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Chapter 15

Preparation of Thin Frozen Sections from Nonfixed and Undecalcified Hard Tissues Using Kawamoto's Film Method (2020)

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See the pictures on the page 278 -281.

Abstract

A method for preparing frozen sections with an adhesive film is described. In order to observe fine structures and weak fluorescence of samples, new types of adhesive films [Cryofilm type 3C(16UF) and 4D(16UF)] are used. The adhesive film is made with very clear and very low autofluorescence. For gene analysis, a very thin adhesive film (LMD film) is used to cut by means of the laser microdissection (LMD). For MALDI mass spectrometry imaging (MALDI-MSI), a conductive adhesive film (Cryofilm type MS) is used to avoid electric charge of the sample. A biological sample is frozen quickly and freeze-embedded. The frozen sample is cut with a very sharp disposable blade made from fine tungsten carbide. The combination of the adhesive films and the blade can generate 3 micrometer thick sections from samples including bone, while it is also possible to generate 1 μm thick sections. The morphology of bone and soft tissues are preserved using this method. Cells such as osteoblasts, fibroblasts, and osteoclasts are clearly observed with an oil immersion lens at high magnification. Sections generated using the Cryofilm type 3C(16UF) shows weak fluorescent signals more clearly than sections generated with the previously reported adhesive films [Cryofilm type 2C(9) and 2C(10)]. Furthermore fluorescence of the fine structures in cells is clearly shown using a super–high-resolution microscope. Several staining and experimental methods such as histology, histochemistry, enzyme histochemistry, immunohistochemistry, and in situ hybridization can be performed on these sections. This method is also useful for preparing frozen sections of large sample such as a whole-body mouse and rat. In gene analysis, gene quality of sample collected from the section made with the LMD film is superior to that of sample made by a conventional method. The Cryofilm type MS makes almost complete section from tissues including hard tissues and large samples. The satisfactory signals are detected from the section with MALDI-MSI.

Key words Frozen-section, Hard tissue, Whole body section, Immunohistochemistry, In situ hybridization, LMD, MALDI-MSI, MAS imaging, Conductive adhesive film, Cryofilm

1 Introduction

It is well known that frozen sections are suitable for immunohistochemistry, for gene analysis using LMD technique, for MALDI-MSI and autoradiography [1–12]. However, there are troublesome samples, which are extremely difficult to make frozen sections by a

conventional method. For example, it is difficult to make high quality frozen sections on glass slides from hard tissues and plant samples. Kawamoto (1990) introduced an adhesive film into the conventional method for making frozen sections from such troublesome samples [13]. The method successfully generates frozen sections from various samples and has been continuously improved to make more complete sections that apply to many types of biological research [14–16]. The improved method allows for the generation of almost complete frozen sections from many types of biological samples without special training. The method makes it possible to prepare thin frozen sections from hard tissues. We reported the method as Kawamoto's film method 2012 in Method in Molecular Biology [17]. The sections prepared by the method are applicable for a variety of techniques, including histology, histochemistry, immunohistochemistry, and in situ hybridization as performed on sections using conventional methods. This method produces excellent results in life science research areas using undecalcified frozen sections and/or nonfixed frozen sections for histochemistry and immunohistochemistry [18–32] (Fig. 1).

However, some problems remain for our original method. The section quality is not high enough for the study of fine tissue structures with weak fluorescence since the section is supported with a plastic film. Normally the plastic film includes small particles within it and the particles disturb the study of detailed tissue structures at high magnification. In addition to this, a conventional plastic film appears to generate autofluorescence, which further disturbs the visualization of fluorescence from green fluorescent protein (GFP) and other fluorescent dyes used in fluorescence immunohistochemistry (FIHC). In order to solve these problems, we have developed new types of adhesive films made with exceptionally clear and very low autofluorescent materials. The new adhesive films allow for the observation of fine structures and faint fluorescence of tissues using a super resolution microscope [33].

It is known that mRNA is degraded by enzymes such as ribonucleases (RNase) and the undesirable degradation appears during sample preparation for gene analysis. The degradation is caused by thawing frozen sections made from fresh samples. Several methods have tried to inhibit this degradation; however, it is difficult to inhibit it completely. We described that the degradation is inhibited by our method [17]. In our method, the section is prepared from a fresh frozen sample and then the frozen section is freeze-dried completely without thawing. The RNase activity is completely inhibited by the freeze-drying process. Samples for gene analysis are collected with an LMD microscope from the freeze-dried section. There is no opportunity for the degradation of mRNA in any step of the sample preparation. We have confirmed that RNA

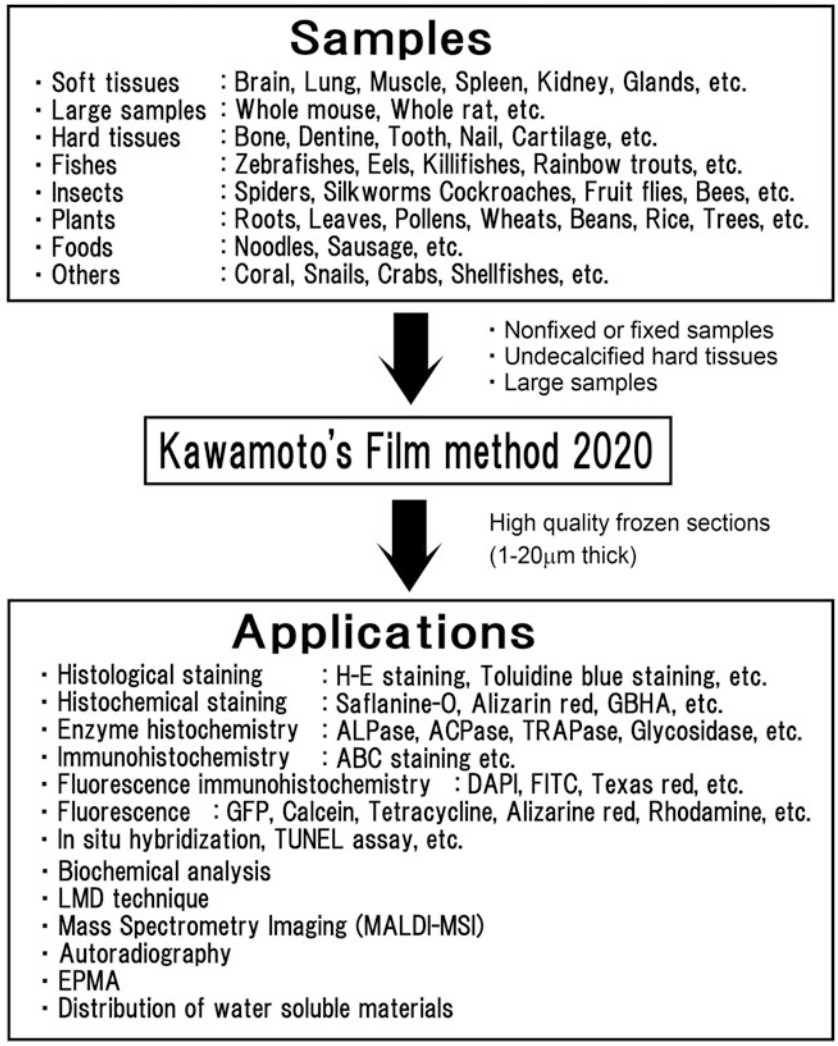

Fig. 1 The figure shows samples and applications used in Kawamoto's film method 2020. *LMD* laser microdissection, *MALDI-MSI* matrix assisted laser desorption ionization MSI, *EPMA* electron probe micro analyzer

integrity values from sample prepared by our method are superior to those prepared by conventional methods [34].

