Supplemental Table

Supplemental Table S1

US Treatment Applied	# of NDL Mice Studied	# of 4T1 mice Studied
Grid	8	0
Circle	38	6
Single	30	4
No US	25	6
Total	101	16

Supplemental Materials and Methods

Doxorubicin hydrochloride (USP grade), copper (II) gluconate, triethanolamine (TEA), solvents, and other reagents were purchased from Sigma-Aldrich unless otherwise noted. Hydrogenated soy phosphatidylcholine (HSPC), cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MPPC), and 1,2 distearoyl-sn-glycero-3-phosphoethanolamine-N-Methoxypolyethyleneglycol-2000 (DSPE-PEG2k), and a mini-extruder were obtained from Avanti Polar Lipids Inc. BAT-PEG-lipid was synthesized by Fmoc solid phase synthesis as previously described (1). $^{64}CuCl_2$ was purchased from Washington University in St. Louis under a protocol controlled by the University of California, Davis. Dulbecco's Phosphate Buffered Saline (DPBS) was purchased from Invitrogen Corporation.

Preparation of ^{64}Cu-liposomes

In brief, lipids in chloroform (0.555/0.39/0.05/0.005 mol/mol/mol/mol, HSPC, cholesterol, DSPE-PEG2k, BAT-PEG-lipid) were transferred to a test tube and solvent evaporated under nitrogen. After overnight lyophilization, lipids were re-suspended in 0.1 M ammonium citrate solution (400 µL, 300 mOsm, pH 5.5), and incubated in a warm bath (60°C) alternating with gentle vortexing for 10 minutes. The lipid mixture was extruded through a 100 nm membrane

filter (Whatman) 21 times at 60°C. After cooling to room temperature, $^{64}CuCl_2$ (18.5 MBq/mg lipid) buffered in 0.1 M ammonium citrate (pH 5.5, 0.1 mL) was added to the extruded lipid solution. The mixture was incubated at 30°C for 40 minutes, and then 0.1 M ethylenediaminetetraacetic acid in water (20 µL) was added and incubated for 10 minutes. ^{64}Cu-liposomes and unbound copper were separated by size exclusion chromatography with Sephadex G75 resin (GE Healthcare) in DPBS. Mean liposomal diameter was 122.5 ± 18 nm as measured by Dynamic Light Scattering (DLS) (Nicomp 380 ZLS, Particle Sizing Systems). Decay corrected labeling yield was greater than 95% by radioactive thin layer chromatography.

Preparation of Dox-LCL

Briefly, lipids containing 0.56/0.39/0.05 mol/mol/mol HSPC, cholesterol, DSPE-PEG2k and 0.86/0.10/0.04 mol/mol/mol DPPC, MPPC, DSPE-PEG2k for long-circulating liposomes (LCL) (2) and temperature-sensitive liposomes (TSL) (3), respectively, were mixed in chloroform. Dried lipids were hydrated in 0.3 mL of 100 mM copper (II) gluconate including 270 mM triethanolamine (TEA), pH 7.4 to prepare LCL and 100 mM copper (II) gluconate including 540 mM triethanolamine (TEA), pH 8.4 to form TSL. The multi-lamellar lipid solution at a final concentration of 50 mg/ml was extruded at 62-64°C through a 100 nm membrane. Copper/TEA-loaded liposomes were then separated from non-encapsulated copper/TEA by passing the extruded liposomal solution through a spin column of Sephadex G-75 (5 × 1 cm) equilibrated with 0.9% sodium chloride. Liposomal diameters measured 119 ± 18 nm with DLS. Doxorubicin hydrochloride was then loaded at drug-to-lipid ratio of 0.2 mg/mg at 37ºC overnight for LCL and 1.5 h for TSL. The resulting liposomes were purified by a spin column of Sephadex G-75.

Preparation of Alexa Fluor 555-liposomes

Alexa Fluor 555 (A555) succinimidyl ester was purchased from Life Technologies and allowed to hydrolyze overnight at room temperature. Lipids were prepared in the same ratio as

Dox-LCL above; however, dried lipid was hydrated with a 1 mM solution of A555 in DPBS. A555-LCL was extruded as above and separated from free A555 by size exclusion chromatography with Sephadex G-75 resin in DPBS. Liposomal diameters measured 113 ± 20 nm with DLS.

