Supplementary Information for “Ultra-High-Frequency-Radio-Frequency-Acoustic Molecular Imaging with Saline Nanodroplets in Living Subjects”

Yun-Sheng Chen, Yang Zhao, Corinne Beinat, Aimen Zlitni, En-Chi Hsu, Dong-Hua Chen, Friso Achterberg, Hanwei Wang, Tanya Stoyanova, Jennifer Dionne, Sanjiv Sam Gambhir

ADDITIONAL DISCUSSION

Comparisons of UHF-RF-acoustic Imaging with Other Acoustic-based Imaging

Advantages of UHF-RF-acoustic imaging: In contrast to photoacoustic imaging that can image only less than a few centimeters inside tissue because of the light attenuation, the biggest advantage of UHF-RF-acoustic imaging is its potential of the deep tissue penetration. The amplitude of UHF-RF waves decay with depth 100 times slower compared to visible light in tissue. For instance, UHF-RF waves can travel 10 mm deep in tissue with a 50% loss in energy\(^1\), whereas microwave travels 1-4 mm and visible to near-infrared light can only travel 0.08-0.1 mm deep in tissue under the same conditions\(^2\). Thus, UHF-RF-acoustic can potentially image much deeper compared to optical, photoacoustic or microwave-acoustic imaging. In addition, quantitative imaging will be relatively easier to achieve in UHF-RF-acoustic imaging than in photoacoustic imaging because of the relatively more uniform RF intensities in tissue than visible light. Due to the large spatial variations in optical properties of tissue, it is not trivial to precisely model the laser intensity distribution inside the tissue\(^3\). The incorrect estimation of laser
intensity would cause errors in estimating the concentrations of imaging targets in photoacoustic imaging for image quantification. On the other hand, because of the overall relatively low attenuation of UHF-RF signals in tissue, it is easier to predict the RF field distribution than its optical counterpart, which will make the image quantification of UHF-RF-acoustic imaging more accurate and reliable than that of photoacoustic imaging.

Disadvantages of UHF-RF-acoustic imaging: compared to photoacoustic imaging, UHF-RF-acoustic imaging has a weak endogenous imaging contrast because of the relatively uniform and low UHF-RF absorption in various types of tissue. In addition, because of the low UHF-RF absorption in most solid materials, UHF-RF-acoustic imaging has been lacking essential contrast agents that generate high acoustic signals. To date, magnetic nanoparticles such as iron oxide nanoparticles have shown the highest RF-acoustic signals, however, these signals are still not strong enough for in vivo applications.

METHODS

Materials

All chemicals in this study were used as received: Krytox 157 FSL (DuPont), polyethylene glycol-amine (Sigma-Aldrich), methoxyperfluorobutane (Sigma-Aldrich), oxalyl chloride (Sigma-Aldrich), poly(propylene glycol)-block-poly(ethylene glycol)-block-poly(propylene glycol)bis(2-aminopropyl ether) (Mn: 900, Sigma-Aldrich), anhydrous dichloromethane (Sigma-Aldrich), sodium chloride (Sigma-Aldrich), sodium hydroxide (Sigma-Aldrich), potassium iodide (Sigma-Aldrich), potassium chloride (Sigma-Aldrich), magnesium chloride (Sigma-Aldrich), and calcium chloride (Sigma9-Aldrich), perfluoro-n-hexane (Fluoromed),
Synthesis of Nanodroplets

To synthesize the nanodroplets, the perfluoro-surfactant was first synthesized for stabilizing the primary emulsion \( \text{NaCl}_{(aq)}/\text{perfluorocarbon liquid} \). The surfactant synthesis used a two-step reaction to PEGylate Krytox 157 FSL, a carboxylic acid functionalized perfluoroether\(^5\). First, the carboxylic acid of the Krytox 157 FSL was converted to acid chloride and then linked with polyethylene glycol (PEG) diamine via nucleophilic addition /elimination reaction. Briefly, under nitrogen, Krytox 157 FSL was added to a flask containing a fluorinated solvent, methoxypervflorobutane. Oxalyl chloride was then added (in 10:1 molar excess to the Krytox 157 FSL) to activate its terminal carboxylic groups. The flask was refluxed and stirred for 24 hours at 50°C under nitrogen. The residue oxalyl chloride in the resulting mixture was removed.
using a rotary evaporator. Second, poly(propylene glycol)-block-poly(ethylene glycol)-block-poly(propylene glycol)bis(2-aminopropyl ether) was dissolved in a mixture of anhydrous dichloromethane and methoxyperfluorobutane (2.3:1 volumetric ratio) and added into the activated Krytox 157 FSL. Similar experimental conditions of the first step were used for this reaction. The resulting copolymer, called Krytox-PEG, was concentrated using a rotary evaporator for 4 hours to remove all the solvent.