Recently, MALDI-MSI has become a powerful tool in biological and medical research. The sections used for MALDI-MSI are normally prepared on conductive glass slides (glass slides coated with Indium-Tin Oxide (ITO)) to avoid electric charge of the sample [8–10]. As described above, for this application it is also extremely difficult to prepare frozen hard tissue or plant sections on conductive glass slides. In order to solve this problem, we have also introduced a conductive adhesive film (Cryofilm type MS) into the section preparation for MALDI-MSI [35]. The conductivity of this

Table 1
The table shows features of the adhesive film used for supporting frozen sections

Type of adhesive film Developed year	Cryofilm type 2C (10) 2003	Cryofilm type 2C (9) 2006	Cryofilm type 3C (16UF) 2016	Cryofilm type 4D (16UF) 2018	LMD film 2006	Cryofilm type MS 2017
Characteristics of the film						
• Available temperature	−35 ~ 40 °C	−35 ~ 110 °C	−35 ~ 110 °C	−42 ~ 110 °C	−30 ~ 100 °C	−30 ~ 100 °C
• Adhesion (at −37 °C)	☆☆☆	☆☆☆	☆☆☆	☆☆☆☆☆	☆	☆
• Adhesion (at −30 °C)	☆☆☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆☆☆	☆☆☆☆
• Optical characteristics	Non-polarization	Polarization	Polarization	Polarization	Polarization	×
• 100% ethanol	☆☆☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆☆☆☆☆	×
• Xylene and acetone	×	☆☆☆☆☆	☆☆☆☆☆	☆☆☆☆☆	×	×
• Background (× 200 <)	☆☆	☆☆	☆☆☆☆☆	☆☆☆☆☆	☆☆	×
• Autofluorescence (×200 <)	☆☆	☆☆	☆☆☆☆☆	☆☆☆☆☆	☆☆	×
• Preservation of stained section	A few days	More than 10 years	More than 10 years	More than 10 years	×	×
Application						
• Histology	☆☆☆	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆	×
• Histochemistry	☆☆☆	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆	×
• Enzyme histochemistry	☆☆☆	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆	×
• IHC	☆☆☆	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆	×
• FIHC	☆☆☆	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆	×
• In-situ hybridyzation	☆	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆	×
• Fluorescence (GFP)	☆☆☆	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆☆☆	×

• Fluorescence (tracer)	☆☆☆	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆	×
• Autoradiography	×	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	☆	×
• LMD	×	×	×	×	☆☆☆☆☆	×
• MALDI-MSI	×	☆☆	☆☆	☆☆	×	☆☆☆☆☆
• EPMA	×	☆☆☆	☆☆☆☆☆	☆☆☆☆☆	×	×
• Polarizing microscope	☆☆☆	×	×	×	×	×

Unsuitable ×, Possible ☆, Good ☆☆☆, Excellent ☆☆☆☆☆
IHC immuno-histochemistry, *FIHC* fluorescence immuno-histochemistry, *LMD*: laser microdissection, *MALDI-MSI* MALDI mass spectrometry imaging, *EPMA* electron probe micro-analyzer

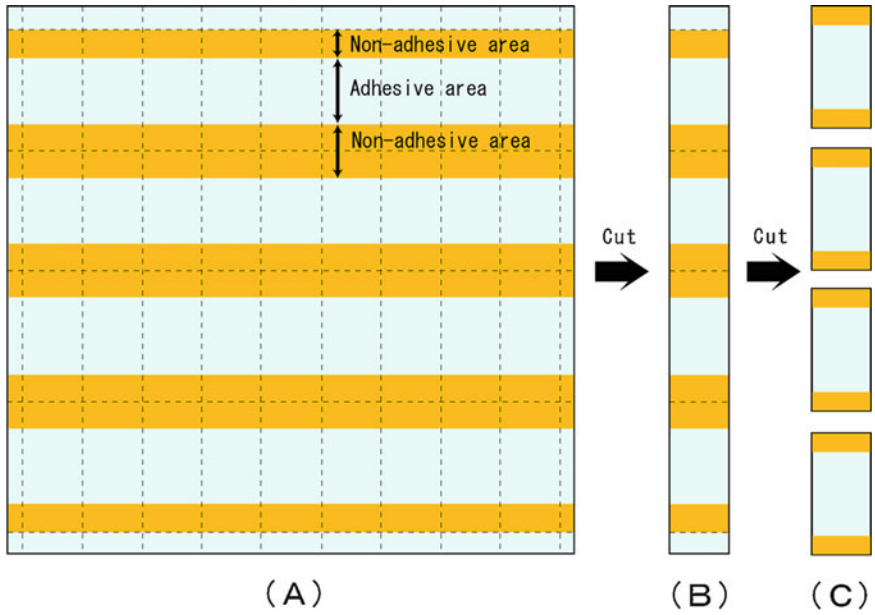

Fig. 2 The figure shows a sheet of Cryofilm (**A**). The sheet is cut into individual pieces successively (**B** and **C**) and the pieces (**C**) are used for supporting frozen sections

adhesive film is nearly the same as that of an ITO glass slide. The conductive adhesive film is very useful for preparing frozen sections of soft tissues and hard tissues similar to that as demonstrated by the Cryofilm (a nonconductive adhesive film).

This chapter describes the latest method named, Kawamoto's film method 2020, and shows some images produced by this method.

2 Materials

Embedding medium (SCEM) and mounting medium (SCMM) used in a previously reported method [17] are also used here; however, a new adhesive film is used to allow for better resolution of fine tissue structures and to observe weak fluorescence signals in these tissues. In addition to this, a conductive adhesive film is used for MALDI-MSI. Materials and tools used for preparing frozen sections are included in a kit (Cryosection Preparation kit, SECTION-LAB Co. Ltd., Japan). Below is a list of the materials and instruments used for this method:

1. Stainless-steel container for embedding frozen samples (included in the kit).
2. Embedding medium (SCEM, SCEM-L1) (included in the kit).
3. Adhesive film (Table 1 and Fig. 2) (some types of film are included in the kit).

4. Cryofilm fitting tool (included in the kit).
5. Mounting medium (SCMM) (included in the kit).
6. UV light for polymerizing the SCMM.
7. Coolant for freezing sample: hexane and dry ice.
8. Cryomicrotome.
9. Disposable tungsten carbide blades (SL-T30UF, 35UF, or 40UF).
10. Blade holder for fixing the disposable blade.
11. 100% ethanol.
12. 4% paraformaldehyde (PFA).
13. Stains (Hematoxylin, Eosin).

3 Methods

3.1 Freeze Embedding

The procedure for preparing frozen sections is almost the same as that described in a previously reported method [17]. The adhesive film sometimes is not available for supporting tissues when the tissue is fixed strongly with PFA, (for instance, muscle, tendon and cartilage, etc.). Therefore, strong fixation should be avoided in this method. The sample should be fixed for less than 1 day if fixation is required to preserve tissue structure. The most important point for preparing good frozen sample is to avoid ice crystal formation during freezing sampls. The artifacts appear more strongly in fresh samples than that in fixed samples (*see* **Notes 1** and **2**).

1. Inject 4% PFA (10–15% v/w of body weight) into the left ventricle of experimental animals.
2. After 20–30 min, dissect a sample from the animal.
3. Place the sample in a mixture of 2% PFA and 10% sucrose kept at a temperature of 0–4 °C for 0.5–24 h.
4. Freeze rapidly the sample in hexane–dry ice (Fig. 3A) (*see* **Note 3**).
5. Put the proper amount of cooled embedding medium (SCEM or SCEM-L1) in the stainless-steel container (Fig. 3B).
6. Place the frozen sample in the embedding medium (Fig. 3C).
7. Quickly move the container into the coolant (hexane–dry ice, or cooled hexane) and freeze the embedding medium completely (Fig. 3D) (*see* **Note 4**).
8. Take the frozen block out of the container (Fig. 3E).
9. Fix the block to the sample holder of the cryomicrotome (Fig. 3F).

