Selective Ablation of Cancer Cells with Low Intensity Pulsed Ultrasound

Supplemental Figures

Figure Sup 1 | **No temperature change induced by LIPUS.** Assessed temperature change using fiber optic hydrophone system at focus of FUS transducer in 24-well plate configuration. LIPUS applied at 0.67 MHz, 1.2 MPa PNP, with 10% duty cycle did not induce any appreciable change (<1 °C) in temperature even with 100 ms pulse duration.

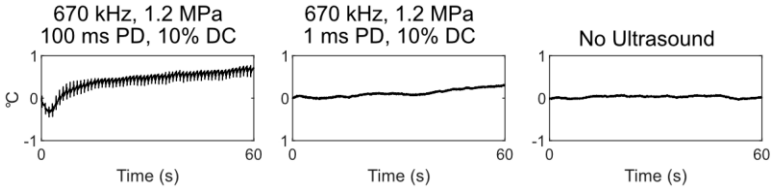


Figure Sup 2 | Flow cytometry gates used for phagocytic, apoptotic analysis. **a,** Ethidium homodimer (Ethd-1) vs AnnexinV stain generates the following quadrants. Q4: live cells, Q3: live cells undergoing apoptosis, Q2-Q1: dead cells. **b,** Ki-67 vs Bcl-2 stains used to identify Bcl2+ and Ki-67+ populations.

