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Speed-of-sound and attenuation-of-sound images by scanning acoustic microscopy

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We use this protocol and it's working

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Abstract

A scanning acoustic microscope (SAM) imaging system calculates the speed of sound (SOS) and attenuation of sound (AOS) through sections and plots the values for each area as a color dot on the screen to create histological images. Because the harder and more viscous tissue shows faster SOS and greater AOS, respectively, SAM can provide data on the elasticity and viscosity of tissues and lesions. Areas with greater SOS and AOS correspond to those with higher concentrations of collagen or other fibers. SAM offers the following benefits: (1) images are acquired in only a few minutes without staining, (2) repeated observations following the same section are possible, (3) the same slide as light microscopy (approximately 10 μm thick) is available with comparable high resolution, (4) digitized SOS and AOS data are statistically comparable among different lesions. This SAM imaging system provides comparable data about not only the mechanical structure but also the functional alterations of organs or lesions.

Image Attribution

Fig. 1. Equipment of scanning acoustic microscopy: 1. Scanner with transducer, 2. Display, 3. Computer

Figure 2: Principles of scanning acoustic microscopy (SAM).

Fig. 3: Screenshot of mouse femoral bone section images, including acoustic intensity (upper left), speed of sound (upper right), attenuation (lower left), and thickness of section (lower right). The right image is the corresponding light microscopic image stained with hematoxylin and eosin.

Fig. 4A shows a captured image with optimal focus, while Figs. 4B and 4C display the images obtained when the focus is shifted forward and backward, respectively.

Fig. 4A. Just focus

Fig. 4B. Prefocus

Fig. 4C Postfocus

Fig. 5A. Over amplitude

Fig. 5B Insufficient amplitude

Fig. 6. SOS images of different fixation conditions: a:Fresh, b:Formalin, c:FFPE

Fig. 6. a: Fresh section, b: Formalin fixed section, c: FFPE section, d: Light microscopy of b, d: Light microscopy of c, A: Artery, B: Bronchiole

a ; Fresh section, b ; Formalin fixed section, c; FFPE section d; Light microscopy of b, d; Light microscopy of c, A;Artery , B ; Bronchiole

Guidelines

Transducer

Just as different objective lenses are used for low and high magnification in optical microscopy, in scanning acoustic microscopy (SAM), the choice of transducer must be tailored to the observation range and desired resolution. For higher magnification observations, transducers with higher frequencies must be selected.

Since transducers are expensive, it is necessary to order the most suitable type from the manufacturer based on the intended purpose.

The resolution is approximately calculated by 1500 m/s (the speed of sound in water) divided by the frequency. If the frequency is 100 MHz, the resolution is about 15 μm .

Amplitude adjustment

It is essential to adjust the amplitude so that it falls within a certain threshold range. Fig. 5A shows an image where the amplitude exceeds the threshold due to excessive adjustment, while Fig. 5B shows an image with a lower amplitude setting. By adjusting the amplitude to a consistent level, optimal imaging can be achieved.

Materials

- Ultrasonic microscope AMS-50SI (Honda Electronics Co., Ltd., Toyohashi, Japan)
- Scanner with transducer
- Display
- Computer
- Glass slides (for sample mounting)
- 10- μm thick sample sections
- Distilled water (as coupling fluid)

Before start

In scanning, a blank area must be included in one of the four corners. This blank area, filled with water, serves as the reference value for measurement.

We can select the size of the observation area from 4.8, 2.4, 1.2, 0.6, and 0.3 mm^2 square.

Before scanning, a copy of the whole or optical microscopic images helps determine the scanning area for SAM in advance.

Microscopy equipment and the principle of measurement

- 1 Simultaneously obtain intensity, speed-of-sound, attenuation-of-sound, and thickness images by scanning the section for a few minutes using the ultrasonic microscope AMS-50SI (Honda Electronics Co., Ltd., Toyohashi, Japan).

