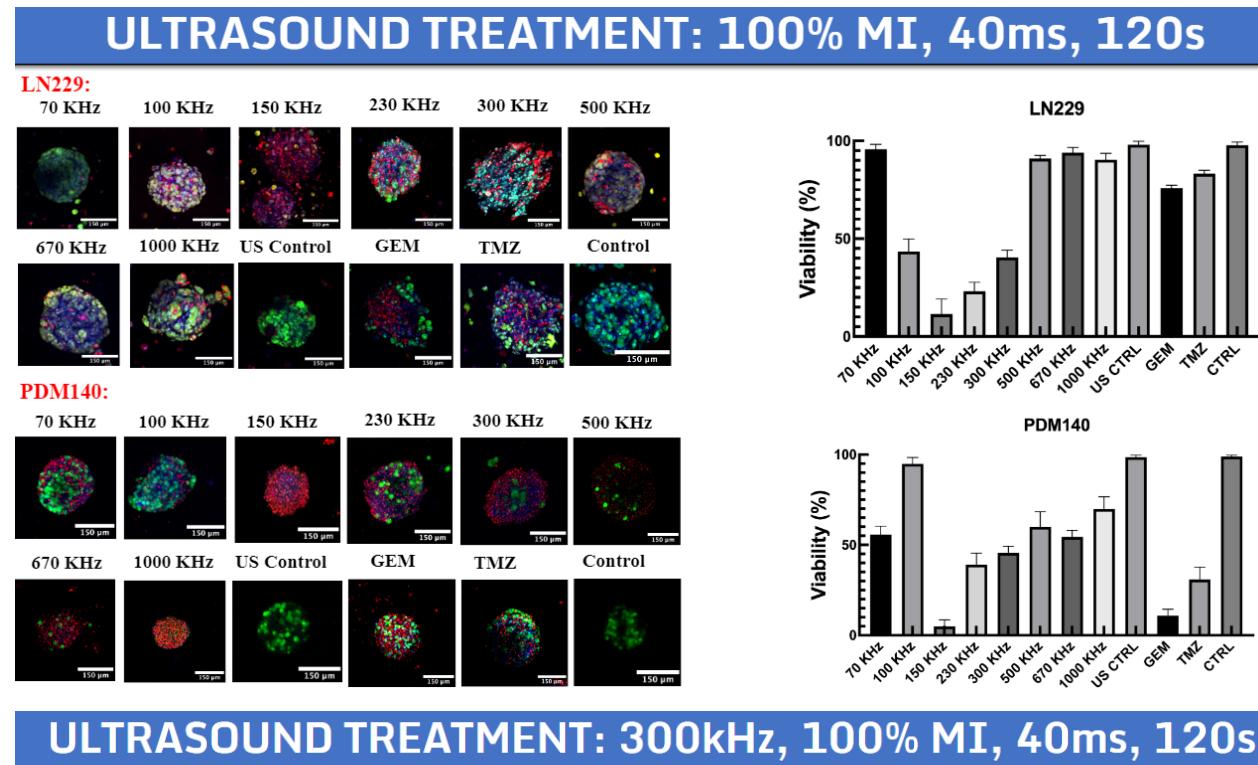


Validation of 7-days experiments timeline



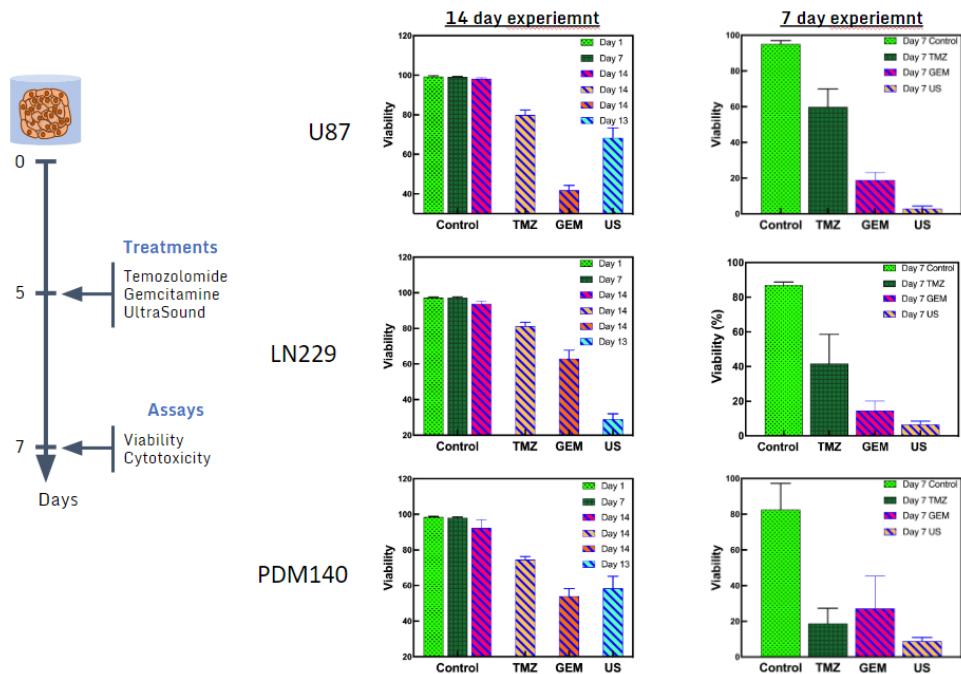
August 5, 2022 - in vitro frequency sweeps. (new single container treatment to mitigate contamination issues)

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

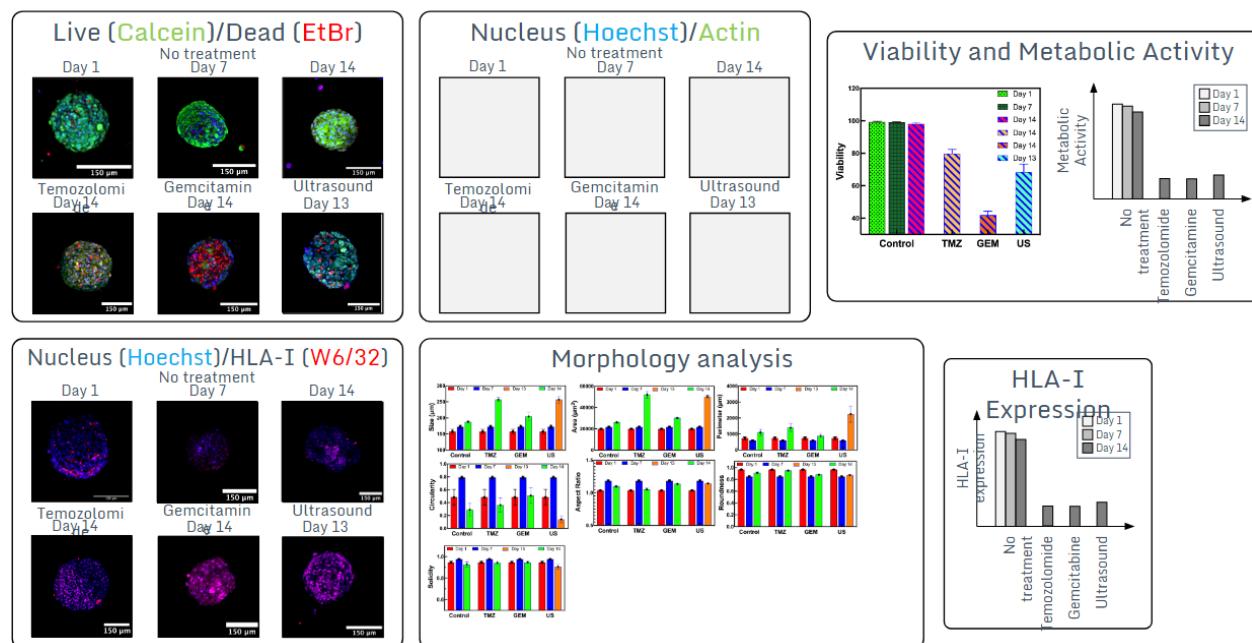


Same pressure, different burst length, same duty-cycle, same total ON time

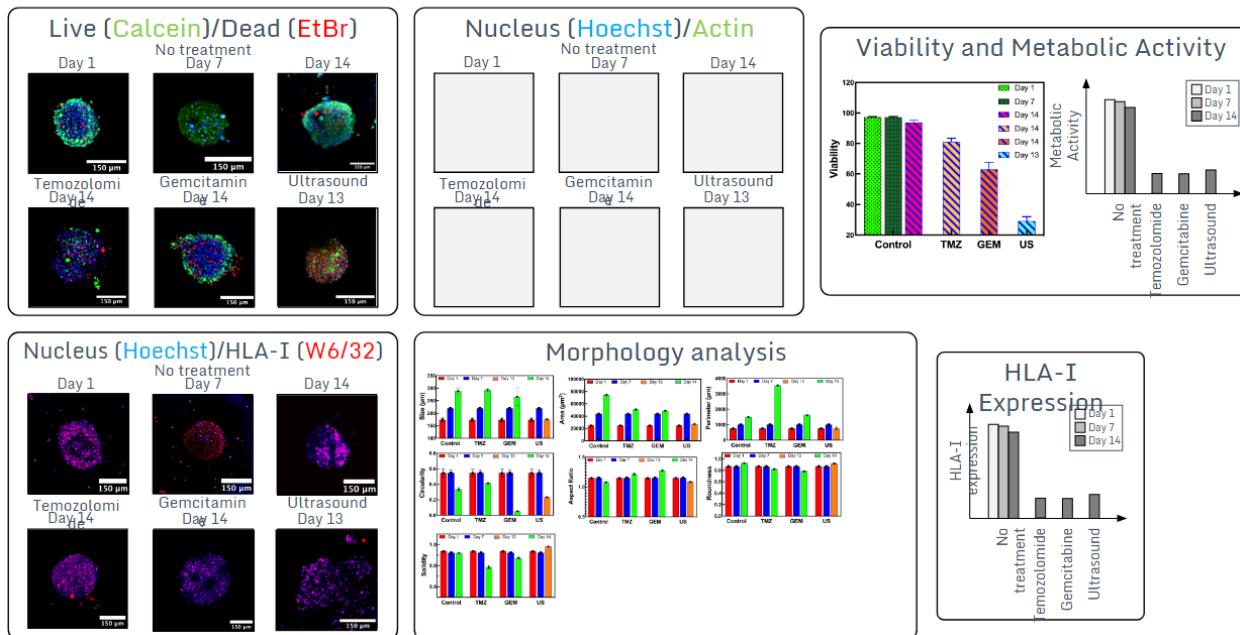
ULTRASOUND TREATMENT: 100% MI, 20ms vs 40ms, 120s



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

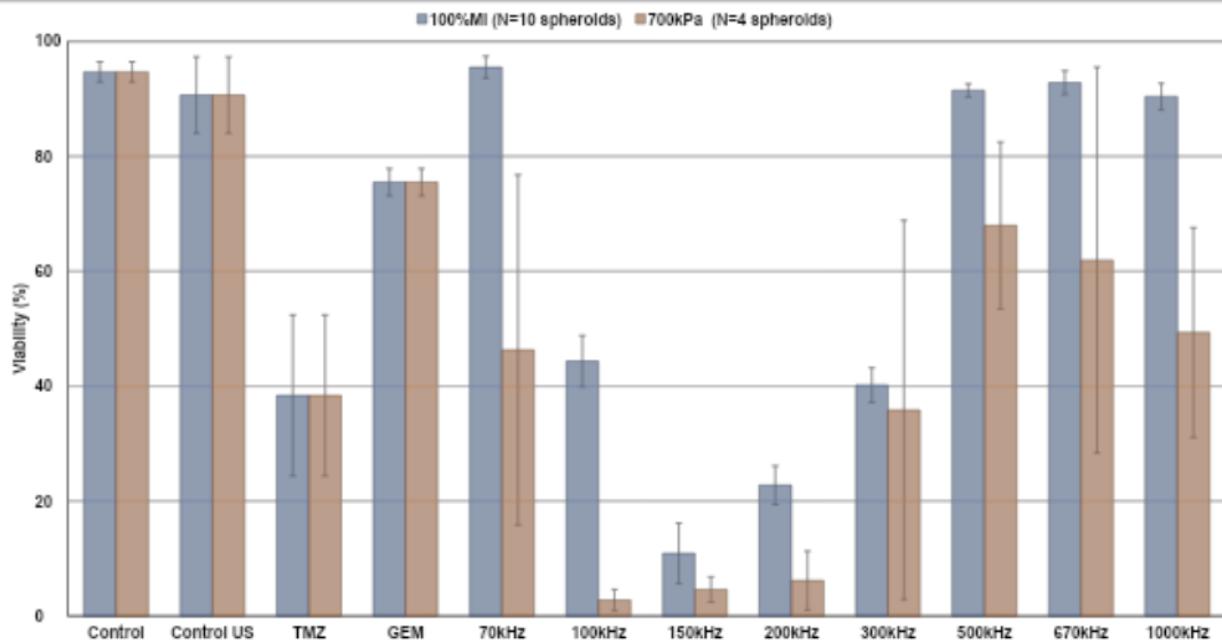


LN229 ULTRASOUND TREATMENT: 100% MI, 20ms, 120s

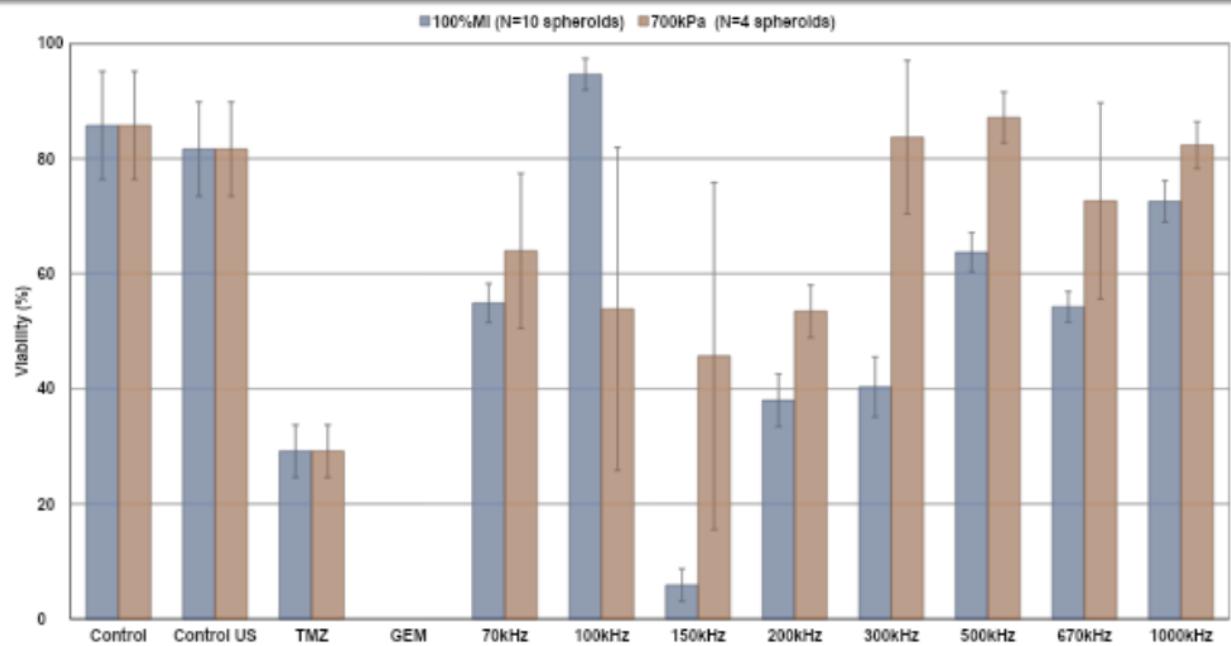


August 10, 2022 - frequency sweep summary graphs with constant pressure and constant MI