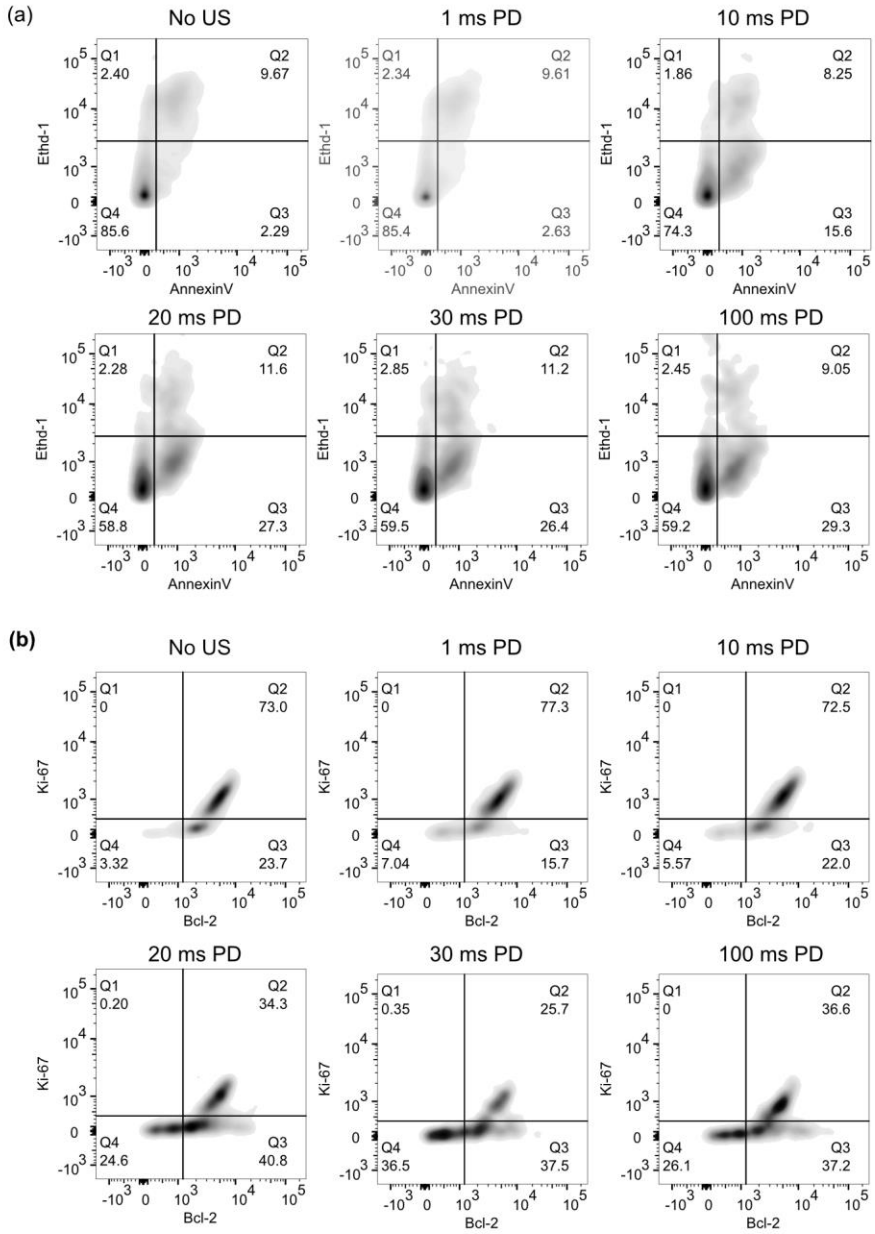


Figure Sup 3 | High speed video demonstrates no large-scale cell deformation during ultrasound insonation. a, Schematic of high frame rate camera setup enabling cellular imaging at 5 Mfps. **b,** 1d trace over time demonstrates translation of cell of ~1 micron after 100ms of 0.67 MHz ultrasound exposure.

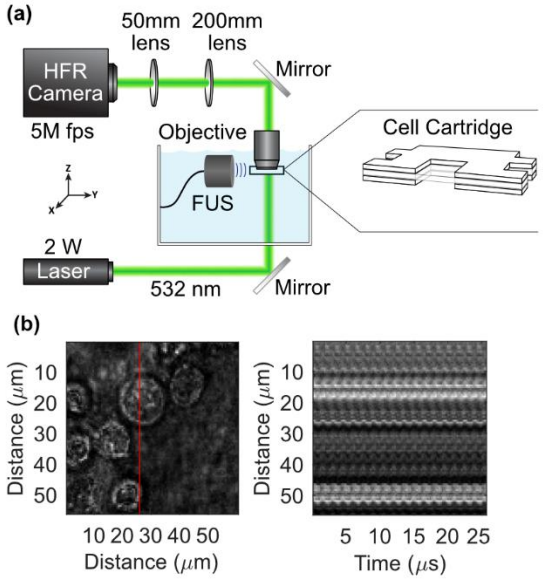


Figure Sup 4 | High speed video demonstrates no large-scale cell deformation during ultrasound insonation. Imaging of cells at 5 Mfps shows no visible deformation, only translation of cells after 100ms of 0.67 MHz ultrasound exposure. Scale bar 20 microns. (Multimedia view)

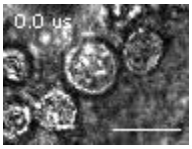
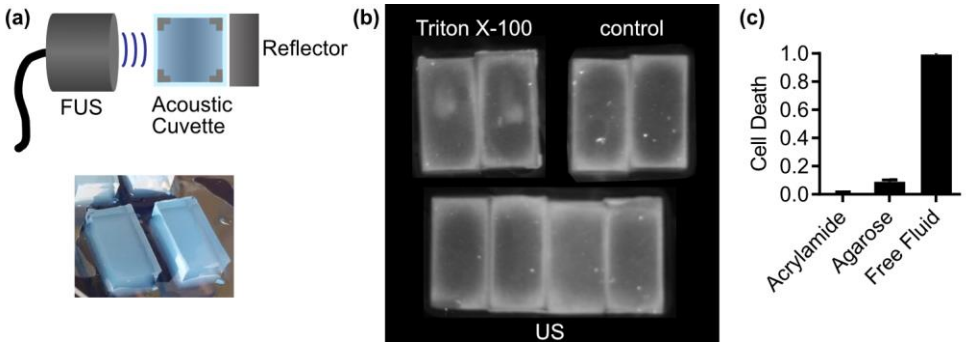