Optical and Viability Studies

Optical and viability studies were performed 24 hours after MRgFUS treatment and injection of A555-liposomes (Supplemental Fig. S3A). Mice were euthanized by cervical dislocation; tumors were removed, immediately placed in OCT compound, and frozen in dry ice and isopropanol. A Leica CM1850 cryostat was used to cut 20 micron serial sections, which were then prepared in three different ways. One set of sections was placed directly onto coverslips and the whole slide images immediately captured with a Zeiss Axio Observer microscope equipped with a Yokogawa spinning disk confocal system, using a 10x objective, a 561 nm solid state laser, and a Cool SNAP HQ camera (Nikon), controlled with Slidebook 5.1 software (Intelligent Imaging Innovations). Another set of sections was fixed with 20% neutral buffered formalin and stained with haematoxylin and eosin. The final set was stained for viability with a preparation of nitroblue tetrazolium and β-NADH. Haematoxylin and eosin as well as nitroblue tetrazolium stained slides were then scanned and digitized with an Aperio ScanScope XT whole slide imaging system with Aperio Spectrum software.

β-NADH slide preparation

Nitroblue tetrazolium (5 mg) was dissolved in 2.5 mL DI water, 1 mL DPBS, and 0.5 mL Ringer's solution. Immediately before use, 2.5 mg β-NADH was dissolved in 1 mL of DI water and added to the nitroblue tetrazolium solution. Then, 100 µL of solution was added to each tissue section and incubated 15 minutes at room temperature. The slides were then washed for thirty seconds in a series of water and acetone washes (0, 30, 60, 90, 60, 30, 0, % acetone in

water). Samples were then mounted with Vectashield aqueous mounting media (Vector Labs) and sealed with clear nail polish.

Simulation of Ultrasound Heating

Simulation of heating was performed in Matlab (R2014a, MathWorks, Inc.), using a finite-difference model based on Pennes' bio heat transfer equation. These simulations guided the development of the scanned US thermal ablation protocol and were used to compare acquired thermometry data with established tissue parameters. Applied ultrasound intensity was modeled as a Gaussian function approximating an acoustic model developed using Fast Object-oriented C++ Ultrasound Simulator (FOCUS, Michigan State University) as previously described (4) as well as measurements performed with a 0.5 mm active diameter Müller-Platte needle hydrophone (HNS-0500, Onda Corporation) and 0.1 mm diameter laser hydrophone (HFO-690, Onda Corporation). Simulated heating was applied to the center of a uniform cube of side length 16 mm, consisting of 100 micron voxels, with time steps of 0.01 seconds, with each boundary face held at 37°C. Tissue parameters used include density of 1050 kg/m^3, specific heat of 3500 J/kg/°C, thermal conductivity of 0.3 W/m/°C, acoustic absorption of 23 Np/m, acoustic intensity of 22 W/mm^2, with perfusion neglected.

T1wMRI of gadoteridol administered before or after MRgFUS

A set of mice were imaged with MR, treated with circle pattern MRgFUS, and injected with intraperitoneal gadoteridol either immediately before or after treatment (0.05 mmol/kg) or with IV gadoteridol before or after treatment. Mice were then imaged with the T1w scan parameters at 0.5, 1.5, 3, and 6 hours following circle pattern MRgFUS ablation. Ablated region and quadriceps were then manually segmented and the T/M ratio measured.

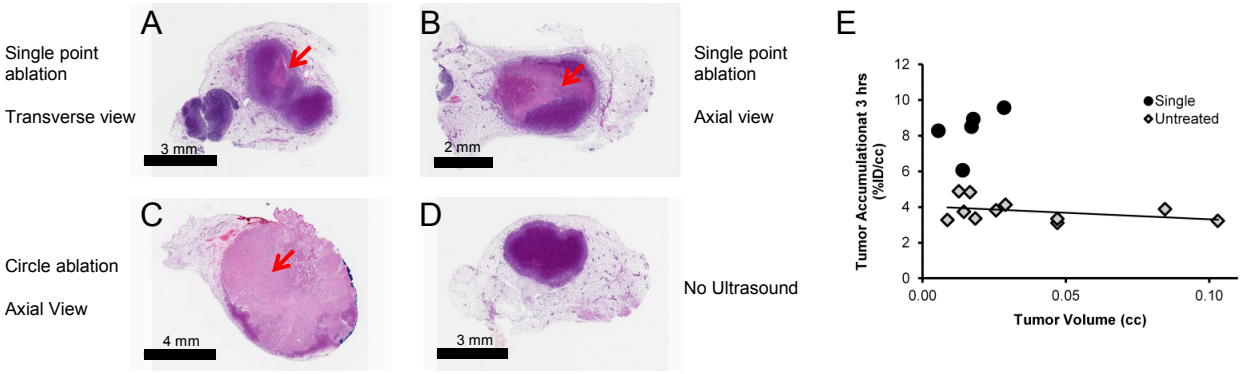
In addition, two control groups of mice were also imaged with T1w MRI, a set treated with circle pattern MRgFUS and but no gadoteridol and a set not treated with ultrasound, but injected with intraperitoneal gadoteridol.