Nanodroplets containing high concentration NaCl(aq) was synthesized with a double emulsion method via ultrasonic agitation. Krytox-PEG (0.3 mg/μL PFC) was first dissolved in 500 μL of perfluoro-n-hexane in a 5 mL microcentrifuge tube. Next, 400 μL of a NaCl(aq) (25%) solution was added drop-wise to the PFC phase under sonication using a microtip (Branson 450, 20 kHz, 3.2 mm diameter, 250 W/cm², continuous mode) and continued for another 30 seconds after the addition. The mixture was emulsified in an ice bath. In a 50 mL Falcon conical centrifuge tube, the primary emulsion was then added drop-wise to 4 mL of Pluronic F-68 (5 mg/mL) under sonication using the same microtip (250 W/cm², continuous mode) and continued for another 30 seconds after the addition. The resulting double emulsion was centrifuged at 50 g for 10 minutes at 4 °C. The supernatant was collected and dialyzed (Slide-A-Lyzer, ThermoScientific, 10K MWCO) against deionized water for two days to remove free NaCl. During the dialysis, water was replaced every 8 hours. After the dialysis, the sample was collected and stored at 4 °C. The same protocol was applied to all the rest perfluorocarbon liquid/saline nanodroplets mentioned in the main text (saline nanodroplets with perfluoropentane, perfluorohexane, perfluoro-15-crown-5-ether, and perfluorodecalin shells). The nanodroplets with diameters 450 nm and 800 nm were produced with the same above-mentioned conditions except for the concentrations of Krytox-PEG (4 mg/mL PFC for both sizes), Pluronic F-68 (2mg/mL for 450 nm and 1 mg/mL for 800
nm), and the power of sonication (200 W/cm² for 450 nm and 150 W/cm² for 800 nm). The referenced saline-soybean oil nanodroplets in aqueous solution were prepared with the same method except that we used soybean oil and Brij 93 as the oil phase and surfactants respectively for the first inversed emulsion.

To allow conjugation of molecular-targeted ligands and indocyanine green (ICG) for molecular targeting and fluorescence visualization, functional groups, thiols, were added to the surface of the pre-formed saline nanodroplets utilizing the post-insertion method. Briefly, 10 mg of DPPC:DSPE-PEG2K-SH:DPPA (with a molar ratio of 0.8: 0.15: 0.05) was dissolved in 2 mL of mixture of chloroform-methanol solution (with a volume ratio of 2:1). The solvent of the mixture was removed by evaporation using a rotary evaporator. The phospholipid film was hydrated using 2 mL of Pluronic F-68 solution (2 mg/mL). The phospholipid suspension was then slowly added into a beaker containing the nanodroplet solution while the solution was gently rotated by a digital shaker (10 RPM, room temperature) continuously at the same speed for 4 hours. The nanodroplets were then purified and solvent exchanged twice by desalting columns (PD-10, Sephadex G-25, GE).

**GRPR Antibody and ICG Dye Conjugation on Nanodroplets**

An anti-GRPR antibody was chosen as a binding moiety for preparing GRPR-targeting nanodroplets. In addition, we also conjugated ICG dyes to the nanodroplets for both cell fluorescence imaging and in vivo epi-fluorescence imaging in tumor bearing mice. We conjugated antibodies to partial thiol groups of PEGs on the nanodroplet surface via a molecular linker, Sulfo-SMCC; at the same time, we conjugated ICG dyes on another partial thiol groups of PEGs on the nanodroplet surface. First, antibody (1 mg/mL) was added to Sulfo-SMCC solution
(20-fold molar excess, in PBS, 4.8 mg/mL, pH 7.2). The mixture was incubated for 2 hours at 4°C. The mixture was then, purified twice to remove free Sulfo-SMCC molecules using a centrifuge desalting column (Bio-Spin 6 Columns, Bio-Rad). Sulfo-SMCC conjugated antibodies and ICG-maleimide were added to nanodroplet solution (10^{11} nanodroplets/mL in PBS pH7.2, the molar ratio of antibodies: ICG: nanodroplets is 1: 9: 7.5) and incubated for 2 hours at 4°C. After the reaction, the nanodroplets were then purified and solvent-exchanged twice with a desalting column (PD-10, Sephadex G-25, GE).

**Characterization of Nanodroplets**

The optical absorption of the ICG-conjugated nanodroplets was characterized using the ultraviolet to visible (UV-Vis) extinction spectroscopy. Extinction spectra were collected from a 0.1 mL nanodroplet suspension in a Microplate Reader (BioTek, Synergy) at room temperature. The fluorescence intensities of the ICG nanodroplet were characterized with epi-fluorescence imaging using IVIS spectrum imaging system (PerkinElmer; see Supplementary Figure S10). The fluorescence images after 60 seconds of exposure (F/stop of 2, medium binning) were recorded with the excitation filter centered at 745 nm and the emission filter centered at 840 nm. The fluorescence radiant efficiency was quantified by Living Image® 4.5 software.

The average size of the nanodroplets was measured with a dynamic light scattering tool (DLS, Zetasizer Nano ZS, Malvern) at room temperature and the size distribution of nanodroplets was determined by the polydispersity value (PDI). The concentrations of the nanodroplets were measured by nanoparticle tracking analysis (Nanosight NS300, Malvern) at 25 °C. The morphology of the nanodroplets was assessed by cryo-EM imaging using a TF20 (Thermo Fisher Scientific, operated at 200 kV) with the nominal magnification of 1,700× and a Gatan K2
Summit direct electron detector in counted mode with 8-second total exposure time and 0.2-second exposure per frame. The total dose for each image is about 0.016 e/Å². The cryo-EM grids were first prepared by applying 3-µL sample (1×10¹⁰ nanodroplets/mL in water) on a 200-mesh Lacey carbon copper grid, then flash plunging into liquid ethane using a Leica EM GP (Leica Microsystems) after 6-second filter paper blotting in a chamber with 95% humidity. The frozen grids were then transferred to TF20 for low-dose imaging with a Gatan 626 cryo-transfer holder and the sample at temperature of -177 degree Celsius.

