In vivo ultrasound-switchable fluorescence imaging using a camera-based system

SHUAI YU,1,2,3 TINGFENG YAO,1,2,3 YANG LIU,1,2 AND BAOHONG YUAN1,2,*

1Ultrasound and Optical Imaging Laboratory, Department of Bioengineering, The University of Texas at Arlington, Arlington, TX 76019, USA
2Joint Biomedical Engineering Program, The University of Texas at Arlington and The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
3These authors contributed equally to this work
*baohong@uta.edu

Abstract: Ultrasound-switchable fluorescence (USF) is a novel imaging technique that provides high spatial resolution fluorescence images in centimeter-deep biological tissue. Recently, we successfully demonstrated the feasibility of in vivo USF imaging using a frequency-domain photomultiplier tube-based system. In this work, for the first time we carried out in vivo USF imaging via a camera-based USF imaging system. The system acquires a USF signal on a two-dimensional (2D) plane, which facilitates the image acquisition because the USF scanning area can be planned based on the 2D image and provides high USF photon collection efficiency. We demonstrated in vivo USF imaging in the mouse’s glioblastoma tumor with multiple targets via local injection. In addition, we designed the USF contrast agents with different particle sizes (70 nm and 330 nm) so that they could bio-distribute to various organs (spleen, liver, and kidney) via intravenous (IV) injections. The results showed that the contrast agents retained stable USF properties in tumors and some organs (spleen and liver). We successfully achieved in vivo USF imaging of the mouse’s spleen and liver via IV injections. The USF imaging results were compared with the images acquired from a commercial X-ray micro computed tomography (micro-CT) system.

1. Introduction
Optical imaging based on fluorophores in biological tissue has been intensively desirable in recent decades due to its high sensitivity and selectivity, non-ionizing radiation, low cost, and capability of multicolor imaging. [1–4] In particular, fluorescence imaging in centimeter-deep tissue has attracted lots of attention in cancer diagnoses because of its ability to track many cellular-level and molecular-level phenomena, such as cancer cell migration, angiogenesis, and cyclic concentration variations in vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs). [5–8] However, fluorescence imaging has encountered a dilemma: Its spatial resolution degrades quickly with the increase of imaging depth, due to the biological tissue’s high optical scattering property. [1] This dilemma prevents it from non-invasively revealing tissue’s microscopic information at centimeter depth.

To overcome the low-resolution limitation caused by strong tissue-optical scattering, researchers have developed several techniques. For example, photoacoustic imaging achieves high resolution by converting optical energy into acoustic energy and has been used for many applications. [9–12] Usually, this technique applies a narrow-pulsed laser to tissue; when the optical energy is absorbed and converted to heat, it triggers thermo-elastic expansion and thus ultrasound emission. Because ultrasound signal has a scattering coefficient two to three orders of magnitude less than the light in tissue [9], photoacoustic imaging provides optical contrast with acoustic resolution. Meanwhile, researchers have developed ultrasound-encoded optical imaging techniques, so-called
time-reversed ultrasonically encoded optical focusing. In this technique, a focused ultrasound (FU) is used to encode or tag photons. These encoded photons are recorded. Using an optical phase conjugation technique, time-revered photons of these encoded photons can be generated. Eventually, these time-revered phones can re-focused back into deep tissue. [13–16] Another technique is aimed at confining fluorescence emission only in a small volume through the interference of a FU and only switching on the fluorophores in the focal volume, so called ultrasound-switchable fluorescence (USF). [17–28] USF adopts a unique fluorescent contrast agent whose fluorescence can be switched on when temperature rises above a threshold (Tth).

When a FU beam is delivered into tissue, it induces a temperature rise in its focus. If there are USF contrast agents in the focus, they will be switched on by thermal energy and release a USF signal. After the FU pulse ends, the temperature cools down and falls below the Tth due to thermal diffusion. Thus, the USF signal will gradually switch off. By scanning the ultrasound focus and repetitively turning on and off the agents, we can acquire a three-dimensional (3D) USF image about the contrast distribution at ultrasound resolution. Because of USF’s excellent detection sensitivity and specificity, high spatial resolution, as well as its potentials in functional and molecular imaging, USF imaging provides a feasible tool for early-stage cancer diagnosis, which is crucial for the survival rate of patients.

Recently, we successfully achieved in vivo USF imaging in mice. [27] We carried out the experiments in a frequency-domain USF imaging system using a single-channel photomultiplier tube (PMT), developed in our previous work. [22] In this work, we demonstrated in vivo USF imaging in a camera-based USF imaging system. The advantages of implementing USF imaging via such a system were improved photon collection efficiency and the fact that the USF scanning area can be planned based on the two-dimensional (2D) image. In addition, the camera system does not require physical contact with the tissue. In this work, we demonstrated in vivo USF imaging with multiple imaging targets in the mouse’s tumor via local injection. Furthermore, we demonstrated that when the USF contrast agents were designed at different particle sizes (70 nm and 330 nm), they would bio-distribute to the mouse’s various organs through intravenous (IV) injections. The results showed that the fluorescence from some organs (spleen and liver) retained high USF properties. Consequently, we successfully achieved in vivo USF imaging of various organs (spleen and liver). All USF imaging results were compared with the images acquired from a commercial X-ray micro computed tomography (micro-CT) system.

In summary, in this work we successfully demonstrated in vivo USF imaging via a camera-based USF imaging system and discussed its advantages. Also, designing USF contrast agents with different sizes made in vivo USF imaging possible in various organs via IV injections. This work will push this technology forward for future applications.

2. Materials and methods

2.1. Chemical materials

N-isopropylacrylamide (NIPAM), acrylamide (AAm), 4-4’-Azobis(4-cyanopentanoic acid) (ACA), sodium dodecyl sulfate (SDS), N,N’-methylenebisacrylamide (BIS), N-tert-butylacrylamide (TBAM), and indocyanine green (ICG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals were used without further purification.

2.2. Animal preparation

The animal protocols were approved by the University of Texas at Arlington’s Animal Care and Use Committee. In this study, we adopted two mouse species: 1) nude mouse (NU/J, female, Jackson Laboratory) and 2) BALB/cJ mouse (BALB/cJ, female, Jackson Laboratory). We used the nude mice for USF imaging in tumors. At age of 7 weeks, the mice were implanted glioblastoma tumors on their right hind legs. At age of 11–14 weeks, they were performed
3D USF imaging and 3D CT imaging when the contrast agents were locally injected in their tumors. We used the BALB/cJ mice (6–8 weeks) for USF imaging of organs. The mice were removed hair and performed 3D USF and CT imaging after the contrast agents were intravenously injected through a lateral tail vein and accumulated in some organs (i.e., spleen and liver). During USF imaging, the mice (NU/J or BALB/cJ) were anesthetized with isoflurane (ISOSOL ISOFLURANE, Miller Veterinary Supply, Ft. Worth, TX, USA). The mice’s respiratory rate was about 1 breath/second under anesthetized conditions. Also, the mice’s body temperature was maintained at about 35–36 °C with a tank of warm water. After USF imaging, the mice were euthanized. Then, the dead mice were performed 3D CT imaging in a commercial micro-CT system.