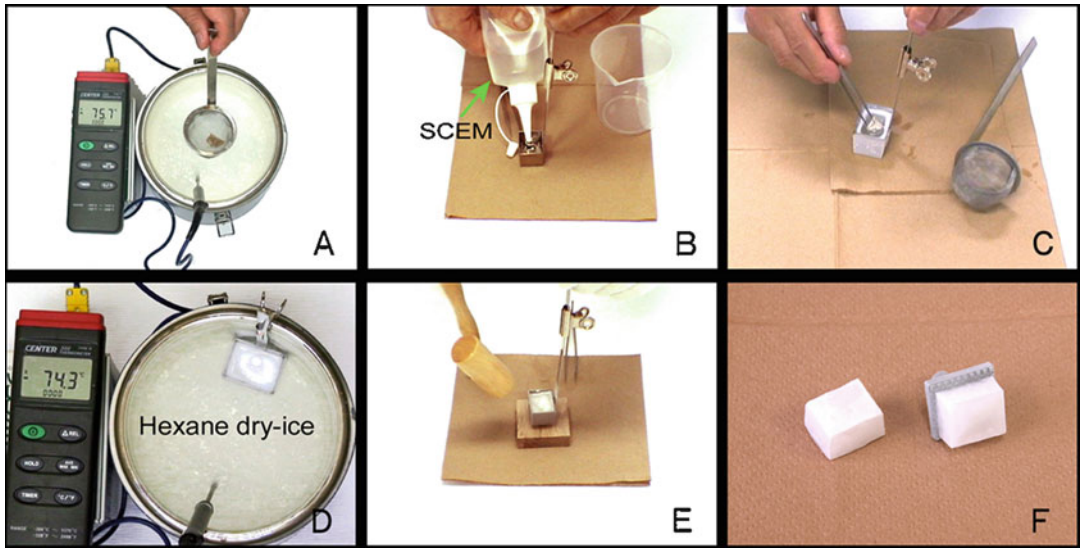

Fig. 3 The figure shows the procedures for preparing frozen sample blocks. (**A**) Freezing the sample, (**B**) embedding medium and stainless-steel container, (**C** and **D**) freeze embedding in hexane–dry ice, and (**E** and **F**) fixing the frozen block to the microtome sample holder

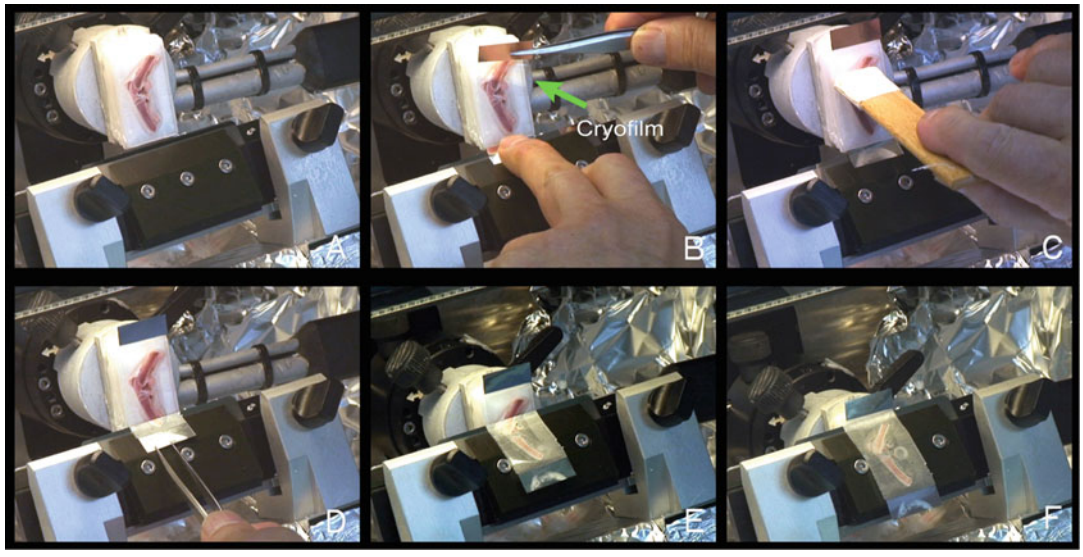

Fig. 4 The figure shows the procedures for preparing frozen sections. The sample is a 7-week-old rat hindlimb. The arrow in panel B indicates the Cryofilm. (**A**) The cut surface (**B** and **C**) applying the Cryofilm to the cut surface, and (**D**, **E**, and **F**) cutting the frozen sample. The thickness: 2 μm

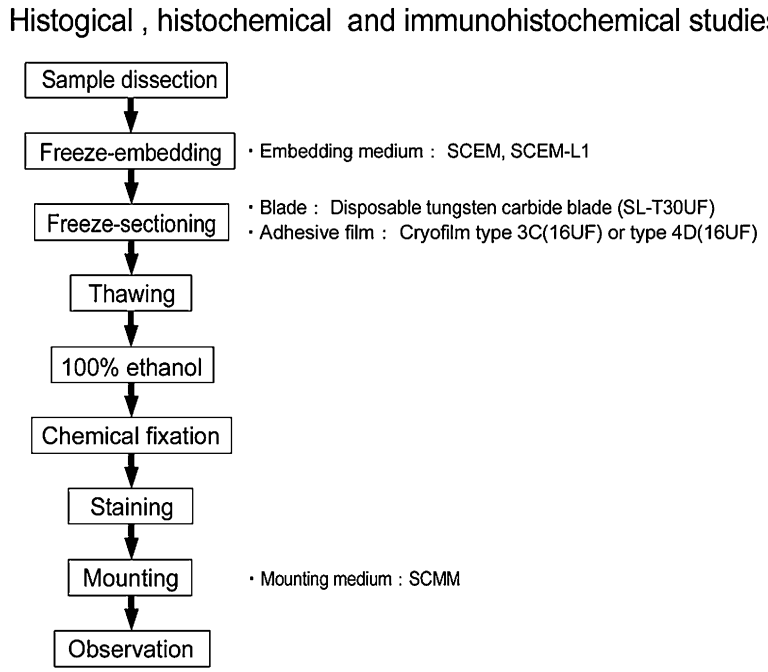

Fig. 5 The flowchart shows the procedures and materials used for preparing frozen sections

3.2 Section Preparation

The temperature of the cryostat is usually kept at −15 to −20 °C. However, the optimal temperature suitable for each sample depends on the type of sample. For example, in the case of adipose tissue, low temperatures are suitable for producing good sections. We sometimes adjust the temperature of the cryostat and the holder chuck to −25 °C and − 35 °C, respectively, when cutting highly mineralized tissues. In order to cut hard tissues, a tungsten carbide blade should be used.

1. Leave the frozen sample block to acclimatize for approximately 10 min after fixing it to the sample holder chuck of cryomicrotome. The temperature of the cryostat is from −20 to −30 °C. The temperature of sample holder chuck is adjusted to −25 to −35 °C (*see* **Note 5**).
2. Trim the sample with a disposable tungsten carbide blade (SL-T30UF or -T35UF) until the area of interest appears on the cut surface (Fig. 4A). After trimming, move a sharp area of the blade into the cut position (*see* **Notes 6** and **7**).
3. Mount the adhesive film [Cryofilm type 3C(16UF) or type 4D (16UF)] to the cut surface (Fig. 4B). Use the LMD film for LMD technique and use the Cryofilm type MS for MALDI-MS (*see* Table 1).
4. Tightly adhere the Cryofilm to the cut surface with a fitting tool (Fig. 4C).