**UHF-RF-acoustic Signal Characterization**

To characterize UHF-RF-acoustic signals of the nanodroplets, we designed a 3D printed sample holder (Supplementary Figure S3) to vertically hold 7 polyethylene tubes (1 mm in diameter). The tubes were placed concentrically to the center of rotation with a radius of 1.5 cm to ensure that each sample will be exposed to the same power of RF wave during the tomography. Nanodroplet solutions and a PBS control solution (physiological saline, 0.9 wt% NaCl) were filled in the tubes, sealed, and placed on the holder for imaging.

We prepared a bovine tissue phantom with tube inclusions to characterize the UHF-RF-acoustic signals of nanodroplets as a function of imaging depth inside the tissue (main text Figure 3e). To do so, we first froze bovine tissue at -20 °C for 4 hours, then drilled six cylindrical holes (with a diameter ~3 mm) using an M3 drill bit. The nanodroplet solutions were mixed with 12% gelatin (1:1 volume ratio) and filled to the holes in the tissue. A 3D-printed holder was built (by 3D system ProJet) to hold the tissue vertically (Supplementary Figure S11). After the tissue thawed completely to room temperature, we started setting up the UHF-RF-acoustic imaging. During imaging, the whole tissue was immersed in a water tank similar to the one used in the in vivo
experiment. The water tank maintains the temperature of the tissue phantom at 37°C, while the phantom was rotated within the imaging plane during the imaging.

To prepare the Stanford logo phantom (main text Figure 3f), a mold of Stanford logo was 3D printed. The mold was used to prepare an agar (5% w/v) phantom with a Stanford logo inclusion (with a diameter ~3 cm). The nanodroplet solutions were mixed with 12% gelatin (1:1 volume ratio) and filled in the space of the inclusion. A 3D-printed holder was built (by 3D system ProJet) to hold the agar phantom vertically.

To prepare the bovine tissue enclosure, the same freezing protocol was used as in the bovine tissue phantom, except that this time a hole saw (with a diameter of 3 cm) was used to drill one cylindrical hole (with a resulting diameter ~3.1 cm). This hole is used to fit the agar gel phantom from the previous step. The tissue was placed in the imaging plane but only the agar gel phantom was rotated during the tomography.

**Confirmation and Quantification of Functionalization**

The concentration of thiol groups on the nanodroplets are confirmed and quantified by a fluorometric thiol quantification assay kit, shown in Supplementary Figure S2.

**Western Blotting**

The expression of GRPR on six prostate cancer cell lines (PC3, C4-2, ARCap, DU145, LnCap, 22Rv1) were evaluated by western blotting with a rabbit polyclonal anti-GRPR antibody (1 μg/mL, predicted molecular weight of 43 kDa and observed molecular weight of 53 kDa) using total cell lysates prepared from prostate cancer cells. Glyceraldehyde 3-phosphate
dehydrogenase (GAPDH) was used as a protein loading control and detected using monoclonal antibody (37 kDa, 0.2 μg/mL) on the same blot. A representative full scan of western blot is shown in Supplementary Figure S6. The semi-quantitative analysis on the western blot is shown in Supplementary Figure S7.

Cell Culture

Human prostate cancer cell lines, PC3-GFP and DU145-GFP cells were purchased from American Type Tissue Collection (ATCC) and cultured on collagen-coated flasks (BD Biosciences) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum per the manufacturer’s recommendation. The cultures were maintained in a humidified incubator with 5% CO₂ / 95% air at 37 °C. The cell lines were authenticated at Stanford functional genomics facility using Short Tandem Repeat (STR) profiling. The cell lines were tested for mycoplasma contamination upon received, after thawed, and monthly during culture using MycoAlert Mycoplasma Detection Kit (Lonza).

Cell Culture Uptake of GRPR-targeted Nanodroplets

1×10⁴ cells PC3 and DU145 cells labeled with green fluorescence protein (PC3-GFP and DU145-GFP cells) were plated in a 96-well plate. Twenty-four hours after seeding, the cell media were removed, and the cells were washed twice with PBS. Then, the cells were incubated with 1×10⁹ nanodroplets in the cell culture medium for 12 hours. After that, cells were imaged by a fluorescence microscope (EVOS FL cell imaging system, Thermo Fisher Scientific) with 20X objective lens, and its GFP filter set and ICG filter set.
Cell Viability Test

PC3 cells were plated in triplicate for each nanodroplet concentrations (4 concentrations $1 \times 10^8$, $1 \times 10^9$, $1 \times 10^{10}$, $3 \times 10^{11}$ and a control 0.9wt% saline) at a density of $1 \times 10^4$ cells per well in a 96-well plate. Cells were allowed to attach for 24 hours, then the wells were washed with PBS. The 100 µL of mixture of nanodroplets in the medium was added to the wells and incubated at a cell incubator for 24 hours. Nanodroplets and medium were then removed from the wells and replaced with 100 µL of medium and 10 µL of Presto Blue. The plate was incubated at 37 °C for 30 min before fluorometer reading at 590 nm (excitation 560 nm, Synergy 4, BioTek).