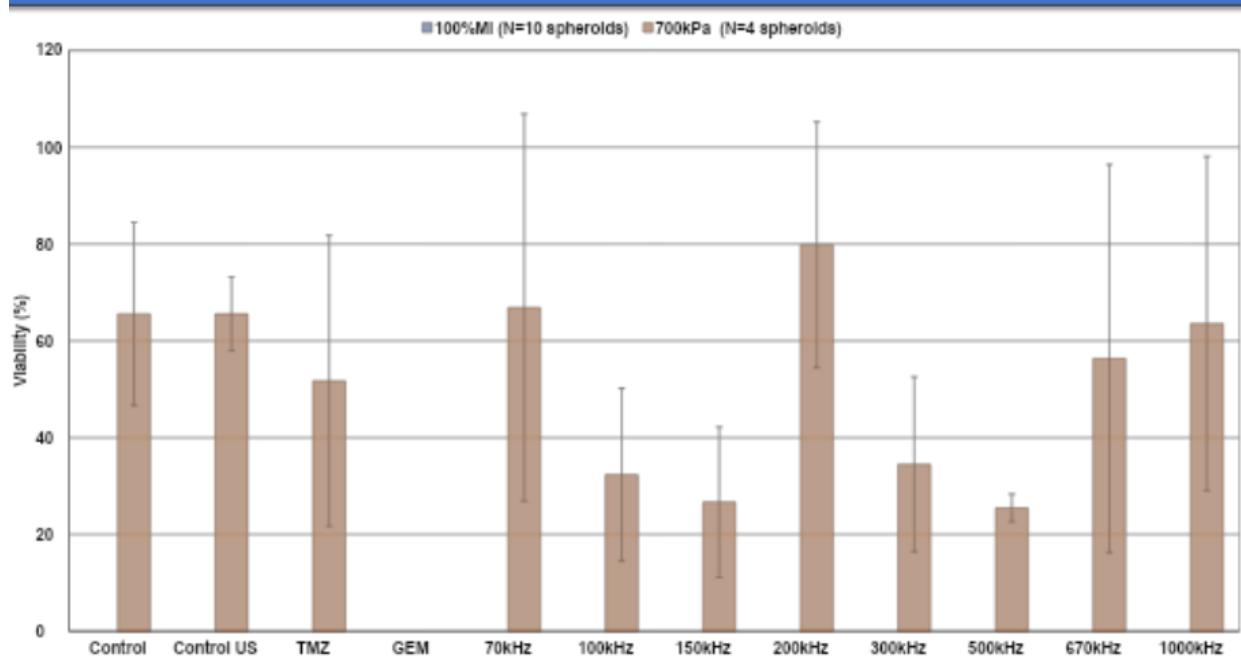
LN229 Summary



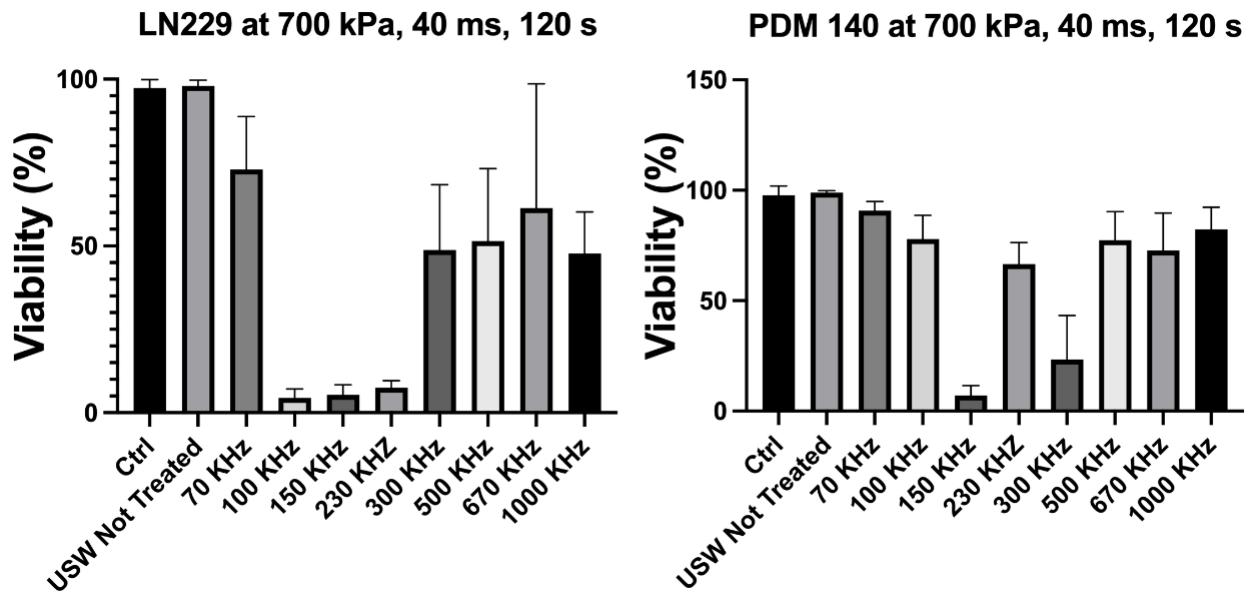
PDM140 Summary



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

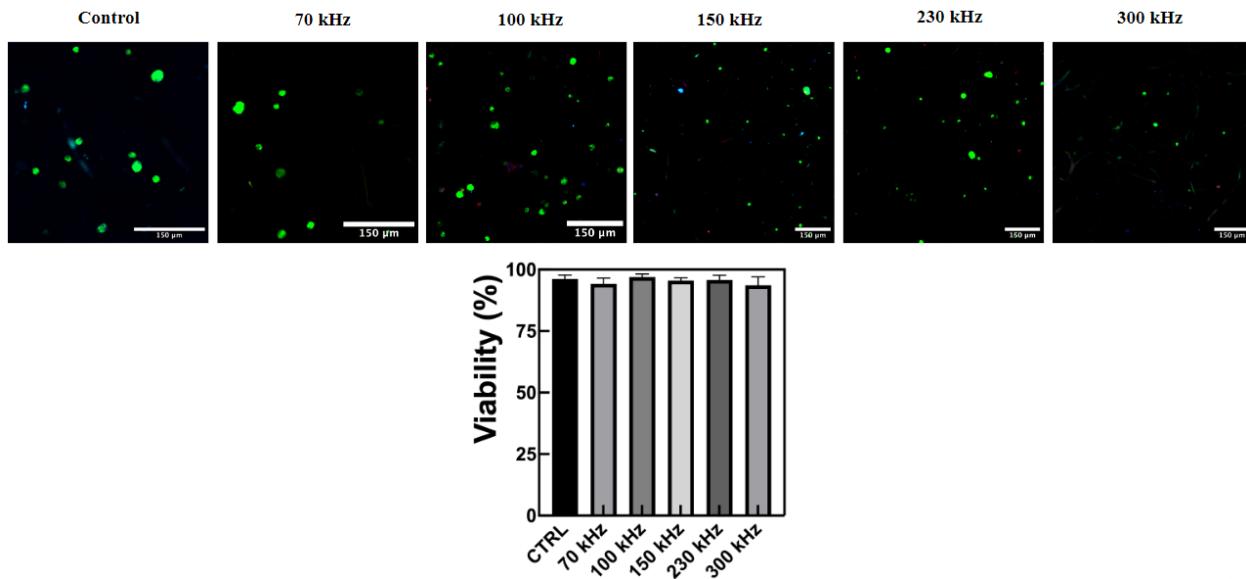
Phase 1.2 - Optimizing Ultrasound Parameters and testing of GBM spheroids and Healthy Cells.

All experiments in this phase use the optimized parameters unless specified

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

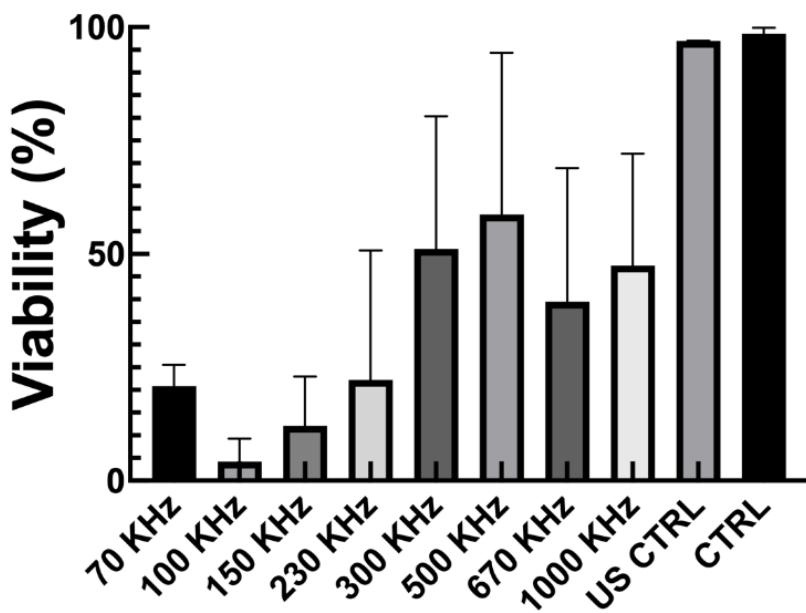
ULTRASOUND TREATMENT: 100% MI, 40ms, 120s

Pericytes



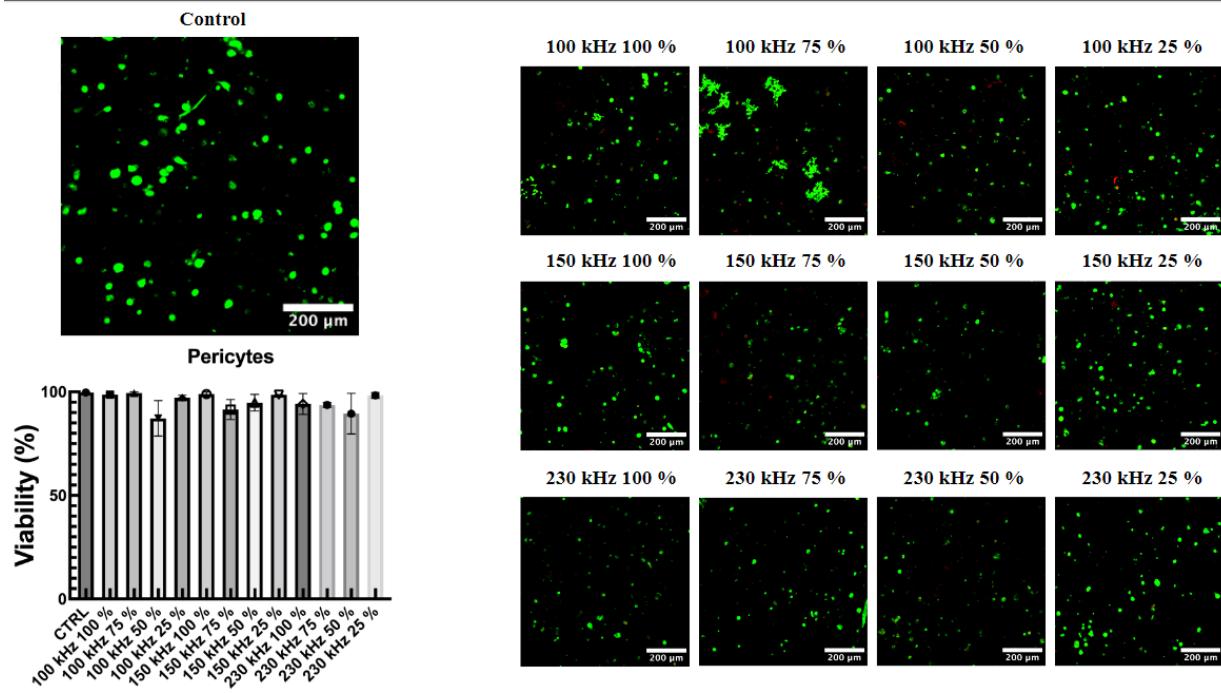
ULTRASOUND TREATMENT: 100% MI, 40ms, 120s

U87



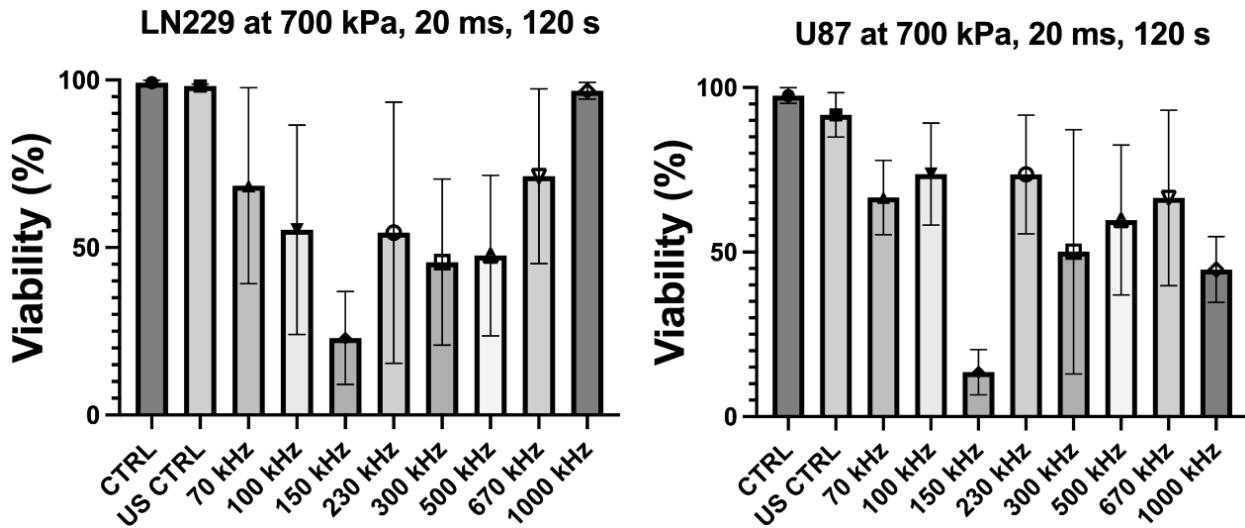
August 31, 2022 - Pericyte frequency and MI sweeps from 100 kHz to 230kHz

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



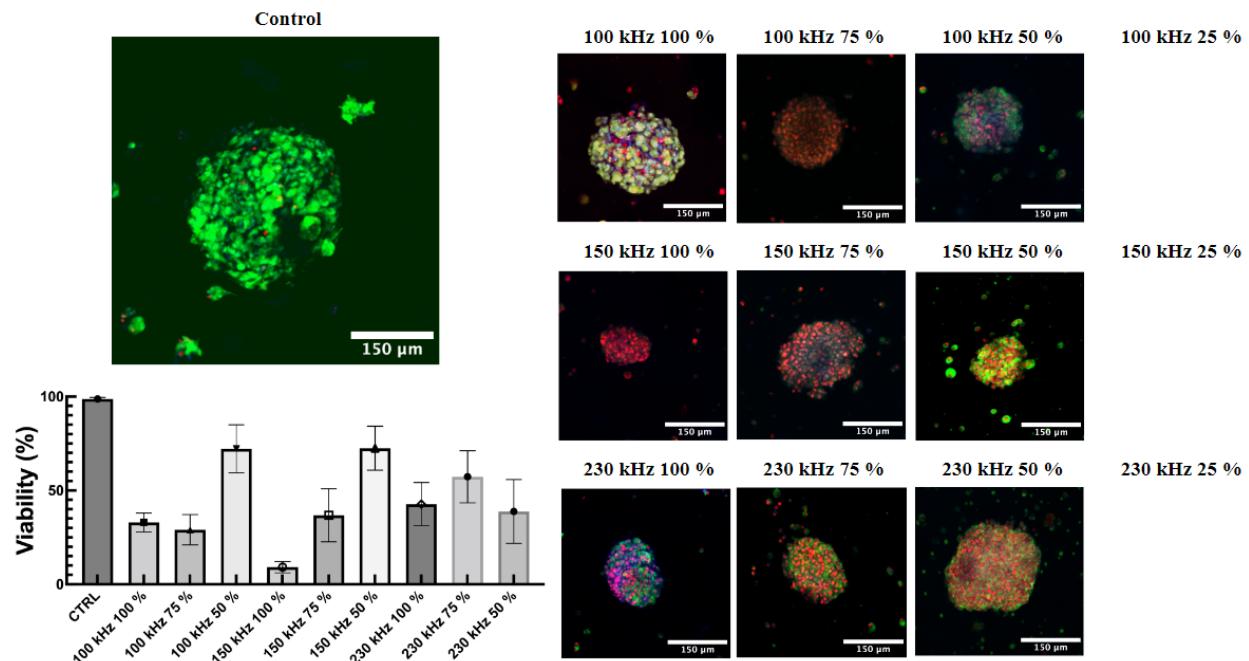
Frequency sweeps of two cell types (LN229 and PDM140) - constant pressure