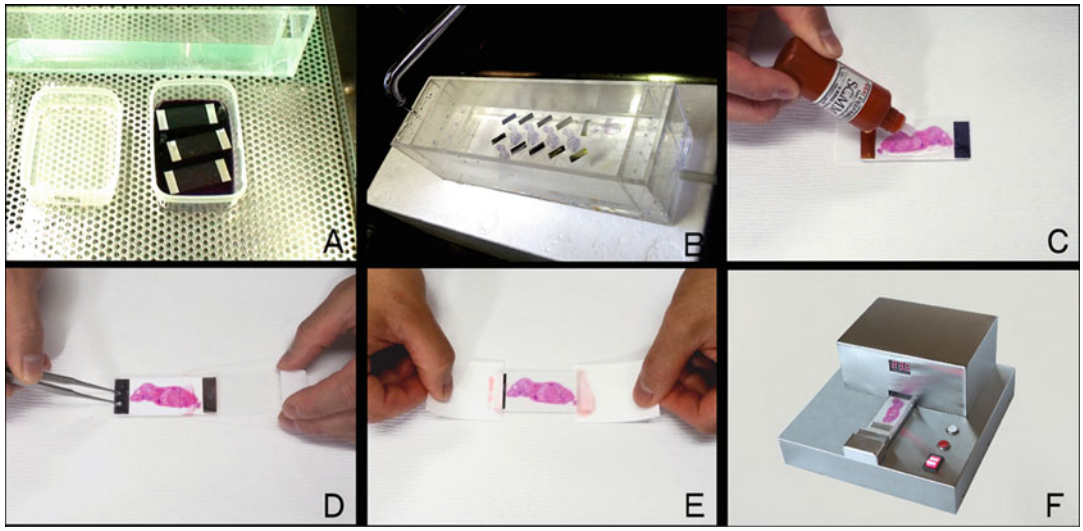

Fig. 6 The figure shows procedures for staining and mounting the sections. (**A**) Staining with hematoxylin, (**B**) washing the section, (**C**) applying the mounting medium (SCMM-R2) on the stained section, (**D**) mounting the section to the glass slide, (**E**) removing excess mounting medium, and (**F**) polymerizing the mounting medium

5. Cut the specimen slowly at a constant speed (Fig. 4D–F).
6. Take the section out of the cryostat and thaw it.
7. Leave it for 10–60 s to dry the section. (The optimum drying time depends on the type of tissue.)
8. Turn the section specimen side down, and immerse the section in 100% ethanol.
9. Then, move the section into the fixative (4% PFA).
10. After sectioning, frozen blocks can be stored at −80 °C (*see* **Note 8**).

3.3 Histological Staining of Sections

For this purpose, sections made with Cryofilm type 3C(16UF) or 4D(16UF) are used. The water soluble mounting medium SCMM is used instead of a conventional xylene-based mounting medium, which causes serious shrinkage and cracking of tissues (*see* **Note 9**). The stained sections are preserved between the adhesive film (Cryofilm) and the glass slide. The sections are treated according to the flowchart (Fig. 5).

1. Stain the section with Hematoxylin for approximately 1 min (Fig. 6A).
2. Carefully wash the section in running water for approximately 4 min (Fig. 6B).
3. Stain the section with water soluble eosin for approximately 10 s.
4. Rinse the section with running water for a few seconds.

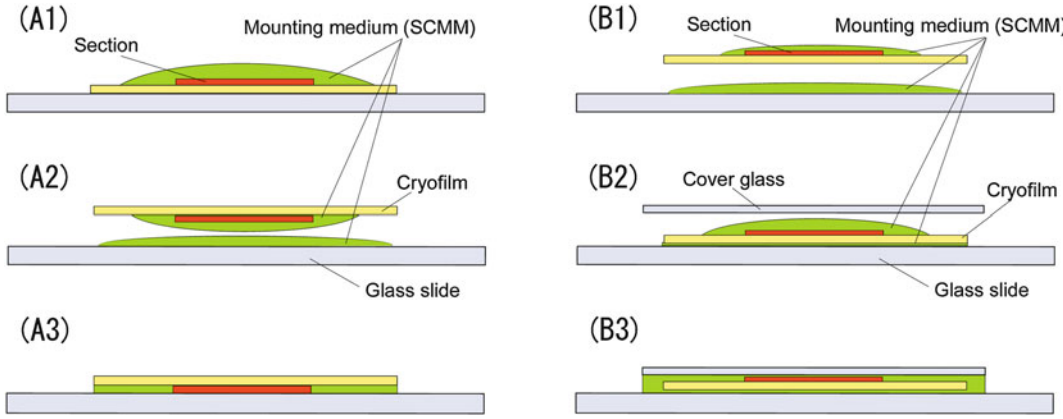

Fig. 7 The schematic diagram shows the procedures for mounting the section. The step **A1**, **A2**, **A3** are normally used for observing the section with a non–oil immersion lens. The step **B1**, **B2**, **B3** are used for observing the section with an oil immersion lens

5. Then rinse it with 100% ethanol for 10 s.
6. Drop the mounting medium (SCMM-R2) on the section (Figs. 6C and 7A1).
7. Turn the section side of the Cryofilm down (Figs. 6D and 7A2) and then place it on a glass slide.
8. Remove the excess mounting medium from the glass slide with filter paper (Figs. 6E and 7A3).
9. Place the glass slide under the UV light to polymerize the mounting medium (Fig. 6F).
10. Wash the glass slide and Cryofilm to remove the unpolymerized mounting medium on the Cryofilm and glass slide.

For observing sections with an oil immersion lens at high power, the following procedures are used.

1. Drop the mounting medium (SCMM-R2) on the glass slide. (Use the mounting medium recommended by manufactures when the sections are used for fluorescence immunohistochemistry.)
2. Also drop the SCMM-R2 mounting medium on the stained section (Fig. 7B1).
3. Place the Cryofilm with the section on the glass slide. In this case, the section side is facing up (Fig. 7B2).
4. Place a glass cover slip on the section.
5. Remove the excess SCMM-R2 mounting media from the glass slide with filter paper.
6. Polymerize the mounting medium (Fig. 7B3).

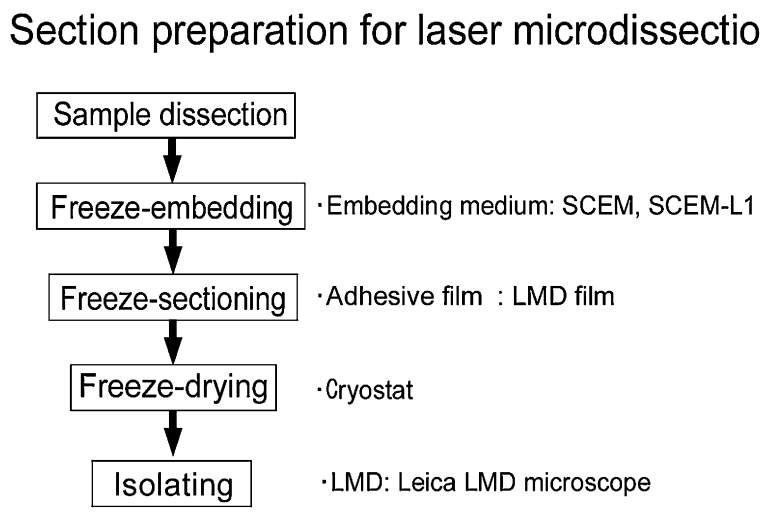

Fig. 8 The flowchart shows the procedures for collecting samples with the LMD microscope

3.4 Enzyme Histochemistry, Immuno-histochemistry (IHC), and In Situ Hybridization

For this purpose, sections are cut on the Cryofilm type 3C(16UF) or 4D(16UF).

