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Preparation of Thin Frozen Sections from Nonfixed and Undecalcified Hard Tissues Using Kawamoto's Film Method (2012)

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Abstract

A method for preparing hard tissue sections by using an adhesive film is described. The method produces very thin (to one-micrometer thick) frozen sections from adult mouse and rat bone. The bone tissue is freeze-embedded with water-soluble medium and then cut with a disposable tungsten carbide blade after mounting the adhesive film onto the cut surface. The sections are stained on the adhesive film and preserved between the adhesive film and glass slide. All the steps including the embedding, cutting, staining, and mounting are completed within only 20 min. The soft and hard tissues are preserved satisfactorily and the bone marrow is also preserved perfectly. Cells such as osteoblasts, fibroblasts, and osteoclasts are clearly identified, and the osteoid layer of bone is clearly observed. The sections are applicable to many types of staining such as histology, histochemistry, enzyme histochemistry, immunohistochemistry, and insitu hybridization. The immunohistochemistry can be carried out with nonfixed and undecalcified sections. In addition to these applications, the sections are used for observing the PGF fluorescence. The sections are also usable for studying the distribution of water-soluble materials in the tissues. Furthermore, the sections are very useful for gene analysis using LMD technique and for imaging mass spectrometry.

Key words:
Mouse, Rat, Hard tissue, Bone,Frozen-section,Freeze-sectioning, Immunostaining,
In situ hybridization, LMD, Mass spectrometry

1 Introduction

It is very important to correlate biochemical results to tissue structures in biological research. Generally, the correlation is carried out using different types of staining such as histology, histochemistry, enzyme histochemistry, immunohistochemistry, and in situ hybridization. Hard tissue sections are often prepared from tissues, which are chemically fixed and decalcified, in order to allow for easy cutting. However, fixation and decalcification cause denaturation of proteins and loss of substances in tissues, thus resulting in reduced enzyme activity and immunoreactivity (1, 2). In order to solve this problem, it is best to prepare frozen sections from nonfixed and undecalcified tissues. How-

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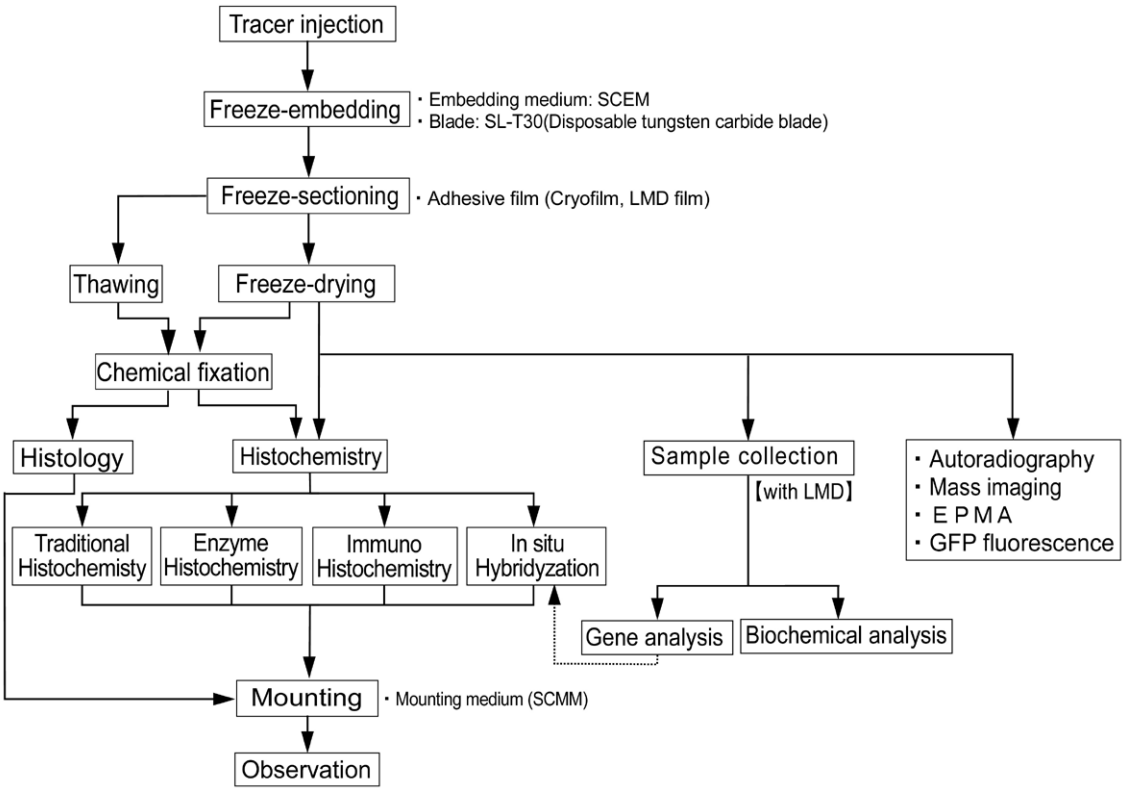

Fig. 1. The figure shows the procedures and materials used for Kawamoto's film method 2012. EPMA: electron probe micro-analysis, LMD: laser microdissection

ever, section preparation from completely mineralized bone is extremely difficult.