ULTRASOUND TREATMENT: 700 kPa, 20ms, 120s

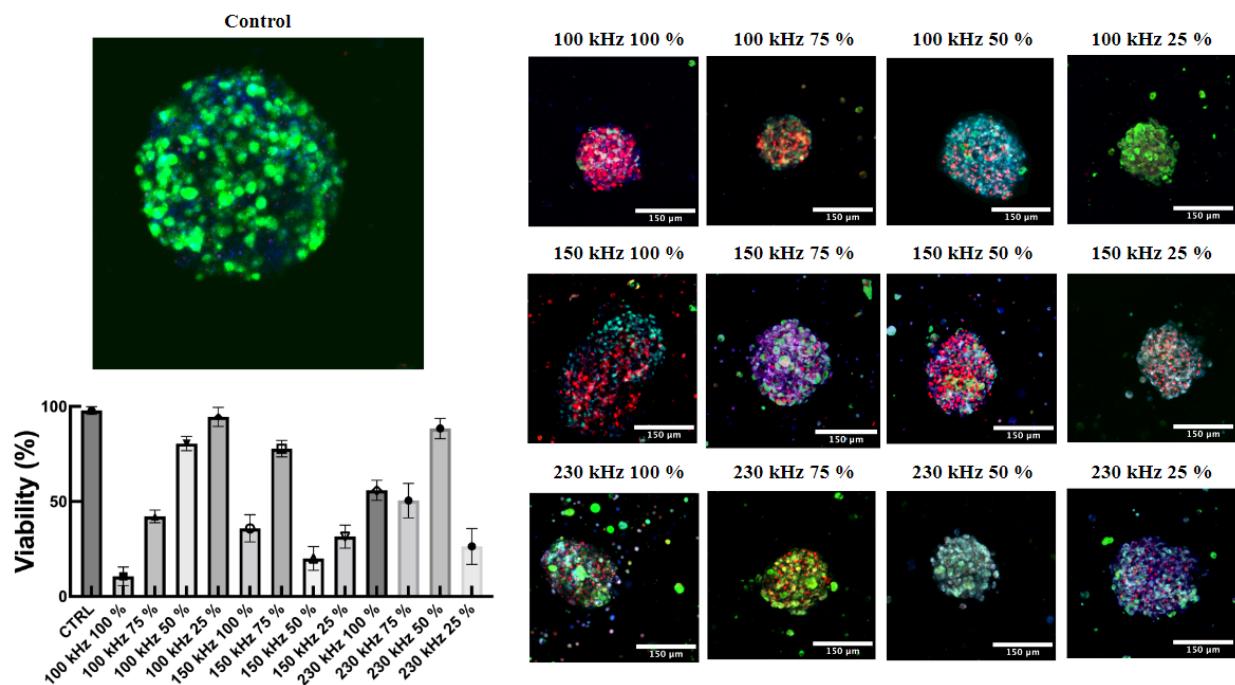


September 7, 2022 - Cell spheroid ultrasound treatment 100kHz - 230kHz
(variable MI)

ULTRASOUND TREATMENT: LN229

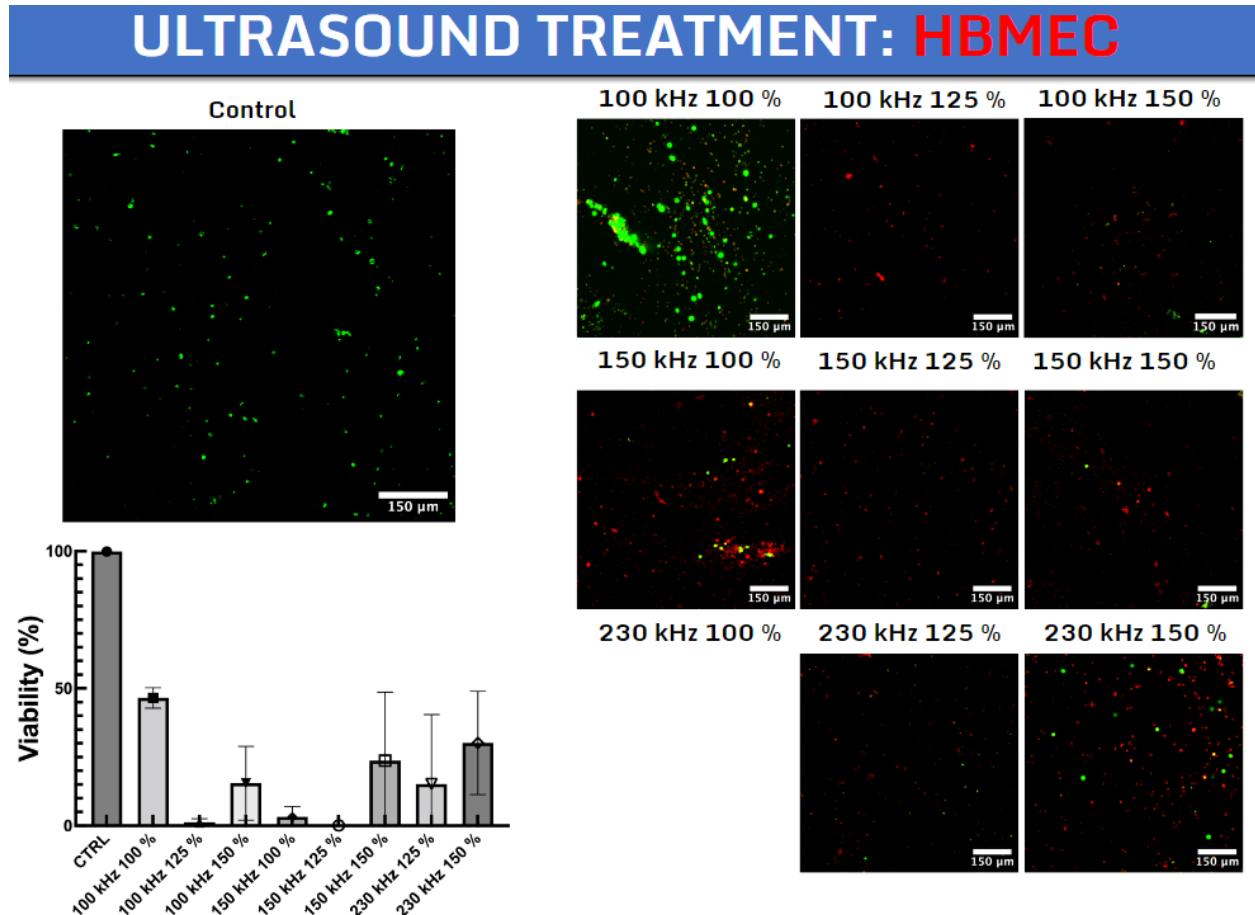


ULTRASOUND TREATMENT: U87

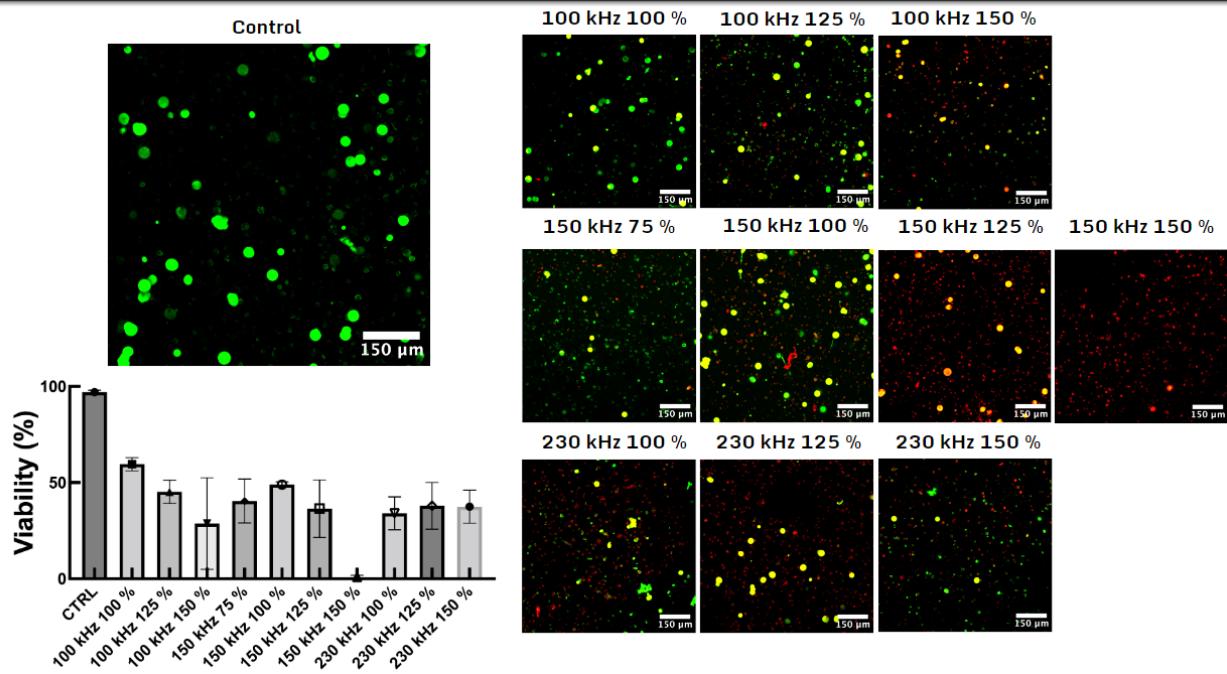


September 14, 2022 - no useful data

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

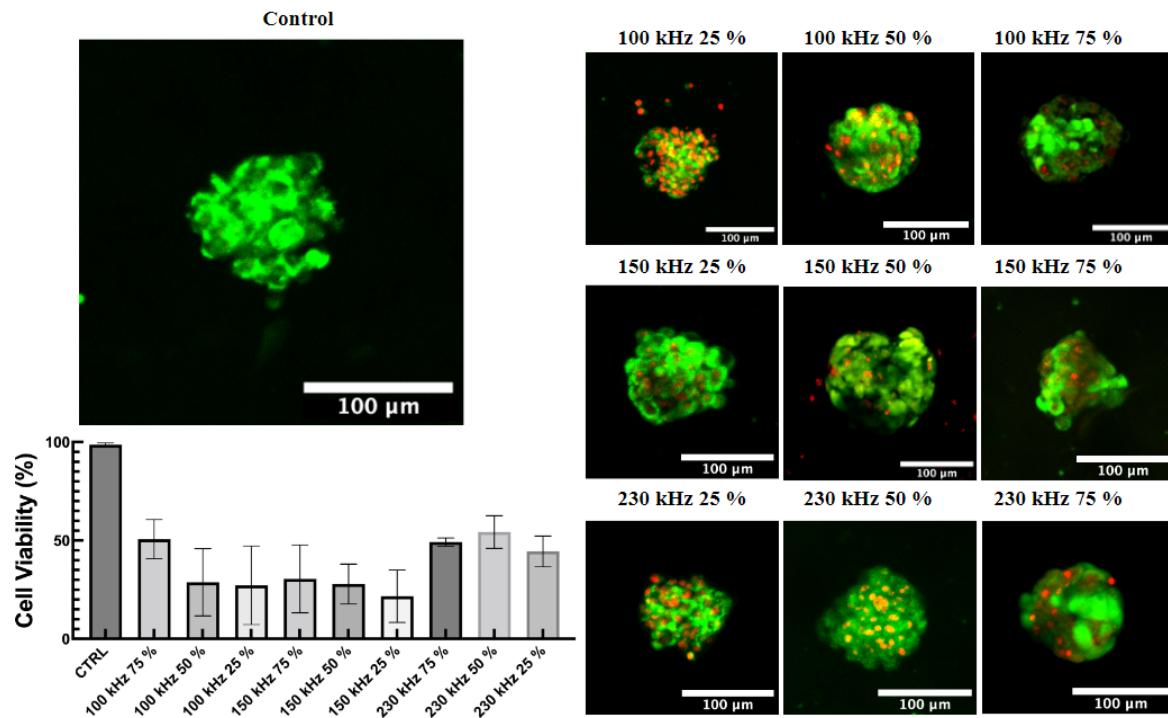


ULTRASOUND TREATMENT: HDF

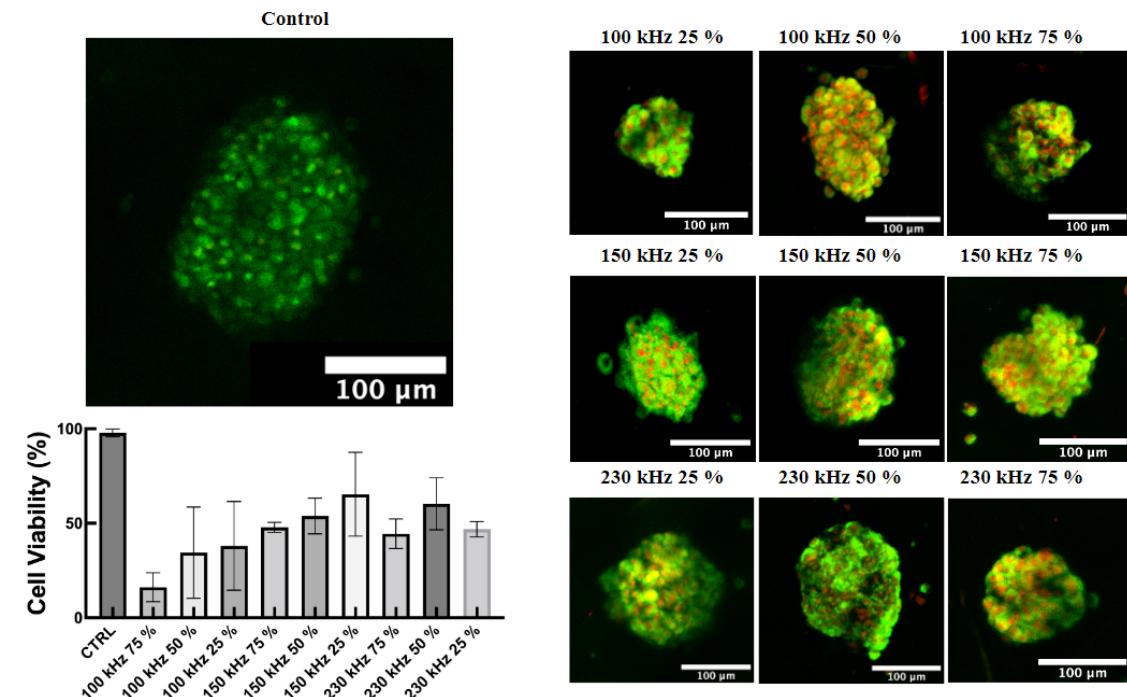


September 28, 2022 - Cell spheroid ultrasound treatment 100kHz - 230kHz (variable MI)