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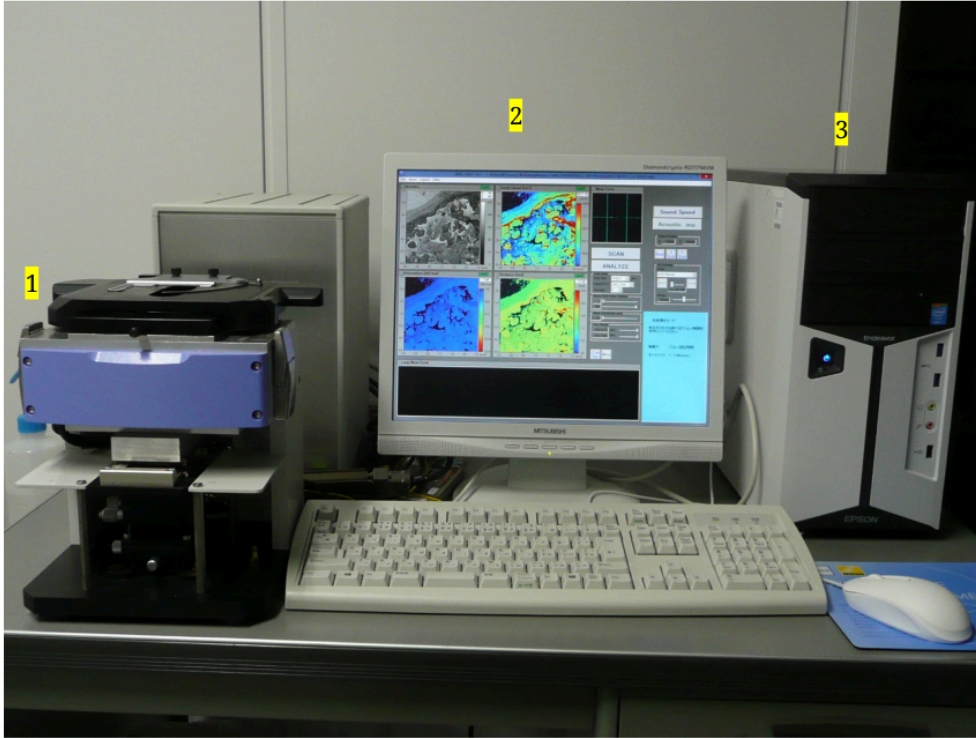


Fig. 1. Equipment of scanning acoustic microscopy 1. Scanner with transducer, 2. Display, 3. Computer

- 3 Place the section on the transducer upside down. Ensure distilled water is present between the transducer and the section to serve as the coupling fluid. The transducer will automatically scan the section, irradiating ultrasonic waves that reflect off both surfaces of the glass slide and the section, then return to the transducer. These waves pass through 10- μ m sample sections with varying ultrasonic properties. The system calculates the speed of sound (SOS), attenuation of sound (AOS), and thickness through each area, collecting data from 300 $\times$ 300 areas in each scan.

