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Non-Ionizing Label-Free Photoacoustic Imaging of Bones

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ABSTRACT X-ray based radiography, the main modality for diagnostic imaging of bone structures and fractures, provides sensitive images, but it inherently involves potentially harmful X-ray exposure. As non-ionizing alternatives, various optical imaging methods have been explored. Here, we demonstrate non-ionizing, label-free, multispectral photoacoustic (PA) imaging of bones in small animals *in vivo*, *in situ*, and *ex vivo*. Using near-infrared light excitation and acoustic detection, the spine and ribs were successfully visualized in high-resolution PA images. PA 3D volume images of the spine and ribs were clearly visualized together with blood vessels and several organs including the spleen, liver, and cecum, without using any exogenous contrast agent nor ionizing radiation. Quantification results of multispectral PA signals from blood vessels and bones were in good agreement with their absorption coefficients. Further, a rib fracture was photoacoustically imaged. Our results demonstrate PA imaging's potential as a non-ionizing and label-free technique for imaging bone tissues.

INDEX TERMS Biomedical imaging, bone imaging, diagnostic radiography, label-free, non-ionizing radiation, photoacoustic imaging, X-ray radiography.

I. INTRODUCTION

Since Röntgen discovered X-rays in 1895, X-ray radiography has been extensively developed as a main diagnostic tool in medicine. Significant technical advances have led to a variety of systems for real-time high-resolution 2D radiography and 3D computed tomography (CT) [4], [5]. X-ray radiographs are used to diagnose maladies in almost any part of the body, including the chest (heart and lung), abdomen, spine, brain, teeth, and pediatric growth plates (bone age), and to image and monitor conditions such as sinusitis and fractures [6]–[12]. X-ray imaging provides particularly high image contrast for organic and inorganic hard target materials, such as bones and metals. Thus, the modality is widely employed in placing implants and determining nerve locations before and after dental implant procedures, in bone fracture and density diagnosis, and in bone fracture internal fixation. However, compared to other diagnostic

modalities, such as magnetic resonance imaging (MRI) and ultrasound (US) imaging, X-ray imaging confronts particular limitations, including weak soft tissue contrast, the provision of only structural information, the use of ionizing radiation, and the difficulty of detecting early microscopic lesions.

Currently, various studies on bone imaging have been conducted using non-ionizing optical imaging techniques, such as bioluminescence imaging, fluorescence imaging, and diffuse optical tomography. Cowey *et al.* monitored bone metastasis in mice with bioluminescent imaging and achieved a sensitivity of 100%, a specificity of 80-90%, and an accuracy of 90-96% compared to histological analysis [13]. Bao *et al.* developed bone-specific pamidronate conjugated near-infrared fluorophores that can provide fluorescence images of the chest, bone, and spine [14]. Niska *et al.* monitored bacterial burden and neutrophil infiltration during orthopedic implant infections, using complementary fluorescence, bioluminescence, and X-ray combined imaging [15]. Xu *et al.* acquired optical absorption and scattering images of chicken bones and human finger joints using diffuse optical

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tomography [16]. However, exogenous contrast agents are unavoidably required for fluorescence imaging in bones, and these pure optical modalities suffer from relatively low resolution in deep tissues. As for non-optical techniques, US has drawn attention thanks to its radiation-free and real-time imaging capability [17]–[19]. There still remain challenges, however, that morphological features including acoustic shadow behind bone and thickness of bone surface are highly dependent on the orientation of US probe with respect to imaging anatomy and functional features on inflammation such as microvasculature are less sensitive in US images [20], [21].

Photoacoustic (PA) imaging is an optical- and acoustic-based hybrid biomedical imaging technique that can noninvasively provide high-resolution optical images of biological tissues [22]–[28]. In principle, materials that absorb pulsed light generate US signals through thermal expansion, and the generated US signals are detected by US transducers. Therefore, fruitful optical contrast with high US resolution can be obtained, even in deep tissues. Aforementioned limitations on US bone imaging can be overcome using PA technique by capturing acoustic signal generated from optical absorbers such as blood (hemoglobin) and bone, not the reflected signal from different tissue layers. Because acoustic signal is generated from bone itself, there are little or no dependencies on probe orientation, angle, and operator. In addition, PA imaging is intrinsically sensitive to oxygenated and deoxygenated hemoglobin and thus can provide not only hyper-vascularization but also hypoxia which are hallmarks of joint/bone inflammation. To visualize bone structures in live animals, Fehm *et al.* developed an *in vivo* whole-body PA scanner that can provide volumetric PA images, and used it to visualize the spine of a mouse [29]. Lee *et al.* acquired PA and US combined images of dental implants in a porcine jawbone and visualized the structure of a jawbone, implants, and soft tissues [30]. Thella *et al.* conducted a simulation study on PA diagnosis for various types of bone including cancerous bone and bone marrow [31]. Wood *et al.* monitored oxygen saturation of murine bone marrow based on PA technique to assess leukemic disease progression [32].