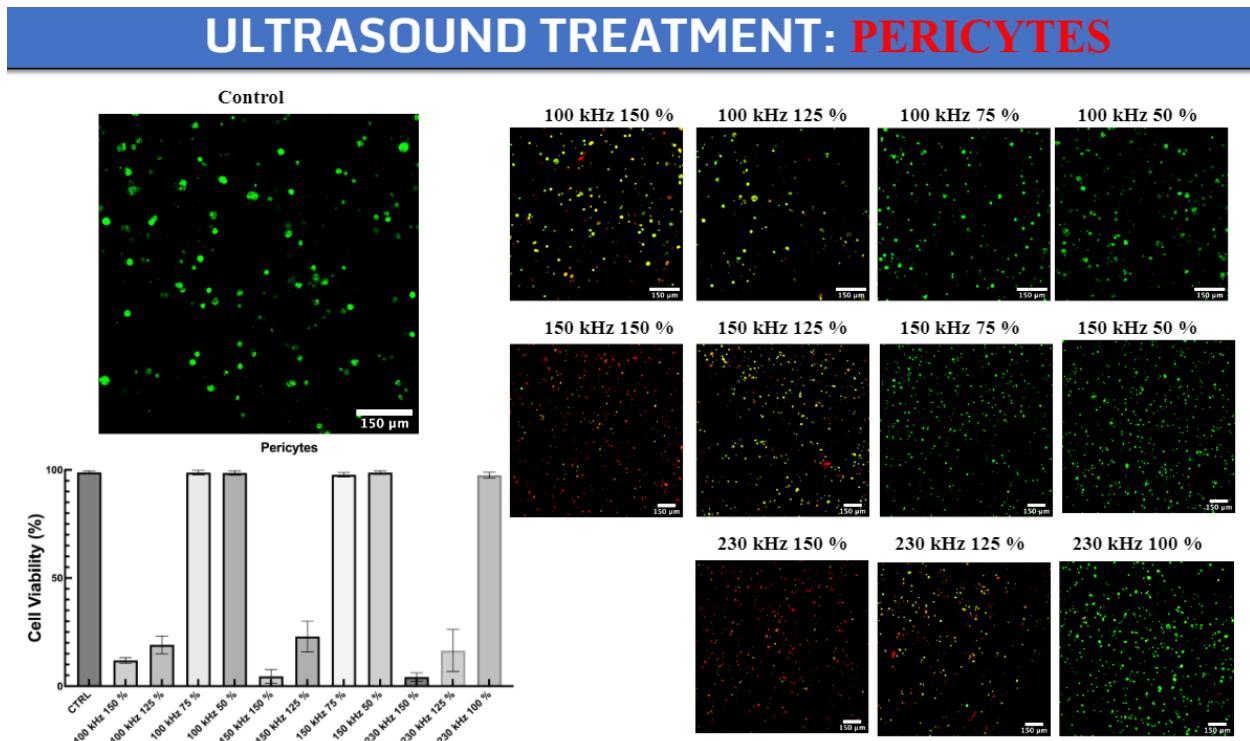
ULTRASOUND TREATMENT: LN229



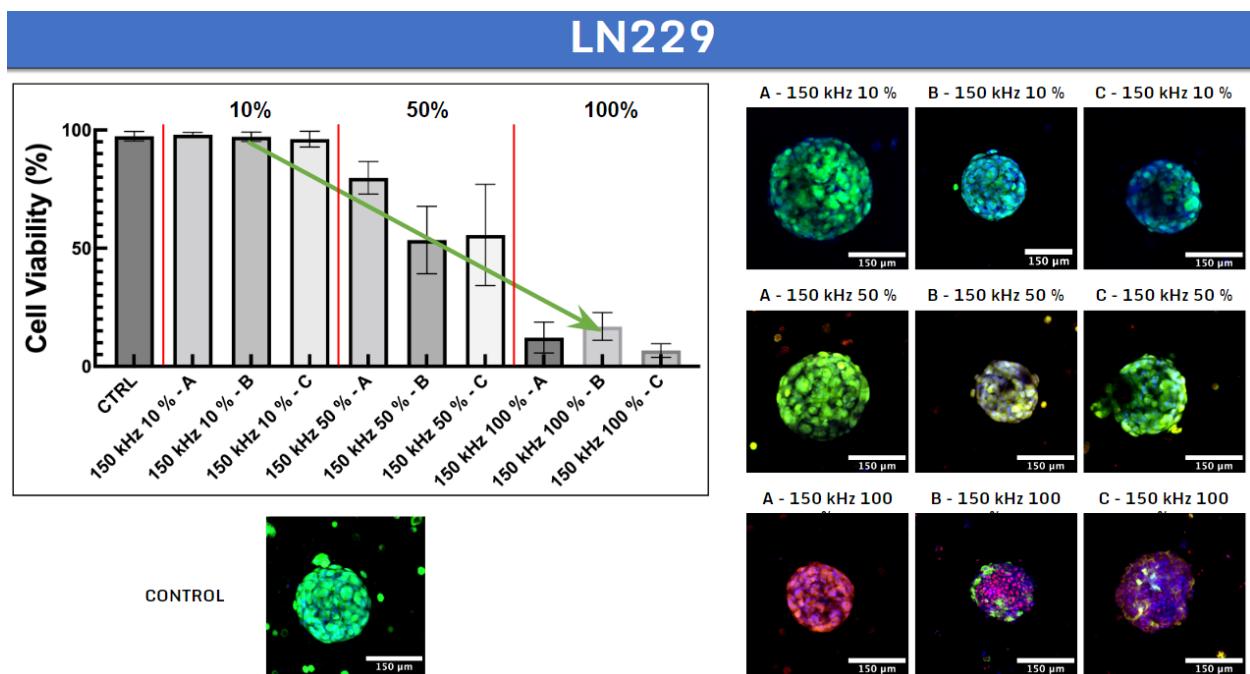
ULTRASOUND TREATMENT: U87



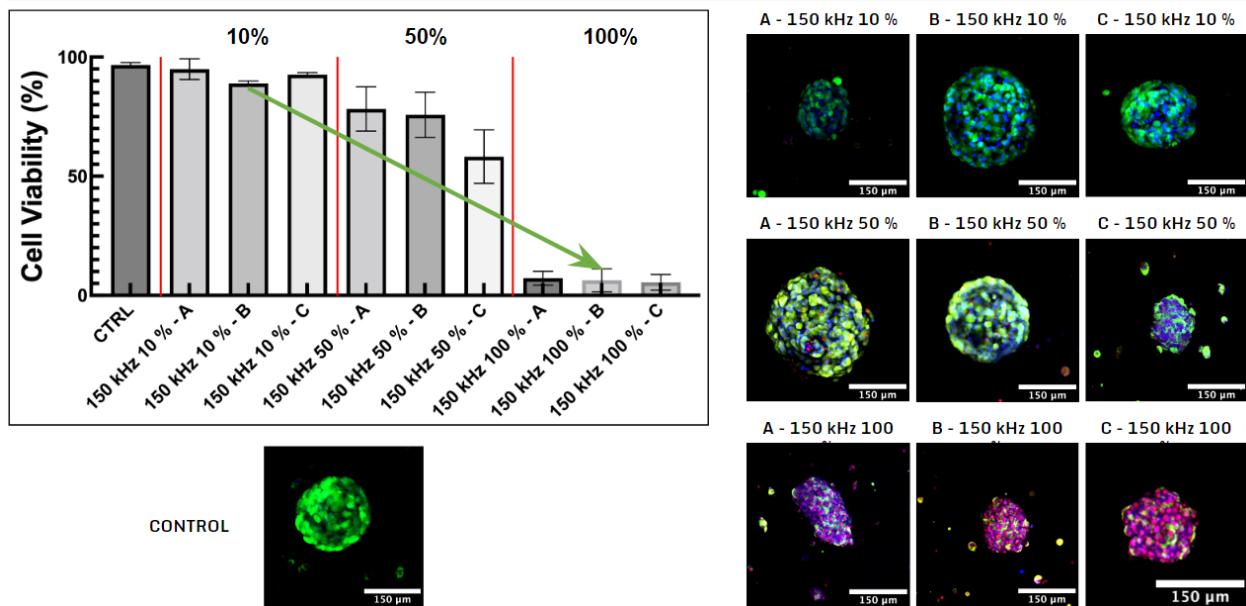
October 12, 2022 - ultrasound treatment slides of pericytes at 50-150% MI



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

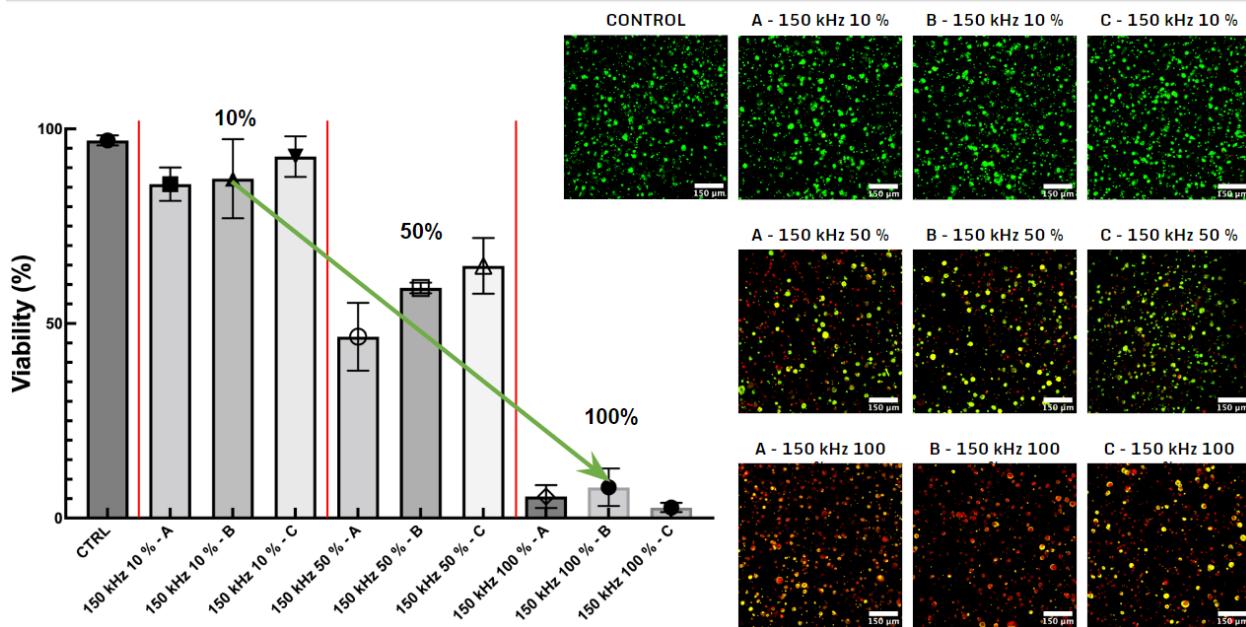


PDM140

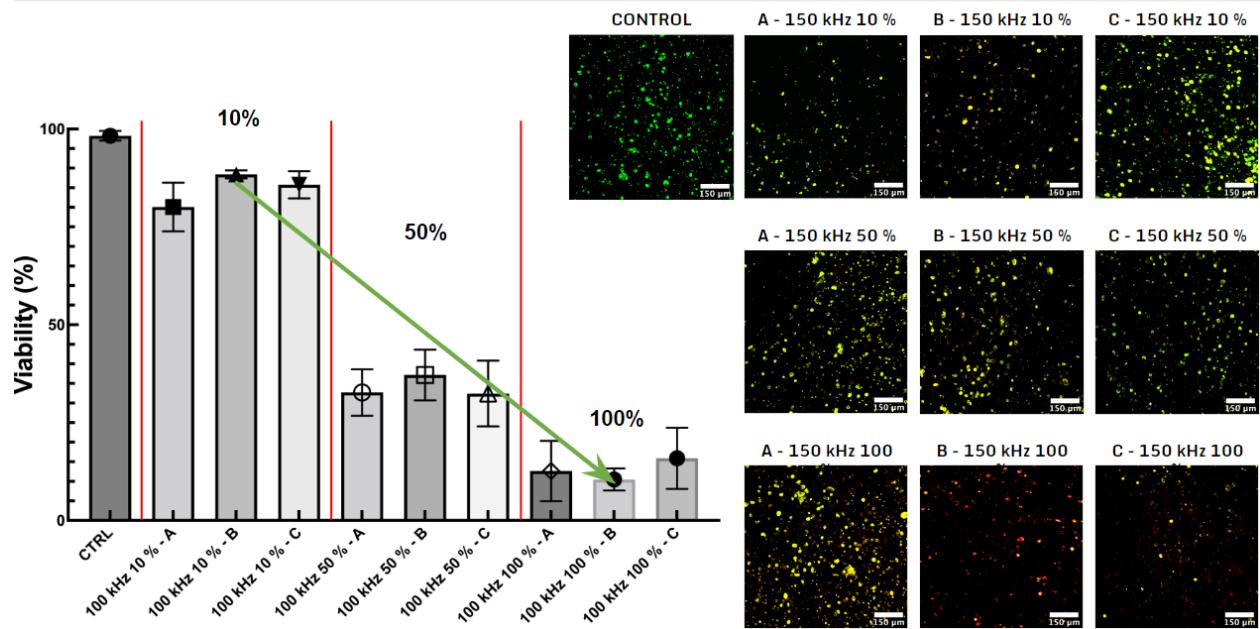


October 26, 2022 - 150kHz cell-laden hydrogel treated with variable MI (10%-100%)

LN229 (cell-laden hydrogel)

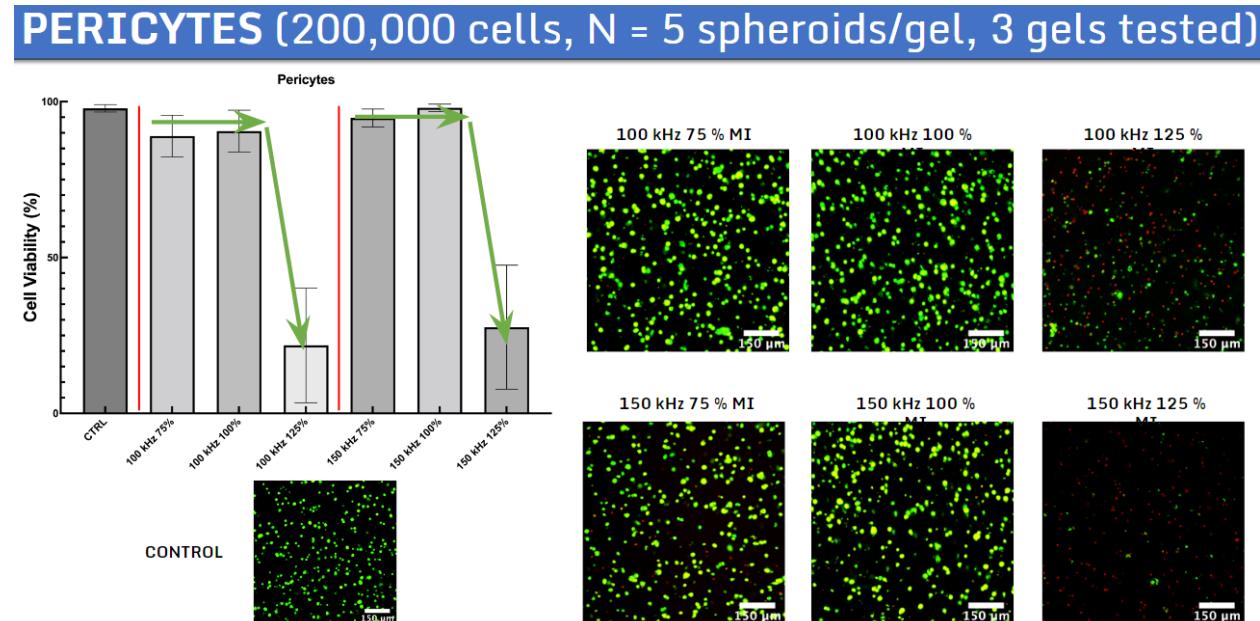


U87 (cell-laden hydrogel)

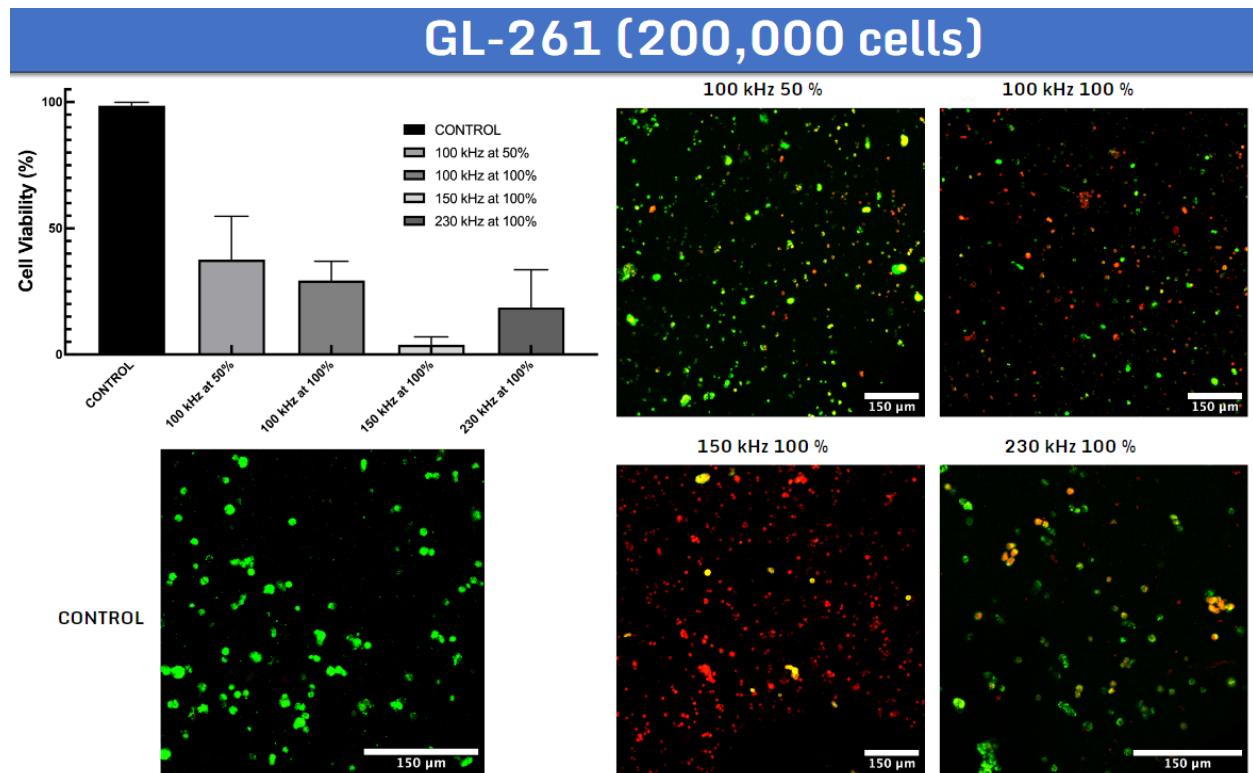


November 2, 2022 - 150kHz cell-laden hydrogel treated with variable MI (10%-100%)

