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Examination of validity of sample preparation method for speed of sound evaluation in ultra-high frequency band

超高周波数帯での音速評価における試料作製方法の妥当性の検討

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1. Background

Scanning acoustic microscopy (SAM) system allows acoustic characterization at the cell size level [1]. However, since ultrasound waves of several hundred MHz are used, the attenuation due to the propagation of ultrasound waves is large, and it is necessary to slice the measurement sample in about 10 μm. There are two standard methods of preparing thin sliced samples, paraffin-embedded and frozen, and we have confirmed that the time of formalin fixation, which is often used in both methods, affects the speed of sound (SoS). In addition, the paraffin-embedded thin sliced samples have the problem of melting fat in the tissues. On the other hand, the freezing method is the absence of heat treatment, so it might be possible to evaluate the SoS of fat.

This paper investigated the effect of the sample preparation on evaluating SoS in the freezing method. In particular, we focused on the half Karnovsky fixation method and examined the effect of the different fixation methods on the SoS. Half Karnovsky fixative contains 0.5 % glutaraldehyde, 2 % paraformaldehyde, 30 mM HEPES buffer and is commonly used in an electron microscope.

2. Materials and Methods

2.1 Target materials

The target organs of this study were a liver and a kidney of a 9-week-old mild fatty liver rat model (Slc:SD, male). In the first step, the target organs were removed from the rat, and multiple blocks were cut out from each organ. Then, as shown in Fig. 1, each block was immersed in formalin or half Karnovsky fixative with or without immersion in 30 % sucrose solution for 24 h. Four types of specimen blocks were prepared, including the fresh-freezing method without any of fixation and replacement. Each block was encapsulated in OCT compound and then freeze-treated, and the sliced samples of 12 μm thickness were prepared with a cryostat. For comparison, a paraffin-embedded sliced samples with the thickness of 7 μm were also prepared separately from the process shown in Fig. 1. It was confirmed that Karnovsky fixation and sucrose replacement reduced the tissue damage during frozen specimen preparation [2].

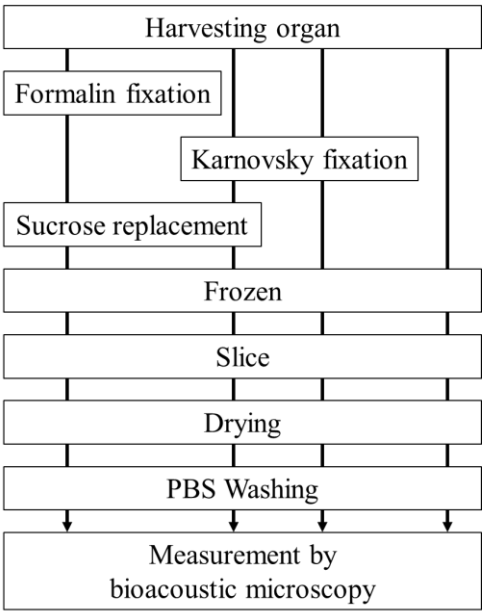

Fig. 1 Process of freezing method [3].

2.2 Data acquisition

A ZnO transducer (HT-400C, Honda Electronics) with a center frequency of 300 MHz and azimuthal resolution of 7 μm was excited by a pulsar (GZ1120ME-03, GEOZONDAS) to observation. Three-dimensional (3D) RF echo data were acquired by scanning the transducer in two-dimensions on each sliced sample of target organs. The received signals were recorded using an A/D board (ATS9373, Alazartech) with a sampling frequency of 4.0 GHz and 12-bit quantization. After the staining of each sliced sample with the Hematoxylin-Eosin method,

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digital histological images (1 μm resolution) were acquired with a virtual slide scanner (NanoZoomer S60, Hamamatsu Photonics), and the histological structures were observed.

2.3 Speed of sound analysis

The RF data of each scan line was up-sampled 10 times, and intensity map was acquired by normalizing with the maximum value of each scan line. In addition, linear tilt correction was performed for each scan line using the intensity and phase differences of the amplitude as indices to correct for the time difference fluctuations in the scanning directions. The echo signal components from the surface and back of the sliced sample were separated by a fifth-order autoregressive model, and SoS was calculated from them using the echo signal of the manually selected glass area as a reference signal [4]. The average SoS of each sliced sample was calculated from several regions of interest (ROI) of 400 μm × 400 μm settled on the SoS map. The position of ROI was selected as keeping correspondence with the digital histological images so that structures such as blood vessels and the sample edges are not included.

3. Results and discussion

Figures 2 and 3 show the results of the SoS evaluation of the liver and the kidney. The relationship between the five methods of sample preparation and the results of SoS evaluation was similar for liver and kidney.

Theoretically, a fresh-frozen sample that has not undergone the fixation process mostly retains its original biological state. In our results, The SoS in those samples was the closest to the known SoS in each organ. However, as mentioned earlier, it is not easy to obtain echo signals from the sample surface because the sample is soft, and the acoustic impedance difference between the sample and the water used for coupling during measurement is slight. Therefore, in many cases, the fresh-frozen method cannot provide stable SoS evaluation. In this study as well, there were not many evaluable areas compared to other samples.

The paraffin-embedded sample had a higher SoS at both organs as well known, and the other three samples without heat treatment had a lower SoS. However, the SoS in the Karnovsky-fixation without sucrose replacement sample is slightly higher than two of scrose replacement samples. In other words, it can be seen that SoS could be evaluated stably under conditions close to the fresh-freezing method by some kind of fixation and sucrose substitution.

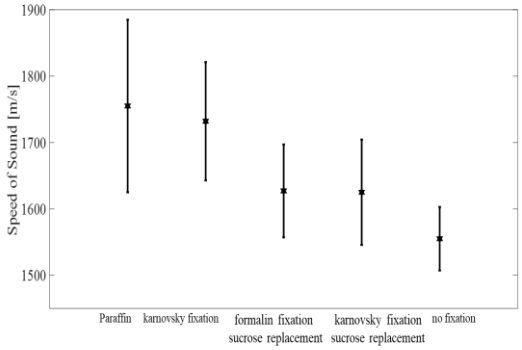

Fig. 2 Speed of sound of rat livers with different sample fixation methods.

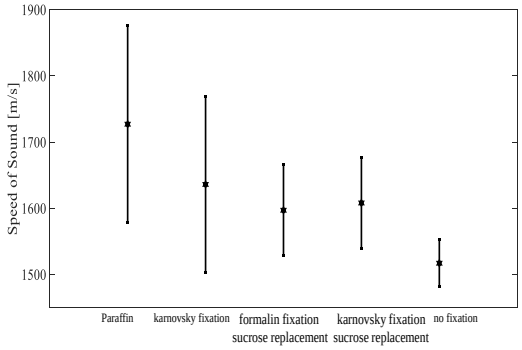

Fig. 3 Speed of sound of rat kidneys with different sample fixation methods.

4. Conclusions

It was confirmed that half Karnovsky-fixation reduced tissue damage to the same extent as formalin fixation, and the absence of heat treatment improved the validity of SoS evaluation. It also confirmed that the sucrose replacement brought the evaluated value of SoS closer to the known value evaluated by low frequency ultrasound. However, there were some unstable factors in the sample preparation methods in this initial study. In the current study, SoS of the organs removed from normal model rats having a homogeneous tissue structure are evaluating.

Acknowledgment

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