In-line X-ray phase-contrast imaging of murine liver microvasculature *ex vivo*

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Abstract Imaging blood vessels is of importance for determining the vascular distribution of organs and tumors. Phase-contrast X-ray imaging can reveal the vessels in much more detail than conventional X-ray absorption method. Visualizing murine liver microvasculature *ex vivo* with phase-contrast X-ray imaging was performed at Shanghai Synchrotron Radiation Facility. Barium sulfate and physiological saline were used as contrast agents for the blood vessels. Blood vessels of <*Φ*20 μm could be detected by replacing resident blood with physiological saline or barium sulfate. An entire branch of the portal vein (from the main axial portal vein to the ninth generation of branching) could be captured in a single phase-contrast image. It is demonstrated that selective angiography based on phase contrast X-ray imaging, with a physiological material of low *Z* elements (such as saline) being the contrast agent, is a viable imaging strategy. Further efforts will be focused on using the technique to image tumor angiogenesis.

Key words Synchrotron radiation, X-ray, Phase-contrast imaging, Microvascular, Liver

1 Introduction

In China, hepatocellular carcinoma (HCC) is the second most common cancer (360,000 cases per year) and causes 350,000 deaths each year[1]. Tumor neovascularization plays a critical role in the development, progression, and metastasis of cancers by providing the tumor with nutrients and oxygen along the blood flow[2,3]. Visualization of the microvascular network is a key step to understand mechanisms of tumor growth, develop better diagnostic strategies, and treat tumors. However, due to weak absorption capabilities of soft tissues, conventional attenuation-based X-ray imaging methods cannot provide clear images of vessels of ≤*Φ*200 μm[4]. Therefore, conventional angiographic techniques cannot detect most hepatic microcirculatory abnormalities associated with malignancies.

Phase-contrast X-ray imaging can do much better in this regard. X-ray scattering is coherent, so changes in the coherent phase of X-rays propagating in soft tissues can be used in diagnostic radiology. Phase-contrast X-ray imaging can show minute differences in a biological object and its sensitivity is approximately 1000 times higher than that of absorption-contrast method[5,6]. It also offers a number of other improvements over conventional attenuation-based radiography. The improvements include a higher contrast that allows visualization of more anatomic detail, reduced dose absorbed by biologic samples, inherent image magnification and high spatial resolution[5].

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Microangiography techniques using synchrotron radiation, along with new techniques such as X-ray interferometry, diffraction enhanced imaging (DEI), and in-line phase contrast imaging (IL-PCI), have been developed and used to study clinical samples since mid-1990s[6]. An X-ray interferometry detected *Φ*50-μm blood vessels without a contrast agent in excised tissue from mice[7]. Using physiological saline as a contrast agent detected *Φ*30 μm vessels in excised liver samples even at a low X-ray dose [8]. DEI imaging was able to discriminate liver vessels down to about *Φ*40 μm without a contrast agent, and synchrotron-based DEI-CT imaging had a vessel detection limit of approximately *Φ*14 μm in murine fibrotic liver tissue[9].

Compared to interferometry imaging and DEI, in-line phase-contrast imaging (IL-PCI), due to its simplicity, is a candidate of great potential for clinical use. On a first-generation synchrotron radiation (SR) facility[10], using murine liver samples infused with physiologic saline, the IL-PCI was able to image vasculature branching of down to the eighth generation, with the smallest vessels measured being about *Φ*30 μm. Murine livers injected with iodine and imaged by *in vivo* microangiography on a third generation SR facility, hepatic arterial and portal venous branches of *Φ*20 μm were visualized[11].

At Shanghai Synchrotron Radiation Facility (SSRF), a third-generation SR facility open to users in 2009, encouraging experimental results have been reported. An iodine-injected (as a contrast agent) mouse was scanned by the X-rays and for the first time the entire vasculature was imaged, with the visible vessels being down to *Φ*20–30 μm[12]. In an *ex vivo* experiments of rat limb using a high resolution CCD camera, the smallest blood vessel detected clearly was approximately *Φ*9 μm[13].

In this paper, we report higher resolution IL-PCI visualizing mouse liver microvasculature, with or without a contrast agent, at SSRF.