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

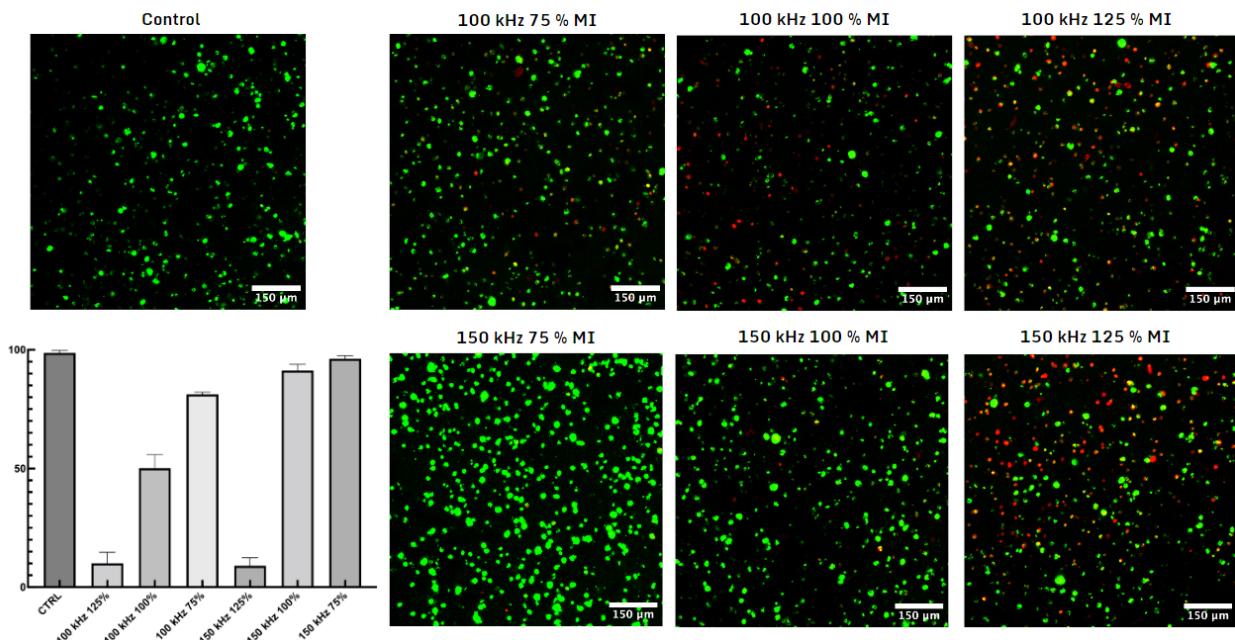


November 9, 2022 - 100kHz, 150kHz, and 230kHz US treatments of GL261 cell-laden hydrogel treated with variable MI (50% and 100%)

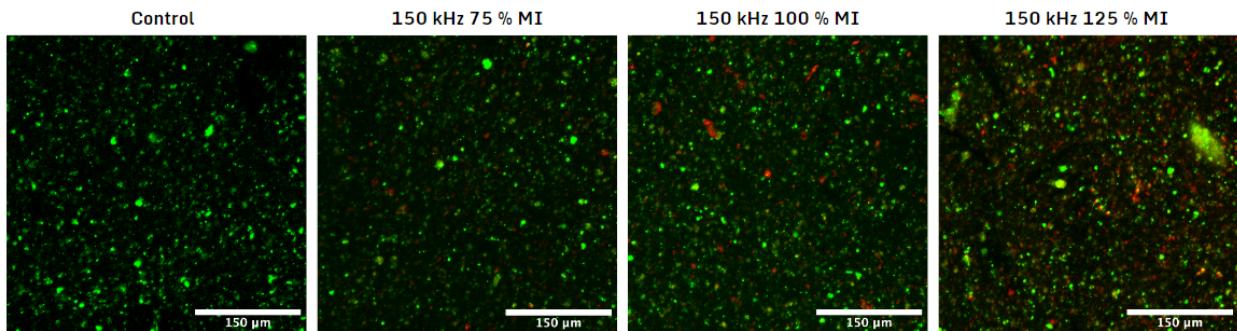


November 23, 2022 - 100kHz and 150kHz US treatments of HBMECs and mouse brain cell-laden hydrogel treated with variable MI (75%, 100%, and 125%)

HBMECs (200,000 cells per hydrogel)

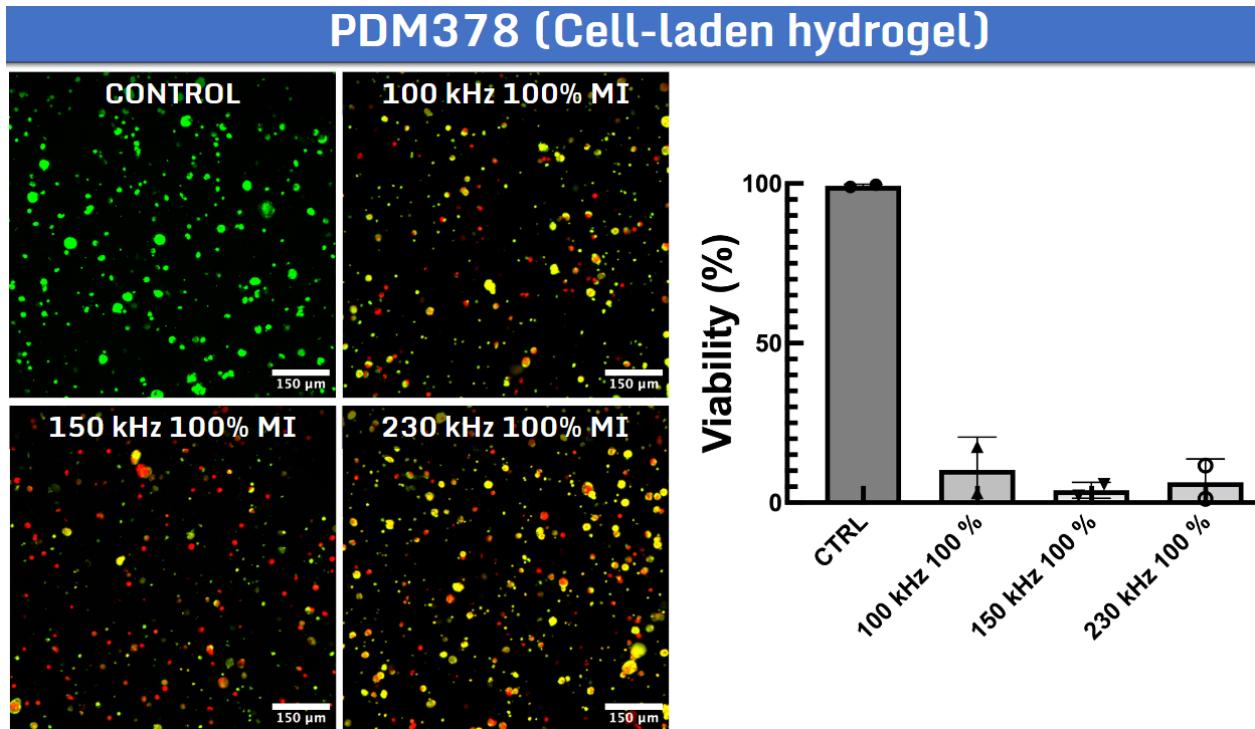


Mouse Brain Cells (400,000 cells per hydrogel)



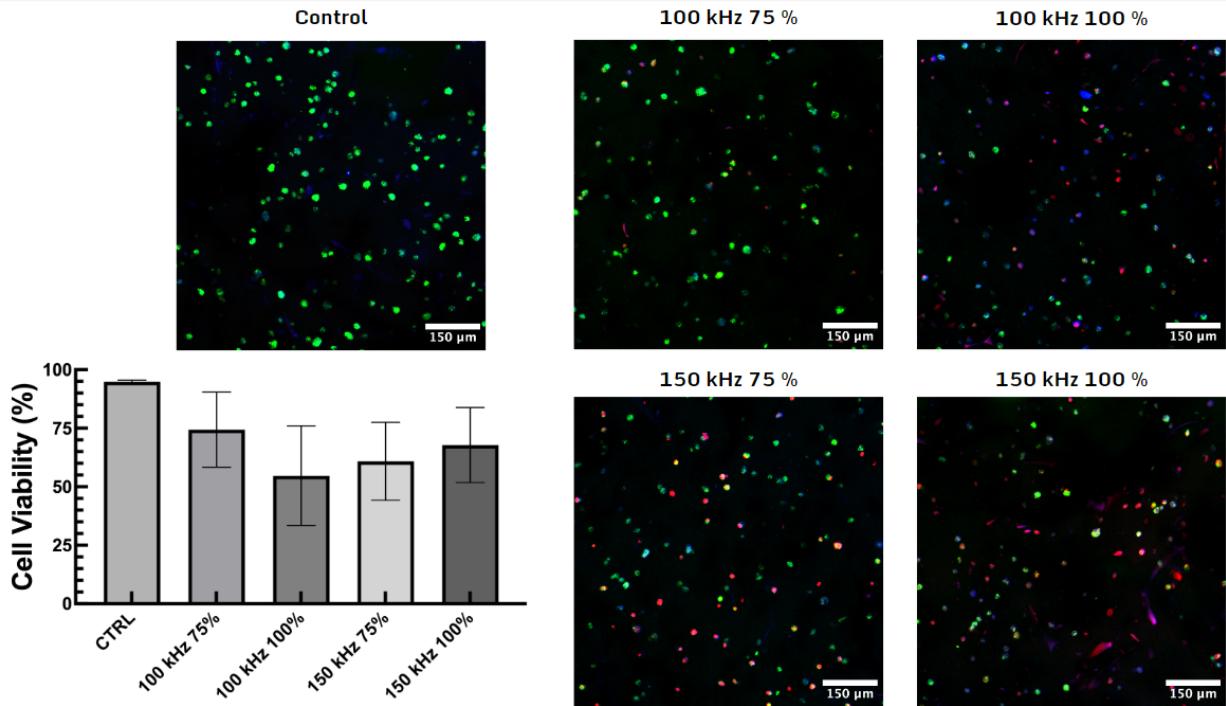
Quantification of viability was not possible due to background noise

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)

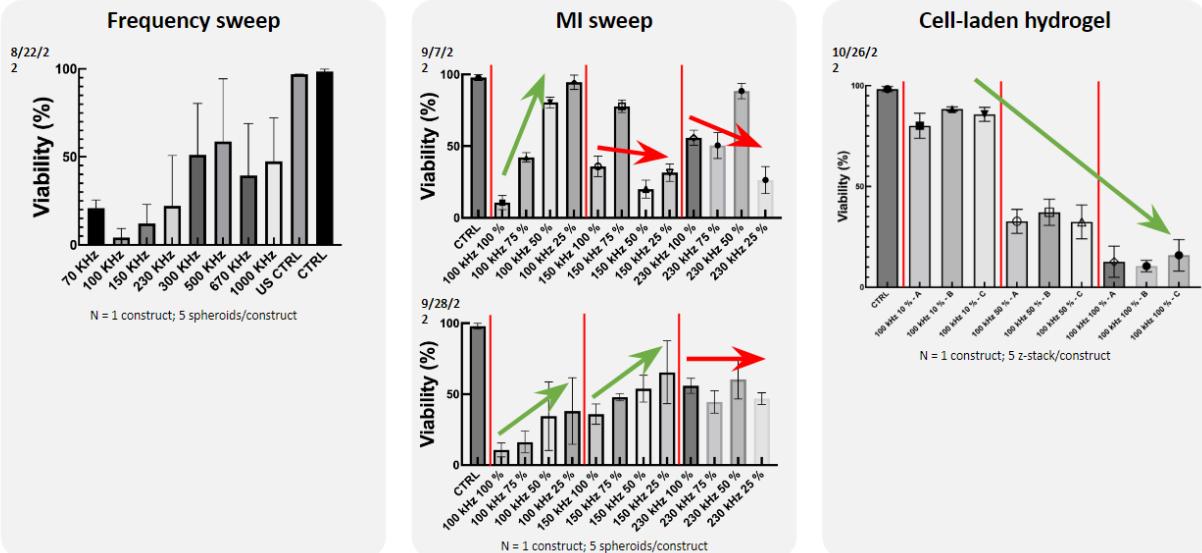


December 21, 2022 - Summary slides of in vitro treatments of both tumor and non-tumor cells

1.2. In vitro evaluation of ultrasonic therapy efficacy

U87

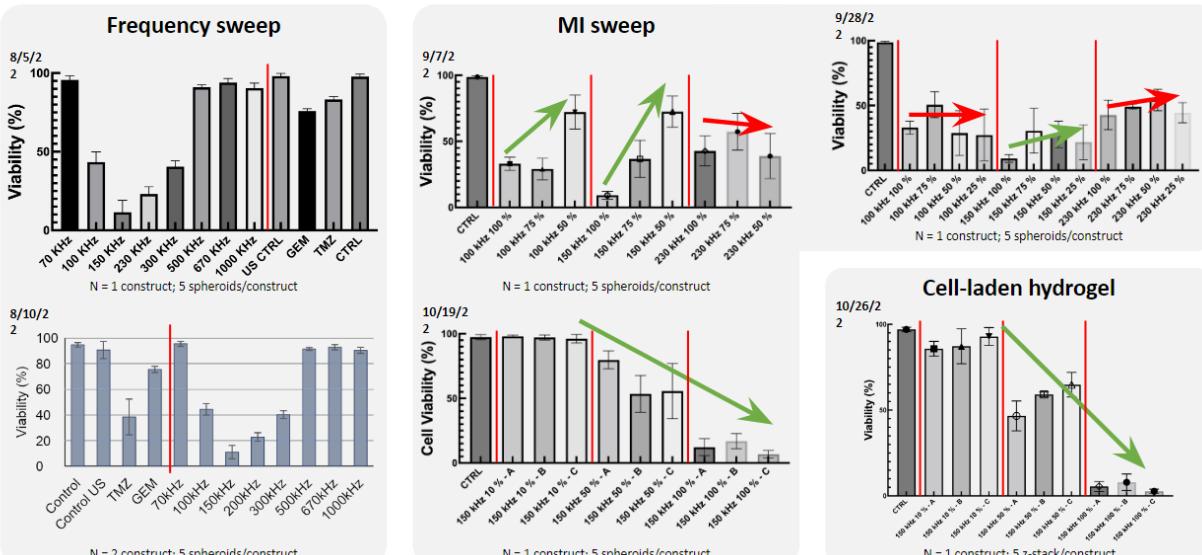
Target parameters: Frequency: 100kHz; MI: 100%; Burst length: 40ms; Treatment : 120s