UHF-RF-acoustic Imaging System

A prototype UHF-RF-acoustic tomography imaging system (with main parts from Endra Inc., Michigan) was used to measure the UHF-RF-acoustic signal from the phantom and the mice. The samples were mounted on a rotational stage that can rotate 360 degrees with 10-degree intervals. During the data acquisition, we rotated the samples at 10-degree intervals. At each angle, 4095 pulses were recorded and averaged to improve the signal-to-noise ratio. A nanosecond 433 MHz RF pulse (pulse duration 160 ns, repetition rate of 1500 Hz) was broadcasted through a pair of customized horn antennas at the near field to the phantom. A 3.5 MHz linear array ultrasound transducer (Acuson L382, bandwidth ~75%, depth of focus 82 mm) measures the generated acoustic waves, which provides 1.2 mm imaging resolution in the transverse plane. Acoustic signals were recorded by an ultrasound imaging system. A filtered back projection algorithm was used to reconstruct each image. The reconstruction software was provided by Endra Inc.
Animal Studies

All animal experiments were performed in compliance with the Guidelines for Administrative Panel on Laboratory Animal Care established by the Stanford University, under the protocol APLAC-13024. Healthy male NSG mice (Jackson laboratory) at age 6 weeks were used in this study. A prostate cancer in mouse model (PC3 and DU145 cancer cell lines) was developed by subcutaneously injecting 100 µL of $5 \times 10^6$ prostate cancer cells mixed with 1:1 volume ratio of growth factor reduced Matrigel (Corning) into the right flank of each mouse. The tumors were allowed to grow to about 1 cm³ before imaging. Mice were anesthetized with 2% isoflurane at 2 liters min⁻¹ of oxygen flow. 100 µL of nanoparticles/phosphate-buffered saline solution (nanodroplets in PBS, $1 \times 10^{11}$ nanodroplets/mL) were injected to the mice through the tail vein.

In vivo UHF-RF-acoustic/Ultrasound Imaging

For in vivo imaging, UHF-RF-acoustic imaging and ultrasound imaging were recorded separately. We used the same UHF-RF-acoustic prototype system with a home-made mouse holder (Supplementary Figure S12). The mouse was held vertically (perpendicular to the imaging plane of the ultrasound transducer) while anesthetized with 2% isoflurane. The mouse body below the neck was immersed in the water at a constant temperature (37 °C). The same imaging condition was used for recording the ultrasound imaging, except that an additional ultrasound imaging system (Vevo® 2100, VisualSonics) with an imaging transducer (LZ250, VisualSonics, central frequency: 21MHz) was used to collect ultrasound imaging of the mouse on the same animal holder. The recorded images are then registered with UHF-RF-acoustic images by the alignment makers on the images.
Nanodroplet Toxicity Studies

We conducted histology studies of tissue, complete blood count, and chemistry panel in three additional three groups of healthy NSG mice (5 mice per group). We injected the same amount of the nanodroplets used in imaging through the tail vein to the first and second groups of mice, and the same volume of PBS to the third group as a control. For the first and second groups, we recorded their vitals and behavior immediately after particle administration. The first group of the mice were sacrificed at 3 days post-injection to collect tissue (liver, kidney, spleen, pancreas, heart) and blood. The second and third groups were sacrificed at 14 days post-injection. The toxicity results show that our nanodroplets neither affect the blood count (blood test assay) nor damage any tissue of the main organs at the dosage used (Supplementary Table S1).

In Vitro Competitive Binding Experiments

PC3 cells (1 × 10^4 cells) were incubated with ICG-GRPR antibodies (0.5 nM) and varying concentrations of GRPR-targeted nanodroplets (0.001 nM to 10 nM) in 100 μL of the binding buffer (RPMI 1640 + 2 mg/mL BSA + 5.2 mg/mL HEPES) at 37°C for 1 hour. Supplementary Figure S9 shows the results from the in vitro competitive binding experiments. Fluorescence signals from the ICG-GRPR antibodies were used as the readout because of their high sensitivity. The half-maximal inhibitory concentrations (IC₅₀) of nanodroplets is at 8.44 nM. The experiment was repeated 5 times.

Blood Half-life of Nanodroplets
In the blood half-life experiment, we injected 100 µL of ICG-nanodroplet solution via the tail vein (1×10¹¹ nanodroplets/mL) to healthy nu/nu mice (N=5). We collected ~3-4 µL of blood using 10 µL pipette via the tail bleeding method at each time point. Blood was collected from each mouse right before the nanodroplet administration, and after the nanodroplet administration at 1, 2, 3, 6, 10, 24, 36, 48, 72, and 92 hours. The drawn blood was mixed with the same volume of Heparin (1000 USP/mL) in a 100 µL capped PCR tube and frozen in -80 °C immediately until analysis. Samples were thawed at room temperature for 30 minutes prior to analysis. Blood with Heparin sample (3 µL) was diluted by 12 µL of PBS then added to a 384-well clear bottom black wall plate and measured by a fluorescence plate reader immediately (excitation 760 nm/emission 800 nm). The fluorescence intensity of the ICG-labeled nanodroplets at each time-point was acquired by subtracting the auto-fluorescence signals of the blood sample (before nanodroplet administration) from the signals of the blood sample (with nanodroplets) of the same mouse. The data was plotted and fitted with the one-phase-decay model (Supplementary Figure S13). The half-life is calculated from the decay constant of the fitted curve.