Here, we demonstrate label-free high-resolution non-ionizing PA imaging of bones in mice. We have successfully acquired multispectral PA images of bone tissues, including ribs and spines, *in vivo*, *in situ*, and *ex vivo*. Further, the PA results are compared with X-ray CT images. In addition, we have photoacoustically imaged broken ribs. Our results indicate that PA imaging can potentially be a useful tool to image bone structures and maladies in preclinical and clinical settings.

II. MATERIALS AND METHODS

A. ACOUSTIC-RESOLUTION PHOTOACOUSTIC MACROSCOPY

To acquire PA images of bone structures in mice, we utilized an acoustic-resolution photoacoustic macroscopy (AR-PAM)

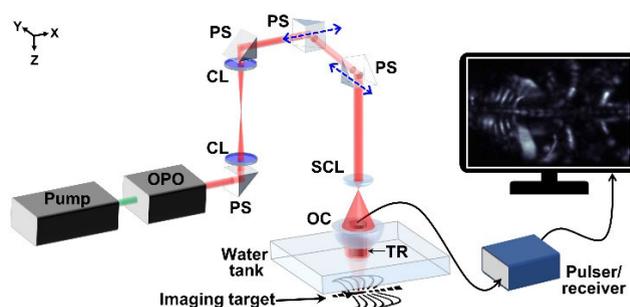


FIGURE 1. Schematic of acoustic-resolution photoacoustic macroscopy. Blue dashed arrows represent the scanning axes. OPO, optical parametric oscillator; PS, prism; CL, collimating lens; SCL, spherical conical lens; OC, optical condenser; TR, ultrasound transducer.

system (Figure 1). The AR-PAM system uses a Q-switched Nd:YAG laser (532 nm; SLII-10, Continuum, USA) and a tunable optical parametric oscillator (OPO) laser (Surelite OPOPLUS, Continuum, USA) for PA excitation. The PA excitation laser provides wavelengths adjustable from 680 nm to 2500 nm, with a pulse width of 5 ns and a repetition rate of 10 Hz. Optical wavelengths of 532 nm, 680 nm, and 850 nm were used for label-free *in vivo*, *in situ*, and *ex vivo* PA imaging, and the optical wavelength of 680 nm was used to monitor a broken rib cage. The PA excitation beam is delivered to a conical lens and a custom-made optical condenser through N-BK7 best form lenses (LBF-254-150, Thorlabs, USA) and 90-angle prisms (PS908, Thorlabs, USA) to make a dark-field ring-shaped beam on the imaging targets. The pulse energy of the laser beam used for PA imaging is ~ 4 mJ/cm², which is well below the American National Standards Institute (ANSI) laser safety limits. To detect the generated PA signal, a single-element focused US transducer with a 5 MHz central frequency (V308, Olympus NDT, USA) and a pulser/receiver (5072PR, Olympus NDT, USA) are used. The US transducer is placed in a custom-made optical condenser at a distance of 1 inch from the imaging target, which is the focal length of the US transducer. The detected PA signals are recorded with a digital oscilloscope (MSO 5204, Tektronix, USA) at 50 MHz sampling rate. For US signal coupling, a custom-made water bath covered with a transparent polyvinyl chloride film is placed between the optical condenser and the imaging target. 3D volumetric PA images are acquired by moving the conical lens and the custom-made optical condenser via two stepper motors in a range of 60 mm (X) \times 40 mm (Y) \times 30 mm (Z). The image acquisition time is about 40 minutes and the axial and lateral resolutions of the AR-PAM system are 145 μ m and 590 μ m, respectively.