1.2. In vitro evaluation of ultrasonic therapy efficacy

LN229

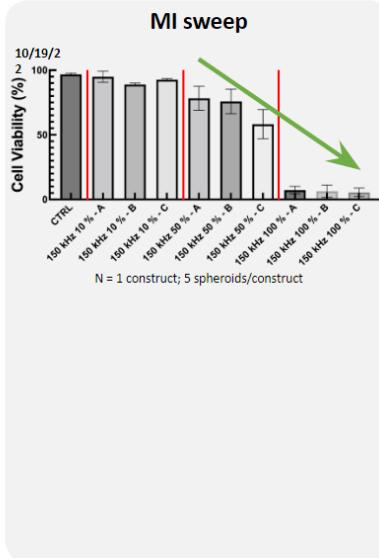
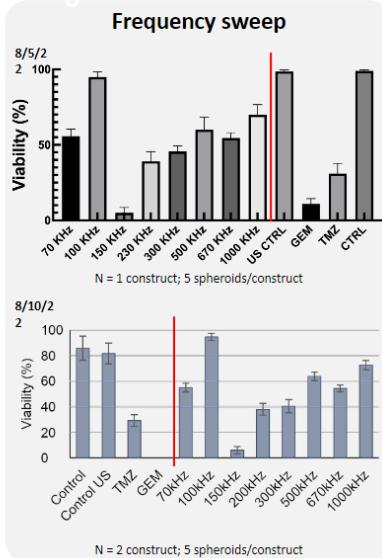
Target parameters: Frequency: 150kHz; MI: 100%; Burst length: 40ms; Treatment : 120s



1.2. *In vitro* evaluation of ultrasonic therapy efficacy

PDM14

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

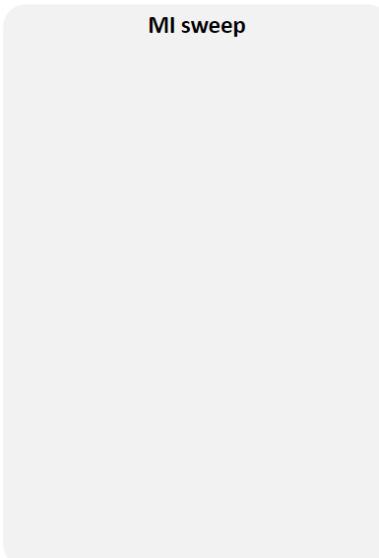
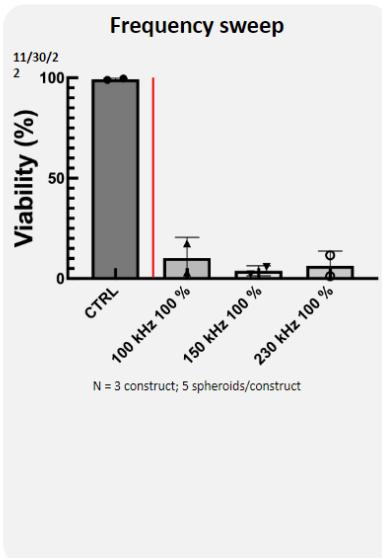


Cell-laden hydrogel

1.2. *In vitro* evaluation of ultrasonic therapy efficacy

PDM378

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

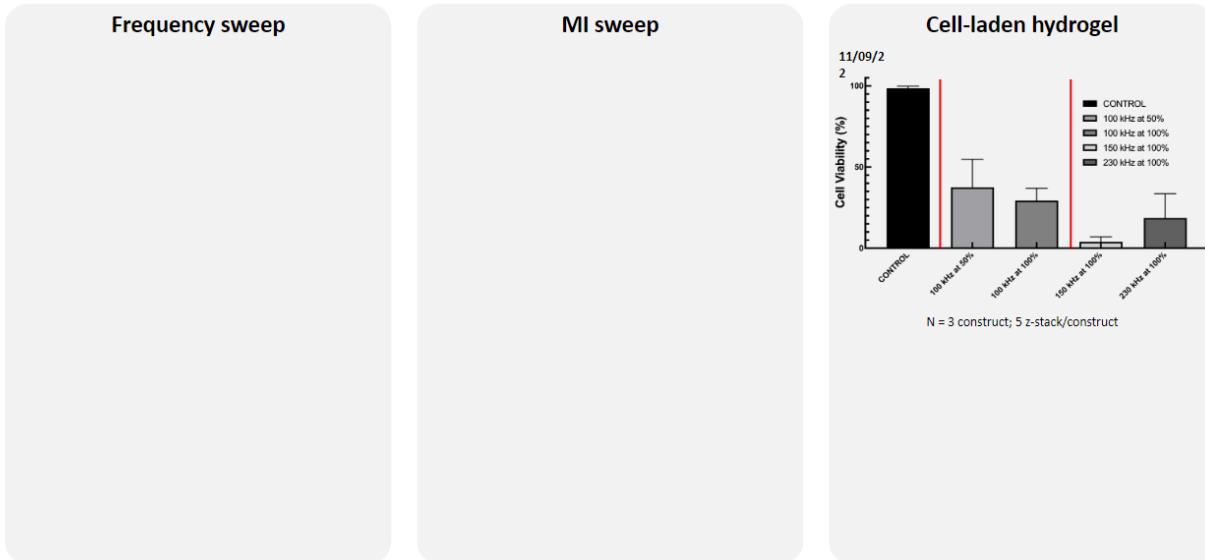


Cell-laden hydrogel

1.2. *In vitro* evaluation of ultrasonic therapy efficacy

GL261

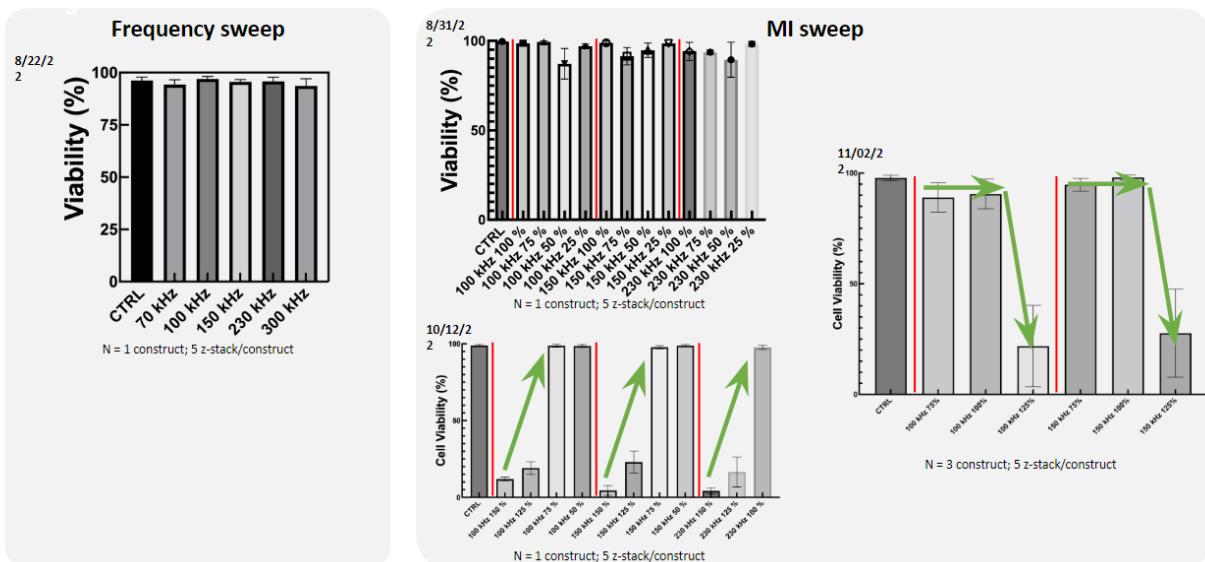
Target parameters: Frequency: **150kHz**; MI: **100%**; Burst length: **40ms**; Treatment : **120s**



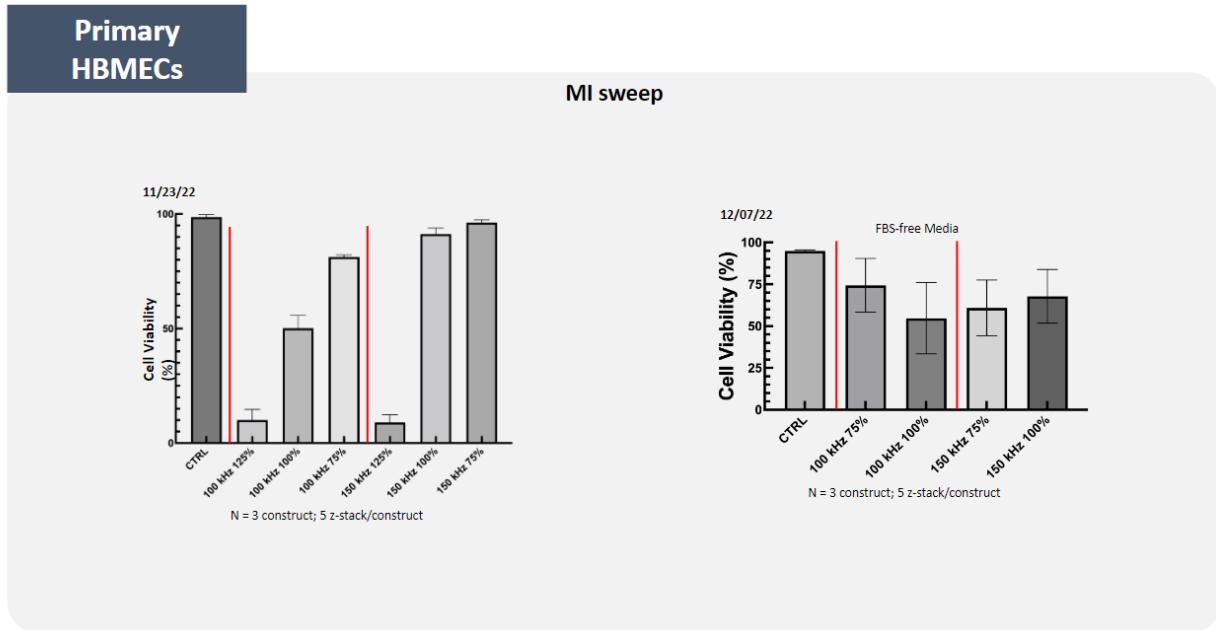
2.2. *In vitro* evaluation of ultrasonic therapy safety

Pericyte

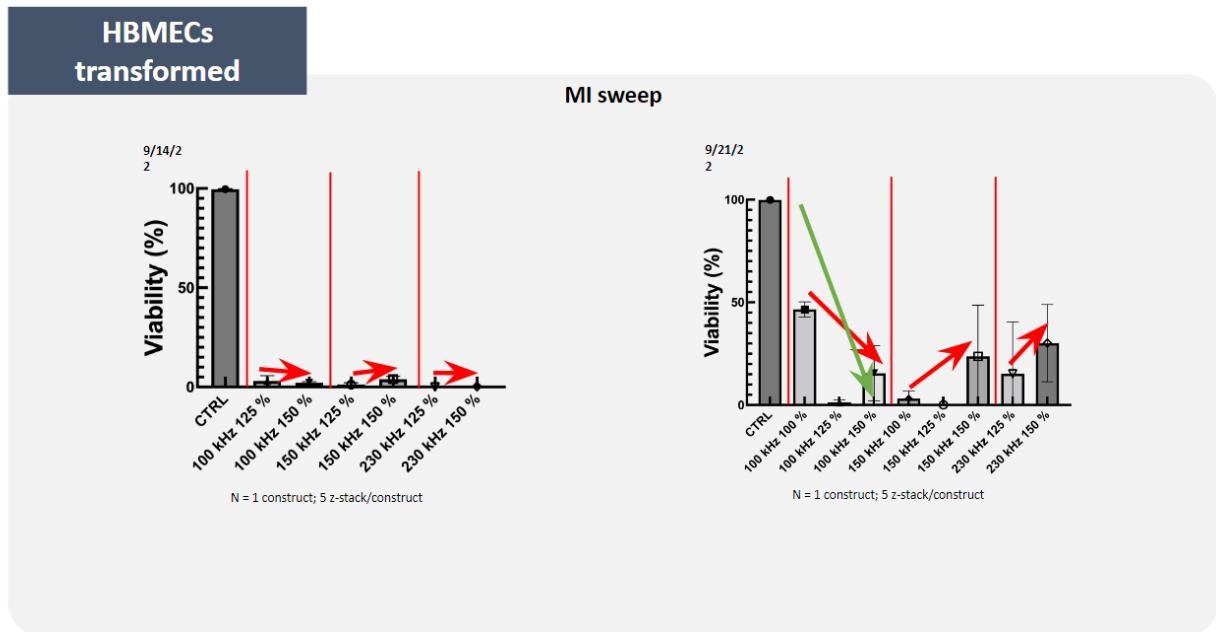
Resistance to **100kHz** and **150kHz** at **100%MI**



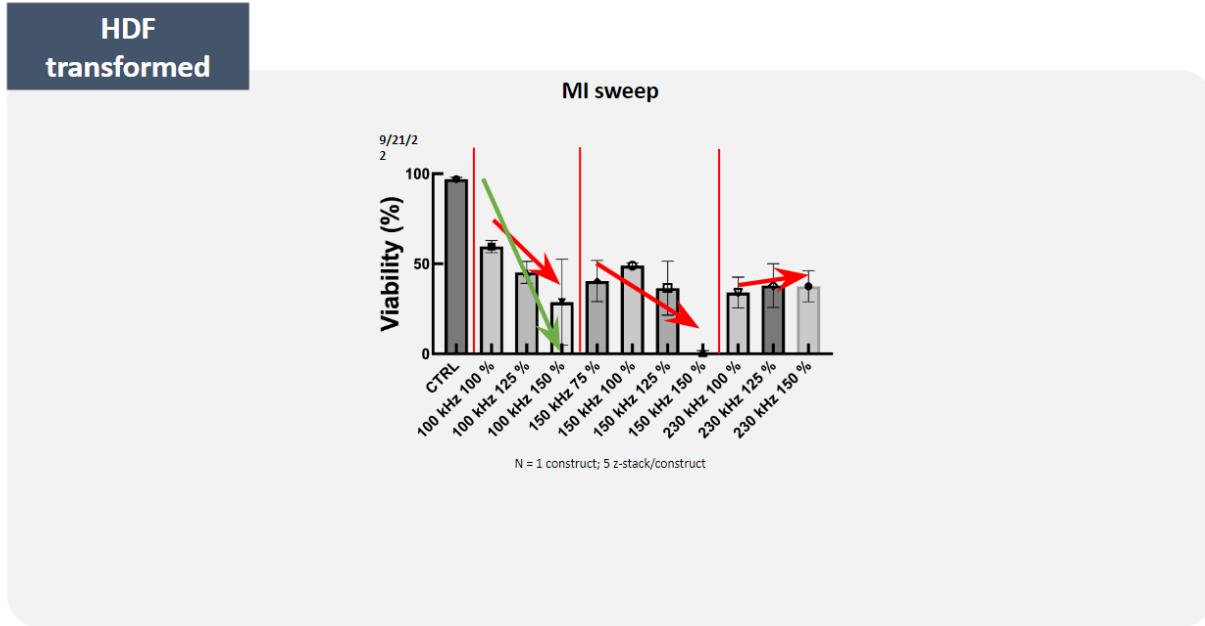
2.2. *In vitro* evaluation of ultrasonic therapy safety