1. Place the section on the glass slide (the sectioned side facing up).
2. Drop the staining solution on the section and stain the section according to standard protocols for enzyme histochemistry, IHC, and in situ hybridization.
3. After staining, preserve the section between the Cryofilm and glass slide according to the above procedures or standard protocols (*see* Subheading 3.3).

3.5 Enzyme Histochemistry and Immuno-histochemistry Using Fresh Frozen Sections

Nonfixed and undecalcified sections are useful when chemical fixation and demineralization impair or damage enzyme activity and immune reactivity. For this purpose, sections are generated using the Cryofilm type 3C(16UF) or 4D(16UF).

1. Prepare sections from a fresh frozen sample using the Cryofilm according to the above procedures (*see* Subheading 3.2).
2. Freeze-dry the section completely in a cryostat kept at lower than −20 °C. (Frozen sections of 5 μm thickness will be freeze-dried for approximately 4 h.)
3. Place sections in an airtight box to avoid condensation on the sections.

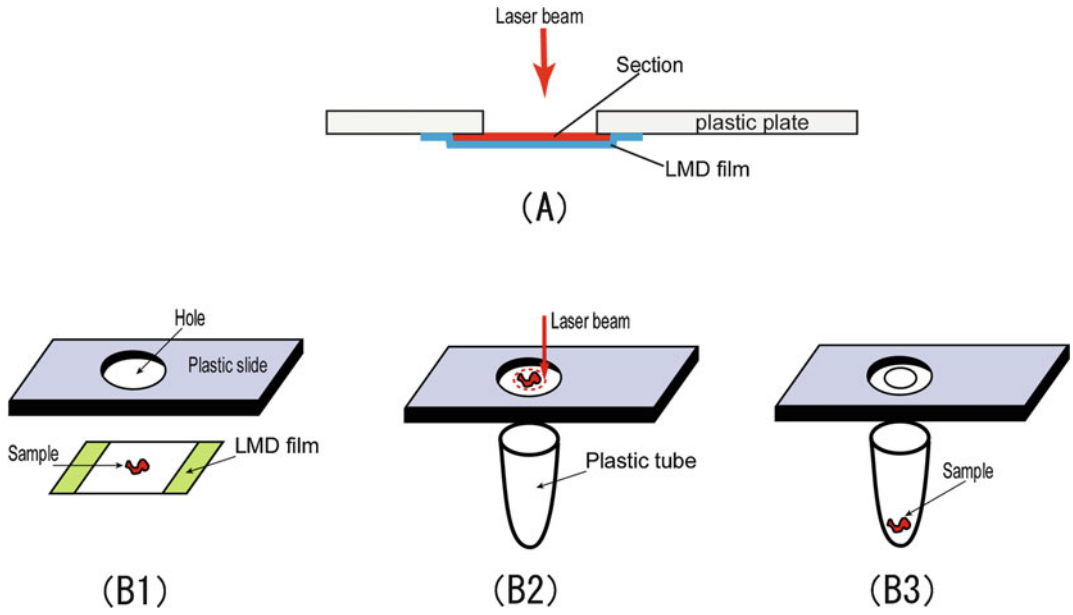

Fig. 9 The schematic diagram shows the sample collections with the LMD microscope. (**A**) The side view of the section fixed under the plastic slide, and (**B1**, **B2**, and **B3**) each step of sample isolation with LMD microscope

4. Take the box out of the cryostat.
5. Stain the sections according to the procedures described above after immersing in 100% ethanol or acetone.

3.6 Sample Collection Using LMD Technique

For this purpose, freeze-dried sections made with an LMD film are used. The sections are treated according to the flowchart (Fig. 8).

1. Prepare sections with the LMD film and freeze-dry according to the procedures described above (*see* Subheading 3.5).
2. Take it out of the cryostat.
3. Fix the section with an adhesive tape to a plastic slide containing a premade hole, making sure to align the sectioned specimen with the premade hole (Fig. 9).
4. Place the slide on the sample stage of LMD device (Leica LMD7, Leica microsystems KK, Germany) and then cut the region of interest with a laser beam. The cut sample falls in the sample collecting tube placed under the slide (Fig. 9B1–B3).
5. Analyze the collected sample according to standard protocols for gene analysis.

3.7 Observation of Fluorescence (Calcein, Alizarin Red S, GFP, etc.)

For this purpose, the sections prepared with Cryofilm type 3C (16UF) or 4D(16UF) are used.

The section is preserved between the glass slide and the Cryofilm without any staining. For GFP fluorescence, the section should be prepared from a chemically fixed sample.

1. Prepare the sections from the frozen sample using the Cryofilm type 3C(16UF) or 4D(16UF) according to the above procedures (*see* Subheading 3.2).
2. Take the section out of the cryostat and thaw it.
3. Then, preserve the section between the Cryofilm and glass slide with SCMM-R2 according to the procedures described above (*see* Subheading 3.3).

3.8 Section Preparation for MALDI-MSI

For this purpose, sections prepared with a conductive adhesive film (Cryofilm type MS) are used. The sections are treated according to the flowchart (Fig. 10).

1. Prepare sections from frozen samples using Cryofilm type MS and Cryofilm type 3C(16UF) according to the above procedures (*see* Subheading 3.2). The section prepared with the Cryofilm type 3C(16UF) is used for examining the results of MALDI-MSI.
2. Take the section out of the cryostat and thaw it. A freeze-dried section should be used if the target substances are degraded during thawing the frozen section.
3. Fix the section on a standard glass slide with a double-sided adhesive tape or a SCMM-MS (Figs. 10 and 11).
4. Deposit a matrix (a-cyano-4-hydroxycinnamic acid, etc.) on the section.
5. Analyze the section by means of MALDI-MSI.
6. Results are examined using serial sections stained histologically or histochemically.

3.9 Studying the Distribution of Water-Soluble Substances (Autoradiography, Water-Soluble Substances, Water-Soluble Tracers, etc.)

For this purpose, sections are prepared from fresh samples using the Cryofilm type 3C(16UF) or 4D(16UF) and the sections are freeze-dried. *See* refs. (11–14, 16).

1. Prepare the frozen sections using the Cryofilm according to the above procedures (*see* Subheading 3.2).
2. Freeze-dry the section in the cryostat and then take it out of the cryostat according to the above procedures (*see* Subheading 3.3).
3. For autoradiography, contact the section to the imaging plate to assess distribution of radiolabeled substances within the tissues.