B. MURINE COMPUTED TOMOGRAPHY

To acquire X-ray CT images of live mice, we used a low dose CT system (Quantum FX microCT, PerkinElmer Inc, USA). The CT system provides a photon energy of 90 kV at 0.2 mA, and has focal spot of 5 \sim 30 μ m. A 127 μ m pixel 14-bit X-ray flat panel detector is used to capture CT signals. The field of view (FOV) of the CT system is cylindrical with a

diameter of 34 mm and a length of 60 mm. For *in vivo* mouse CT imaging, the mouse was anesthetized by a vaporized-isoflurane system (XGI-8, PerkinElmer Inc, USA).

C. ANIMAL PREPARATION

Six-week old female normal balb/c mice (weight: ~ 25 g) were prepared for PA bone imaging. All animal experimental procedures were conducted following the laboratory animal protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Pohang University of Science and Technology (POSTECH) and in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals. For *in vivo* PA and CT imaging, the mouse was anesthetized with a vaporized-isoflurane system (1L/min of oxygen and 0.75% isoflurane). Then the mouse was sacrificed for *in situ* imaging with and without skins, and for *ex vivo* imaging. To image bone fracture, we initially euthanized the animal, broke ribs, and then photoacoustically imaged the fractured rib cage *in situ*.

D. IMAGE SEGMENTATION

For a quantitative analysis, we segmented the PA images into blood vessels, bones, organs (including spleen, cecum, and liver), and background regions. For each region, a polygonal region-of-interest (ROI) was manually created on a maximum amplitude projection (MAP) image, and then a binary mask was created from pixels inside the ROI with values greater than a manually defined threshold. Then, using 3D PHOVIS software developed in [33], each volume region was rendered with different color maps of hot-, blue-, copper-, and gray-scale for blood vessels, bones, organs, and background regions, respectively.

III. RESULT AND DISCUSSION

In vivo CT images of a mouse were acquired to compare with the PA images. Bones and lungs, lending themselves well to X-ray radiography, are clearly visualized in the CT volumetric image (Figure 2a) and cross-sectional images (Figure 2b-d). Gas-containing organs such as the intestine and cecum can be read based on anatomical information. Other internal structures, including the liver, spleen, and blood vessel are invisible and typically require a radiocontrast agent to be seen.

PA images were then acquired under different conditions, such as *in vivo*, *in situ* with and without skin, and *ex vivo*. Figure 3 shows label-free PA MAP images and 3D volume-rendered images captured at various optical wavelengths. A gray scale represents the PA amplitude of the background, a blue scale indicates the PA amplitude in bones (ribs and backbone), a hot scale (from deep red to yellow) shows the PA amplitude in blood vessels, and a copper scale indicates the PA amplitude in organs (spleen, cecum, and liver). Movie clips of 3D volume-rendered PA images are presented in Supplementary Video 1-9. The 532 nm *in vivo* PA MAP image (Figure 3a_{ii}) shows primarily blood vessels near the skin. As the optical wavelength becomes

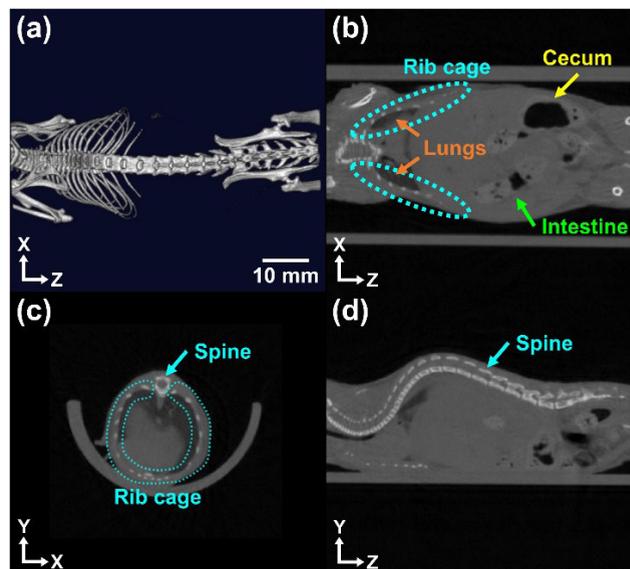


FIGURE 2. *In vivo* CT images of a mouse. (a) 3D volume, (b) coronal plane, (c) axial plane, and (d) sagittal plane.