Figure Sup 5 | Cells suspended in gel medium undergo attenuated cytodisruption in response to our targeted LIPUS parameters. a, Schematic diagram and picture demonstrating 1 cm thick 1% agarose gels containing K562 cells and their placement between the 0.5 MHz transducer and the metal reflector. **b,** Cytodisruption was assessed using ethidium homodimer assessed in gel reader, positive control had injection of Triton X-100 in center of gel. **c,** Cell death is completely attenuated in acrylamide gels compared to free fluid condition. Cell death is attenuated, but still significant in agarose gels. Observed using ethidium homodimer-1 fluorescence after ultrasound at 0.5 MHz, PD 100 ms.



Methods

High Throughput Ultrasound Experiments

Acoustically transparent 2.5 μm mylar film (Chemplex #100) was placed on the bottom of 24-well no-bottom plates (Greiner Bio-One #662000-06) that have been painted with a thin film of Sylgard 184 PDMS (Fisher Scientific # NC9285739) and heat treated at 60 °C for 24 hours. The resulting 24 well plates are watertight and have acoustically transparent bottoms. The plates were sterilized and loaded with cell samples as required for ultrasound exposure.

24 well plates were placed on a metal stage such that the mylar film was in contact with a water bath. One of the three available FUS transducers (0.3 MHz: Benthowave BII-7651/300, 0.5 MHz: Benthowave BII-7651/500, and 0.67 MHz: Precision Acoustics PA717) was attached to a metal arm angled 20 degrees from the normal of the water bath. A Velmex X-Slide motorized positioning system allowed the 3d motion of the arm allowing the transducers to be targeted at each well individually. The transducers were aligned using a Precision Acoustics fiber optic hydrophone to target the bottom center of well A1 on the 24 well plate. A MATLAB script controlled a signal generator (B&K #4054B) which generated a unique RF signal for each well of the plate and the Velmex positioning system. This signal was then amplified (AR #100A250B) and sent to drive the FUS transducers. The water bath was filled with distilled water which was degassed by a water conditioning system (ONDA #AQUAS-10) and heated to 37 degrees Celsius prior to experiments.

Fiber optic thermometry was used to measure the effect of insonation at the highest frequency 0.67 MHz and highest pressure 1.2 MPa PNP tested to confirm that no heating would occur in LIPUS experiments. Fiber was placed at ultrasound focus within acoustically transparent 24 well plate and temperature measurements were made for 1 ms and 100 ms pulse duration insonations. (**Fig. Sup 1**)

For the parameter search experiments (**Fig. 1d**) K562, U-937, or T cells were spun down and carefully resuspended in vacuum degassed PBS containing 2 μM ethidium homodimer-1 (Ethd-1) at 2 million cells in 2 mL PBS in each well of an acoustic 24 well plate. On each plate, 2 wells were loaded with 0.1% Triton X-100 as a positive control (pos) and 2 wells were un-insonated as a negative control (neg). Immediately after insonation, cell death for each well was estimated as Ethd-1 signal (s) as measured through plate reader as: cell death = $(s_{well} - s_{neg}) / (s_{pos} - s_{neg})$.

For the broad cell panel experiments (**Fig. 1e**), $2x10^6$ 4T1, CT26, MCF7, SK-BR-3 or MDA-MB-231 cancer cells were mixed with $2x10^6$ PBMCs in 2 mL degassed PBS respecitively and loaded into each well of an acoustic 24 well plate. After insonation, $2x10^4$ cells are cultured on 96 well plates for 2 days, and resuspended in PBS with 2 μM Ethd-1 prior to analysis with flow cytometry. For immune cell surface marker analysis, single-cell suspensions were stained with antibodies in PBS containing 2% fetal bovine serum. Antibodies to CD3(UCHT1), CD4(SK3), CD8(RPA-T8), CD19(SJ25-C1), CD33(P67.6) and CD56(5.1H11) were used to gate the CD4 T cells, CD8 T cells, B cells, myeloid cells and NK cells respecitively. Myeloid cells, which are largely undifferentiated cells with similar mechanical properties as cancer cells and comprise <1% of the PBMC cells, were excluded from analysis. Cell death for each subpopulation was determined from the count of cells that did not uptake Ethd-1 in comparison to untreated control.