2 Materials and methods

2.1 Preparation of contrast agents

The contrast agents used were physiological saline and barium sulfate. Barium sulfate (Sachtleben Micro, Sachtleben Chemie GmbH, Duisburg, Germany) was suspended in glycerol (50% water solution) to form a solution with a concentration of 1 g·mL^{-1}.

2.2 Animals and surgical procedures

All animals were obtained from the Experiment Animal Department of Fudan University and handled following the guidelines provided by the Animal Care and Use Committee of Zhongshan Hospital, Fudan University. Nine 6-week old female Kunming (KM) mice (25–23 g) were randomized into three groups (three per group) and anesthetized by intraperitoneal injection of sodium pentobarbital. The Group 1 mouse livers imaged were in the presence of the resident blood. For Group 2, the group of physiological saline, a 26-gauge catheter (Surflo-W; Terumo Europe N.V., Leuven, Belgium) was inserted into the portal vein, and physiological saline was injected into the vein by hand pressure and drained from the inferior vena cava until all blood in the portal and hepatic veins was replaced. For Group 3, barium sulfate was infused through the portal veins catheter by hand pressure. After the contrast adding, the inferior vena cava, superior vena cava, and the hepatic pedicle (including the arteries, hepatic veins, and common bile ducts) near the porta hepatis were ligated, and the livers were excised and fixed with formalin for imaging.

2.3 Synchroton radiation system and image acquisition

The samples were imaged on beamline BL13W with 200–300 mA@3.5 GeV beams. X-ray of 16 keV was used, with a beam spot of 45 mm (horizontal) × 5 mm (vertical) on a sample, which was placed approximately 30 m downstream of the source. The X-rays penetrating the sample were detected with a CCD camera of 4008×2672 pixels in pixel size of approximately 13 μm (VHR1:1; Photonic Science Ltd., Robertsbridge, UK). The sample-to-detector distance, which is precisely controlled by a motor, was 1200 mm as was optimized in advance. The exposure time was 4 ms per frame.

Synchrotron radiation-based micro-computed tomography (SRμCT) was also performed in *ex vivo* specimens infused with physiological saline and barium sulfate. The parameters used were as follows:

16 keV X-ray for phase-contrast imaging and 36 keV X-ray for absorption-contrast imaging; sample-detector distance, 1200 mm; exposure time, 7 ms per projection; number of projections, 1200 for 180°. X-ray CT images were reconstructed with a voxel size of 0.010 mm ×0.010 mm×0.009 mm.

2.4 Image evaluation

The minimum diameter of a visualized blood vessel was measured directly on a PC using Image-Pro plus 6.0 (Media Cybernetics, USA). Two peripheral vessels were chosen in each quadrant of the image, and the diameter of each vessel at the branch point was separately measured by two of the authors manually[13]. Minimum vessel diameter was expressed as mean ±*SD*.

2.5 Statistical analysis

Independent samples *t* test was used for data statistical analysis. $P < 0.05$ was considered significant.

3 Results

The IL-PCI was able to visualize the morphology and branching of liver blood vessels to the micron level. The structures observed in the non-contrast and physiological saline groups were confirmed to be portal and hepatic veins. High level contrast was achieved at an object-detector distance of 1200 mm, and nine generations of vasculature branching could be observed with good contrast against liver tissue in livers infused with physiologic saline (Fig.1a). The vessel diameter decreased with increasing generation of branching and distance from the main axial vein. The smallest visible vessels were Φ(17.4± 4.0) μm.