4T1 tumor model

4T1 cells were obtained from American Type Culture Collection and cultured in IMDM complete medium (Iscove's Modified Dulbecco's medium containing 0.584 g/L L-glutamine and 25 mM HEPES and supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin) at 37°C in an humidified 5% CO2 incubator. For passaging, at 70-80% confluency, cells were removed from plates via dissociation with TrypLETM Express (Invitrogen), and split at a subculture ratio of 1:3 to 1:6. Cells between passage 4 through passage 7 were used for primary tumor initiation in mice. 7 week old female BALB/c mice (10 mice total) were injected in the mammary fat pad with 2 x 10^4 cells in an injection volume of 30 µL PBS. These were allowed to grow for 2 weeks following injection, to a size of 4-6 mm in longest dimension prior to treatment with US and ^{64}Cu-LCL.

1. Seo JW, Zhang H, Kukis DL, Meares CF, and Ferrara KW. A novel method to label preformed liposomes with 64Cu for positron emission tomography (PET) imaging. *Bioconjug Chem.* 2008;19(12):2577-84.
2. Kheirolomoom A, Mahakian LM, Lai CY, Lindfors HA, Seo JW, Paoli EE, Watson KD, Haynam EM, Ingham ES, Xing L, et al. Copper-doxorubicin as a nanoparticle cargo retains efficacy with minimal toxicity. *Mol Pharm.* 2010;7(6):1948-58.
3. Kheirolomoom A, Lai CY, Tam SM, Mahakian LM, Ingham ES, Watson KD, and Ferrara KW. Complete regression of local cancer using temperature-sensitive liposomes combined with ultrasound-mediated hyperthermia. *J Control Release.* 2013;172(1):266-73.
4. Fite BZ, Liu Y, Kruse DE, Caskey CF, Walton JH, Lai CY, Mahakian LM, Larrat B, Dumont E, and Ferrara KW. Magnetic Resonance Thermometry at 7T for Real-Time Monitoring and Correction of Ultrasound Induced Mild Hyperthermia. *PLoS ONE.* 2012;7(4).

Supplemental Figure Legends:



Supplemental Figure S1: Additional information on the size of ablated regions and the effect of initial tumor diameter on accumulation in the NDL tumor model. A-D) H&E images of treated tumors (A-C) for single point ablation (A, transverse view and B, axial view) and C) circle ablation, D) no ultrasound. E) Quantitation of tumor accumulation of ^{64}Cu-LCL following ablation. Tumor diameter did not correlate with tumor accumulation of ^{64}Cu-LCL at 3 hours. A linear regression line (slope of -0.0074 %ID/cc tumor accumulation per 0.01 cc tumor volume and Pearson's R^2 of 0.14) was added to the plot to examine the relationship between tumor size and tumor drug accumulation in untreated tumors. n = 16 tumors. Red arrow indicates the ablated region For A and D, scale bar = 3 mm, for B scale bar = 2 mm, for C scale bar = 4 mm.