Heparinized bovine red blood cells (Sierra for Medical Science), were diluted to 10% hematocrit in degassed PBS, then insonated as described above. After ultrasound, RBCs were centrifuged so that samples of supernatant could be assessed for hemoglobin release (Abcam ab234046). RBC death in resposne to LIPUS calculated as hemoglobin release compared to positive control 0.1% Triton X-100 and negative control of no ultrasound. (**Fig. 1e**)

Biomolecular mechanism experiments

For CT26 cell apoptosis and proliferation marker analysis, 2 days after ultrasound treatment, CT26 cells were stained with anti-Calreticulin (Abcam) 30 min at room temperture. Annexin V binding buffer (Biolegend) was used for Annexin V staining. Fixation and permeabiliztion was performed with BD Cytofix/Cytoperm buffers (BD Biosciences) for Ki-67 and Bcl-2 intracellular antibodies staining. **(Fig. 2a)** Cell death was determined from the count of cells that did not uptake Ethd-1 in comparison to untreated control. The percentage of apoptotic cells reported is the fraction of Annexin V positive and Ethd-1 negative cells measured through flow cytometry. Cell signaling pathway markers Bcl-2 and Ki-67 were the fraction of Bcl-2 or Ki-67 stain positive and Aqua (fixable dead cell stain) negative cells. The pro-phagocytic marker calreticulin percentage reported was the fraction of calreticulin stain positive in all cells, live or dead.

For confocal experiments, CT-26 cells were allowed to settle on PDL-coated 1.5 μm coverslips for 1 hour. Slides were treated with fixable LIVE/DEAD stain (ThermoFisher #L34971), fixed in 4% paraformaldehyde and stained with phalloidin (Cayman #20549). Coverslips are then mounted with mountant containing DAPI (ThermoFisher #P36971). Four color confocal images were acquired with a 100x oil immersion objective at the Caltech Beckman Imaging Facility. **(Fig 2b)** To quantify, the actin signal intensity was measured on 12 cells imaged in each ultrasound treatment condition.

Standing Wave Experiments

Pressure measurements performed using the fiber optic hydrophone positioned using the Velmex X-Slide. Color scale pressure maps represent peak negative pressure at each position within well or acoustic cuvette. Acoustic cuvettes were 1 cm x 1 cm 3d printed chambers with walls made of mylar film fixed with super glue. The cuvette was mounted to the bottom of the water tank and surrounded by distilled, degassed water. The center of cuvettes were aligned with the FUS transducer focus using fiber optic hydrophone and Velmex positioning system. A 3”x3”x0.5” rectangular prism block of aluminum was used as an acoustic reflector and positioned directly opposite from the transducer next to the cuvette (**Fig 3a)**. K562 were loaded into the acoustic cuvette at 1 M cells / mL containing 0.2 μM Ethd-1. A 3d printed imaging chamber submersed in a water bath positioned the 0.5 MHz transducer a fixed position from an acoustic reflector, such that fluorescent imaging of a compartment containing GFP-labeled K562 at the focal point of the transducer could be achieved. Imaging was obtained through a 4x air objective (Olympus) (**Fig. 3c**).