Quantification of Bio-distribution of Nanodroplets

We quantified the nanodroplet distribution in tissue of the main organs with epi-fluorescence imaging. For the bio-distribution, mice were sacrificed 48 hours post-injection of the nanodroplets. Epi-fluorescence imaging of the excised organs was carried out using an IVIS spectrum imaging system (PerkinElmer) or a Spectral instruments imaging system (Lago) with an excitation filter centered at 745 nm and an emission filter centered at 840 nm with 60 seconds of exposure time. We quantitatively analyzed the images using Living Image 4.5 software using the radiant as the read-out.
In the longitudinal bio-distribution of nanodroplets study, we injected 100 µL of GRPR-targeted ICG-labeled nanodroplet solution via the tail vein (1×10^{11} nanodroplets/mL) to mice (N=5, NSG mice with GRPR-positive PC3 tumor). After injection, the in vivo epi-fluorescence imaging of the mice was recorded at 0.5, 2, 4, 17, 24, 41, 72 hours using Spectral Instruments Imaging system (Lago). As a control, a group of the healthy mice (N=5, nu/nu mice) were injected the same amount of GRPR-targeted ICG-nanodroplet solution. Using the same imaging conditions of the tumor-bearing mice, both dorsal and ventral sides of the mice were imaged at 1, 6, 10, 24, 28, 48, 72 hours after injection. The longitudinal fluorescence images are analyzed using Aura software (Supplementary Figure S14).

To study time-dependent bio-distribution of nanodroplets in tumor and main organs, we injected 100 µL of GRPR targeted ICG-labeled nanodroplet solution via the tail vein (1×10^{11} nanodroplets/mL) to 5 groups of mice for each time point (N=5, NSG mice with PC3 tumor). After injection, each group of the mice were euthanized at 3, 5, 24, 48, 72 hours respectively. The excised tumors, liver and spleens where nanodroplets mainly accumulated were ex vivo imaged using Spectral Instruments Imaging system (Lago). The fluorescence intensities at each site were analyzed using Aura software (Supplementary Figure S15).

**Numerical Simulations**

We conduct analytical calculations and numerical simulations to estimate the temperature rise of the nanodroplets and the surrounding tissue, as well as the electric fields near the nanodroplets.

To calculate the temperature profile, we first calculate the UHF-RF absorption cross-section of the nanodroplets using dipole approximation with Matlab R2019b. The saline core contains 25
wt% of salt, we use an electrical conductivity of 22.2 S/m. Our dipole approximation estimates that the absorption cross-section of one nanodroplet at 433 MHz is $1.475 \times 10^{-5}$ µm$^2$.

To estimate the temperature profile across the nanodroplets, we conduct numerical simulations using the Finite Element Method (COMSOL Multiphysics 5.4, Heat Transfer Module). The UHF-RF pulse has a Gaussian temporal profile, a temporal width of 160 ns at full-width-at-maximum, and each pulse carries an energy of approximately 1 mJ. From the experimental configuration, one pulse is split into two and emitted by two UHF-RF antennae each with an area of ~50 cm$^2$. The antenna has a divergence angle of ~9°, and mice are placed at ~5 cm away from the antenna. Therefore, the area of illumination in the free-space is ~0.015 m$^2$, and the time-averaged illumination intensity is ~100.12 W/m$^2$. By taking into account the electromagnetic wave impedance of tissue and that of the free-space, the time-averaged intensity is ~36.88 W/m$^2$ inside the tissue. The temporal profile of one UHF-RF pulse is shown in Supplementary Figure S16 (a), the peak intensity of the pulse inside the tissue is $1.44 \times 10^5$ W/m$^2$. We take a temporal step of 6.4 ns to ensure a high temporal resolution and numerical convergence. The initial temperature is assumed to be 310 K to simulate the body temperature of mice in the in vivo experiments. Due to the much higher conductivity of the saline-core of the nanodroplet than tissue, it absorbs the UHF-RF energy at 433 MHz strongly, therefore the nanodroplet acts as a major heat source. A continuous boundary is assumed between the nanodroplet and the surrounding tissue, and a 310 K-temperature boundary is assumed as the outer tissue domain.

The nanodroplet has a diameter of 250 nm, including the saline core ($d = 150$ nm) and a 50-nm-thick perfluorocarbon shell. These dimensions are extracted from our experimental results and confirmed with cryo-transmission electron microscopy imaging (main text, Figure 2e).
Supplementary Figure S16 (b) shows the temperature elevation as a function of time at the center and the surface of the nanodroplet. At the nanodroplet’s center, the peak temperature rise is 13.2 µK; at the nanodroplet/tissue interface, the peak temperature rise is 1.2 µK. At the peak temperature, Supplementary Figure S16 (c) shows one-dimensional (1D) and two-dimensional (2D) temperature profile across the nanodroplet. The temperature decays dramatically away from the nanodroplet/tissue interface, at 70 nm away from the nanodroplet, the elevated temperature is ~0.4 µK. We also estimate the macroscopic tissue temperature rise using analytical method with the following parameters: a tissue relative permittivity of 53, a tissue conductivity of 1.43 S/m, an electromagnetic wave impedance of 42.23 Ω at 433 MHz inside tissue. With the same RF pulse width and intensity as in the COMSOL simulation, the temperature increase of macroscopic tissue is 0.4 µK.

To estimate both global and local Specific Absorption Rate (SAR), we analytically calculate the time-averaged electric field in tissue and numerically simulate the local electric field distribution across a nanodroplet (Supplementary Figure S16 (d)). For the perfluorocarbon shell, we use a relative permittivity of 2 without electric conductivity; for the saline core, we use a relative permittivity of 22.4 and electric conductivity of 22.2 S/m. Using the energy of each UHF-RF pulse (1 mJ), the source repetition rate 1500 Hz, the area of illumination 0.015 m², as well as the tissue impedance, we estimate that the time-averaged electric field inside tissue is $|E|=39.47$ V/m. The estimated global SAR is defined as $\sigma|E|^2/\rho$, where $\sigma$ is the electric conductivity of tissue and $\rho$ is the tissue density (1090 kg/m³). The global SAR is estimated to be 2.04 W/kg.