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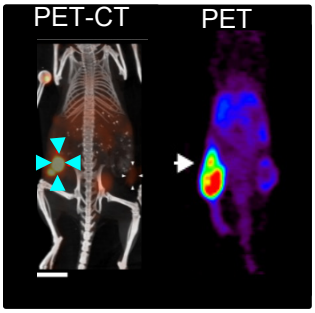
A



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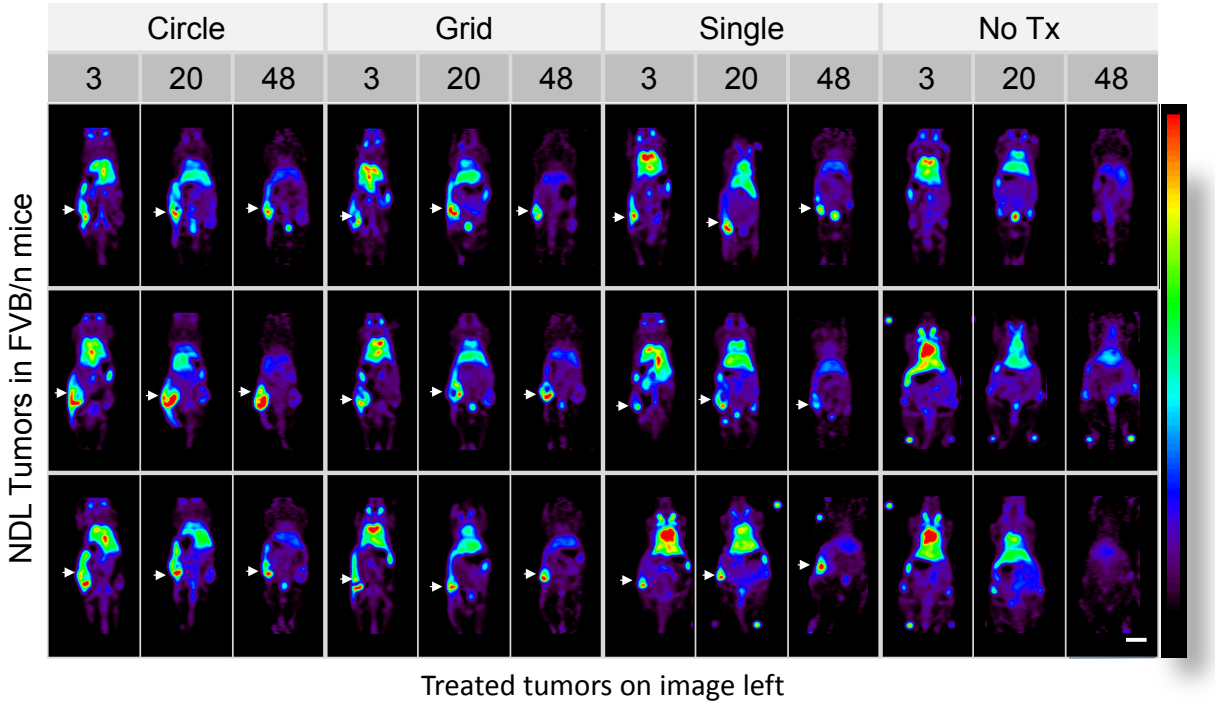
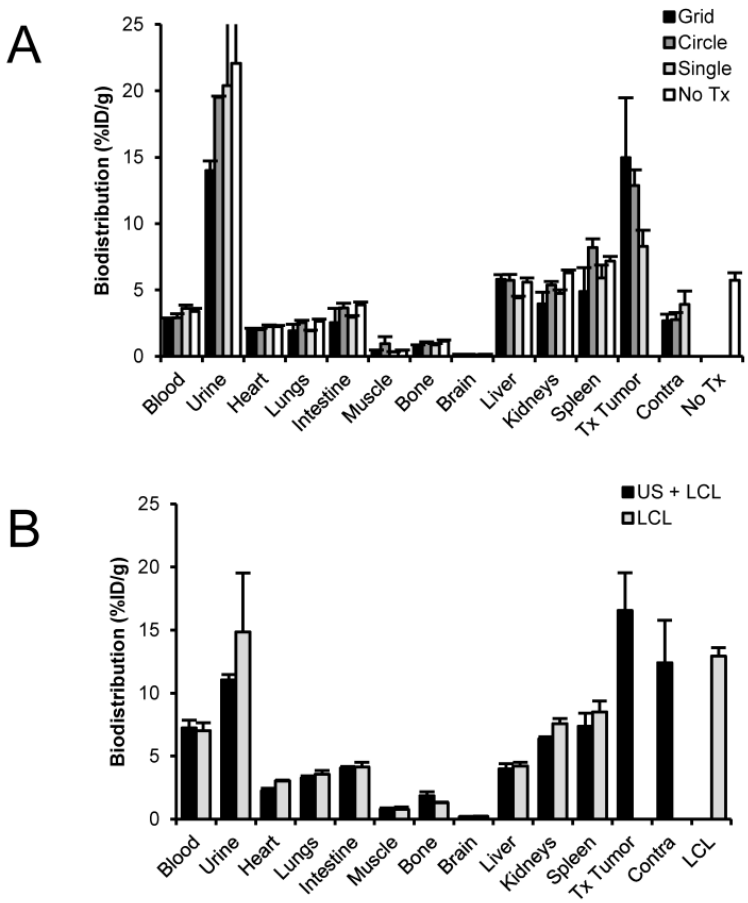
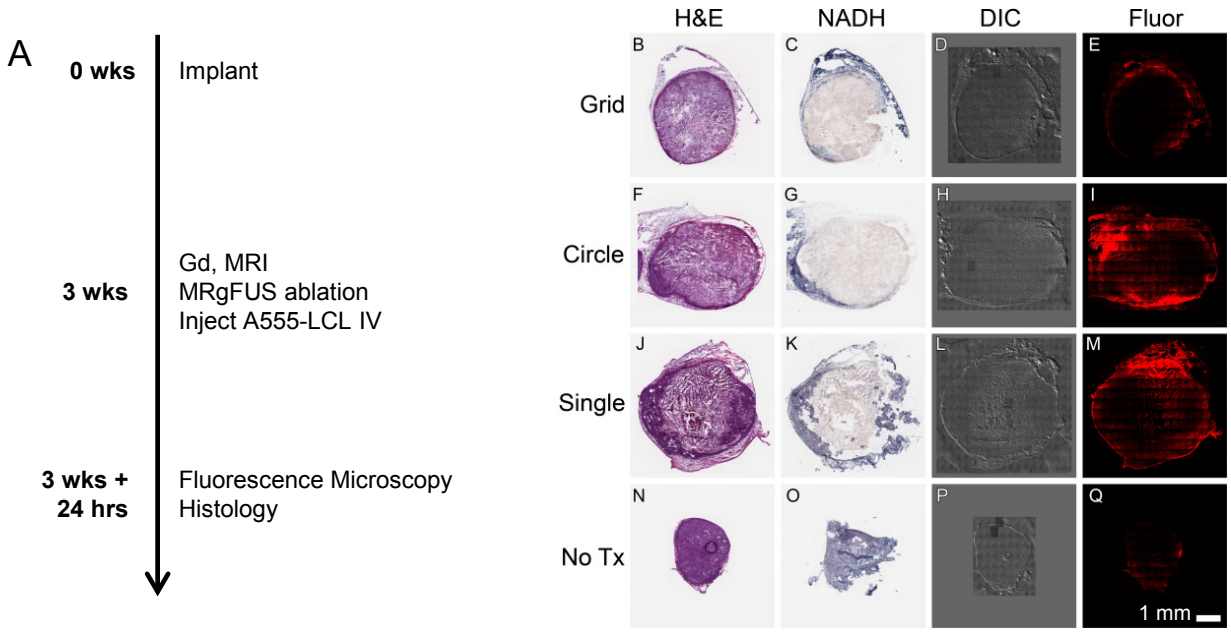