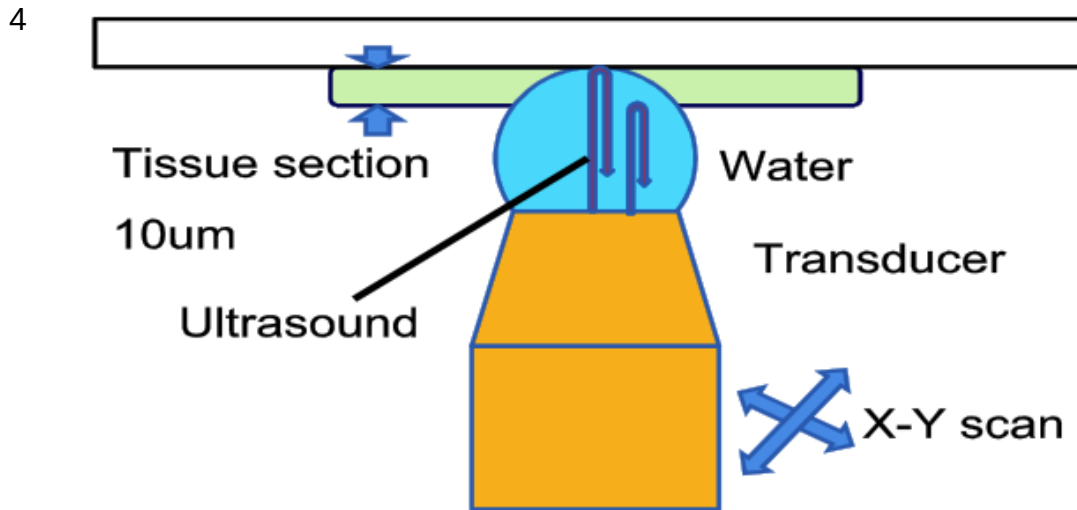


Figure 2: Principles of scanning acoustic microscopy (SAM).

- 5 In scanning, a blank area must be included in one of the four corners. This blank area, filled with water, serves as the reference value for measurement.
- 6 Select the size of the observation area from 4.8, 2.4, 1.2, 0.6, and 0.3 mm² square.
- 7 Before scanning, use a copy of the whole or optical microscopic images to help determine the scanning area for SAM in advance.

Captured image on the screen

- 8 Fig. 3 shows a screenshot of mouse femoral bone section. It contains acoustic intensity (upper left), SOS (upper right), AOS (lower left), and thickness (lower right) images. The vertical bar on the left and the horizontal bar at the bottom of each figure indicate the distance (mm) on the slide. The vertical color scale on the right side of each image indicates SOS (m/s), AOS (dB/mm), and thickness (µm) values.

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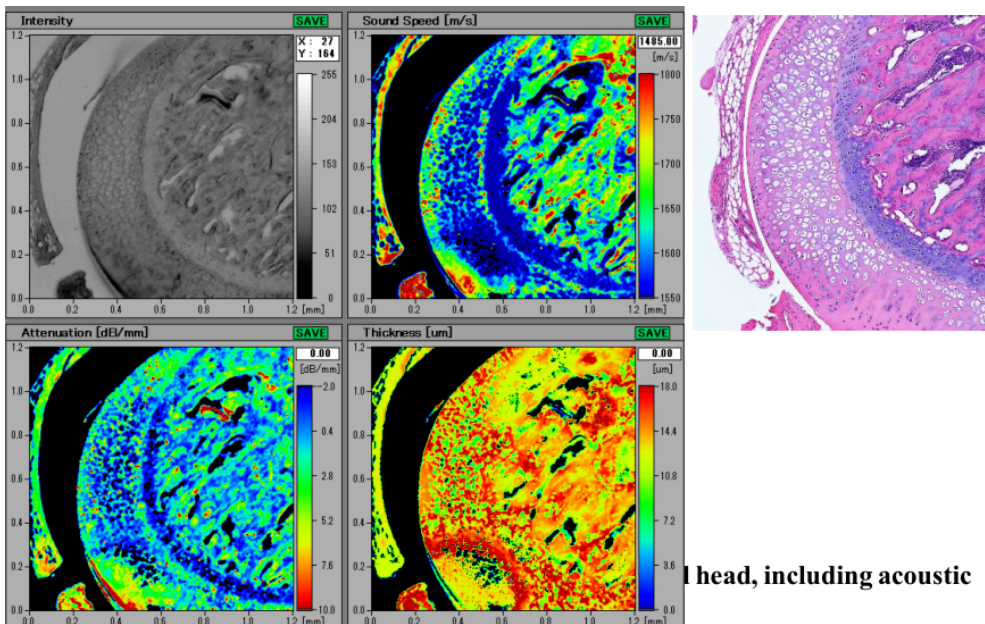


Fig. 3: Screenshot of mouse femoral bone section images, including acoustic intensity (upper left), speed of sound (upper right), attenuation (lower left), and thickness of section (lower right). The right image is the corresponding light microscopic image stained with hematoxylin and eosin.