2.2. *In vitro* evaluation of ultrasonic therapy safety



2.2. *In vitro* evaluation of ultrasonic therapy safety



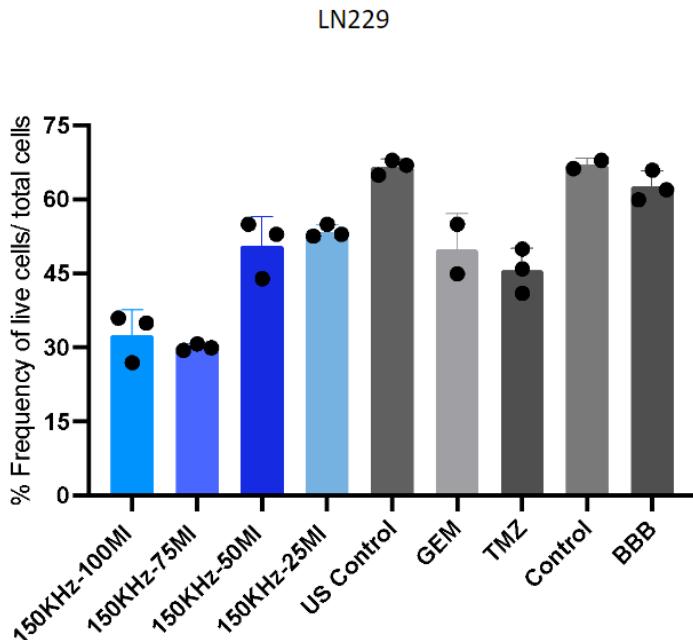
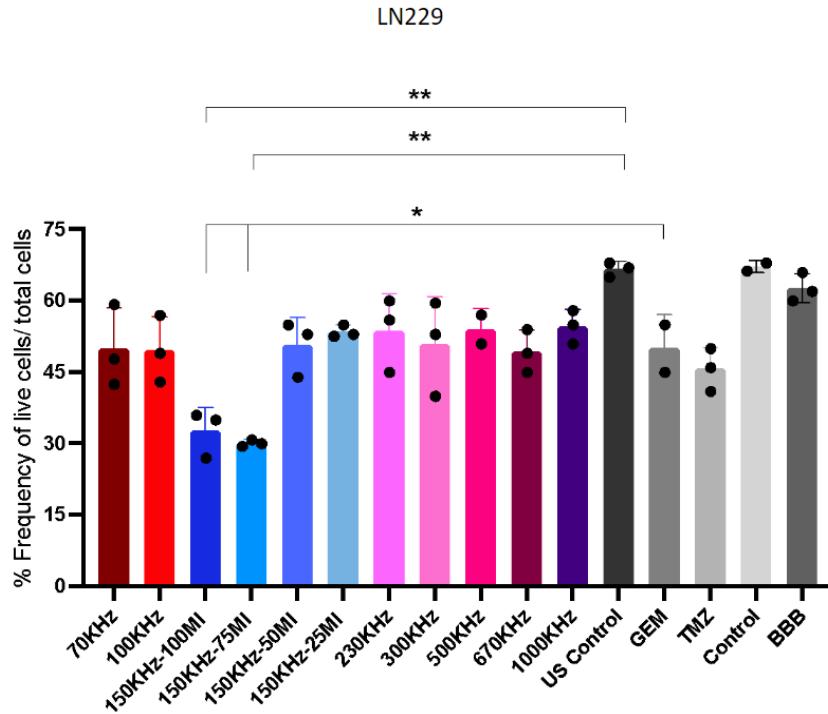
Summary of in vitro experiments done in Phase 1.1 and 1.2

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						
		150								
		125								
		100	40	120		99	98	99		0.7
		75				100	100	99		0.5
		50				79	86	96		8.5
	100	25				96	99	97		1.4
		150								
		125								
		100	40	120		98	100	99		0.9
HBMECs	150	75				93	96	86		4.8
		50				96	98	90		4.0
		25				99	99	99		0.1
		150								
		125								
	230	100	40	120		98	89	96		5.0
		75				95	94	92		1.2
		50				98	79	92		9.8
		25				100	97	99		1.4
		300	100	40	120					
HDF	100	150				17	28	1		13.4
		125				1	3	0		1.3
		100	40	120		47	50	43		3.7
		75								
		50								
	150	25								
		150				21	0	50		24.9
		125				0	0	0		0.0
		100				7	1	2		3.7
		75								
230	230	50				31	11	49		18.8
		25				0	44	1		25.3
		150								
		125								
		100	40	120						
	100	75								
		50								
		25								
		150								
		125								

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

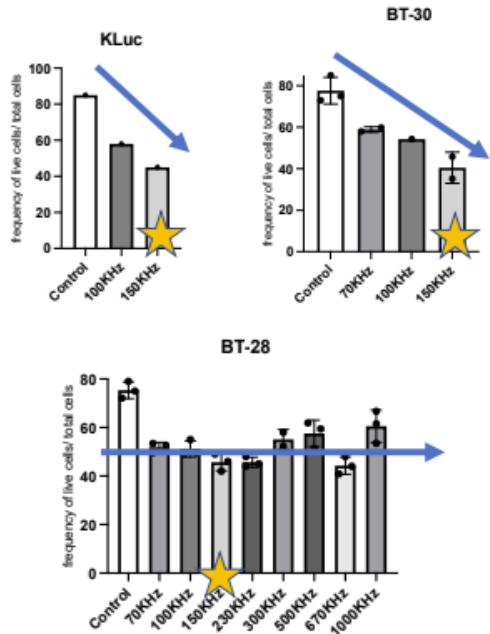
Feb 15, 2023 in vitro dome preparation protocol development. Protocol development went through May 15, 2023

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

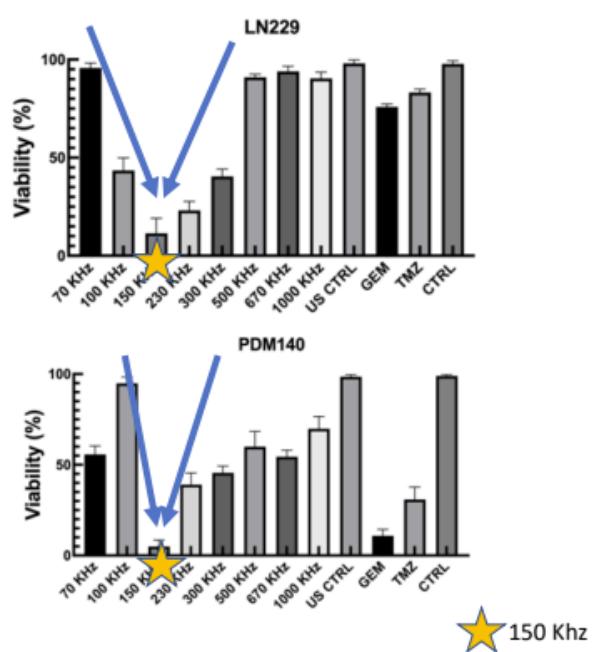


Apr 26, 2023 and May 3, 2023 -

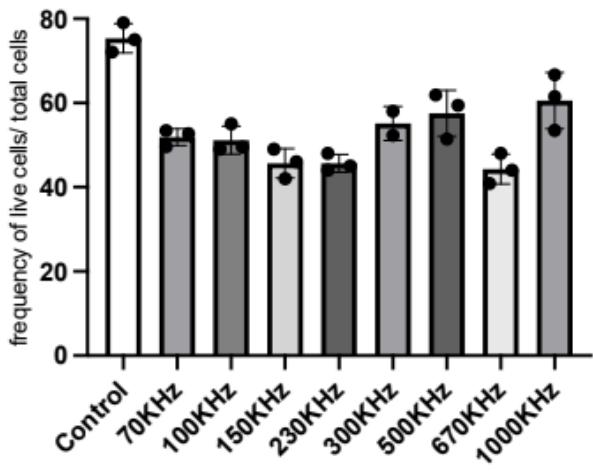
New Screen



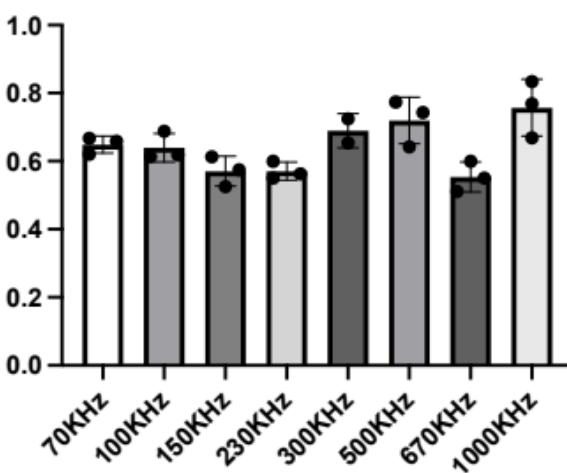
Prior Screen



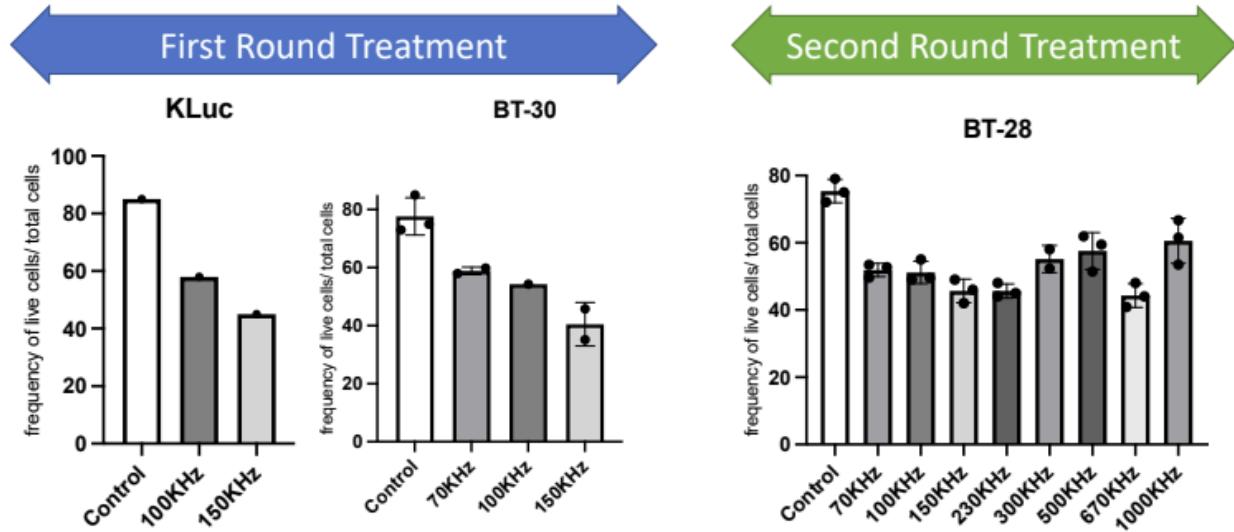
BT-28



BT28-second experiment (normalized)



Ultrasonic Treatment: Results

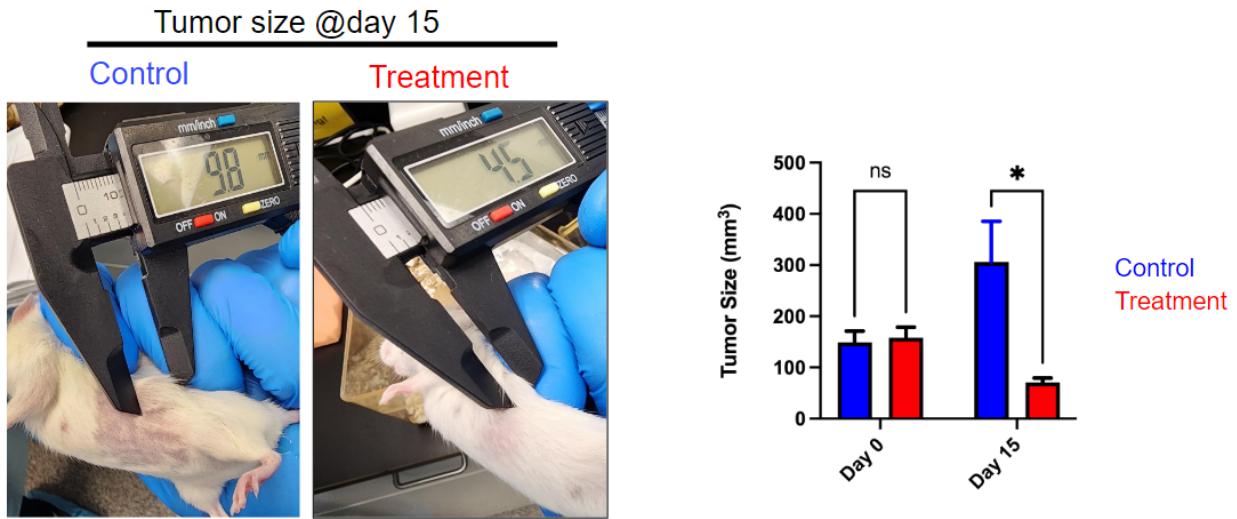


Phase 3: In Vivo - Mouse Flank

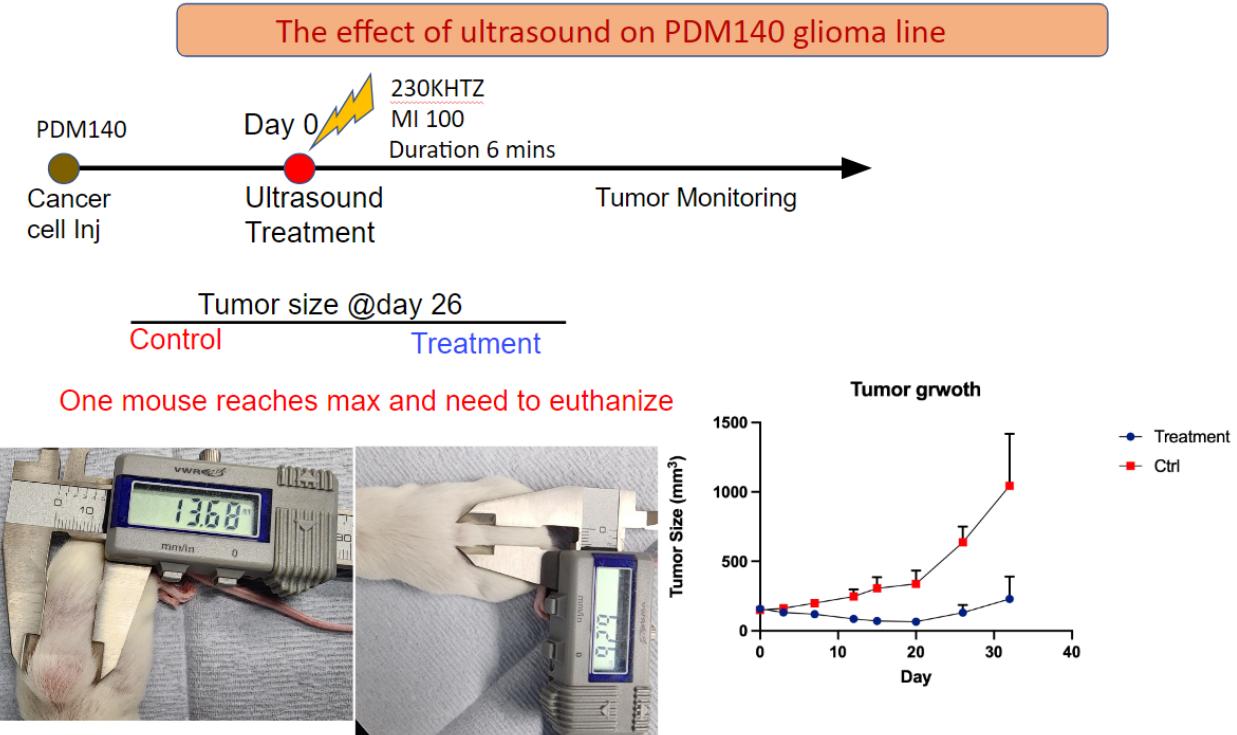
Mar 16, 2023 - in vitro dome preparation protocol development - in vivo treatment result (1 mouse?)