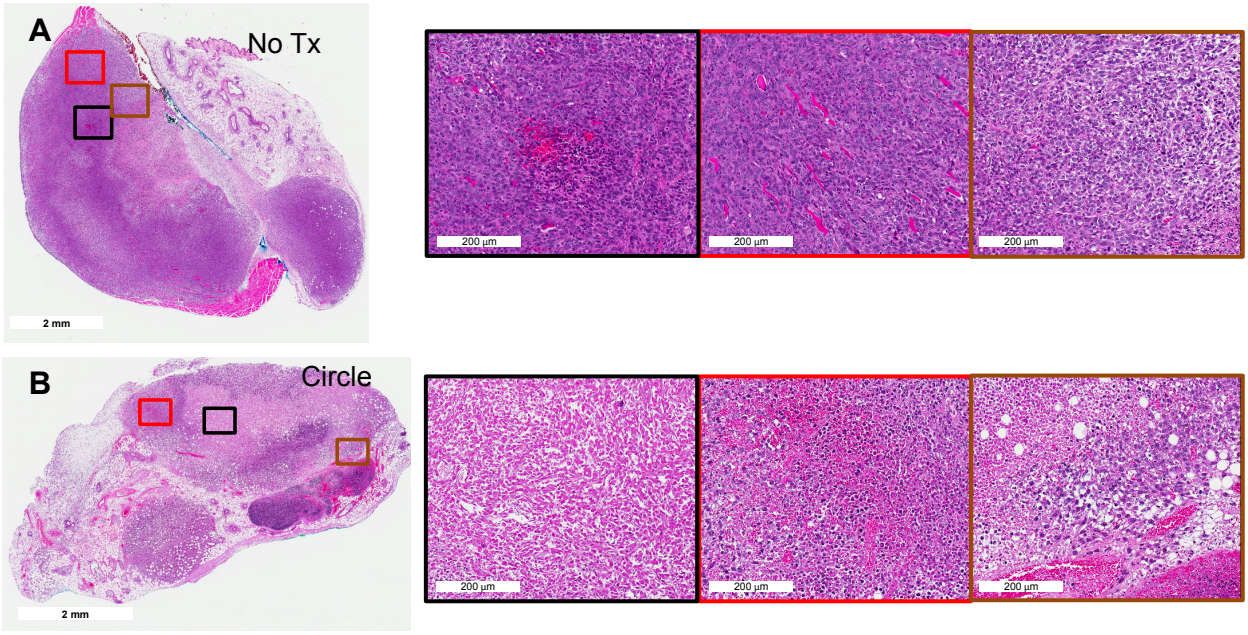
Supplemental Figure S2: A) Example of PET-CT maximum intensity projection image provided for anatomical reference, as compared with PET-only slice image. B) Additional PET slice images of ^{64}Cu-LCL distribution for NDL tumors in FVB/n mice provided to visualize the entire cohort. Either the left or right tumor was ablated; however for visualization, some images have been flipped horizontally such that the ultrasound treated tumor is on the left side of the image in all cases. White arrows indicate insonified tumors. Scale bar = 1 cm. Color bar ranges from 0 to 25 %ID/cc.