2.3. Contrast agent preparation

In USF imaging, we adopted ICG-encapsulated poly(N-isopropylacrylamide) (PNIPAM) nanoparticles (ICG-NPs, the 2nd generation) as USF contrast agents [21]. The protocols were described in our previous work. [21] We used three subtypes in the experiments: (1) ICG-NPs with its temperature threshold ($T_{th}$) = ∼26 °C and particle size = ∼330 nm, suitable for ex vivo USF imaging; (2) ICG-NPs with $T_{th}$ = ∼36 °C and particle size = ∼330 nm, suitable for in vivo USF imaging in tumor via local injection, as well as in vivo USF imaging in a mouse’s spleen via IV injection; and (3) ICG-NPs with $T_{th}$ = ∼39 °C and particle size = ∼70 nm, suitable for in vivo USF imaging in a mouse’s liver via IV injection. The $T_{th}$ of ICG-NPs was controlled by adjusting the quantity and ratio of NIPAM, TBAM, and AAm. It is worth mentioning that, because the contrast agent will only be switched on when temperature rises over its $T_{th}$, different $T_{th}$s were adopted in different experiments. That is, in an ex vivo scenario where the background temperature ($T_B$) = ∼23.5 °C, $T_{th}$ was selected 26 °C, slightly above $T_B$ for best USF acquisition efficiency. In an in vivo scenario, when the mouse’s body temperature (i.e., $T_B$) = ∼35–36°C under an anesthetized condition, $T_{th}$ was selected at ∼36–39°C. Note that ICG-NPs (2) and (3) have different $T_{th}$s (36°C or 39°C) because they adopted slightly different synthesis protocols; nevertheless, both worked excellent as in vivo USF contrast agents. More details are discussed in the Methods 4.2.

The particle size of ICG-NPs was controlled by adjusting the quantity of surfactant SDS. In consideration of selecting an appropriate particle size, we adopted ICG-NPs (1) and (2) in local injection experiments because they had a relatively large size (∼330 nm) and could stay in tissues or tumors for up to a few hours without a significant change in distribution. In IV injections, we controlled the ICG-NPs’ bio-distribution to the mouse’s different organs by adjusting their particle sizes: ICG-NPs (2) ($T_{th}$ = ∼36 °C, particle size = ∼330 nm) would mostly accumulate in the mouse’s spleen, whereas ICG-NPs (3) ($T_{th}$ = ∼39 °C, particle size = ∼70 nm) would mostly accumulate in the mouse’s liver and kidneys. Note that the particle size of each ICG-NP solution was represented by the median value, measured by dynamic light scattering (DLS) at room temperature. In micro-CT imaging, we adopted a commercial CT contrast agent ExiTron nano 12000, which could stay in tissues or tumors for up to a few hours without significant change in distribution when locally injected. When intravenously injected, ExiTron nano 12000 would mostly accumulate in the mouse’s spleen and liver after a few hours. In our experiments, we adopted an aqueous mixture of USF contrast agents and CT contrast agents, and injected the mixture solution locally in the tumor or intravenously through the mouse’s lateral tail vein. The mixture ratio and the volume varied in each experiment, as described in the Results section. By so doing, we had the same imaging target for both USF and CT imaging. Through 3D co-registration of a USF image and a CT image, we could compare the two modalities.

2.4. A camera-based USF imaging system

In this work, we carried out USF imaging experiments in an intensified charge-coupled device (ICCD) camera-based imaging system. The system setup was similar to the electron multiplying
CCD (EMCCD) camera-based imaging system developed in our previous work. [26] Fig. 1(a) shows a schematic diagram of the system. The tissue sample (or animal subjects) was placed on a platform for imaging. The platform was fixed above a water tank. A FU transducer (central frequency = 2.5 MHz) was submerged in the water tank and sent a FU beam into the tissue sample. The FU transducer was mounted on a motorized 3D translation stage. The sample was illuminated by a diode laser (wavelength: 785 nm; total output power: 2 Watts) through a bifurcated optic fiber bundle cable. The laser was working in direct-current (DC) mode. One band-pass interference filter (F1, center wavelength: 785 nm, bandwidth: 62 nm) was adopted for excitation filtering. On the top, the ICCD camera with a lens system captured the emitted fluorescence. The camera was also working in DC mode. The camera was placed at two different heights so that two fields of view (FOVs) were available for imaging: FOV 1 and FOV 2. The FOV 1 had a diameter of 6.5 cm, relatively large and appropriate for imaging the mouse’s whole body in a bio-distribution study. The FOV 2 had a diameter of 2.6 cm, appropriate for USF imaging. In FOV 1, four long-pass interference filters (F3–F6, 830 nm long pass filters, BLP01-830R-50; Rochester, NY, USA) were placed in front of the lens system for emission filtering. Note that in FOV 1 there is no F2 filter. In FOV 2, one long-pass interference filter (F2, 830 nm long pass filters, BLP01-830R-25; Rochester, NY, USA) was placed behind the lens system and four long-pass interference filters (F3–F6, 830 nm long pass filters, BLP01-830R-50; Rochester, NY, USA) were placed in front of the lens system for emission filtering.

![Fig. 1.](image)

A pulse delay generator (PDG 1) followed by a function generator (FG) was triggered by the camera via its internal trigger (i.e., the image trigger). The FG generated a sinusoidal signal (2.5 MHz), further amplified by a radiofrequency power amplifier (RF AMP, 50 dB amplification) and finally delivered to the FU transducer via a matching network (MNW). An ultrasonic wave was generated from the transducer and focused into the sample or tissue. A second pulse delay generator (PDG 2) was triggered by PDG 1 and sent a pulse to the translation stage for FU
scanning. It is worth mentioning here that the location of the FU’s focus on the 2D image (i.e., its \( x, y \) position on the \( x-y \) plane) could be detected by acquiring a USF signal from a single silicone tube under the camera. Briefly, before each USF imaging experiment, we placed a silicone tube (which was submerged in water) in the camera’s FOV. The tube was filled with the USF contrast agents. The FU transducer was focused on the tube at a certain location \((x_0, y_0)\). When a FU signal was sent to the tube, the agents at location \((x_0, y_0)\) would be switched on. Because the tube was presented in a clear medium (i.e., water), the USF signal was not scattered and its location \((x_0, y_0)\) was captured by the camera. Correspondingly, the location of the FU focus was determined. Based on the location of the FU’s focus, we could control the scanning points and plan the USF scanning area on the 2D image.

Figure 1(b) shows the time sequence diagram of acquiring a USF signal (in FOV 2). The first row shows that the camera acquired two images. The time interval between the two images was 5.0 s (i.e., the camera trigger rate = 0.2 Hz). The camera exposure time (yellow region) was 1.0 s with a readout time of 0.5 s (green region). The acquisition of the first image finished at \( t = 1.5 \) s, which was considered as background because no ultrasound was applied. In the second row, the ultrasound driving signal was delayed by 4.6 s from the start. The duration of the ultrasound signal was 0.4 s, so it ended right before the start of the acquisition of the second image. The acquisition of the second image finished at \( t = 6.5 \) s. Both USF photons and background photons were acquired in this image. We then subtracted the first image from the second, and the remainder was an image of the USF signal only. We summed the USF photons in the subtracted image within a selected region of interest (ROI) based on the location of FU focus, and counted the summation as the USF signal strength. Typically, the selected ROI was a circled region with its center at the FU focus and 10 mm in diameter. In the third row, the trigger of the translation stage started at \( t = 7.0 \) s. It was for moving the FU transducer to the next point. After 30.0 s from the start, the camera repetitively followed the same time sequence to acquire the USF signal at the next point. The reasons that we determined FU pulse duration = 0.4 s and camera exposure time = 1.0 s for signal acquisition are listed below. (1) The thermal relaxation time constant of a typical soft tissue can be estimated by \( L^2/4D \), where \( L \) is the characteristic linear dimension of tissue volume being heated and \( D \) is tissue’s thermal diffusivity. [29] In our experiments, \( L=0.5 \) mm is the lateral acoustic focal size. Based on literature [29], a typical value of the thermal diffusivity for most soft tissues is \( D=1.4 \times 10^{-3} \) cm\(^2\)/s. Thus, the thermal relaxation time constant is 0.446 s, which is longer than our ultrasound exposure time of 0.4 s. (2) By setting the camera exposure time after the FU pulse ended we avoided a possible motion artifact from ultrasound-tissue vibration. (3) According to our previous work [28], by setting an appropriate FU pulse duration (i.e., 0.4 s) and a long camera exposure time (i.e., 1.0 s) we could achieve a high signal-to-noise ratio (SNR) while at the same time maintaining a good spatial resolution. Meanwhile, we selected a time interval = 30.0 s between each USF signal acquisition because this interval should be sufficient for the heated tissue region to cool down (through thermal diffusion) so that it would not affect the next scan, as investigated in our previous work. [26] Through raster scanning of the FU transducer and acquisition of USF signals at each scan point, we acquired a 3D USF image based on a point-to-point scanning method. The 3D USF image was normalized and interpolated.