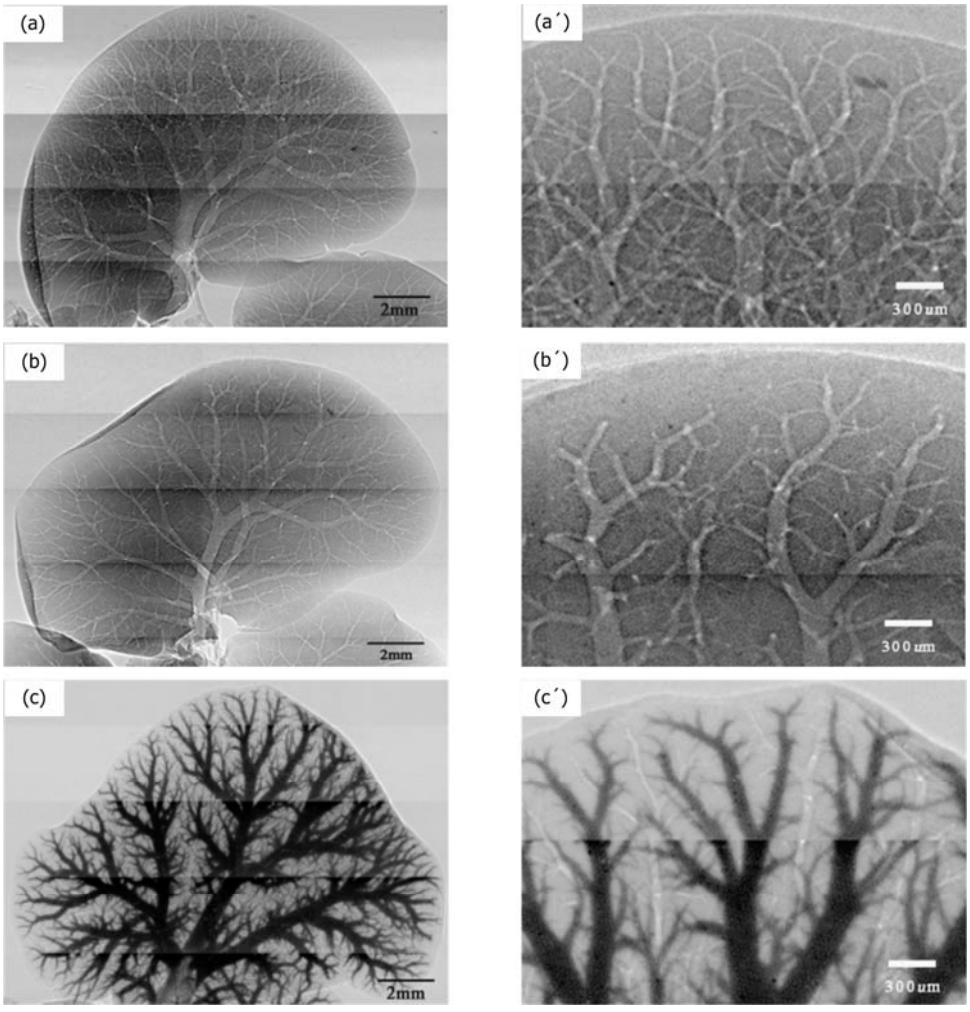

Fig.1 Phase-contrast images of mouse liver blood vessels with physiological saline (a), blood (b) and barium sulfate (c). (a'), (b') and (c') are enlargements of upper middle part in Fig.1(a), (b) and (c). The smallest vessels visualized were approximately 17.4, 27 and 18 μm in Fig.1(a'), (b') and (c'), respectively. The sample-detector distance was 1200 mm.

For Group 1 mice, the blood-containing vessels could be visualized to the eighth generation of branching (Fig.1b) with the smallest visualized blood vessel being Φ(27.1±4.2) μm in diameter, and the vessel edges were less clear than that of the physiologic saline-infused vessels (Fig.1a). Almost no blood vessel branching was observed in the liver periphery in Group 1 images, and they provided fewer details of the blood vessels than the images of Group 2.

Absorption contrast X-ray images with barium sulfate contrast demonstrated nine generations of portal vein branching (Fig.1c). The smallest vessels observed were Φ(18.6±2.9) μm, a size similar to the smallest visible saline-infused vessels in livers imaged with phase-contrast imaging (P>0.1).

Images of SRμCT indicated that 3D morphology of the blood vessel tree could be clearly visualized at the micrometer scale over the entire thickness of the mice liver (Fig.2). A dense microvascular network was visible.