Transducer

- 10 Just as different objective lenses are used for low and high magnification in optical microscopy, in scanning acoustic microscopy (SAM), the choice of transducer must be tailored to the observation range and desired resolution. For higher magnification observations, transducers with higher frequencies must be selected.
- 11 Since transducers are expensive, it is necessary to order the most suitable type from the manufacturer based on the intended purpose.
- 12 The resolution is approximately calculated by 1500 m/s (the speed of sound in water) divided by the frequency. If the frequency is 100 MHz , the resolution is about $15 \text{ }\mu\text{m}$.

Observation procedure

- 13 Section the formalin-fixed and paraffin-embedded blocks at a thickness of $10 \text{ }\mu\text{m}$. To compare the images with light microscopic images, prepare adjacent sections.
TIP: The sections should be cut flat to avoid irregular reflection. Correct measurements of SOS and AOS need some distance for sound travel. On the contrary, long distances cause energy loss as the sound passes through sections. Therefore, the best thickness

is approximately 10 μm . The exact thickness of each area is displayed on the screen after the analysis.

- 14 Soak the sections in 100% xylol to remove the paraffin, immerse them in 100% ethanol, and dip them in lower concentrations of ethanol in a step-wise fashion and then in 100% distilled water.
- 15 Keep the sections in distilled water before observation.
TIP: Fresh-frozen sections can be used for these observations. However, sections are soaked in water to dissolve unfixed components. Therefore, fixation is necessary for tissue observation. We recommend using cross-linking fixatives, such as formalin, rather than dehydration fixatives, like ethanol.
- 16 Set the transducer and apply a drop of distilled water on the transducer as a coupling fluid.
- 17 Place the section upside down on the stage above the transducer.
TIP: Water expands between the slide and the transducer. Avoid bubbles that will interfere with the sound transmission.
- 18 In order to adjust the focus and amplitude, move the slide glass to locate a blank area on the transducer. (SEE the next section on focus adjustment and amplitude adjustment)
- 19 Switch on the "SCAN" button. Adjust the distance between the transducer and the glass slide so that the reflected sound waves appear on the bottom screen.
TIP: Several waves appear on the screen, but the desired wave is usually the greatest one. The optimal distance between the transducer and the slides is determined for each transducer, and this information can be stored on the computer.
- 20 In order to adjust the slope of the slide, switch on the "X scan" button and turn the X slope knob to place the sound wave within the center zone between the 2 vertical bars that are blue in color. Then, switch on the "Y scan" button and turn the Y slope knob to adjust the position of the wave in the center zone.
- 21 Relocate the slide to set the region of interest on the transducer, and switch on the "SCAN" button. Include a blank area in at least one corner of the screen as a reference area.
- 22 During mechanical X-Y scanning, an image of acoustic intensity gradually appears on the left-upper screen.
- 23 You can adjust the location of the slide, scan size (region of interest: 0.3, 0.6, 1.2, 2.4, or 4.8 mm^2).
TIP: To save time orienting the sections, first try rough scans, and then do more precise scans.

- 24 Then, push the "ANALYZE" button.
TIP: SOS values from each point on the section are calculated and plotted on the screen to create two-dimensional, color-coded images (Figure 2, upper right). The vertical bar on the left and the horizontal bar at the bottom of each figure indicate the distance (mm) on the slide. The vertical color scale on the right side of the figure indicates the average SOS of each square area on the section.
Other data, such as sound attenuation (Figure 2, lower left) and section thickness (Figure 2, lower right), are also displayed on the screen.
- 25 For the statistical analysis, the values of SOS, attenuation, and the thickness of each area are displayed on the screen by placing the cursor on the area.

Focus adjustment

- 26 It is necessary to adjust the position of the transducer to capture the reflected waves from both the glass slide and the tissue section surface. This adjustment has a significant impact on sound speed and attenuation values.
- 27 Fig. 4A shows a captured image with optimal focus, while Figs. 4B and 4C display the images obtained when the focus is shifted forward and backward, respectively.