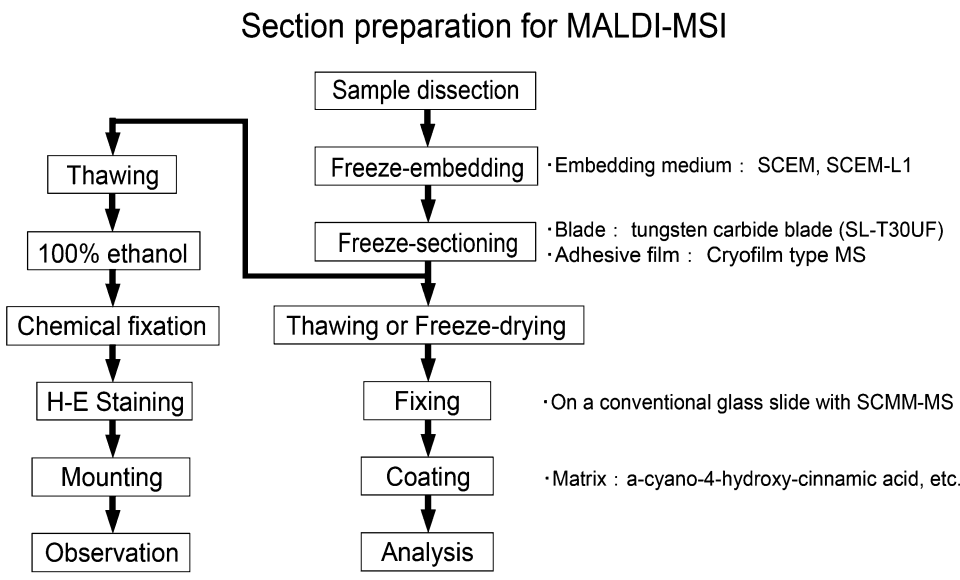

Fig. 10 The flowchart shows the procedures of sample preparation for MALDI-MSI

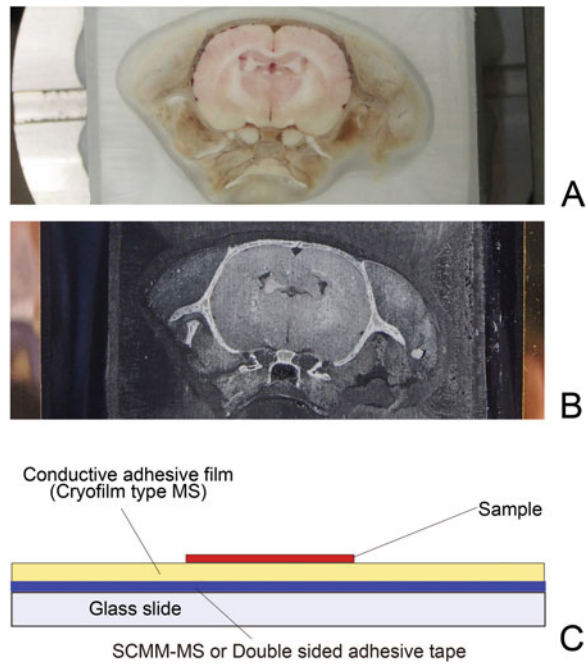

Fig. 11 The figure shows a 10 μm thick section used for MALDI-MSI. The frozen section was prepared from the undecalcified head of 10-week-old rat. Embedding medium: SCEM, blade: SL-T30UF, conductive adhesive film: Cryofilm type MS. (**A**) The cut surface, (**B**) the section prepared with the Cryofilm type MS, and (**C**) the side view of the section and Cryofilm fixed on the glass slide. The H-E stained section is shown in the Fig. 16

4. For elemental analysis, fix the section on the sample holder of an electron probe micro-analyzer (EPMA) with a double-sided adhesive tape and analyze the section with EPMA.

4 Notes

1. Cracks appear on the frozen blocks when the embedding medium (SCEM) is frozen with a very low temperature coolant such as liquid nitrogen. The problem is solved by using dry ice–hexane.
2. Optimal cutting temperature (OCT) compound is widely used for freeze-embedding samples; however, the compound is not suitable for cutting at very low temperatures (for instance a temperature lower than −30 °C) and the Cryofilm does not adhere strongly to the cut surface. The embedding medium (SCEM) is a different type of mounting medium than conventional embedding medium, and it has been optimized to this method. The SCEM-L1 is used for cutting at lower temperatures. The temperature suitable for cutting is as follows:
   (a) SCEM: −15 to −35 °C.
   (b) SCEM-L1: −27 to −50 °C.

   Both embedding media allows smooth cutting of frozen samples at above temperature and the Cryofilm adheres tightly to the cut surface.
3. Pentane and isopentane cooled with liquid nitrogen are also useful for freezing samples.
4. Do not use liquid nitrogen to freeze the embedding medium within the mold. Use of liquid nitrogen to freeze the SCEM will result in cracks within the frozen block.
5. The temperatures of the cryostat and sample holder chuck are important for producing high quality sections, especially the temperature of the chuck. We usually adjust the temperature from −25 to −30 °C when cutting bone tissues. Occasionally we cut bone samples at lower than −35 °C.
6. The blade is a very important tool in preparing quality sections. The SL-T series blades were specially designed for this method with a fine tungsten carbide. The lowest angle blade (SL-T30UF) produces excellent sections from soft and hard tissues. However, the SL-T30UF is inferior to other types (SL-T35UF and T40UF) of blades in durability though it is used for cutting mineralized bone. The SL-T35UF or T40UF should be used when cutting highly mineralized bones and teeth. The cutting angle of each blade is following;

(a) SL-T30UF: 30°.

(b) SL-T35UF: 35°.

(c) SL-T40UF: 40°.

7. The disposable blade is fastened to the specially designed holder and then the holder is fixed to the cryomicrotome. The angle of blade holder clamp has to be adjusted to the angle suitable for each blade.
8. The frozen sample blocks are preserved in a deep freezer (approx. −80 °C) for later investigation after covering the cut surface with the Cryofilm for protection. Serious desiccation does not appear on samples embedded with the SCEM. These frozen samples can be used for at least 2–3 years.
9. The mounting medium is also an important material in order to generate beautiful images. Some troubles appear when mounting the sections with conventional mounting medium; serious cracks occur in the tissues and/or stains leach out of the tissue during mounting. Therefore, mounting mediums suitable for stains have to be used and include the following:

(a) SCMM-G1 (drying type): For hematoxylin and eosin staining.

(b) SCMM-R1 (drying type): For enzyme histochemistry, immunohistochemistry (ABC method).

(c) SCMM-R2 (polymerizing type): For hematoxylin and eosin staining, histochemical staining, Immunohistochemistry (depends on the stains) et al., fluorescence immunohistochemistry (depends on the antibody).

(d) SCMM-R3 (polymerizing type): For toluidine blue staining.

(e) SCMM-MS (polymerizing type): For MALDI-MSI

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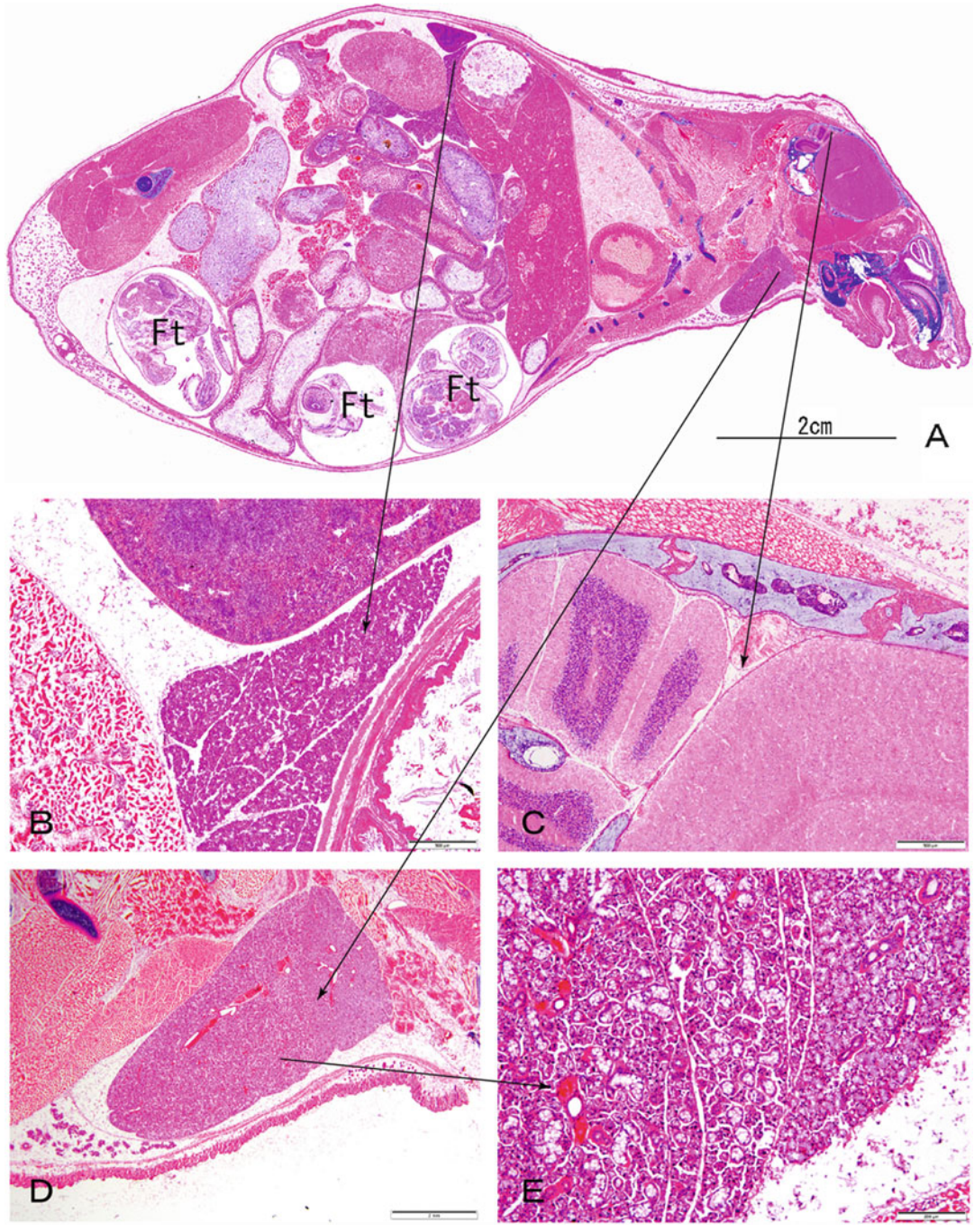