### 2.5. CT imaging

We acquired the 3D CT images in a commercial micro-CT system (Skyscan high-speed X-ray \textit{in vivo} micro-CT, Bruker, Billerica, MA). The images were reconstructed and processed via the software (NRecon and CTAn) provided by the manufacturer of the system. The 3D CT image was presented in maximum intensity projection mode.
2.6. Image processing and 3D co-registration

This section involved two main tasks. The first was to quantify the distribution of USF and CT contrast agents. The second was to co-register their distributions. In this study, we directly adopted the USF signal strength to represent distribution of the USF contrast agents at each location. To quantify the distribution volume, we segmented the 3D USF image via a 2D Otsu method [30]. Briefly, we selected one representative slice of the 3D USF image on the x-y plane and applied the 2D Otsu method. Thus, we found the threshold differentiating the object from the background. By segmenting the whole 3D USF image with the threshold value, we obtained the USF contrast distribution. Similarly, we quantified the CT contrast distribution volume by the same method. In 3D co-registration, by co-localizing spatial coordinates, we marked the 3D USF imaging volume in the CT image so that the same region of CT data was extracted.

3. Results

3.1. Ex vivo USF imaging of the contrast agent distribution in a porcine heart tissue phantom via a local injection

To test the ICCD camera-based USF imaging system, we first adopted a porcine heart tissue and conducted an ex vivo USF imaging experiment. The thickness of the tissue was \( \sim 10 \) mm. A total of 20 \( \mu \)L aqueous mixture of USF contrast agents (ICG-NPs, \( T_{th} = \sim 26^\circ C \), particle size = \( \sim 330 \) nm) and CT contrast agents was locally injected from the center of its top surface. The volume ratio of the USF to CT contrast agent was 3/1. Figure 2(a) displays the 2D normalized fluorescence strength distribution on the tissue’s top surface in FOV 2 with a threshold of 0.25, acquired from the ICCD camera. The top left corner contains a white-light photo of the tissue sample. The red solid square in Fig. 2(a) represents the FU scanning region on the x-y plane. The total scanning volume was \( 10.16 (x) \times 12.192 (y) \times 6.096 (z) \) mm\(^3\). The lateral step size along the x and y directions was 1.016 mm, and the axial step size along the z direction was 2.032 mm, respectively. Figure 2(b) represents the top view of the 3D CT image of the tissue phantom. The red dashed square highlights the CT contrast agents’ distribution in the tissue. Figure 2(c) shows how to acquire a USF signal in FOV 2. In this example, the FU driving peak-to-peak voltage from the FG was 160 mV. The FU location was marked as a black cross on the first image (\( x = -0.743 \) mm, \( y = +0.392 \) mm, and \( z = +3.048 \) mm). The first image was acquired at \( t = 1.5 \) s from the tissue surface before the exposure of ultrasound; it represents the background image. The second image was acquired at \( t = 6.5 \) s immediately after the exposure of ultrasound, representing the fluorescence image. The third image represents the image subtracted between the second and the first one, representing USF photons generated from the ultrasound focal zone and received from the tissue surface. As described in the Methods section, we considered the summation of the USF photons within a selected ROI on the third image as the USF strength at this FU scan point. By scanning the FU transducer, we acquired a 3D USF image. Figure 2(d) shows the normalized USF image on the x-y planes at different z positions.

After acquiring both USF and CT images, we processed the data for 3D co-registration. Figure 2(e) represents the normalized (value range: 0–255) USF contrast volume in a 3D view, a left (YZ) view, a top (XY) view, and a front (XZ) view. A transparency displaying threshold of 93 was used here to remove the background, the same as that used in USF image segmentation. Similarly, Fig. 2(f) represents the normalized CT contrast volume (value range: 0–255), extracted from the CT image shown in Fig. 2(b) and with the same spatial coordinates as the USF image. A transparency displaying threshold was 163, the same as that used in CT image segmentation. It is worth mentioning here that the segmentation thresholds in USF and CT image were different because the thresholds were determined independently when the 2D Otsu method was applied to each image. Figure 2(g) represents their 3D co-registration result. The green plus red volume represents the distribution of the USF contrast, which was 108.62 mm\(^3\); the blue plus red volume
Fig. 2. (a) Normalized 2D planar fluorescence image of the porcine heart tissue in FOV 2. (b) Top view of the 3D CT image of the porcine heart tissue. (c) An example of acquired USF signals from the porcine heart tissue. (d) Acquired USF images on x-y plane at different z locations. The data was normalized and interpolated. (e) The USF image of the USF contrast volume from 3D view, left side view, top view, and front view. (f) The CT image of the CT contrast volume from 3D view, left side view, top view, and front view. (g) 3D co-registration results of the USF and CT image. The green volume represents the USF contrast only. The blue volume represents the CT contrast agents only. The red volume represents their overlapped area. (h) The co-registration results on x-y plane at different z locations.
represents the distribution of the CT contrast only, which was 47.44 mm$^3$; and the red volume represents their overlapped area, which was 44.15 mm$^3$. Figure 2(h) shows the 2D $x$-$y$ planar cross-sections of the co-registered volumes at different $z$ positions. The results indicate that the two modalities have similar distribution of contrast agents.

3.2. In vivo USF imaging of the contrast agent distribution in a glioblastoma tumor on a mouse via a local injection

In this section, we adopted a nude mouse as the imaging subject. We implanted a glioblastoma tumor in the right hind leg, which grew to a size of a maximum diameter $\approx 1.5$ cm for the experiment. An aqueous mixture of USF contrast agent (ICG-NPs, $T_{th} \approx 36^\circ C$, particle size $\approx 330$ nm) and the CT contrast agent was locally-injected into the tumor, with a total volume of 50 uL and a volume ratio ICG-NPs/ExiTron nano 12000 = 3/1. A 3D USF image was acquired first and then a 3D CT image was scanned. Figure 3(a) shows the normalized 2D fluorescence image of the mouse in FOV 1. At top left it shows a corresponding white-light photo of the mouse (taken after euthanasia). The yellow dashed square in Fig. 3(a) represents FOV 2 of the camera. Figure 3(b) represents the normalized 2D fluorescence image in FOV 2, which highlights the fluorescence distribution in the tumor area. Both Fig. 3(a) and (b) display the fluorescence at a threshold $> 0.25$. In Fig. 3(b), the red solid square represents the USF scanning area on the $x$-$y$ plane. The 3D USF scanning volume was $11.176 (x) \times 12.192 (y) \times 6.096 (z)$ mm$^3$. The lateral step size along the $x$ and $y$ directions was 1.016 mm, and the axial step size along the $z$ direction was 2.032 mm. Figure 3(c) represents the top view of the CT image of the mouse. The red dashed square highlights the distribution of CT contrast agents in the tumor area. Figure 3(d) represents a single USF signal acquired at a location of FU focus: $x = +1.015$ mm, $y = +1.542$ mm, and $z = 0.000$ mm (marked as a black cross in the first image). The FU driving voltage was $V_{pp} = 220$ mV. Similarly, the first image ($t = 1.5$ s) represents the background image, the second image ($t = 6.5$ s) represents the fluorescence image after the exposure of a FU signal, and the third image was obtained by subtracting the first image from the second. The third image clearly shows a USF signal in the form of a 2D scattering spot emitted on the tumor’s top surface. Its location was close to the FU focus projected on the horizontal $x$-$y$ plane. Figure 3(e) shows the USF image on $x$-$y$ planes at different depths of $z$.