To calculate the local SAR, we use the COMSOL AC/DC Module to simulate the local electric field around the nanodroplet. At the nanodroplet/tissue interface the highest electric field is 57.94
V/m, which leads to a local SAR of 4.40 W/kg. Both global and local SARs meet the IEEE safety guideline of 2-4 W/kg and 20-40 W/kg, respectively.12

**Data Analyses, Statistics, and Reproducibility**

For data analysis, we used MATLAB to process the images acquired with the Vevo imaging system. The ultrasound images are shown in dB scale, and the UHF-RF-acoustic images in linear scale. The two-dimensional (2D) tube phantom images shown in Figure 2 (main text) are the maximum intensity projections of the 3D volumetric images to the plane of lateral axis and elevational axis of the transducer. Data plot, average, and standard deviation were computed in Origin pro 2009.

To test the nanodroplet uptake in the cell culture study, we calculated the mean and standard deviation of fluorescence intensities by first summing the fluorescence intensities of ICG from 30 cells (red channel) and then normalizing it with the cell footprint. This footprint was defined as the area covered with GFP (green channel), indicating the viability of the cell. Image J was used here for signal analyses. For bio-distribution of the nanodroplets in the in vivo studies, we summed the fluorescence intensities within the region of interest (Figure 5e in main text). The region of interest was identified by the footprint of each organ from the photographic images. We then normalized the summation with the footprint to obtain the mean and standard deviation. In this research, we calculated the two-tailed $p$-value using an unpaired student $t$-test to determine the significance. We considered our data to be statistically significant with $p < 0.05$.

The Cryo-EM experiment shown in main text figures 2e was repeated 3 times independently with similar results; the Western blot analysis for GRPR expression in different prostate cancer
cell lines shown in main text figure 4b was repeated 5 times independently with similar results; the optical fluorescence cell imaging shown in main text figure 4d was repeated 3 times independently with similar results; the immunohistochemical experiment shown in figure 5g was repeated 3 times independently with similar results.
Supplementary Figure S1 | Fluorescence intensity as a function of the thiol concentration.

The open dots represent the different concentrations of the standard thiol sample provided by the thiol quantification kit (white filled circles). The relation between the intensity and the thiol concentration was fitted with a linear regression fitting (red line, $R^2 = 0.97$, data are presented as mean values +/- standard deviation, N=6). The nanodroplets ($1.5 \times 10^{15}$ nanodroplets/mL, black filled circle) contain $2.7 \pm 0.5$ μmole of thiols which correspond to $(6.5 \pm 1.2) \times 10^3$ thiols per nanodroplet. The experiment was replicated twice with the similar results.
**Supplementary Figure S2**| Dynamic light scattering (DLS) measurement of UHF-RF-acoustic contrast agents. Size distribution of the UHF-RF-acoustic nanodroplets used in this study confirms their averaged diameter of 248 ± 34 nm. The experiment was replicated 3 times with the similar results.
Supplementary Figure S3 | Customized tube phantom holder for UHF-RF-acoustic characterization of nanodroplets. a, Three-dimensional schematic of the customized tube-phantom holder. Dimensions of the customized holder for calibration of nanodroplets in main text Figure 2. b, The transverse view shows that the total width of the holder is 20 mm, and c, the vertical view shows that each tube phantom is 1 mm in diameter, and the thickness of the tube is 250 µm. d, A photograph of the assembled tube phantom.
Supplementary Figure S4: UHF-RF-acoustic amplitude as a function of iron oxide nanoparticle concentration. The open dots represent the UHF-RF-acoustic amplitude at each concentration, data are presented as mean values +/- standard deviation (N = 5), and the dashed line represents the linear regression fitting of the data ($R^2 = 0.997$). The experiment was replicated twice with similar results.
Supplementary Figure S5| UHF-RF-acoustic image resolution. a, The UHF-RF-acoustic image of the Stanford Logo phantom from main text Figure 3. The red and blue bars are drawn across the smallest features of the image (i.e., the branches of the redwood tree) to show the 1D signal (line profiles) in panel (b). b, The line profiles from the image at the locations indicated by the red and blue bars in (a), displaying the spatial resolution ~1.2 mm in our imaging technique, the “0” in the Distance axis corresponds to the top of the red/blue bars.
Supplementary Figure S6| A representative image of a western blot scan. The scan shows lysates of 6 human prostate cancer cell lines. Polyvinylidene difluoride (PVDF) membranes were probed with a rabbit polyclonal anti-GRPR antibody (1 μg/mL) to detect GRPR expression of each cell line. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control was probed by mouse monoclonal GAPDH antibody (0.2 μg/mL). Specific bands were detected for GRPR and GAPDH at approximately 37 kDa and 53 kDa (as indicated).
**Supplementary Figure S7| Semi-quantitative analysis of Western blot.** The intensity of GRPR/GAPDH of PC3 is $2.82 \pm 0.81$ times higher than that of DU145 (data are presented as mean values +/- standard deviation; $*, p=0.0013, N=5$).
Supplementary Figure S8| Fluorescence intensities of ICG from 30 cancer cells incubated with nanodroplets. PC3 cells with the GRPR-targeted nanodroplets show 2.5-fold higher ICG fluorescence signals ($p=0.002$, $N=30$ analyzed in both cases, data are presented as mean values +/- standard deviation), compared with the non-targeted (PEG) nanodroplets. As expected, the negative control cell line DU145 shows much lower fluorescence signals from both targeted and non-targeted nanodroplets due to the relatively low expression of GRPR. The signal from the targeted PC3 is statistically significant (2.15 ± 0.57 times higher) that that from targeted DU145.