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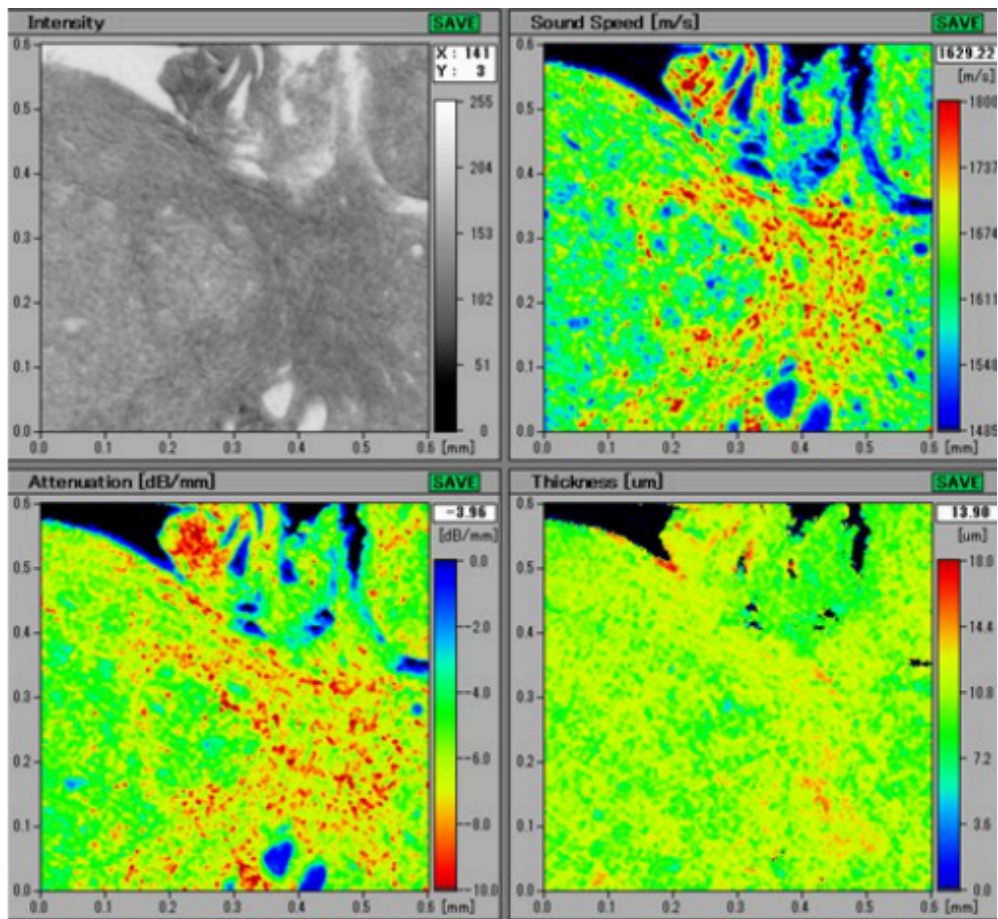