Following the same co-registration principle, we represented the 3D USF contrast volume, the 3D CT contrast volume and their co-registered volume. Figure 3(f) shows a 3D view, a left ($YZ$) view, a top ($XY$) view, and a front ($XZ$) view of the normalized (value range: 0–255) USF contrast volume. A transparency threshold of 120 was applied to remove the background. Correspondingly, Fig. 3(g) shows a 3D view, a left ($YZ$) view, a top ($XY$) view, and a front ($XZ$) view of the normalized (value range: 0–255) CT contrast volume within the same spatial coordinates. A displaying threshold of 129 was applied to remove its background. Figure 3(h) represents the co-registration results. The green plus red volume represents the distribution of the USF contrast, 207.91 mm$^3$; the blue plus red volume represents the distribution of the CT contrast, 48.94 mm$^3$; and the red volume represents their overlapped area, 37.857 mm$^3$. Figure 3(i) represents 2D $x$-$y$ planar cross-sections of the co-registered volumes at different $z$ positions. The results indicate that in vivo USF imaging was successful in the tumor and that the two modalities provided a similar distribution of contrast agents.

3.3. In vivo USF imaging of the contrast agent distribution in two glioblastoma tumors on a mouse via two separate local injections

In this section, we demonstrated in vivo USF imaging of two separate clusters of contrast agents distributed in two adjacent tumors on a nude mouse, via two separate local injections. We implanted two glioblastoma tumors into the nude mouse in adjacent positions on its right hind leg. An aqueous mixture of USF contrast agents (ICG-NPs, $T_{th} \approx 36^\circ C$, particle size $\approx 330$ nm)
Fig. 3. (a) Normalized 2D planar fluorescence image of the mouse in FOV 1. The yellow dash square highlights the fluorescence from the tumor. (b) Zoomed-in normalized 2D planar fluorescence image of the mouse’s tumor area in FOV 2, which corresponds to the yellow dash square in (a). (c) Top view of the 3D CT image of the mouse’s dorsal side. The red dash square highlights the distribution of the CT contrast agents in the tumor. (d) An example of acquired USF signals from the tumor. (e) Acquired USF images on x-y plane at different z locations. The data was normalized and interpolated. (f) The USF image of the USF contrast volume from 3D view, left side view, top view, and front view. (g) The CT image of the CT contrast volume from 3D view, left side view, top view, and front view. (h) 3D co-registration results of the USF and CT image. The green volume represents the USF contrast only. The blue volume represents the CT contrast agents only. The red volume represents their overlapped area. (i) The co-registration results on x-y plane at different z locations.
and CT contrast agents (ExiTron nano 12000) was locally injected into each tumor. The mixture volume ratio of ICG to CT contrast agent was 3/1. Each injection volume was 50 μL. The distance between two injection spots was ∼1.5 cm. Figure 4(a) shows the normalized 2D fluorescence planar image of the mouse in FOV 1. The fluorescence was displayed with a threshold of 0.5. The top left shows a white-light photo of the mouse. The yellow dashed square represents FOV 2 of the camera. Figure 4(b) shows the normalized 2D fluorescence planar image in FOV 2, highlighting the tumor area. The fluorescence was displayed with a threshold of 0.25. The red solid square represents the USF scanning area on the x-y plane. The 3D scanning volume was 13.716 (x) × 24.384 (y) × 10.668 (z) mm³. The lateral step size (x and y) was 1.524 mm and the axial step size (z) was 3.556 mm. Figure 4(c) shows the CT image of the mouse in a top view. The CT contrast in the two tumors were highlighted in the red dashed square in the figure. Figure 4(d1) and (d2) represent an example of a USF signal acquired at a specific location in each tumor, respectively. The FU driving voltage was fixed at Vpp = 220 mV. Figure 4(d1) represents a USF signal acquired at the upper location of the scanned area (i.e., near the first local injection). The position of the FU focus was at x = +1.905 mm, y = –9.144 mm, and z = 0.000 mm (marked as a black cross in the first image in [d1]). Figure 4(d2) represents a USF signal acquired at the lower location of the scanned area (i.e., near the second local injection). The position of the FU focus was at x = +0.762 mm, y = +6.096 mm, and z = 0.000 mm (marked as a black cross in the first image in [d2]). In both Fig. 4(d1) and (d2), the first image represents the background fluorescence image, the second image represents the fluorescence image immediately after the exposure of a FU signal, and the third image represents a subtraction of the first image from the second, showing acquired USF signals from the tumors’ top surfaces. Comparing Fig. 4(d1) and (d2), it clearly shows that the USF signal, in either case, only occurred near the location where a FU signal was focused. It is worth mentioning that their sizes were different, depending on the depths and the tumor tissue’s scattering coefficients. Figure 4(e) shows the USF images on x-y planes at different z positions. It clearly shows the presence of two separate clusters of USF signals at the upper and lower locations.

Using the same method to process the images, we acquired the 3D USF contrast volume, the 3D CT contrast volume, and their co-registration results. Figure 4(f) shows a 3D view, a left (YZ) view, and a front (XZ) view of the normalized (value range: 0–255) USF contrast volume. A displaying threshold of 78 was applied to remove the background. Figure 4(g) shows a 3D view, a left (YZ) view, a top (XY) view, and a front (XZ) view of the normalized (value range: 0–255) CT contrast volume with a displaying threshold of 74. Figure 4(h) shows their 3D co-registered images. The USF volume (green plus red) had a size of 1242.34 mm³; the CT volume (blue plus red) had a size of 141.48 mm³; and their overlapped volume (red) had a size of 116.639 mm³. Figure 4(i) shows the co-registered images on x-y planes at different z positions. In this experiment, we successfully achieved in vivo USF imaging with two separate imaging targets. Also, the results show that the two modalities provided a similar distribution of contrast agents.