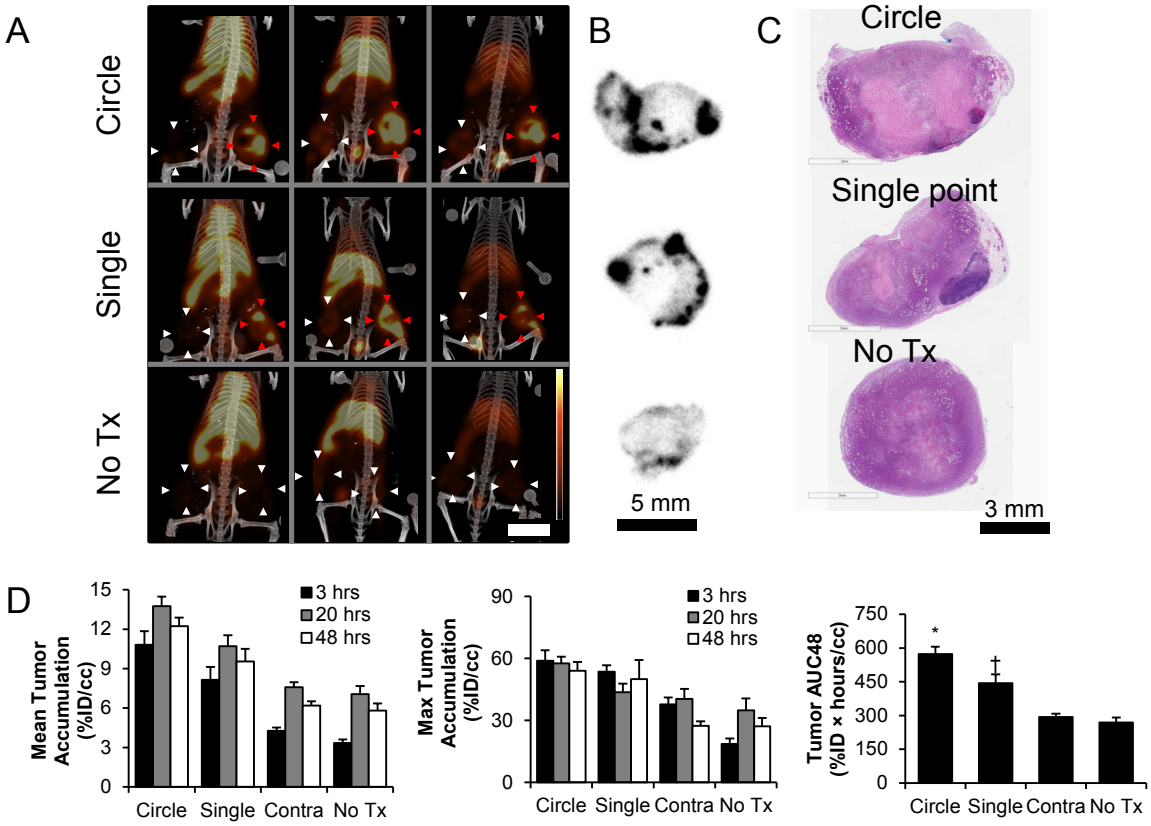
Supplemental Figure S3: MRgFUS has local effects on tumor distribution of LCL while systemic effects are small. In NDL model, organ biodistribution for mice treated with A) ^{64}Cu-LCL and Grid, Circle and Single point MRgFUS protocols, as well as B) two weeks of biweekly CuDox-LCL and then ^{64}Cu-LCL (LCL) or two weeks of weekly single point MRgFUS and biweekly CuDox-LCL and then single point MRgFUS and ^{64}Cu-LCL (US + LCL). After PET/CT imaging at 48 hour time point, mice were sacrificed and organs removed for gamma counting. Tracer distribution is comparable between each treatment type, in each organ, with the exception of treated tumors, which accumulate ^{64}Cu-LCL.