Fig. 4A. Just focus

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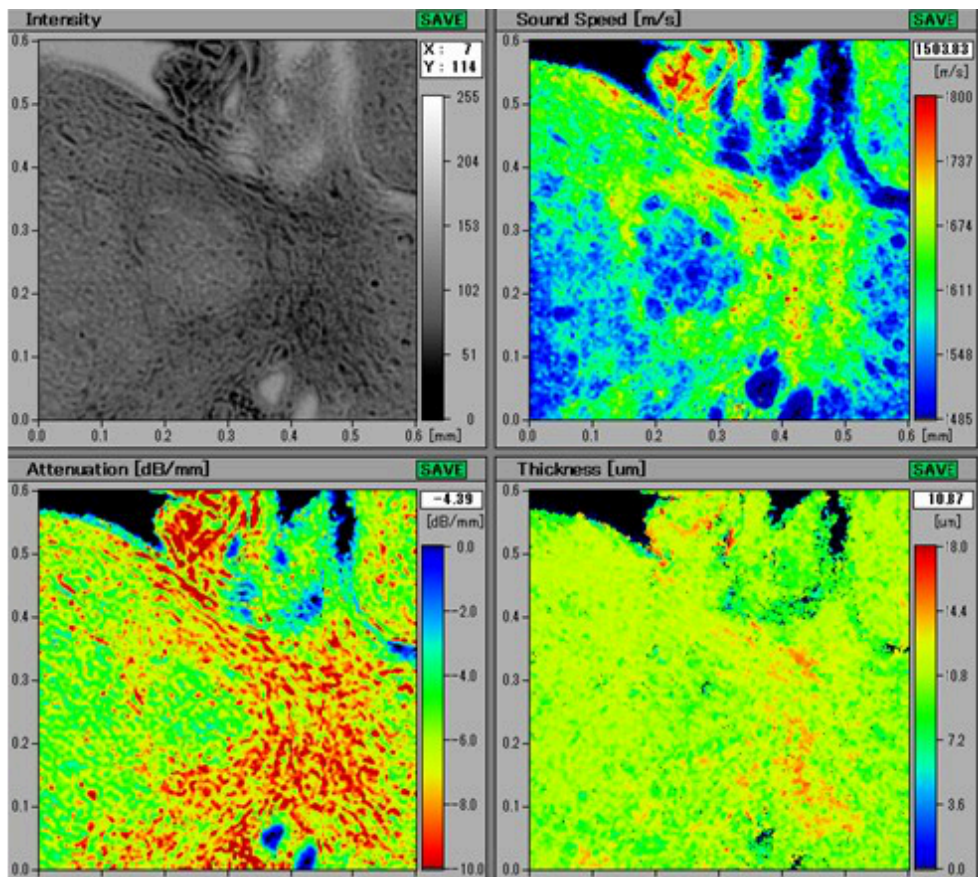


Fig. 4B. Prefocus

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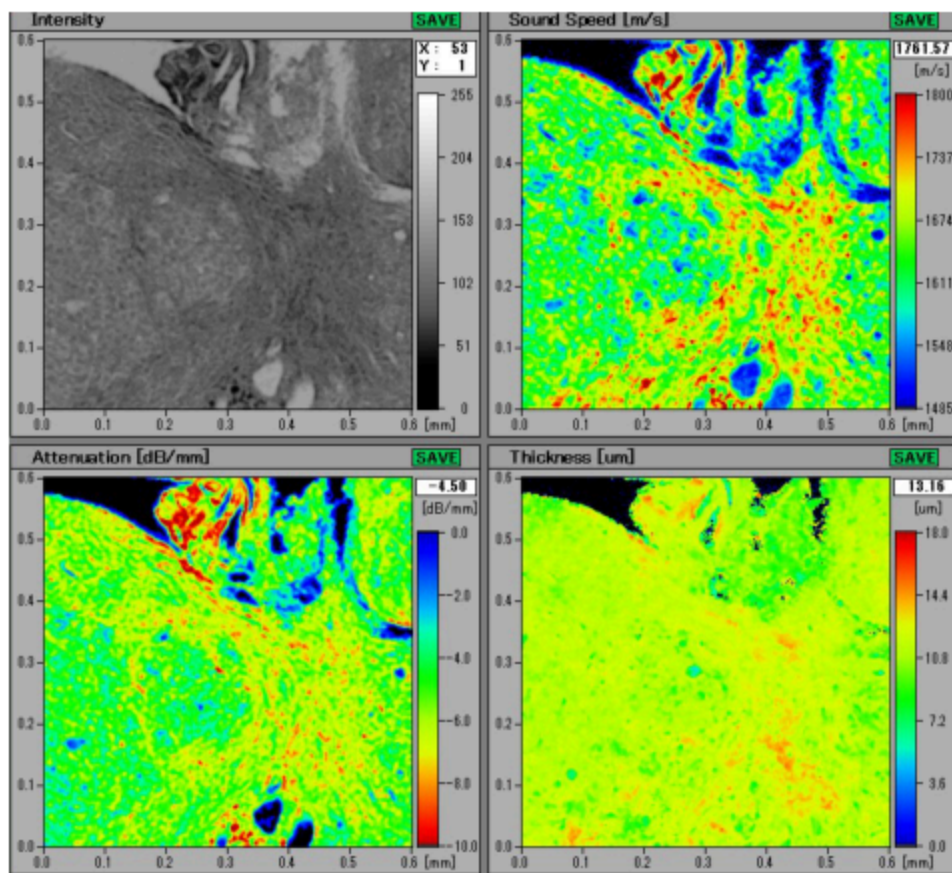