*, $p=0.002$; **, $p=0.006$; ***, $p=0.005$; ****, $p=1.7\times10^{-15}$. 
Supplementary Figure S9 | In vitro competitive binding experiments. The in vitro competitive binding experiments show that the half-maximal inhibitory concentrations (IC₅₀) of nanodroplets is at 8.44 nM, data are presented as mean values +/- standard deviation, the error bars are standard deviation of 5 replicates.
Supplementary Figure S10| Fluorescence intensity of ICG-saline nanodroplets as a function of concentration. a, Epi-fluorescence images of ICG-saline nanodroplets. The concentration of nanodroplet from left to right is $(1 \pm 0.21) \times 10^{11}$ nanodroplets/mL, $(2 \pm 0.27) \times 10^{11}$ nanodroplet/mL, $(3 \pm 0.34) \times 10^{11}$ nanodroplets/mL, $(4 \pm 0.53) \times 10^{11}$ nanodroplets/mL, $(5 \pm 0.79) \times 10^{11}$ nanodroplets/mL, respectively. b, Fluorescence intensity of ICG-saline nanodroplets as a function of concentration. The open dots are the averaged sum of fluorescence intensity over the area of a single well in 96 well plate (ROI =0.3 cm², N=5). The x error bar is the standard deviation of concentration (N=5), the y error bar is the standard deviation of 5 independent measurements at each concentration. Data are presented as mean values +/- standard deviation. The red dashed line is the linear regression fitting of the intensity data, $R^2 = 0.97$. 
**Supplementary Figure S11** | Bovine tissue holder for UHF-RF-acoustic tomography.  

*a*, schematic illustration of the bovine tissue holder.  

*b*, The photograph of the tissue holder with bovine tissue mounted in the vertical direction. The two black tubes are the tissue supporting rod. The bovine tissue has a thickness of 3 cm.  

*c*, Illustration of the bovine tissue cross section. The six white dots show the locations of the six tubes containing nanodroplets; the two black dots show the locations of the two tissue supporting rods.  

*d*, The photograph of the bovine tissue, showing the six drilled holes for the locations of the six tubes. The two white arrows point towards the two tissue supporting rods (the black rods in panel (b), also shown as the yellow circles in the main text Figure 3e).
Supplementary Figure S12| Customized animal holder for UHF-RF-acoustic tomography.

a, Three-dimensional schematic illustration of the customized animal holder. b, A photograph of the customized animal holder produced with 3D printing. c, A photograph of one of the mice during experiments mounted on the customized animal holder. d, Setup for UHF-RF-acoustic tomography imaging with a mouse mounted during the experiment.
**Supplementary Figure S13| Elimination half-life of nanodroplets in blood.** Measured fluorescence intensity of ICG-nanodroplets in blood as a function of time (data are presented as mean values +/- standard deviation, error bars represent standard deviation of 5 replicates). The red curve represents the one-phase-decay fitting of the measured data. The half-life is calculated from ln2 divided by the elimination rate constant. The half-life of nanodroplets is 21.31 ± 4.14 hours.
Supplementary Figure S14 | Analysis of the in vivo fluorescence signals showing the nanodroplet pharmacokinetics. a, Representative images of GRPR-positive (PC3) tumor baring NSG mice (N=5) with GRPR-targeted ICG-nanodroplets at 0.5, 4, 17, 24, 41, and 72 hours after injection. b, Representative images of the healthy nu/nu mice (N=5) with GRPR-targeted ICG-nanodroplets at 1, 6, 10, 24, 48, and 72 hours after injection. c, Analysis of the in vivo fluorescence signals in PC3 tumor (N=5, black) and tissue (N=5, red). The black dashed curve is the guide to the eye. The half-life of GRPR-targeted ICG-nanodroplets in tumors is
38.16 ± 21.00 hours. *p = 0.764, **p = 0.635, ***p = 0.037. d. Analysis of the in vivo fluorescence tissue signals in the dorsal side of the healthy nu/nu mice (N=5). The half-life of nanodroplets is 29.29 ± 14.79 hours. For both (c) and (d), data are presented as mean values +/- standard deviation. The decay-fitting curves represent the one-phase-decay fitting of the measured data. The half-life is calculated from ln2 divided by the elimination rate constant. When comparing the two half-lives in (c) and (d), the p value is 0.232; this difference is not statistically significant.
Supplementary Figure S15 | Analysis of ex vivo tissue and organs showing the nanodroplet pharmacokinetics. a, Fluorescence signals of harvested tumors from mice with GRPR-targeted ICG-labeled nanodroplets at each time point, showing the nanodroplet accumulation after 24 hours and the fluorescence signals remain at the peak value until 48 hours in PC3 tumors. *p = 0.742, **p = 0.0005. The analysis of ex vivo fluorescence signals in b, liver and c, spleen at each time-point, showing the half-life is 27.79 ± 14.97 hours in liver and 14.87 ± 2.52 hours in spleen. The decay-fitting curves represent the one-phase-decay fitting of the measured data. The half-life is calculated from ln2 divided by the elimination rate constant. Data in (a), (b) and (c) are presented as mean values +/- standard deviation (N=5).
Supplementary Figure S16 | Simulated temperature profile and electric field distribution near a nanodroplet in tissue using COMSOL. The simulation domain contains one nanodroplet. **a**, Illumination intensity of the UHF-RF pulse. The UHF-RF pulse is assumed to be Gaussian with a full width at half maximum of 160 ns and peak intensity of $1.44 \times 10^5$ W/m$^2$. **b**, Simulated temperature elevation with a pulse energy of 1 mJ at two locations: the center (blue) and surface (red) of the nanodroplet. The nanodroplet has a diameter of 250 nm with a 25wt% saline core and a perfluorocarbon (PFC) shell. The PFC shell thickness is 50 nm. The inset shows the core-shell structure of the nanodroplet, where the blue dot locates at the center of the nanodroplet, indicating the location where the blue temperature curve is evaluated; the red dot
locates at the nanodroplet/tissue interface, indicating the location where the red temperature curve is evaluated. e, Simulated two-dimensional (2D) and one-dimensional (1D) temperature profile across a nanodroplet at the time when the temperature reaches its peak. The 1D plot is evaluated across the center of the nanodroplet. d, Simulated 2D and 1D electric field profile across a nanodroplet. The 1D plots of electric field are across the x and y axis, respectively. The highest local electric field at nanodroplet/tissue interface is 57.94 V/m.
Supplementary Table S1: Complete blood count. NSG mice (N=5 per group) were tail-vein injected with nanodroplets and hematological and biochemical measurements were taken at two weeks post injection. Values represent the mean ± standard deviation. Clinical pathology reference ranges are from The Jackson Laboratory unless otherwise indicated. a: Stanford VSC Clinical Lab mouse reference value. b: Reference ranges adapted from Knibbe-Hollinger et al.\textsuperscript{14} WBC: white blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; MPV: mean platelet volume; PLT: Platelet count; and PCT: plateletcrit ALT: alanine transaminase; Alk Phos: alkaline phosphatase; BUN: blood urea nitrogen; T. Protein: total protein; TIBC: total iron-binding capacity.