### 3.4. Bio-distribution of the USF contrast agents in mice

In this section, we studied the bio-distribution of two USF contrast agents: ICG-NPs (2) with T<sub>th</sub> = ∼36°C and particle size = ∼330 nm; and ICG-NPs (3) with T<sub>th</sub> = ∼39°C and particle size = ∼70 nm. We adopted BALB/cJ mice (female, 6–8 weeks). The mice’s hair was removed and the contrast agent was intravenously injected via the lateral tail vein. The mice were placed in different body positions (i.e., dorsal, ventral, left lateral, and right lateral) under the ICCD camera system for in vivo 2D planar fluorescence imaging in the FOV 1. Thereafter, we investigated whether the fluorescence distributed in organs still had some USF properties. Thus, we euthanized some mice after in vivo fluorescence imaging and conducted ex vivo fluorescence imaging of their organs. Figure 5 shows the bio-distribution of ICG-NPs (2) (T<sub>th</sub> = ∼36°C and particle size = ∼330 nm). First, one mouse was intravenously-injected 100 μL ICG-NPs (2) and conducted in vivo
Fig. 4. (a) Normalized 2D planar fluorescence image of the mouse in FOV 1. (b) Zoomed-in normalized 2D planar fluorescence image of the mouse’s tumor area in FOV 2. (c) Top view of the 3D CT image of the mouse’s ventral side. (d) Examples of acquired USF signals from each tumor. (d1) represents the USF signal acquired from the first tumor at the upper location. (d2) represents the USF signal acquired from the second tumor at the lower location. (e) Acquired USF images on x-y plane at different z locations. (f) The USF image of the USF contrast volume from 3D view, left side view, top view, and front view. (g) The CT image of the CT contrast volume from 3D view, left side view, top view, and front view. (h) 3D co-registration results of the USF and CT image. (i) The co-registration results on x-y plane at different z locations.
fluorescence imaging over 24 hours. Figure 5(a) shows the normalized fluorescence distribution on the left lateral side of the mouse’s body after injection. Other positions of the mouse were also recorded (i.e., dorsal, ventral, and right lateral; not shown). We found that the strongest fluorescence signal occurred after 3 hours on the left lateral side around the mouse’s spleen. To verify whether the strong fluorescence signal was from the spleen, we conducted the same procedures using another mouse, euthanizing the mouse 3 hours after the injection of contrast agents. The first 3 images in Fig. 5(b) show the in vivo fluorescence images on the mouse’s left lateral side. The last image in Fig. 5(b) shows the fluorescence image of the ex vivo organs taken from the euthanized mouse. Clearly the spleen shows the strongest fluorescence signal, while the liver, kidney, stomach, and intestine show much weaker fluorescence signals. To investigate whether the fluorescence in the spleen preserved the USF properties (i.e., the switch on/off of the fluorescence as a function of temperature), we imaged the fluorescence strength of the spleen at different temperatures. The spleen was placed in a mortar filled with warm water. Figure 5(c) shows the fluorescence images when the water temperature was cooling down to 50°C, 43°C, 34°C, 28°C, and 25°C. The first image in Fig. 5(c) is a white-light photo of the sample. When the temperature was higher than the ICG-NPs’ Th (≈ 36°C), the fluorescence strength was a few times higher than when the temperature was lower than the Th. As an example, the ratio of the fluorescence strength at 50°C to that at 25°C was ≈2.3. These results indicate that the fluorescence strength in the spleen changed as the temperature changed, retaining an excellent temperature sensitivity.

In comparison, Fig. 6 shows the bio-distribution of ICG-NPs (3) (Th ≈ 39°C and particle size = ~70 nm) in mice. Similarly, the first mouse was intravenously injected 150 µL ICG-NPs (3) and conducted in vivo fluorescence imaging over 24 hours. Figure 6(a1) shows the normalized
Fig. 6. (a) 24 hours bio-distribution of one mouse after it was intravenously injected 150 µL ICG-NPs with its $T_b = \sim 39$ °C and particle size = \sim 70 nm. (a1) Normalized fluorescence intensity variation on the dorsal side of the mouse over 24 hours. (a2) Normalized fluorescence intensity variation on the ventral side of the mouse over 24 hours. (b) 3 hours bio-distribution of a second mouse after it was intravenously injected the same ICG-NPs solution. (b1) Normalized fluorescence intensity variation on the dorsal side of the mouse over 3 hours. (b2) Normalized fluorescence intensity variation on the ventral side of the mouse over 3 hours. (b3) Normalized fluorescence from the mouse’s organs after sacrifice at 3 hours. (c) The fluorescence intensity change from the second mouse’s liver and kidney as a function of temperature. (c1) The first image is a white photo of the liver sample. The next five images show its normalized fluorescence intensity change as a function of temperature. (c2) The first image is a white photo of the kidney sample. The next five images show its normalized fluorescence intensity change as a function of temperature.
fluorescence distribution of the mouse’s dorsal side, and Fig. 6(a2) shows that of the mouse’s ventral side. Other positions (i.e., left lateral and right lateral, not shown in the figure) were recorded as well. The strongest fluorescence signals were in the areas of the kidney and liver 3 hours after the injection. To validate this result, we conducted the same procedures in the second mouse. The mouse was euthanized 3 hours after the injection. Figure 6(b1)–(b2) show the in vivo bio-distribution. Figure 6(b1) represents the dorsal side and Fig. 6(b2) the ventral side. The ex vivo organs were imaged after euthanasia in Fig. 6(b3). The kidney and liver show the strongest fluorescence signal, in agreement with the results in Fig. 6(a1–a2 and b1–b2). Figure 6(c1) and (c2) respectively show the variation of the fluorescence strength in the liver and kidney at different temperatures (50°C, 43°C, 34°C, 28°C, and 25°C) in the water bath. In the liver, the ratio of the fluorescence strength at 50°C to that at 25°C was ~2.1. In the kidney, the ratio of the fluorescence strength at 50°C to that at 25°C was ~1.1. These results indicate that the fluorescence in liver retained a good temperature sensitivity, appropriate for USF imaging. In contrast, the fluorescence showed a lower temperature sensitivity in the kidneys.

In summary, the ICG-NPs (2)’s fluorescence most accumulated in the mouse’s spleen after IV injection. The fluorescence in the spleen retained the temperature sensitivity, so the spleen is appropriate for USF imaging. The ICG-NPs (3)’s fluorescence most accumulated in the mouse’s liver and kidneys after IV injection. The fluorescence in the liver retained the temperature sensitivity. Thus, USF imaging should also work in the liver. In contrast, the temperature sensitivity in the kidneys degraded. A possible explanation is that the fluorescence in the kidneys may mainly come from the small ICG-attached polymer/nanoparticle fragments or even free ICG molecules when ICG-NPs (3) were metabolized and decomposed, since kidneys usually did not take particles bigger than 10 nm [31]. Thus, USF imaging should not work in the kidneys. In the next two sections, we will demonstrate how we achieved in vivo USF imaging in the mouse’s spleen and liver.

3.5. In vivo USF imaging of the contrast agent distribution in a mouse’s spleen via an intravenous injection

Similarly, a BALB/cJ female mouse was intravenously-injected with an aqueous mixture of USF contrast agent ICG-NPs (2) (T$_{th}$ = ~36°C and particle size = ~330 nm) and CT contrast agent (ExiTron nano 12000) via a lateral tail vein. The volume ratio of the USF/CT agent was 5/1, and the total volume was 120 µL. Figure 7(a) shows the normalized 2D planar fluorescence image in FOV 1 of the mouse’s left lateral side 3 hours after IV injection. The fluorescence displaying threshold was 0.25. At the top left is a white-light photo of the mouse (taken after euthanasia). The yellow dash square in Fig. 7(a) represents FOV 2 of the camera. Figure 7(b) shows the normalized 2D planar fluorescence image in FOV 2, with the same displaying threshold at 0.25. The red solid square represents the USF scanning area on the x-y plane. The USF scanning volume was 13.208 (x) × 12.192 (y) × 6.096 (z) mm$^3$, with a lateral step size of 1.016 mm and an axial step size of 2.032 mm. Figure 7(c) shows the CT image of the mouse from its top view after USF imaging. The red dash square highlights the spleen area. Figure 7(d) shows a single USF signal acquired near the spleen, at a FU focus location where $x$ = -0.469 mm, $y$ = +1.000 mm, and $z$ = 0.000 mm (marked as a black cross in the first image). The third image shows a USF signal acquired on the x-y plane. The FU driving voltage was fixed at Vpp = 200 mV. Figure 7(e) shows the normalized USF image on the x-y planes at different z positions.