longer, stronger PA signals are obtained from the ribs and spine, located deeper below the skin than the blood vessels (Figure 3a_{iii} and iv). This result demonstrates that the longer the optical wavelength, the smaller the optical scattering in the tissue, resulting in deeper light transmission. Additionally, the optical absorption coefficient of hemoglobin in the near-infrared region (680 nm and 850 nm) is less than that in the 532 nm wavelength. Therefore, the PA signals from blood vessels near the skin are weaker with near-infrared illumination than those at 532 nm. Next, we acquired *in situ* PA MAP images (Figure 3b). Compared to the *in vivo* PA MAP images with 532 nm excitation, the PA signal from blood vessels in dead mice is decreased (Figure 3b_{ii}). After death, the heart stops beating and the capillary circulation ceases, and blood vessels near skin become less visible in the PA MAP images. As blood drains into the large vessels or settles in the lowermost vessels by gravity, large blood vessels near the thigh and the tail become more visible [34]. The spine and the rib cage are clearly visible in the PA MAP images captured at optical wavelengths of 680 nm and 850 nm (Figure 3b_{iii} and iv).

After skin removal, the spine and the rib cage are more clearly visible (Figure 3c). In the PA MAP with 532 nm excitation (Figure 3c_{ii}), blood vessels are invisible and internal organs, including the liver, spleen, and cecum, are seen. Penetration depth gets deeper as the optical wavelength gets longer [35], and thus the spine, rib cage, and large arteries near the thigh and tail are clearly visible (Figure 3c_{iii} and iv). Next, we acquired PA MAP images of the spine and rib cage after they were removed from the body (Figure 3d). As with the *in situ* and skin-removed conditions, the spine and rib cage are more clearly visualized, and the nodes of the spine appear in the PA MAP images with 680 nm and 850 nm excitation. PA signal of *ex vivo* bones obtained at 532 nm (Figure 3d_{ii}) are weaker than that at 680 nm (Figure 3d_{iii}) despite higher absorbance (TABLE 1). This might be due to

