Ethanol fixed brain imaging by phase-contrast X-ray technique

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Abstract. The two-crystal phase-contrast X-ray imaging technique using an X-ray crystal interferometer can depict the fine structures of rat’s brain such as cerebral cortex, white matter, and basal ganglia. Image quality and contrast by ethanol fixed brain showed significantly better than those by usually used formalin fixation at 35 keV X-ray energy. Image contrast of cortex by ethanol fixation was more than 3-times higher than that by formalin fixation. Thus, the technique of ethanol fixation might be better suited to image cerebral structural detail at 35 keV X-ray energy.

1.  Introduction

X-ray imaging is an indispensable technique to visualize the inner structure of objects in medicine, biology and various sciences. Since X-rays have a nature of wave, the amplitude changes of the wave and wave shifts (called as phase-shift) occur when X-rays pass through an object. The sensitivity of the phase-shift is up to 1000-fold greater than that of the absorption for low atomic-number elements such as hydrogen, carbon, nitrogen, and oxygen [1, 2]. Then, phase-contrast X-ray imaging technique, to depict the difference of minute X-ray phase-shifts, is now being developed. Four phase-contrast X-ray imaging techniques such as the Bonse-Hart type crystal interferometer-based technique [3, 4], refraction-based technique using crystal diffraction (diffraction enhanced imaging) [5], the propagation-based technique [6], and the grating (Talbot) interferometer technique [7, 8], are being used. The Bonse-Hart type crystal interferometer-based technique works best for small- or smooth-phase gradient such as soft tissues [9-14]. In phase-contrast X-ray imaging of biological samples, the formalin is most commonly used to fix the samples [15]. Recently, ethanol fixation was used to obtain superior immunostaining and comparable histology [16]. The fixation method (to use formalin or ethanol) of biomedical soft tissue might cause the density change of soft tissue. Here, we examine the difference of image quality fixed by formalin and ethanol, and determine the best fixation method for brain imaging in phase-contrast X-ray technique at 35 keV X-ray energy because high X-ray energy is
important to acquire the large object such as the whole brain of rat, and to reduce the X-ray exposure of the sample.

2. Materials and Methods
This study used 10 weeks-old male rats. All rats were anesthetized, and cannulation to the left ventricle was carried out surgically. The physiological saline was firstly injected from the apex of left ventricle, and the whole blood in vessel was replaced to eliminate the artifact of blood coagulation. Then, the 10 % formalin (a solution of paraformaldehyde in water) or 100 % ethanol was perfused for fixation.

Phase-contrast X-ray CT system consisted of an asymmetric silicon crystal (220) to generate a two-dimensional beam, two crystal interferometer [17, 18], a phase shifter, and an X-ray CCD camera. The X-ray CCD camera had an 18 μm x 18 μm pixel size and 14 bit dynamic ranges. In phase-contrast X-ray imaging, the specimen was placed in a 15-mm thick cell (Fig.1) filled with formalin for formalin-fixed brain and ethanol for ethanol-fixed brain, respectively. The cell was inserted in the beam path between the X-ray interferometer. Experiments were carried out at vertical wiggler beam line BL14C2 of the Photon Factory in Tsukuba, Japan.

The X-ray energy was set 35keV, and 1.4*10^7 cps/mm^2 in front of cell at the typical beam current was 450 mA with 2.5 GeV using top-up operations. The beam exposure time was 10 s per projection and the total number of projections was 250 over 180°. Image reconstruction was performed by fringe-scan data acquisition technique. The visibility of the interference fringe was about 60%. The phase-contrast X-ray CT images were analyzed by using the NIH image 1.63. In statistical analysis, values are expressed as means ± SD. Differences between groups were analyzed using Student’s unpaired t test. P<0.05 was considered statistically significant.

This experiment was approved by the Medical Committee for the Use of Animals in Research of the Kitasato University, and it conformed to the guidelines of the American Physiological Society.

3. Results and Discussions
Inner structures of rat’s brain such as cerebral cortex, basal ganglion (thalamus), hippocampus, and white matter can be depicted by both ethanol- and formalin-fixed brain of rat at 35 keV X-ray energy (Fig.2). Image quality and contrast differentiation of the ethanol-fixed brain were significantly better than those of the formalin-fixed brain.
Cerebral structures fixed by ethanol (left) can be depicted more detail comparing to that fixed by formalin (right). Cortex had significant high density in ethanol-fixed brain.

In ethanol fixation, cortex had significant high density shown as white, whereas white matter in external capsule had low density shown as dark. The thalamus showed gently higher density comparing to white matter. In formalin fixation, density of cortex and thalamus had almost same values, and the white matter showed low density.

Semi-quantitative analysis showed that the relative contrast ratio (RCR) of cortex was 3.51 by ethanol-fixed brain and 1.12 by formalin-fixed brain, respectively (Fig.3). The RCR of thalamus was
2.60 in ethanol-fixed brain and 1.30 in formalin-fixed brain. Thus, the contrast by ethanol-fixed brain was 3.13 and 2.00 higher than that by formalin-fixed brain in cortex and thalamus, respectively.

The brain is composed of nerve cell in cortex and nerve fibers with myelin sheaths in white matter. The ethanol fixation caused strong dehydration of the nerve cell in cortex, whereas myelin sheath might block the dehydration of axon. Then, the cortex has significant high density, and area of white matter showed the relatively low density. The difference of dehydration in cerebral tissue enabled to enhance the contrast differences of cerebral structures. Especially small fiber tract of mammillothalamic tract was depicted well by ethanol-fixed brain.

The image quality and contrast differentiation of the ethanol-fixed brain was significantly better than that of the formalin-fixed brain, and the ethanol-fixed technique was thought to suit for cerebral imaging at 35 keV X-ray energy.

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