Figure 7(f) shows the 3D, left (YZ), top (XY), and front (XZ) views of the USF contrast volume (value range: 0–255). Its segmentation and displaying threshold was 95. Figure 7(g) shows the corresponding CT contrast volume (value range: 0–255) of the spleen, with a segmentation and displaying threshold of 99. Figure 7(h) shows their 3D co-registration results. The USF volume (green plus red) was 137.30 mm$^3$. The CT volume (blue plus red) was 73.67 mm$^3$. The overlapped volume (red) was 29.99 mm$^3$. Figure 7(i) represents the co-registered results on the
Fig. 7. (a) Normalized 2D planar fluorescence image of the mouse on its left side in FOV 1. The fluorescence mainly accumulated in the mouse’s spleen. (b) Zoomed-in normalized 2D planar fluorescence image of the mouse’s spleen area in FOV 2. (c) Top view of the 3D CT image of the mouse’s left side. (d) An example of acquired USF signals from the spleen area of the mouse. (e) Acquired USF images on x-y plane at different z locations. (f) The USF image of the USF contrast volume from 3D view, left side view, top view, and front view. (g) The CT image of the CT contrast volume from 3D view, left side view, top view, and front view. (h) 3D co-registration results of the USF and CT image. (i) The co-registration results on x-y plane at different z locations.
Fig. 8. (a) Normalized 2D planar fluorescence image of the mouse on its ventral side in FOV 1. The fluorescence mainly accumulated in the mouse’s liver. (b) Zoomed-in normalized 2D planar fluorescence image of the mouse’s liver area in FOV 2. (c) Top view of the 3D CT image of the mouse’s ventral side. (d) An example of acquired USF signals from the liver area of the mouse. (e) Acquired USF images on x-y plane at different z locations. (f) The USF image of the USF contrast volume from 3D view, left side view, top view, and front view. (g) The CT image of the CT contrast volume from 3D view, left side view, top view, and front view. (h) 3D co-registration results of the USF and CT image. (i) The co-registration results on x-y plane at different z locations.
x-y planes at different z positions. The results indicate that we successfully achieved in vivo USF imaging of the mouse’s spleen.

3.6. In vivo USF imaging of the contrast agent distribution in a mouse’s liver via an intravenous injection

In this experiment, we used ICG-NPs (3) ($T_{th} \approx 39^\circ C$ and particle size $\approx 70$ nm) and the same CT contrast agent. The USF/CT contrast volume ratio of the mixture solution was 5/1. The total volume was 180 $\mu$L. The other procedures were the same as in section 3.5. Note that the ICG-NPs (3) had a smaller particle size than the ICG-NPs (2) used in section 3.5. In addition, the CT contrast agent of ExiTron nano 12000 also accumulated in the mouse’s liver. As found in section 3.4, the ICG-NPs (3) retained the temperature sensitivity in the liver. Thus, in this experiment we aimed to image the mouse’s liver via both USF and CT modalities. Figure 8(a) shows the normalized 2D fluorescence image in FOV 1 of the mouse’s ventral side 3 hours after IV injection. The mouse was placed in a supine position (i.e., displaying its ventral side). The inset at the top left corner is a white-light photo (taken after euthanasia). It shows the agent mainly accumulated in the liver. The yellow dashed square represents FOV 2. Figure 8(b) shows the normalized 2D fluorescence image in FOV 2 of the mouse’s liver area. Both Fig. 8(a) and (b) have a fluorescence displaying threshold of 0.75. The red solid square in Fig. 8(b) represents the USF scanning area on the x-y plane. The 3D USF scanning volume was $15.24 \times 12.192 \times 4.064$ mm$^3$, with a lateral step size of 1.016 mm and an axial step size of 2.032 mm.

Figure 8(c) is the CT image of the mouse from a top view after USF imaging. It shows that the mouse’s liver had a relatively high contrast (highlighted by a red dashed square). Figure 8(d) shows a single USF signal acquired in the liver, at a FU focus location where $x = -2.545$ mm, $y = +0.048$ mm, and $z = +2.032$ mm (marked as a black cross in the first image). The last image clearly shows increased fluorescence after a FU signal was exposed. The FU driving voltage was fixed at $V_{pp} = 200$ mV. Figure 8(e) shows the normalized and interpolated USF image on x-y planes at different z positions.

Figure 8(f) shows the normalized 3D, left (YZ), top (XY), and front (XZ) view of the USF contrast volume (value range: 0–255). Its segmentation and displaying threshold was 82. Correspondingly, in the same spatial coordinate, Fig. 8(g) shows the 3D, left (YZ), top (XY), and front (XZ) view of the CT contrast volume. Its segmentation and displaying threshold was 120. Figure 8(h) shows their co-registered images. The USF volume (green plus red) was 254.47 mm$^3$. The CT volume (blue plus red) was 286.14 mm$^3$. The overlapped volume (red) was 128.871 mm$^3$. Correspondingly, Fig. 8(i) represents their co-registration on x-y planes at different z positions. The results indicate that in vivo USF imaging of the mouse’s liver was successful.

4. Discussion

4.1. Bio-distribution of USF contrast agents in tumor and organs

In this work, we injected the contrast agents (i.e., ICG-NPs and ExiTron nano 12000) by two pathways: local injection in the tumor and IV injection through a mouse’s lateral tail vein. First, when we locally injected the ICG-NPs ($T_{th} = \sim 36^\circ C$, particle size $\sim 330$ nm) and ExiTron nano 12000 into the tumor, they were likely to stay in the tumor’s intercellular space for at least a few hours. The 3D micro-CT image and 2D planar fluorescence image before and after the USF imaging (not shown in the Results) serve as proof of this assertion. Second, the bio-distribution study of ICG-NPs (through IV injection) showed that the large ICG-NPs ($\sim 330$ nm) mainly accumulated in the spleen, and the small ICG-NPs ($\sim 70$ nm) mainly in the liver and kidneys for up to 24 hours without significant losses of fluorescence. The agents in both the spleen and the liver retained the USF property. The CT contrast agent (ExiTron nano 12000) could stay in the spleen and the liver up to 9 hours after IV injection. The slow change of bio-distribution made it
possible to carry out 3D USF and CT imaging and process their co-registration without much variation in the contour of the imaging target. Therefore, the co-registration results were reliable, and the CT imaging validated the USF imaging.

4.2. \( T_{th} \) of the USF contrast agents

In our previous work, [21] we have demonstrated that the \( T_{th} \) of USF contrast agents should be slightly higher than the \( T_B \), either \textit{ex vivo} or \textit{in vivo}, in order to most efficiently acquire the USF signal. In this work, we adopted ICG-NPs as contrast agents. In the USF imaging of the porcine heart tissue phantom (i.e., an \textit{ex vivo} scenario), we selected the \( T_{th} \) of ICG-NPs at 26°C when the \( T_B \) was at room temperature (= ~23.5°C). In \textit{in vivo} USF imaging, we selected the \( T_{th} \) of ICG-NPs at ~36–39°C when \( T_B \) was equal to the mouse’s body temperature (~35–36°C, under an anesthetized condition). In our previous work, [27] we also demonstrated that it was difficult to acquire a USF signal from a dead mouse when the \( T_B \) (~23.5°C) was much lower than the \( T_{th} \) (~36°C) of the contrast agent.

4.3. Bio-stability of USF contrast agents in tumors and organs

The bio-stability of USF contrast agents played a vital role in the success of USF imaging in live animals. For successful acquisition of a USF signal, the contrast agents \textit{in vivo} should retain two key USF properties: (1) the USF contrast agents should retain the same \( T_{th} \); (2) the USF contrast agents should retain a high fluorescence intensity on-to-off ratio (i.e., a high \( I_{on}/I_{off} \)). We demonstrated that ICG-NP was an excellent USF contrast agent with a long shelf life and a high stability in biological environments. [21,27] In this study, the agents appeared highly stable after being locally injected in a mouse’s tumor or intravenously injected through the tail vein. We also found that the ICG-NPs (2) and (3) had more efficient accumulation in the spleen and liver while also maintaining the USF properties. The fluorescence \( I_{on}/I_{off} \) retained a good value of ~2.3 in the spleen and ~2.1 in the liver, respectively, good enough for USF imaging in the current system. By contrast, the fluorescence signal acquired from the kidneys showed a low \( I_{on}/I_{off} \) (~1.1). The possible reason is that the kidneys usually took up small particles (e.g., < 10 nm) in bio-distribution [31] so that most of the small ICG-attached polymer/nanoparticle fragments or free ICG molecules accumulated in the kidneys. As a result, the fluorescence from the kidneys might have lost most of its temperature sensitivity.