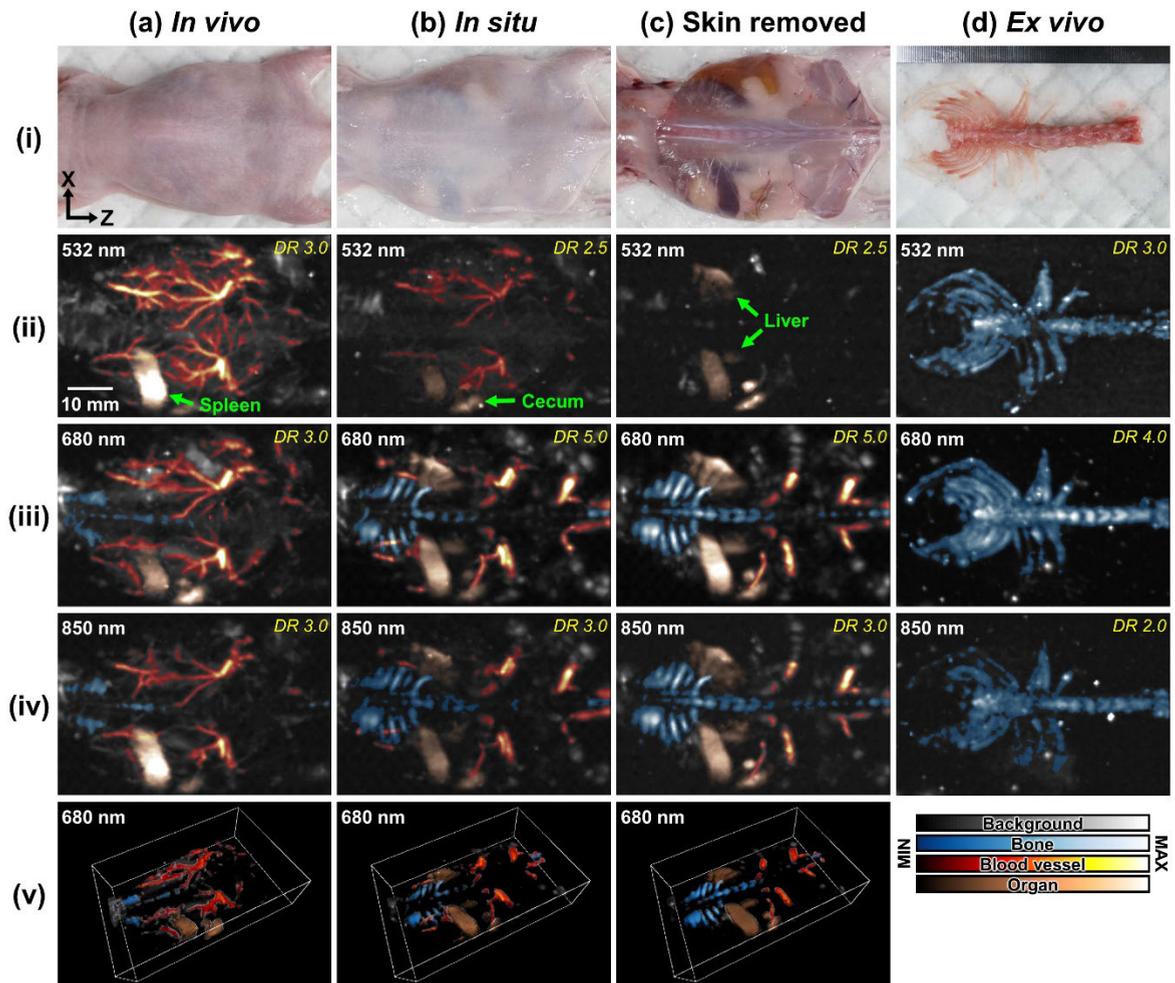


FIGURE 3. (i) Photographs of mouse in different conditions: (a) *in vivo*, (b) *in situ*, (c) skin removed, and (d) *ex vivo*, and corresponding label-free PA (ii-iv) MAP and (v) 3D volume-rendered images captured using different excitation laser wavelengths. The gray-, blue-, hot-, and copper-scale images respectively represent PA amplitudes in the background, bone, blood vessels, and organs. The minimum and maximum values of the colormaps are 0 and DR, respectively. MAP, maximum amplitude projection; PA, photoacoustic; DR, dynamic range.

strong optical scattering in remaining biological tissues on excised bones at the shorter optical wavelength. Although the bone images are not as clean as in CT images, bone images can be successfully obtained from PA images non-invasively, without using ionizing radiation and any contrast agent. Imaging in the NIR-II window, such as 1064 nm, might contribute to achieving deeper imaging depth.

We further compared multispectral PA signals from bone and blood vessels under *in vivo*, *in situ*, skin-removed, and *ex vivo* conditions (Figure 4b and d) with the absorption coefficients of bone and blood (TABLE 1 and Figure 4a and c). The quantified PA amplitudes in Figure 4b and d are average signals within each ROI (colored volume data in Figure 3: bone in blue-scale and blood vessel in hot-scale) and their standard deviation. As can be seen, for *in situ* and skin-removed conditions, as capillary circulation collapses and the blood sinks down into the large vessels, PA signals from capillaries near the skin significantly decrease at 532 nm. Correspondingly, compared to the *in vivo* PA signals,

TABLE 1. Absorption coefficients of bone and blood at 532,680, and 850 nm wavelengths. Bone data are from [1] and [2], and blood data are calculated using data from [3]. Absorption coefficients of bone and blood at 532,680, and 850 nm wavelengths. Bone data are from [1] and [2], and blood data are calculated using data from [3].

Wavelength	532 nm	680 nm	850 nm
Bone [1]	1.11 cm ⁻¹	0.48 cm ⁻¹	0.20 cm ⁻¹
Bone [2]	-	0.28 cm ⁻¹	0.27 cm ⁻¹
Artery (SO ₂ 98%)	234.60 cm ⁻¹	1.71 cm ⁻¹	5.63 cm ⁻¹
Vein (SO ₂ 70%)	229.66 cm ⁻¹	4.91 cm ⁻¹	5.08 cm ⁻¹

PA signals from large vessels near the thigh and the tail greatly increase at 680 nm. With the reduced absorption from blood in dead mice, PA signals from bone markedly increase at 680 nm excitation, to about 2.6 and 2.0 times higher than those at 850 nm in the *in situ* and skin-removed conditions, respectively. These values match well with the absorption coefficients of bone in TABLE 1.