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.**

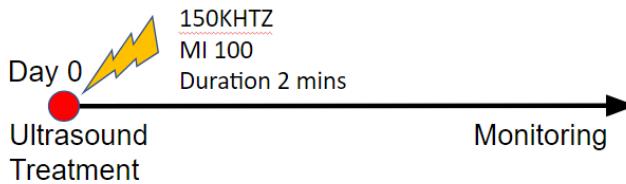
In Vivo tumor growth after a single treatment



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

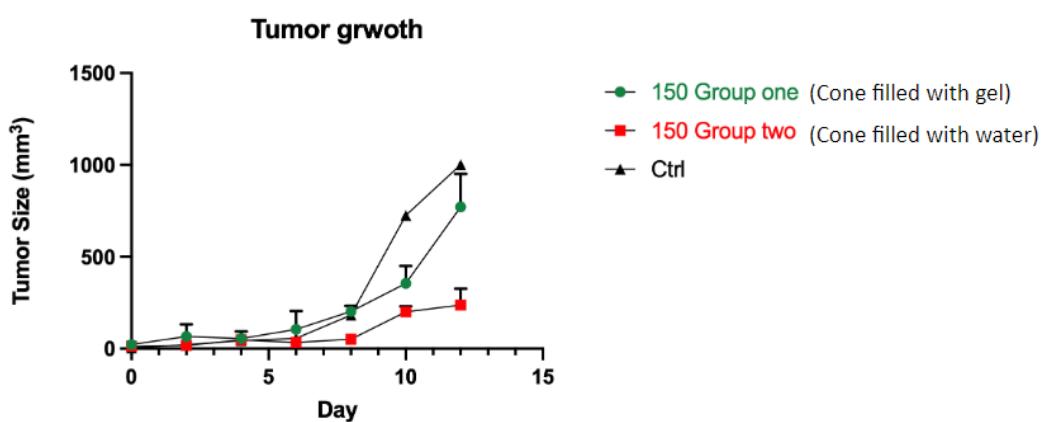
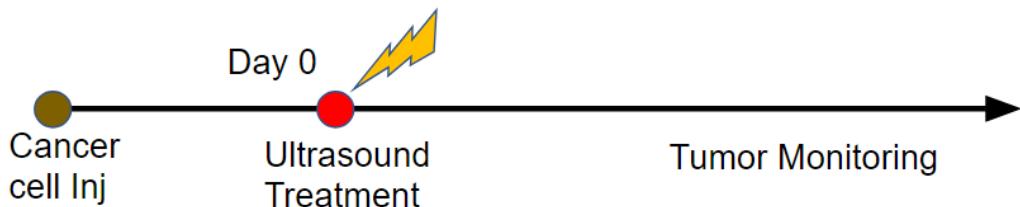


Healthy mouse treatments: No side effect, Vet tech Check

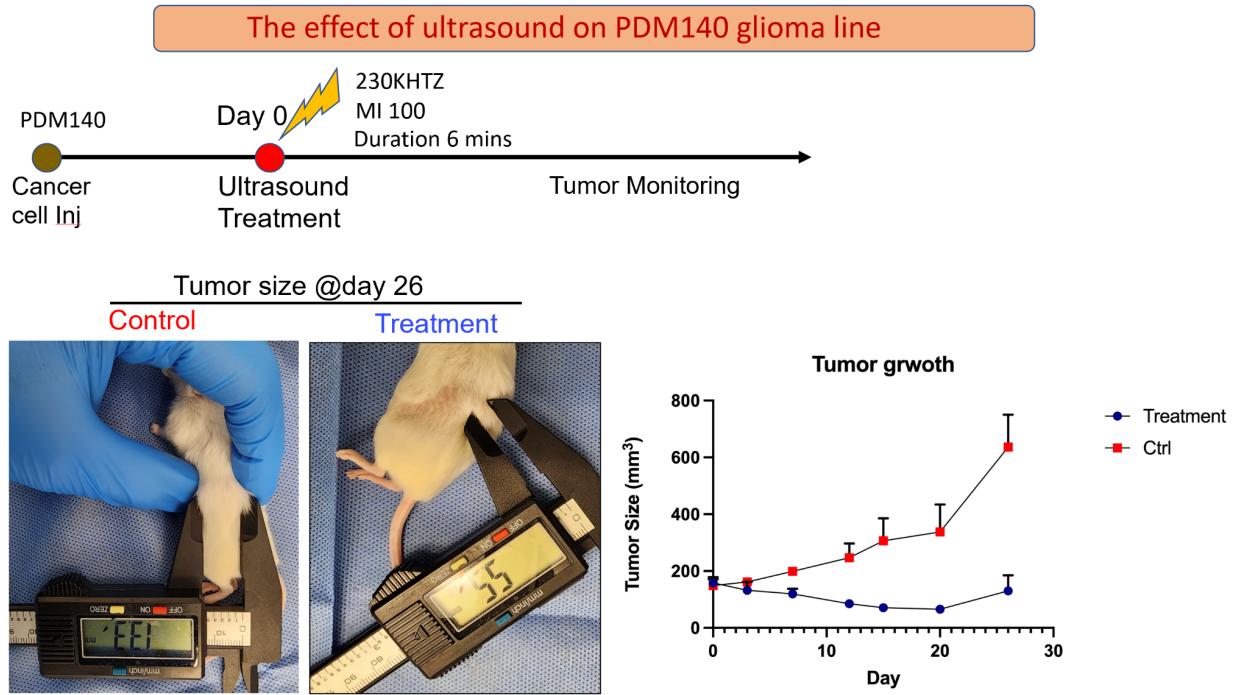


The cone filled with gel had a lot of air in it so it likely did not transmit the ultrasound completely

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



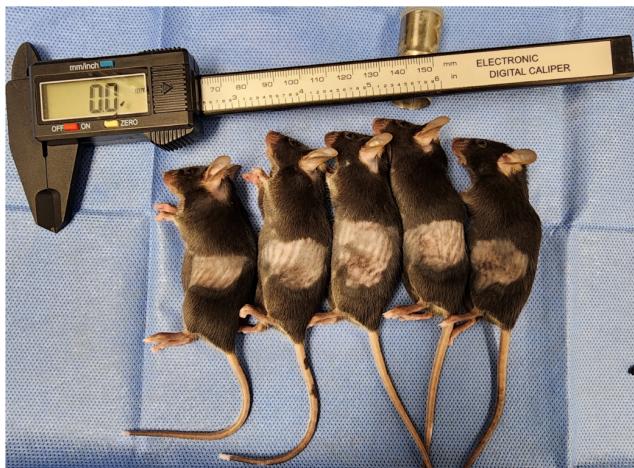
Mar 28, 2023 in vivo treatment results of 1 mouse @ 230kHz, MI 100% for 6 minutes, and some mice @ 150kHz, MI 100%, and 2 minutes.



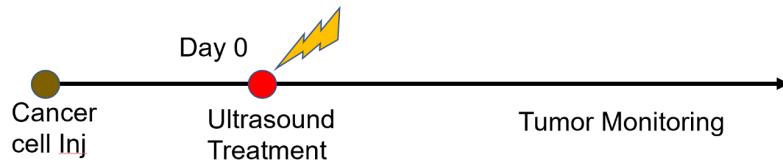
The effect of ultrasound on healthy mice (no tumor engraftment)



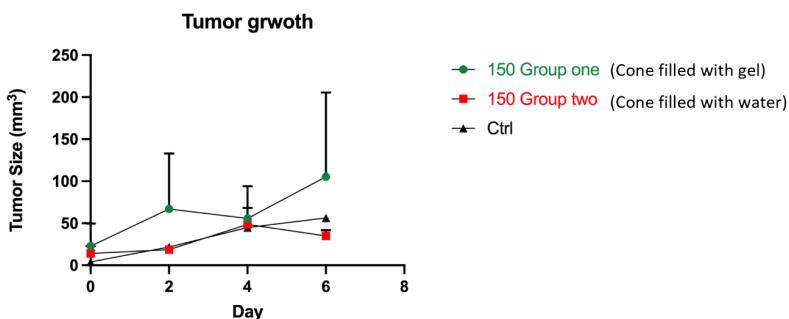
Healthy mouse treatments: No side effect



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

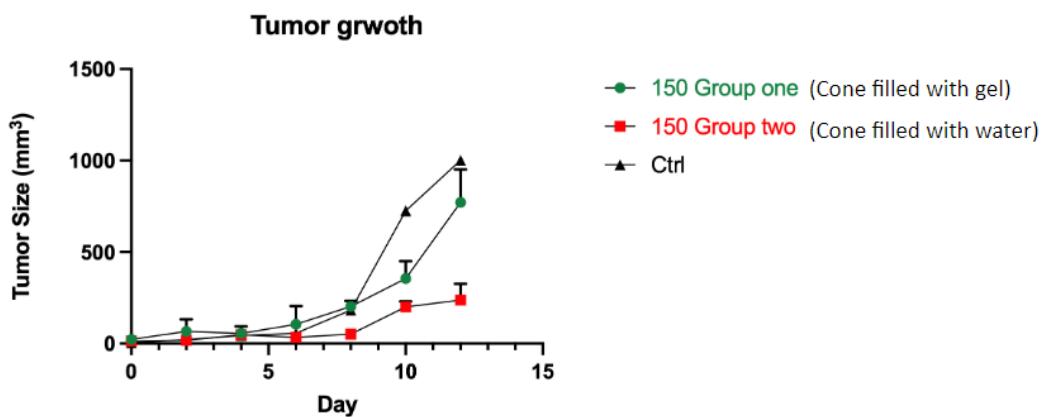


One mouse from group 150 one has issue



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

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



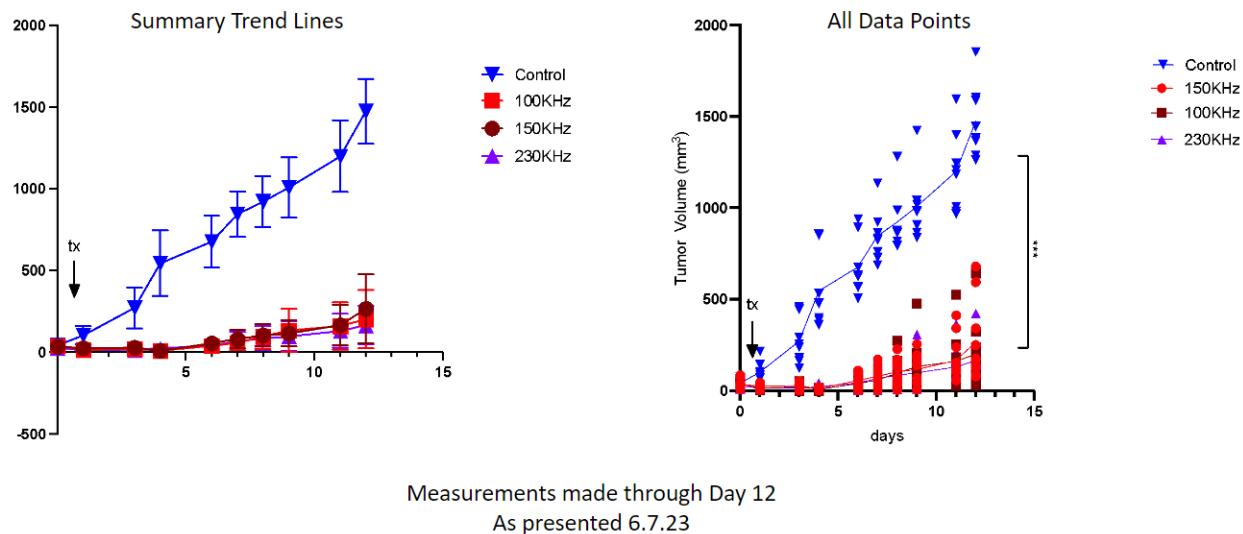
Apr 19, 2023 - no data - just planning meeting slides

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)

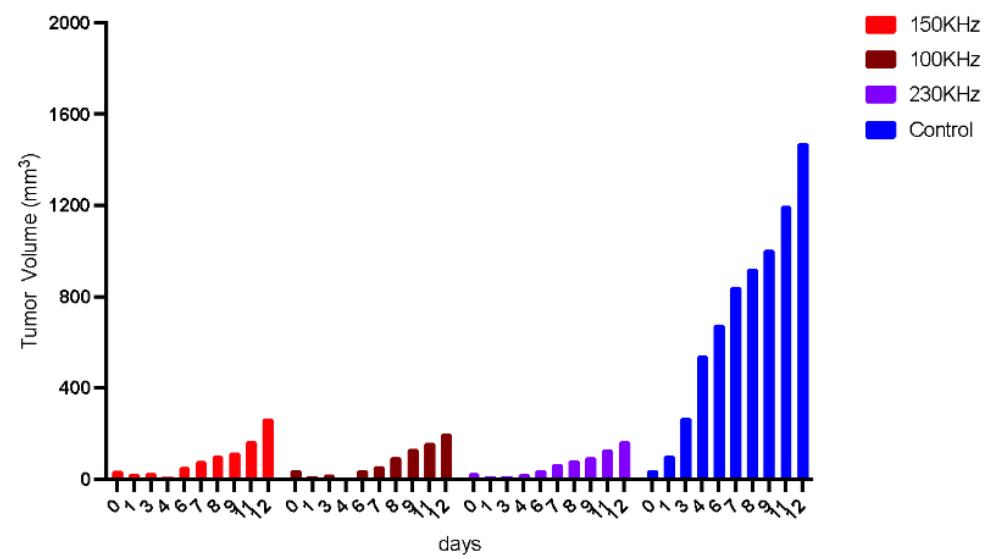
Treatment Date: 5/25



Flank Tumor Growth Measurements (GL261)

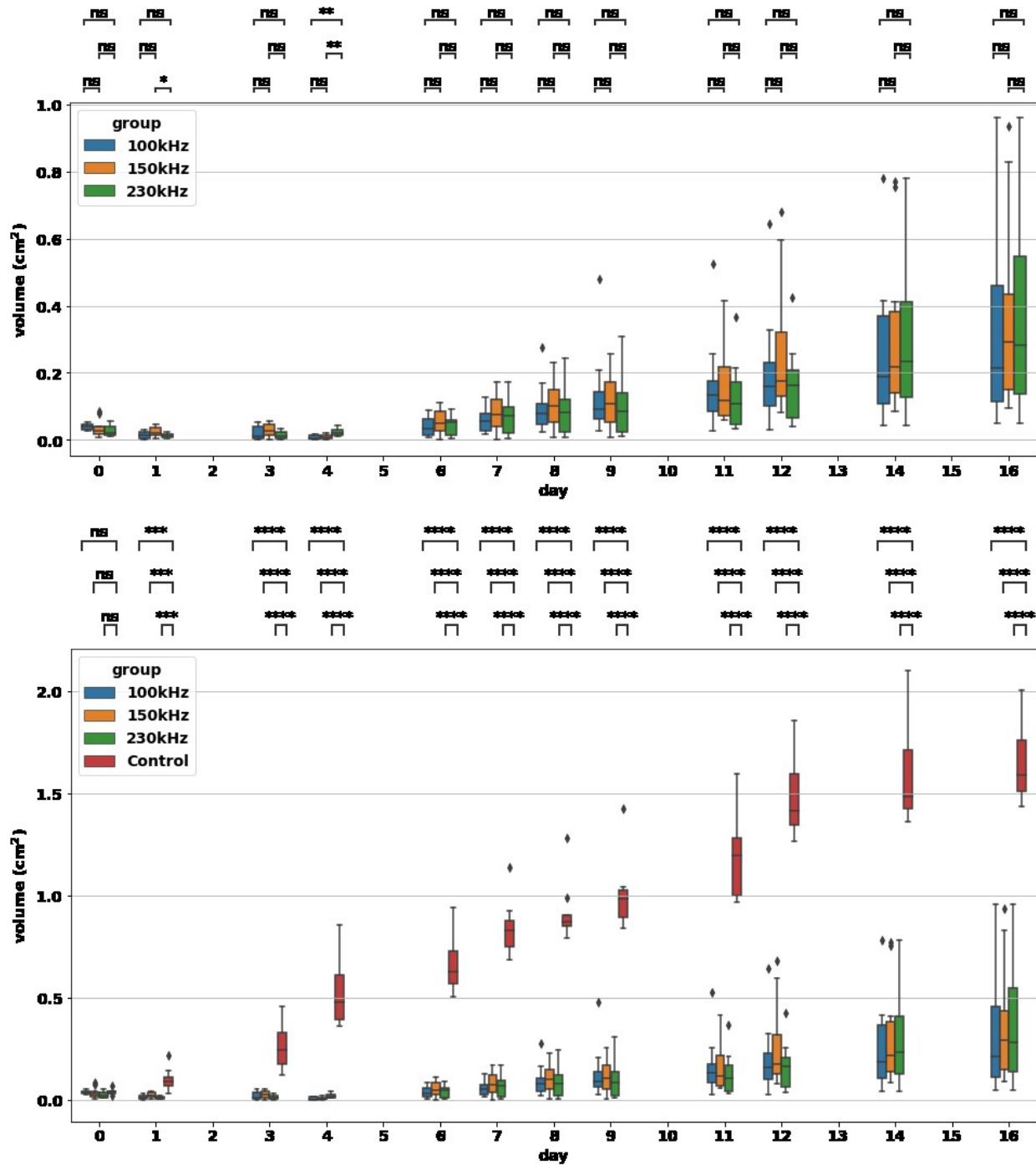
Injection Date: 5/17 (N=38 mice; N=10 in each treatment arm, N=8 control)

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):

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

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		

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 cel	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 / 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	1 / 5		
Well circumscribed							0 / 5	P	P	P		3 / 5	P	P	P	P	P	4 / 5				P	P	2 / 5	
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:

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.