4.4. The camera-based USF imaging system for \textit{in vivo} study

Compared with our previous USF imaging system [22], which usually used an optic fiber bundle cable to collect and a photomultiplier (PMT) to detect USF photons, there are several advantages of adopting a camera-based system for \textit{in vivo} study. First, the ICCD camera is near-infrared (NIR) enhanced, and it has a high sensitivity in detecting NIR USF photons. Moreover, the camera’s intensifier was on DC mode so that it had the highest detection sensitivity for \textit{in vivo} USF signals. Second, the camera captured the USF signal in a 2D space (\( x-y \) plane), with a relatively large FOV. Because the USF photons mainly came from centimeter-deep tissue and were usually significantly scattered and appeared as a spread spot on the top surface of the tissue, the camera could help collect more USF photons from the top surface, which meant the camera provided high collection efficiency. More importantly, the 2D scattering spot of the USF signal could be visualized on the camera’s FOV, which clearly represented the projection of the source of the USF signal on the tissue surface (note that the ultrasound focus is known). It is very easy to locate the USF signal based on the 2D camera images and then determine whether the acquired signal was a USF signal, based on the fact that only the USF signal is ultrasound-correlated, while other background noise is independent of ultrasound. For example, in the USF imaging of the two glioblastoma tumors, when a FU signal was applied to the tumor at the upper location, the 2D scattered USF signal only showed up at the upper tumor’s top surface (Fig. 4 [d1]). By contrast,
when the FU was applied on the tumor at the lower location, the 2-D scattered USF signal only showed up at the lower tumor’s top surface (Fig. 4[d2]). Thus, the increased fluorescence in the subtracted image was highly correlated to the location of the FU and thus not likely caused by fluorescence fluctuation (such as noise). This advantage is a unique feature in a camera-based USF imaging system. Third, the camera captured the USF signal by accumulatively collecting photons within a long exposure time (such as 1 second in this study), helpful in reducing the noise caused by the animals’ breath. Especially, in USF imaging of the spleen or liver, the live mice had strong breathing motions, causing fluorescence fluctuation in their organs. This fluctuation would significantly add noise to the acquired USF signal. In our previous work, we removed this noise by fitting the USF signal and correlating it with a reference signal. [27] In this work, instead, the camera accumulated all the USF photons over a long exposure time, smoothing out this motion-caused fluctuation. Note that the mice’s breathing rate was controlled at ∼1 breath/second (as described in the Methods section), and the camera exposure time was also selected at 1 second, so that the fluorescence fluctuation caused by the mouse’s breath could be mostly averaged and removed. Table 1 compares the performances between the PMT-based and the ICCD camera-based USF imaging system.

| Performances                  | PMT-based USF system | ICCD camera-based USF system |
|-------------------------------|----------------------|-----------------------------|
| Detector’s sensitivity        | High                 | High                        |
| Signal collection             | Through an optic fiber bundle in our previous system | Through the camera’s lens system in current system |
| Signal detection FOV          | 0.3 cm fiber core diameter adopted in our previous system | 2.6 cm in diameter in current system |
| USF signal 2D visualization   | No                   | Yes                         |
| Temporal resolution           | Up to nanoseconds    | From milliseconds to hundreds of milliseconds |
| Removal of breath motion noises| Yes, by signal fitting | Yes, by signal accumulation |

4.5. Spatial resolution, ultrasound power and detection sensitivity

The spatial resolution of a USF image is determined by the size of thermal focal volume generated by the FU beam. [28] It depends on several parameters, such as ultrasound pulse duration, power, focus size and tissue’s acoustic (such as absorption coefficient and its nonlinearity) and thermal (such as diffusion coefficient) properties. [18] Because our previous works have quantified the relationship between ultrasound input and spatial resolution in several tissue models. [18,22,27,28], we did not repeat it in this work considering we used the same ultrasound transducer as in our previous studies. It is worth mentioning that in this work the spatial resolution varied in each experiment because we adopted different FU power (or driving voltage). The FU driving voltage for USF imaging in ex vivo porcine heart tissue was 160 mV Vpp, in a live mouse’s tumor was 220 mV Vpp, in a live mouse’s spleen was 200 mV, and in a live mouse’s liver was also 200 mV. A higher FU power provided a better SNR and a worse resolution. [28] Because the living tissues (e.g., liver and kidney) usually have a lower ultrasound absorption coefficient than ex vivo tissue samples or phantoms as they contains more fluids including blood, we adopted higher Vpps. In addition, increasing imaging depth also degrades SNR. [22] In this study, we adopted the highest Vpp in the tumor because its imaging depth (up to 1 cm) is larger than that in the organs (spleen and liver are located relatively close to the skin). It is common in practice starting with a low ultrasound driving voltage and gradually increasing until observing a USF signal with an acceptable SNR. Thus, a minimal driving power is found and
meanwhile it can avoid overheating. The detection sensitivity of the system is based on the minimum detectable concentration of USF contrast agents when all other experimental conditions are fixed. According to the synthesis protocol [21], the original solution of ICG-NPs has a dye concentration of 87.7 µM. Because the system was able to detect USF signals from in vivo organs, where the agent was likely at a lower concentration due to the dilution caused by intravenously injection, the system should have a detection sensitivity even higher.

4.6. Current limitations and future approaches

Because the system adopted a point-by-point mechanical scanning method, the imaging speed was slow. [26] Increasing imaging speed and reducing data-acquisition time is especially important in in vivo studies. There are several possible ways to address this issue, such as using a camera-based fast scanning technique [26] and adopting a transducer array (will be published somewhere else). A second limitation stems from the current USF contrast agents. The SNRs of some in vivo USF images in this study were relatively low. This is because the contrast agents were diluted after the IV injection. The ICG-NPs have demonstrated that they have excellent USF properties such as an adjustable $T_{th}$, a good $I_{on}/I_{off}$, and a high bio-stability, as well as an adjustable particle size. However, it would be desirable to develop more contrast agents with higher performance and better SNR. A third limitation results from the motion artifacts caused by the animals’ breath. We addressed this problem by smoothing the signal (or signal-averaging) in the current study and by signal-fitting in the previous study [27]. Nevertheless, the motion artifacts still possibly degraded the quality of the USF image because the location of the ultrasound focus varied when the organ was moving quickly. Similarly, the motion artifacts also existed in the CT imaging of a live mouse. One way to reduce the motion artifacts in in vivo USF imaging is to use a respiratory trigger, so that the signal is acquired at a fixed respiratory phase. Another method is to significantly increase the imaging speed and currently we are working on this topic.

5. Conclusion

In this work, we successfully achieved in vivo 3D USF imaging in live mice via a camera-based USF imaging system. We demonstrated in vivo USF imaging in two glioblastoma tumors implanted in a mouse. We also achieved in vivo USF imaging of the mouse’s spleen and liver. The results were validated by micro-CT images. This work showed the feasibility of in vivo USF imaging using the camera-based system. The ICG-NPs showed bio-stability in the mice’s tumors as well as the organs, such as the spleen and the liver, proving to be a suitable USF contrast agent for in vivo USF imaging. In addition, the advantages of a camera-based USF imaging system were discussed for in vivo imaging. We expect that USF imaging techniques will have many biomedical applications in future.

Funding

Cancer Prevention and Research Institute of Texas (RP170564); National Science Foundation (CBET-1253199).

Acknowledgements

Thanks to Dr. Kytai Nguyen and Dr. Yi Hong for allowing us to use some equipment in their labs to synthesize and characterize some contrast agents.