Fig. 12 The figures show a picture of 3 μm thick frozen section (**A**) prepared from a fresh pregnant mouse and the enlarged pictures (**B**, **C**, **D**, and **E**) of the section. Staining: hematoxylin and eosin, embedding medium: SCEM, mounting medium: SCMM-R2, blade: SL-T30UF, adhesive film: Cryofilm type 4D(16UF). Ft: fetus

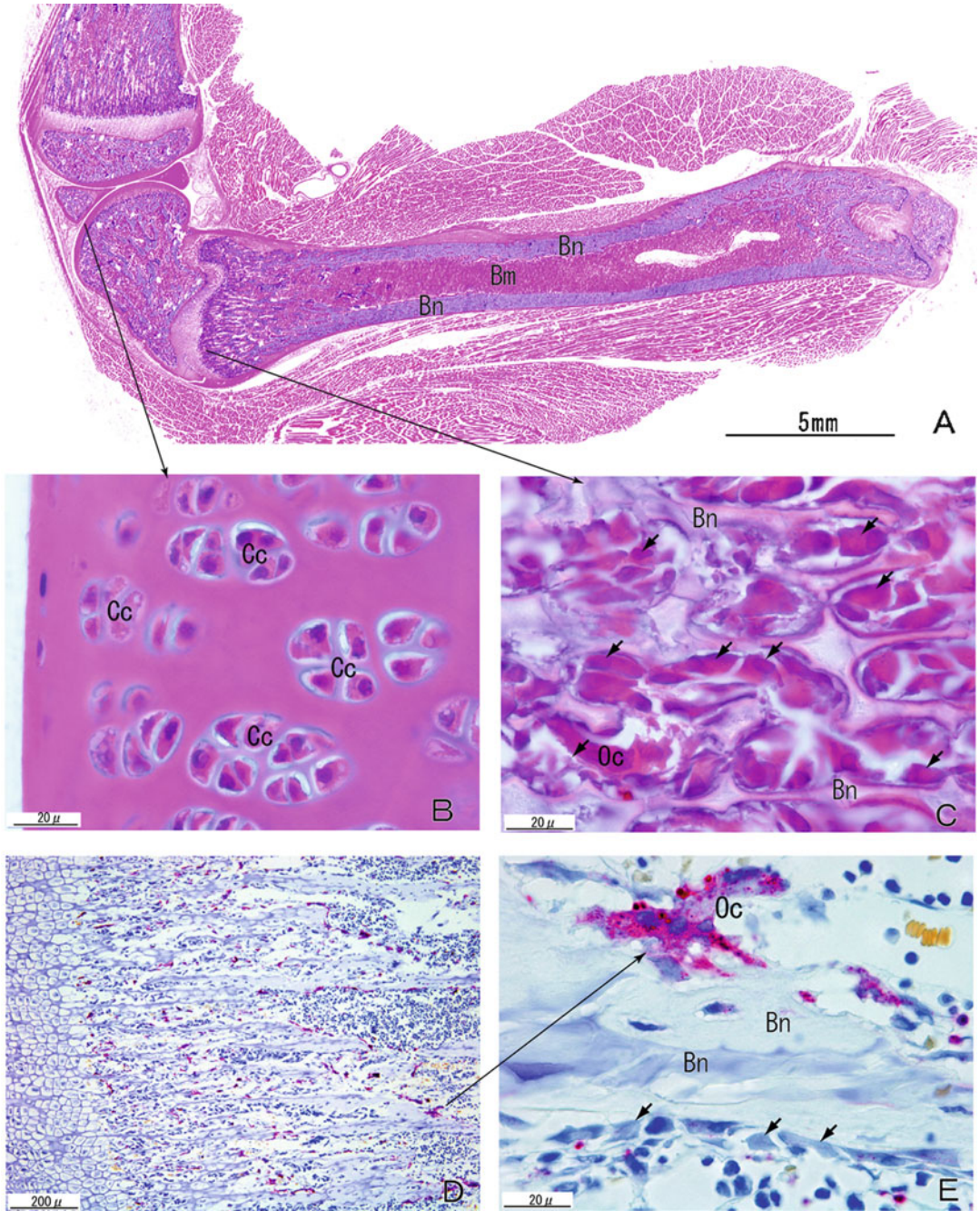

Fig. 13 The figure shows a 3 μm thick frozen section prepared from an undecalcified hindlimb of a 7-week-old rat. The pictures (**B**, **C**, and **E**) were taken with an oil immersion lens. Staining: hematoxylin and eosin (**A**, **B**, and **C**), and TRAP staining (**D**, **E**), embedding medium: SCEM-L1, mounting medium: SCMM-R2, blade: SL-T30UF, adhesive film: Cryofilm type 4D(16UF). *Bn* bone, *Bm* bone marrow, *Oc* osteoclast, *Cc* chondrocyte, and arrows in the panel C: osteoblasts