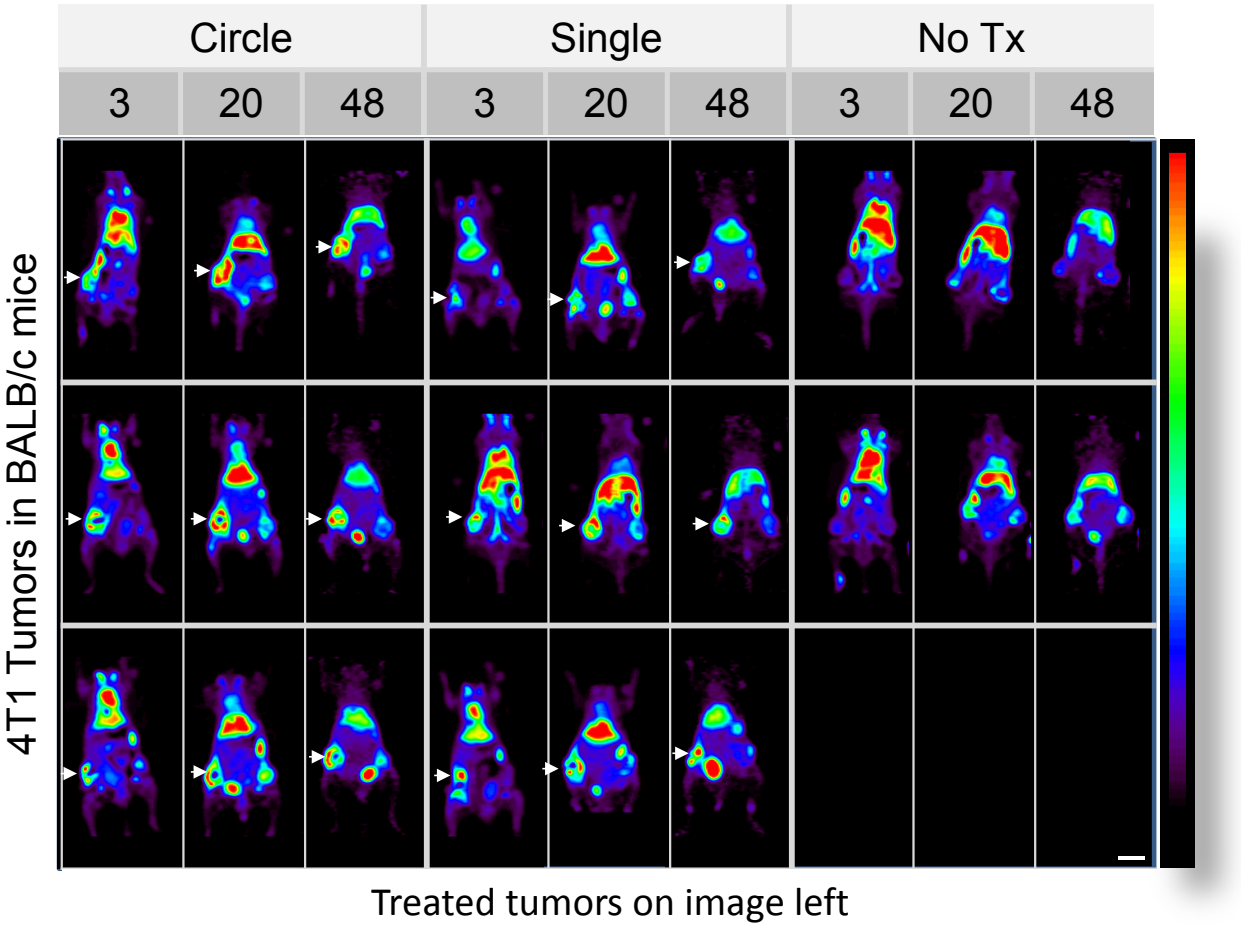
Supplemental Figure S4: Fluorescence microscopy confirmed MRgFUS enhancement of accumulation within remaining viable tumor. A) 3 weeks after implant with NDL mammary carcinoma, mice were treated with MRgFUS and then intravenously injected with A555-LCL. After 24 hours, mice were sacrificed and tumors frozen sectioned and imaged with H&E, NADH, differential interference contrast (DIC), and Fluorescence imaging (columns). Rows represent serial sections of the same tumor treated with B-E) grid protocol, F-I) circle protocol, J-M) single point ablation, and N-Q) no treatment. Scale bar = 1 mm.