Cavitation Experiments

Using the same setup for standing wave experiments, a 10 MHz single element transducer (Olympus #U8421024) was positioned orthogonally to the FUS transducer, also aligned using the fiber optic hydrophone (**Fig. 4a**). The signal from the single element transducer was captured directly by an oscilloscope (Keysight InfiniiVision 3000 X-series) without pre-amplification. 4M data points were acquired during the signal transmission period of LIPUS. Frequency spectral analysis was then performed to generate logarithmic spectral graphs of frequency intensity at various representative time points during the pulse (**Fig. 4b)** and quantification of total signal energy **(Fig. 4c).** Samples were loaded into the acoustic cuvette. Vacuum degassed PBS used as negative control. 10 μL of freshly resuspended Definity microbubbles (Lantheus Medical Imaging, Inc.) in degassed PBS used as positive control. Aluminum reflector used as described above to introduce standing waves. K562 or PBMC cells loaded at 1 M cells / mL in degassed PBS. Liposomes were generated from 14.0-18.0 PC Avanti Polar lipid suspended in chloroform which was lyophilized to remove chloroform, rehydrated using degassed 300 mOsm sucrose solution, sonicated for 10 minutes, heated at 40°C for 10 minutes, then degassed. Liposomes solution resuspended in degassed PBS to approximate lipid concentration in cell samples. 10 μM polystyrene beads at 1 M beads / mL also measured. Cavitation energy assessed in relative units by integrating the square of the pressure signal over time (**Fig 4c)**. Image of cavitation bubbles taken using camera facing the single element transducer, with plane of laser illumination generated from laser light source and 1D diverging lens positioned above the acoustic cuvette (**Fig 4d).** A pressure chamber was constructed by attaching a compressed air line with a gauge pressure of 400 kPa onto an acoustically transparent plastic pipet bulb. Acoustic transmission through the pipet bulb was confirmed using hydrophone measurements. 1 M/mL K-562 cells loaded in degassed PBS containing 2 μM Ethd-1 into the pipet bulb. Cavitation from in samples loaded into the pipet bulb in place of the acoustic cuvette could be measured as described above. Cell death assessed using Ethd-1 signal **(Fig 4e)**.

Gel Experiments

Agarose gels were prepared by mixing 2% agarose in vacuum-degassed PBS at 65 °C with 2 M/mL K562 cells in PBS, adding 2 μM Ethd-1, and poured into 1 cm x 1 cm x 2.5 cm molds. Acrylamide gels were prepared as in this reference [1] with a final concentration of 1 M/mL K562 and 2 μM Ethd-1. Gels were insonated in the acoustic cuvette as described above. Cell death was calculated as magnitude of Ethd-1 signal observed at LIPUS focus on gel reader in comparison to signal from gels injected with 0.1% Triton X-100 and gels not treated with LIPUS.

High Speed Camera Experiments

We assembled a high-speed microscopy setup capable of directly visualizing the effect of ultrasound on K562 cells. Our setup used a 2 W 532-nm laser (CNI, MLL-F532-2W) controlled by an optical beam shutter

(Thorlabs SH05, KSC101). Right angle prism mirrors directed the laser light through a water bath and into a sample chamber containing the imaged samples. K562 cells were loaded into a custom-made acrylic cartridge containing an inner pocket surrounded by mylar film. A 3d printed holder positioned the cartridge such that the inner pocket was at the focus of the 0.5 MHz transducer. The cells were freely floating between two acoustically transparent films near an acoustic reflector that generate standing waves. A 100x water immersion Plan Fluor objective (Olympus) was used to image the target cells. A series of prism mirrors and converging lenses with focal lengths of 200 mm and 50 mm delivered the image into a Shimadzu HPV-X2 camera, which acquired 256 images over 51.2 μs, at a sampling rate of 5 million frames per second. To account for acoustic propagation through water, the camera was externally triggered to begin acquisition 100 ms after the start of the ultrasound pulse. A single pulse of 100 ms at 0.5 Mhz and 0.7 MPa PNP was used to insonate the sample in these experiments.

References

1. Tse, J.R. and A.J. Engler, *Preparation of hydrogel substrates with tunable mechanical properties.* Curr Protoc Cell Biol, 2010. **Chapter 10**: p. Unit 10 16.