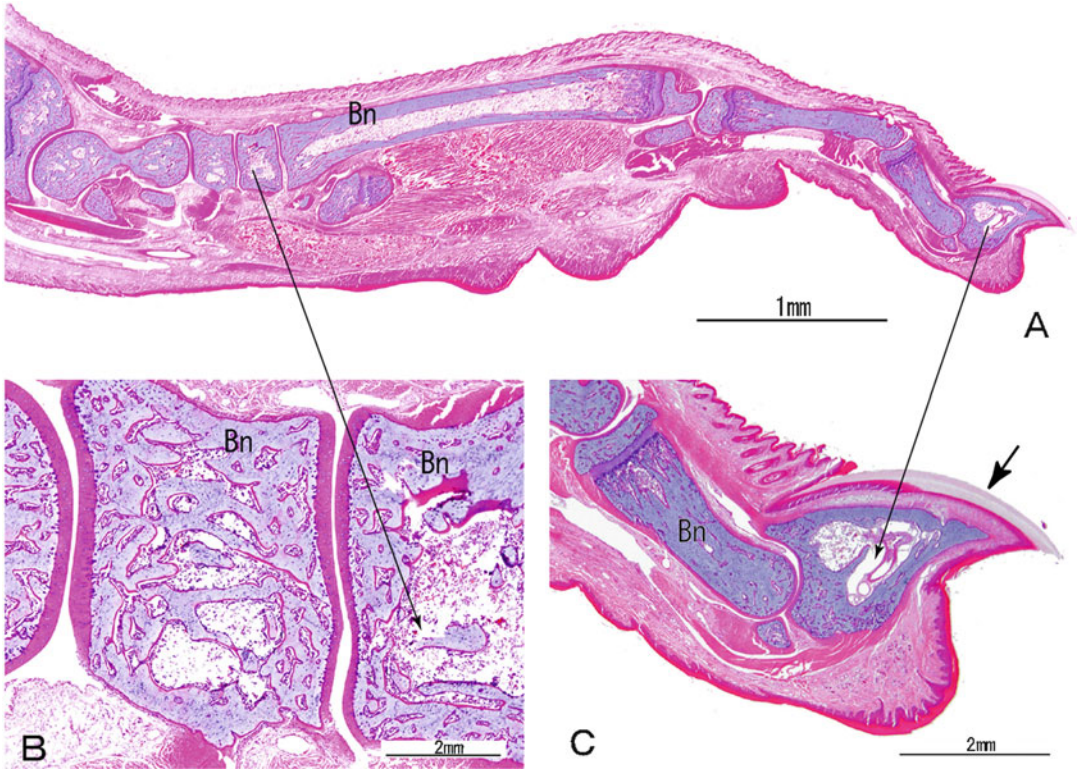

Fig. 14 The figures show a 3 μm thick frozen section prepared from an undecalcified 7-week-old rat foot. Staining: hematoxylin and eosin, embedding medium: SCEM-L1, mounting medium: SCMM-R2, blade: SL-T30UF, adhesive film: Cryofilm type 4D(16UF). *Bn* bone and arrow in the panel C: nail

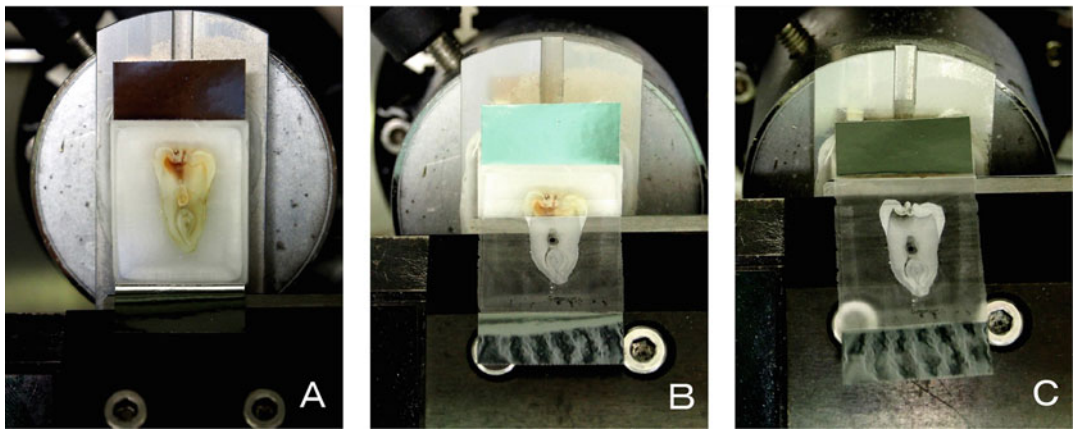

Fig. 15 The figure shows a 3 μm thick frozen section prepared from an undecalcified human molar tooth. Embedding: Embedding medium: SCEM, blade: SL-T30UF, adhesive film: Cryofilm type 4D(16UF), (**A**) the cut surface, (**B**) the section on cutting, and (**C**) the cut section

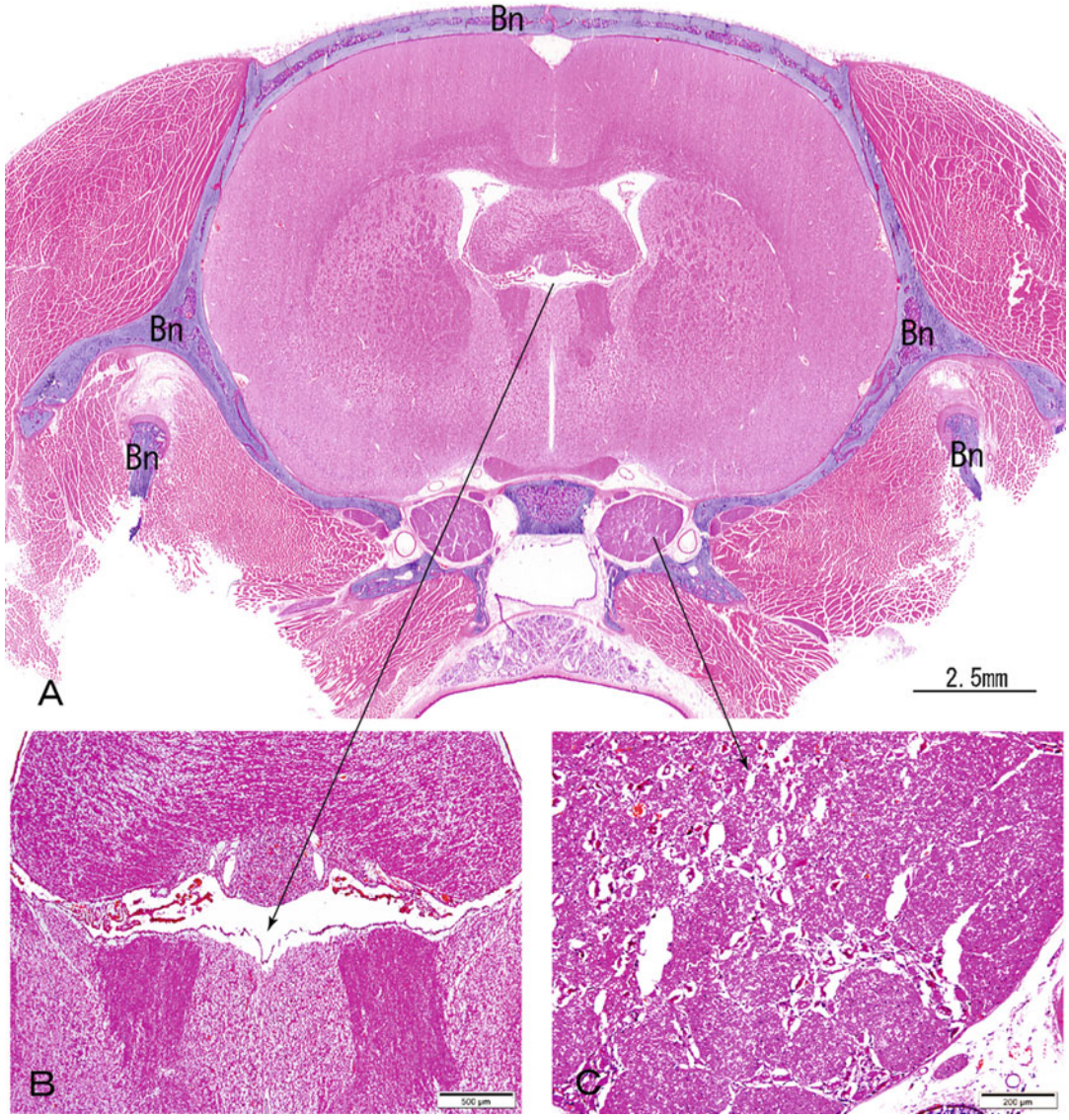

Fig. 16 The figure shows a 3 μm thick frozen section prepared from an undecalcified 10-week-old rat head. Staining: hematoxylin, embedding medium: SCEM-L1, mounting medium: SCMM-R2, blade: SL-T30UF, adhesive film: Cryofilm type 4D(16UF). *Bn* bone