Fig. 4C Postfocus

Amplitude adjustment

- 31 It is essential to adjust the amplitude so that it falls within a certain threshold range. Fig. 5A shows an image where the amplitude exceeds the threshold due to excessive adjustment, while Fig. 5B shows an image with a lower amplitude setting. By adjusting the amplitude to a consistent level, optimal imaging can be achieved.

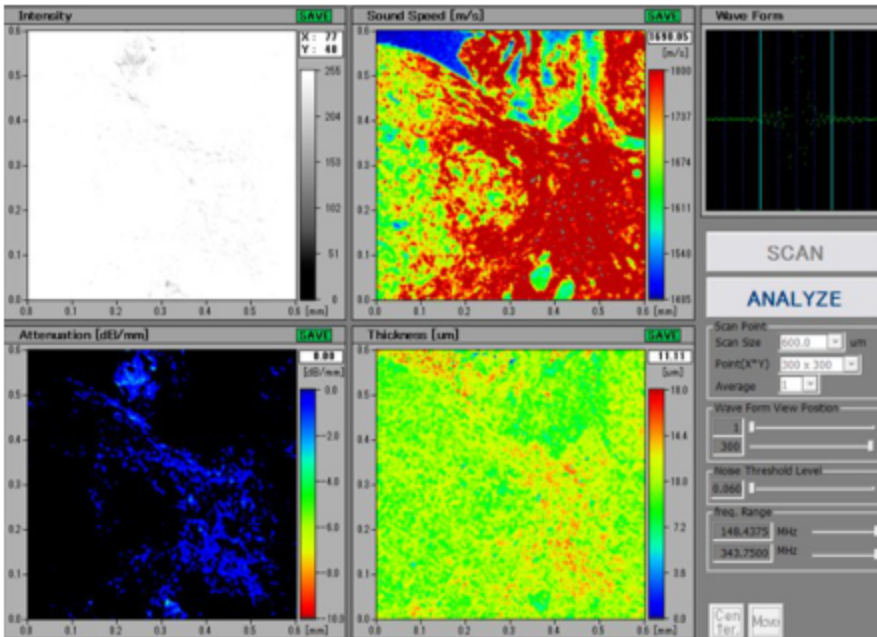


Fig. 5A. Over amplitude

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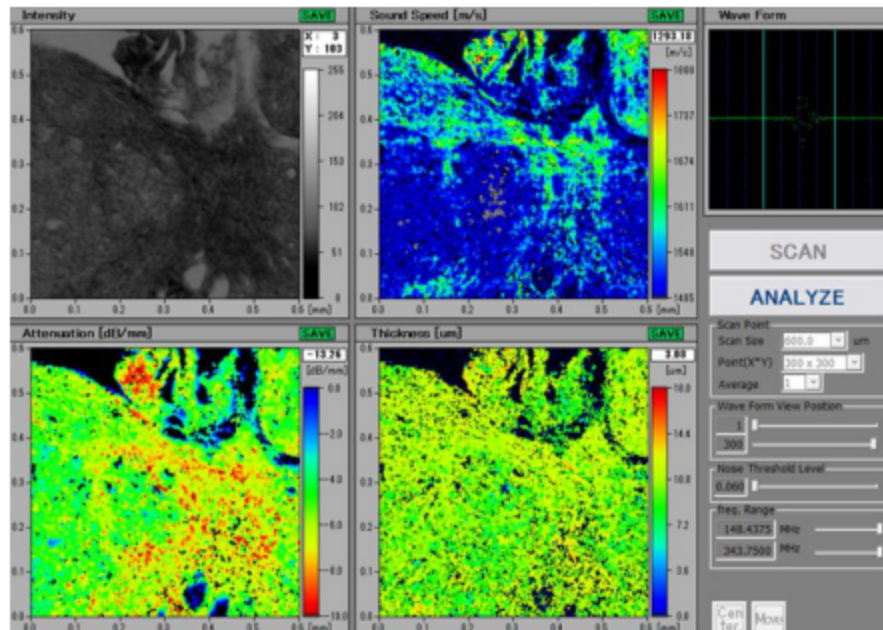