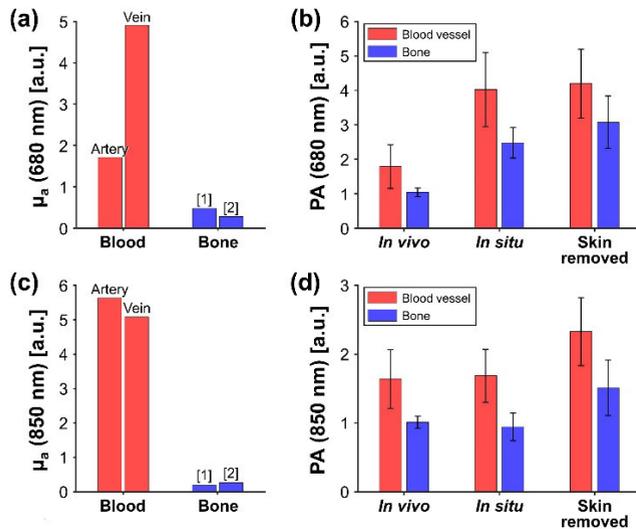


FIGURE 4. Absorption coefficients (μ_a) of bone and blood at 680 nm (a) and 850 nm (c). PA amplitudes in bone and blood vessels at (b) 680 nm and (d) 850 nm, for different mouse conditions.

To investigate the feasibility of imaging bone fractures, we photoacoustically examined a rib cage fracture in situ. As a control, before the ribs were broken, we acquired in situ PA MAP images at 680 nm (Figure 5a), in which the spine and rib cage are clearly visualized. Then, we broke left ribs and repeated the PA imaging experiments. The fractured parts of the ribs in the PA MAP image are clearly visualized at 680 nm (yellow dashed circle region in Figure 5b). Additionally, the ribs and spine are clearly visualized in PA cross-sectional images (Figure 5c), of the red arrow regions in Figure 5b. The B-mode image at line 4 in Figure 5c clearly shows the missing rib signal at the fractured site. These results demonstrate that PA imaging can both show bones and reveal bone fracture without using ionizing radiation.

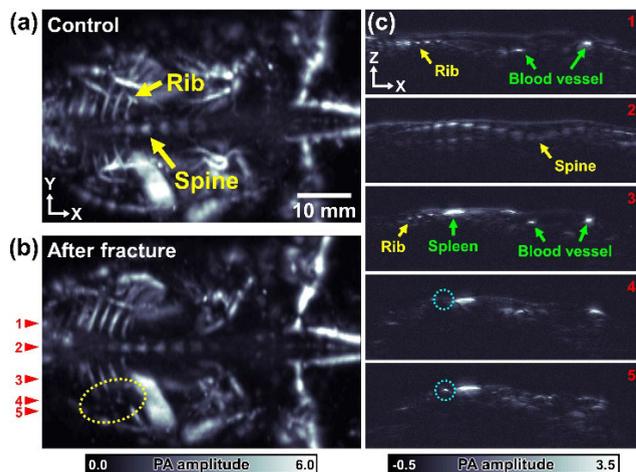


FIGURE 5. *In situ* rib cage fracture monitoring with 680 nm excitation before (a) and after (b) a fracture. The yellow dotted circle in (b) contains the fracture site. (c) PA cross-sectional images of red arrow regions in (b). The cyan dotted circles in (c) 4 and 5 enclose fractured (missing rib) and normal ribs, respectively.

IV. CONCLUSION

In this study, we acquired PA bone images of mice *in vivo*, *in situ*, with the skin removed, and *ex vivo*, using an AR-PAM system with optical wavelengths of 532 nm, 680 nm, and 850 nm. Bones, especially the spine and the rib cage, were visualized in PA MAP images with near-infrared wavelengths. Additionally, a rib fracture was imaged at 680 nm. Longer wavelengths reduced image perturbations by veins near the skin and thus showed better images of bones deep below the skin. In addition, because blood flow had ceased in dead mice, large arteries and bones were more clearly visualized. Finally, a rib fracture was successfully visualized with PA imaging. Although PA imaging does not have much higher bone contrast than conventional X-rays, it can provide bone images non-invasively and without using ionizing radiation. PA imaging can also be used for inflammatory arthritis diagnosis and treatment monitoring using strengths that can provide various information such as soft tissue and blood vessel around bones [36], [37]. These results demonstrate that the PA imaging can visualize bones inside the body without ionizing radiation exposure and can be a vital tool for diagnosing bone conditions in orthopedics.

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