| Tests              | Result                  | Reference Range |
|--------------------|-------------------------|-----------------|
|                    | Mouse 1 | Mouse 2 | Mouse 3 | Mouse 4 | Mouse 5 | Averaged |                   |
| WBC (K/μL)         | 3.78    | 5.45    | 6.35    | 8.86    | 11.01   | 7.09±1.43 | 5.5 - 9.3\textsuperscript{a} |
| RBC (M/μL)         | 9.25    | 9.11    | 9.1     | 8.6     | 8.79    | 8.97±0.133 | 7.0 - 8.8 |
| HGB (gm/dL)        | 14.1    | 14.3    | 13.9    | 12.9    | 13.6    | 13.76±0.273 | 13.7 - 16.4 |
| HCT (%)            | 49.8    | 46.4    | 46.5    | 44.4    | 45.4    | 46.5±1.016 | 39 n - 47 n |
| MCV (fL)           | 53.8    | 50.9    | 51.1    | 51.6    | 54.6    | 52.4±0.843 | 52.0 - 68.7 |
| MCH (pg)           | 15.2    | 15.7    | 15.3    | 15      | 15.5    | 15.34±0.135 | 18.4 - 19.6 |
| MCHC (g/dL)        | 28.3    | 30.8    | 29.9    | 29.1    | 30      | 29.6±0.476 | 34.0 - 36.0 |
| Platelet Count (K/μL) | 1633 | 1261 | 844 | 1595 | 1435 | 1353.6±160.318 | 675 - 1338 |
| RDW (%)            | 20.2    | 19.5    | 22.2    | 20.9    | 19      | 20.36±0.627 | 16.9 - 23.5 |
| MPV (fL)           | 6.4     | 7       | 7       | 7.1     | 6.5     | 6.8±0.167 | 6.5 - 8 |
| PCT (%)            | 1.05    | 0.88    | 0.59    | 1.14    | 0.93    | 0.918±0.105 | 0.3 - 0.9 |
| Reticulocyte Count (%) | 5.11 | 6.07 | 6.62 | 6.41 | 3.66 | 5.57±0.608 | 3.00 - 5.8 |
| Neutrophils (%)    | 85      | 29      | 32      | 35      | 40      | 44.2±11.583 | 50 - 75\textsuperscript{5} |
| Lymphocytes (%)    | 10      | 49      | 51      | 50      | 43      | 40.6±8.69 | 10.5 - 40\textsuperscript{5} |
| Monocytes (%)      | 1       | 22      | 17      | 15      | 17      | 14.4±3.96 | 5 - 14.5 |
## Supplementary Table S2: Thermophysical properties of perfluorocarbon liquid\textsuperscript{15-17}

| Property               | Perfluoropentane | Perfluorohexane | Perfluorodecalin | Perfluoro-15-crown-5-ether |
|------------------------|------------------|-----------------|------------------|---------------------------|
| Molecular weight (g mol\textsuperscript{-1}) | 288.04           | 337.90          | 461.90           | 580.01                    |
| Density (kg m\textsuperscript{-3})          | 1.63             | 1.68            | 1.93             | 1.78                      |
| Boiling point (°C)     | 26.00-36.00      | 58.00-60.00     | 144.00           | 145.00                    |
| Vapor pressure (kPa)   | 83.99            | 29.41           | 1.02             | 0.92                      |
| Water solubility       | $4.00 \times 10^6$ | $2.70 \times 10^{-7}$ | $9.90 \times 10^9$ | No data                   |
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