Supplemental Figure S5: Histopathology of treated and control tumors of 4T1 model. A) no treatment and B) post-circle ablation. A) Black and red highlighted regions: even in the absence of ultrasound, highly vascular tumors have small regions of hemorrhage and vessel dilatation, consistent with the higher level of accumulation via the EPR effect. Brown highlighted region: 4T1 tumors demonstrate a mesenchymal-transition phenotype with highly proliferative spindle-shaped tumor cells that were observed to infiltrate the fat pad. B) Post-circle ablation, large central regions of necrosis were observed (black highlighted region), hemorrhage (red highlight) and small regions of viable cells (brown highlight) were observed at the periphery. For A and B, scale bar = 2 mm, for insets scale bar = 200 µm.



Supplemental Figure S6: In 4T1 mammary carcinoma, the accumulation of nanoparticles was enhanced following ultrasound thermal ablation in BALB/c mice in a manner similar to that observed in NDL tumors. A) Circle and single point ultrasound protocols resulted in enhanced accumulation of nanoparticles within tumor, while distribution within other organs remained comparable to untreated mice (red arrows delineate insonified tumor, white arrows delineate contralateral). B) Autoradiography demonstrated intense uptake of nanoparticles at the tumor periphery in tumors treated with ablation. C) H&E of circle ablation (top), single point ablation (middle) and control (lower). A thin rim of viable tumor surrounding a necrotic core was visible in tumors treated with circle ablation a. D) Mean and maximum accumulation of nanoparticles and tumor AUC calculated over 48 hours were increased by ablation in a manner comparable to that observed in NDL tumors with much of the accumulation observed at 3 hours after ablation. Without ultrasound, accumulation is greater in 4T1, as compared with NDL, tumors. * $P < 1 \times 10\text{-}4$, vs untreated and contralateral tumors and $P < 0.05$ vs single point ablation, † $P < 0.01$ vs untreated and contralateral tumors. Color bar ranges from 0 to 25 %ID/cc. n = 4, 3, 3 mice for circle, single point, and no treatment (No Tx) groups in PET study. For A, scale bar = 1 cm, for B, scale bar = 5 mm, for C, scale bar = 3 mm.



Supplemental Figure S7: Additional images of ^{64}Cu-LCL distribution for 4T1 tumors in BALB/c mice. Either the left or right tumor was ablated; however for visualization, some images have been flipped horizontally such that the ultrasound treated tumor is on the left side of the image in all cases. White arrows indicate insonified tumors. Scale bar = 1 cm. Color bar ranges from 0 to 25 %ID/cc.