Fig. 5B Insufficient amplitude

Sample preparation

- 33 The specimens used for SOS and AOS images are tissue sections or cells mounted on glass slides as used in optical microscopy. Ideally, the sections should be smooth, without wrinkles or scalpel marks, and approximately 10-μm thick. An accurate

measurement requires a certain propagation distance, and too thin sections, typically 3–4 μm , which are commonly used for optical microscopy, are not suitable for measurements. Additionally, calcifications or uneven surfaces in the section can cause reflection or refraction of sound waves, making measurement difficult.

- 34 Neither frozen sections nor fresh cells are suitable for observation because tissue components may dissolve in water during measurement, leading to unstable results. Moreover, when handling clinical specimens, infection and contamination risks exist, so unfixed sections or cells must be fixed before observation.
- 35 Ethanol, a dehydrating fixative that does not crosslink proteins, penetrates quickly but may cause shrinkage or hemolysis. In contrast, formalin, which crosslinks proteins, penetrates more slowly (approximately 1 cm per hour), maintaining the stable structure of tissues or cells, and allows for the easy creation of formalin-fixed paraffin-embedded (FFPE) sections, typically 10- μm thick (Fig. 6). However, crosslinking and hot paraffin-embedding make the tissue harder and increase its hydrophobicity. In our previous study, the order of stiffness in each section showed no significant difference before and after fixation.

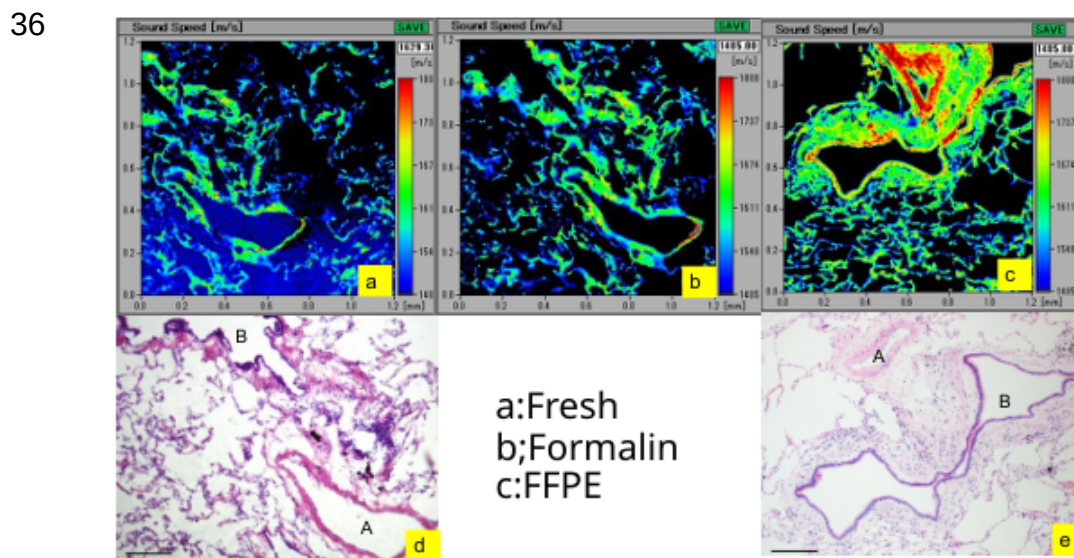


Fig. 6. SOS images of different fixation conditions a ; Fresh section, b ; Formalin fixed section, c; FFPE section d; Light microscopy of b, d; Light microscopy of c, A; Artery , B ; Bronchiole

- 37 a ; Fresh section, b ; Formalin fixed section, c; FFPE section d; Light microscopy of b, d; Light microscopy of c, A; Artery , B ; Bronchiole