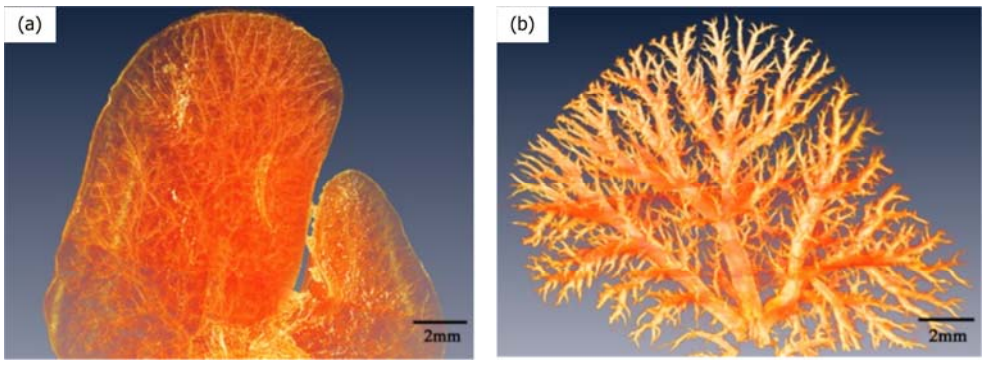

Fig.2 3D vessel images of mouse liver at a sample-detector distance of 1200 mm using SRμCT. Vessels imaged and reconstructed with physiological saline (a) and barium sulfate (b).

4 Discussion

From this study, visualizing murine liver microvasculature on SSRF and high-definition CCD camera show an enhancement in sensitivity and superior image quality over previous work[7–10]. Also, either physiological saline or barium sulfate is a good contrast agent that enhanced the details of the liver blood vessels. Zhang, *et al.*[10] showed that without any contrast agent the smallest liver vessels they visualized with a second-generation SR facility was Φ30 μm and the finest level of branching was about the eighth generation, while using the third-generation SR facility we visualized liver vessels of about Φ27 μm without a contrast agent and detected vessel branching down to the ninth generation.

Blood can function as a contrast medium because iron atoms in hemoglobin can cause a large phase shift[14]. It seems that an extra contrast agent is redundant. However, from a diagnostic point of view, the finer the vessels that could be visualized, the better diagnostic information one gets. Since contrast agents enhance sensitivity, they could reveal more details of the liver vessels together with the phase-contrast[7].

Barium sulfate enhances the phase-contrast X-ray images. It has been used as a contrast agent for vessel imaging in animal brains, auricles, hearts, lungs, kidneys, and other organs[13,15-18]. The average particle size (~0.7 μm) of high purity synthetic barium is small enough to allow its entering into capillaries, but the relatively large particles do not diffuse through the blood wall easily, allowing their retaining in the vessel[15]. With barium sulfate, vessels of Φ17.4 μm could be detected (Fig.1a). However, the use of barium sulfate may cause serious side effects in mice. This has not been done on living animals.

Physiological saline enhances phase-contrast X-ray images, too. As the difference in refractive indices between physiological saline and liver tissue is greater than that between blood and liver tissue, contrast of the saline-infused vessels is better than that of the blood-containing vessels[8] showing more generations of vessel branching and finer details of vessels in sizes of down to Φ17.4 μm.

Since physiological saline causes the X-ray phase to shift sufficiently, the smallest vessels visualized were similar in diameter for the saline-infused and barium sulphate-infused livers (Φ17.4 μm vs Φ18 μm). Without any contrast agents that contain heavy elements, the resolution becomes worse, but the side effects can be eliminated[8].

The optimum sample-to-detector distance depends highly on resolution of the detector, but a suitable free space between the sample and detector must be ensured[19]. The best IL-PCI quality is obtained using an X-ray CCD of 13-μm pixel size at a sample-to-detector distance of 1200 mm.

5 Conclusion

Our findings suggest that IL-PCI with a third-generation SR source is useful in the evaluation of murine liver microvasculature *ex vivo*. Blood vessels of Φ17.4 μm could be visualized with a higher contrast in livers with saline infused or barium sulfate infused vessels. Our results have shown that the third-generation SR source together with physiological saline could be valuable for future studies on the angiogenesis of tumor in living animal models.

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