Disclosures

The authors declare no competing interests related to this article.
References

1. J. V. Frangioni, “In vivo near-infrared fluorescence imaging,” Curr. Opin. Chem. Biol. 7(5), 626–634 (2003).
2. S. A. Hilderbrand and R. Weissleder, “Near-infrared fluorescence: application to in vivo molecular imaging,” Curr. Opin. Chem. Biol. 14(1), 71–79 (2010).
3. N. Yamamoto, H. Tsuchiya, and R. M. Hoffman, “Tumor imaging with multicolor fluorescent protein expression,” Int. J. Clin. Oncol. 16(2), 84–91 (2011).
4. C.-D. Hu and T. K. Kerppola, “Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis,” Nat. Biotechnol. 21(5), 539–545 (2003).
5. A. Becker, C. Hessenius, K. Licha, B. Ebert, U. Sukowski, W. Semmler, B. Wiedenmann, and C. Grötzing, “Receptor-targeted optical imaging of tumors with near-infrared fluorescent ligands,” Nat. Biotechnol. 19(4), 327–331 (2001).
6. R. Weissleder, C.-H. Tung, U. Mahmood, and A. Bogdanov Jr, “In vivo imaging of tumors with protease-activated near-infrared fluorescent probes,” Nat. Biotechnol. 17(4), 375–378 (1999).
7. M. V. Backer, Z. Levasheva, V. Patel, B. T. Jehning, K. Claffey, F. G. Blankenberg, and J. M. Backer, “Molecular imaging of VEGF receptors in angiogenic vasculature with single-chain VEGF-based probes,” Nat. Med. 13(4), 504–509 (2007).
8. A. G. T. van Scheltinga, G. M. van Dam, W. B. Nagengast, V. Ntziachristos, H. Hollemia, J. L. Herek, C. P. Schröder, J. G. Kosterink, M. N. Lab-de Hoog, and E. G. de Vries, “Intraoperative near-infrared fluorescence tumor imaging with vascular endothelial growth factor and human epidermal growth factor receptor 2 targeting antibodies,” J. Nucl. Med. 52(11), 1778–1785 (2011).
9. L. V. Wang, “Multiscale photoacoustic microscopy and computed tomography,” Nat. Photonics 3(9), 503–509 (2009).
10. L. V. Wang and S. Hu, “Photoacoustic tomography: in vivo imaging from organelles to organs,” Science 335(6075), 1458–1462 (2012).
11. L. V. Wang and J. Yao, “A practical guide to photoacoustic tomography in the life sciences,” Nat. Methods 13(8), 627–638 (2016).
12. X. Deán-Ben, S. Gottschalk, B. Mc Larney, S. Shoham, and D. Razansky, “Advanced optoacoustic methods for multiscale imaging of in vivo dynamics,” Chem. Soc. Rev. 46(8), 2158–2198 (2017).
13. X. Xu, H. Liu, and L. V. Wang, “Time-reversed ultrasonically encoded optical focusing into scattering media,” Nat. Photonics 5(3), 154–157 (2011).
14. Y. Liu, P. Lai, C. Ma, X. Xu, A. A. Grabar, and L. V. Wang, “Optical focusing deep inside dynamic scattering media with near-infrared time-reversed ultrasonically encoded (TRUE) light,” Nat. Commun. 6(1), 5904 (2015).
15. B. Judkewitz, Y. M. Wang, R. Horstmeyer, A. Mathy, and C. Yang, “Speckle-scale focusing in the diffusive regime with time reversal of variance-encoded light (TROVE),” Nat. Photonics 7(4), 300–305 (2013).
16. K. Si, R. Fiolka, and M. Cui, “Fluorescence imaging beyond the ballistic regime by ultrasound-pulse-guided digital phase conjugation,” Nat. Photonics 6(10), 657–661 (2012).
17. B. Yuan, S. Uchiyama, Y. Liu, K. T. Nguyen, and G. Alexandrakis, “High-resolution imaging in a deep turbid medium based on an ultrasound-switchable fluorescence technique,” Appl. Phys. Lett. 101(3), 033703 (2012).
18. B. Yuan, Y. Pei, and J. Kandukuri, “Breaking the acoustic diffraction limit via nonlinear effect and thermal confinement for potential deep-tissue high-resolution imaging,” Appl. Phys. Lett. 102(6), 065703 (2013).
19. Y. Pei, M.-Y. Wei, B. Cheng, Y. Liu, Z. Xie, K. Nguyen, and B. Yuan, “High resolution imaging beyond the acoustic diffraction limit in deep tissue via ultrasonically switchable NIR fluorescence,” Sci. Rep. 4(1), 4690 (2015).
20. B. Cheng, M.-Y. Wei, Y. Liu, H. Pitta, Z. Xie, Y. Hong, K. T. Nguyen, and B. Yuan, “Development of ultrasound-switchable fluorescence imaging contrast agents based on thermosensitive polymers and nanoparticles,” IEEE J. Sel. Top. Quantum Electron. 20(3), 67–80 (2014).
21. S. Yu, B. Cheng, T. Yao, C. Xu, K. T. Nguyen, Y. Hong, and B. Yuan, “New generation ICG-based contrast agents for ultrasound-switchable fluorescence imaging,” Sci. Rep. 6(1), 35942 (2016).
22. B. Cheng, V. Bandi, M.-Y. Wei, Y. Pei, F. D’Souza, K. T. Nguyen, Y. Hong, and B. Yuan, “High-resolution ultrasound-switchable fluorescence imaging in centimeter-deep tissue phantoms with high signal-to-noise ratio and high sensitivity via novel contrast agents,” PLoS One 11(1), e0156993 (2016).
23. B. Cheng, V. Bandi, S. Yu, F. D’Souza, K. Nguyen, Y. Hong, L. Tang, and B. Yuan, “The mechanisms and biomedical applications of an NIR BODIPY-based switchable fluorescent probe,” Int. J. Mol. Sci. 18(2), 384 (2017).
24. J. Kandukuri, S. Yu, T. Yao, and B. Yuan, “Modulation of ultrasound-switchable fluorescence for improving signal-to-noise ratio,” J. Biomed. Opt. 22(7), 076021 (2017).
25. J. Kandukuri, S. Yu, B. Cheng, V. Bandi, F. D’Souza, K. Nguyen, Y. Hong, and B. Yuan, “A dual-modality system for both multi-color ultrasound-switchable fluorescence and ultrasound imaging,” Int. J. Mol. Sci. 18(2), 323 (2017).
26. T. Yao, S. Yu, Y. Liu, and B. Yuan, “Ultrasound-Switchable Fluorescence Imaging via an EMCCD Camera and a Z-Scan Method,” IEEE J. Sel. Top. Quantum Electron. 25(2), 1–8 (2019).
27. T. Yao, S. Yu, Y. Liu, and B. Yuan, “In vivo ultrasound-switchable fluorescence imaging,” Sci. Rep. 9(1), 9855 (2019).
28. S. Yu, T. Yao, and B. Yuan, “An ICCD camera-based time-domain ultrasound-switchable fluorescence imaging system,” Sci. Rep. 9(1), 10552 (2019).
29. L. V. Wang and H.-I. Wu, Biomedical Optics: Principles and Imaging (John Wiley & Sons, 2012).
30. L. J. L. Wenqing, “The Automatic thresholding of gray-level pictures via two-dimensional otsu method [J],” Acta Automatica Sinica 1, 325–327 (1993).

31. W. H. De Jong, W. I. Hagens, P. Krystek, M. C. Burger, A. J. Sips, and R. E. Geertsma, “Particle size-dependent organ distribution of gold nanoparticles after intravenous administration,” Biomaterials 29(12), 1912–1919 (2008).