This method for the preparation of frozen sections from nonfixed and undecalcified hard tissues was originally developed to study calcium transport mechanisms during biological calcification(3-9). The fundamental procedures of this method were established in 2003(10).

In order to produce high-quality sections from hard tissues, the materials such as embedding medium, blades, adhesive films, and mounting medium have been continuously improved(11-13). The method using the latest materials can produce one micrometer thick sections from calcified bone (rat and mouse) and large specimens. The sections have great possibilities in biological research as shown in Fig. 1. Sections prepared from nonfixed and/or undecalcified tissues produce very useful data for enzyme histochemistry and immunohistochemistry (14-24). In addition to those applications, it is shown that the sections can be useful for studying the distribution of GFP labeled substances(25) and for imaging mass spectrometry(26-28).

Recently, the Laser Microdissection (LMD) technique has become a powerful tool in gene analysis (29-31) because it permits the collection of samples from a very small area within a tissue. The sample for

gene analysis is usually collected from sections prepared by a conventional freeze-sectioning method. Using traditional methods, the frozen section is thawed to fix it to the glass slide; however, the thawing causes an undesirable result in gene analysis. RNA is easily decomposed by enzymes such as ribonucleases. The mRNA from traditional frozen sections is often degraded by these enzymes during thawing of the section, and as a result the gene expression information is decreased or lost. It is well known that enzyme activities are effectively inhibited by removing the water surrounding the enzymes. The method presented here completely solves this problem since the frozen sections on the adhesive film can be dried (freeze-dried) without thawing. We have confirmed that the freeze-drying of the section significantly inhibits the decomposition of mRNA. Therefore this method becomes a very useful tool for gene analysis using the LMD technique.

This chapter describes the latest method (we are continuously improving the method, so we named the latest version as Kawamoto's film method 2012) and shows some images produced by this method.

2 Materials

The procedures are almost the same as the method described in the review (10) but all the important materials are improved to make high quality sections from hard tissues and large samples. The first material to be improved was the embedding medium. The medium has to allow smooth cutting of the frozen sample at very low temperatures such as -35℃ and to ensure the strong adhesion of the adhesive film to the cut surface. The second is the adhesive film, which is the most important material. The adhesive film has to maintain a strong adhesion at low temperature such as -35℃. Furthermore, the film has to permit many types of staining used in the histology, histochemistry and immunohistochemistry. As a matter of course, the section supported with the film has to be observed in detail. The third is the blade for cutting hard tissues. The blade has a very sharp edge and has to permit smooth cutting of the tissues, including hard tissue. The fourth material is the mounting medium. The mounting medium that provides the least tissue shrinkage is the best for this method because the shrinkage causes serious damage to the tissues.

The present sections (Fig. 8, 9, 10, 11, 12, 13, and 14), including hard tissues, were prepared using a kit (Multipurpose Cryosection Preparation kit, SECTION-LAB Co. Ltd., Japan). The kit includes most of the tools and materials needed for the procedure, which includes: embedding medium (SCEM), an adhesive film (Cryofilm, LMD film),

and mounting medium (SCMM). The blade is not included. In cutting frozen hard tissue, a cryomicrotome (CM3050, Leica Microsystems KK, Germany) and a disposable tungsten carbide blade (SL series, SECTION-LAB Co. Ltd.) are used. Given below is a list of these and other materials that may be needed depending on the specific experiment:

1. Hexane.
2. Dry ice.
3. Stainless steel container for freeze-embedding samples.
4. Embedding medium (SCEM).
5. Cryofilm fitting tool.
6. Adhesive films (Cryofilm and LMD film).
7. Tungsten carbide blades (SL-T30).
8. Cryomicrotome.
9. 100% ethanol.
10. 4% paraformaldehyde (PFA).
11. Stains (Hematoxylin, Eosin, Alizarin red S,Safranin-O).
12. Airtight box.
13. Acetone.
14. Antibody kits and reagents.
15. Mounting medium (SCMM).
16. UV light.
17. LMD microscope.
18. Fluorescent microscope or conforcal laser scanning microsopoe (CLSM)
19. Imaging plate
20. Electron probe micro-analyzer (EPMA)

3 Methods

3.1. Freeze Embedding

The adhesive film supports strongly the frozen sections when the fresh sample and the lightly fixed sample are used. However the adhesive film sometimes is not available for supporting the tissues when the tissue is fixed strongly with PFA, (for instance, muscle, tendon, and cartilage). As the results, serious damages appear on the tissues when the section is stained and/or mounted. Therefore, long fixation should be avoided in this method. The sample should be fixed for less than 1 day. During freeze embedding, the most important point is to avoid the artifact of ice crystal formation during the freezing of tissues. These artifacts are not so apparent when using the fixed samples, but is quite obvious in fresh samples. (see Notes 1 and 2).

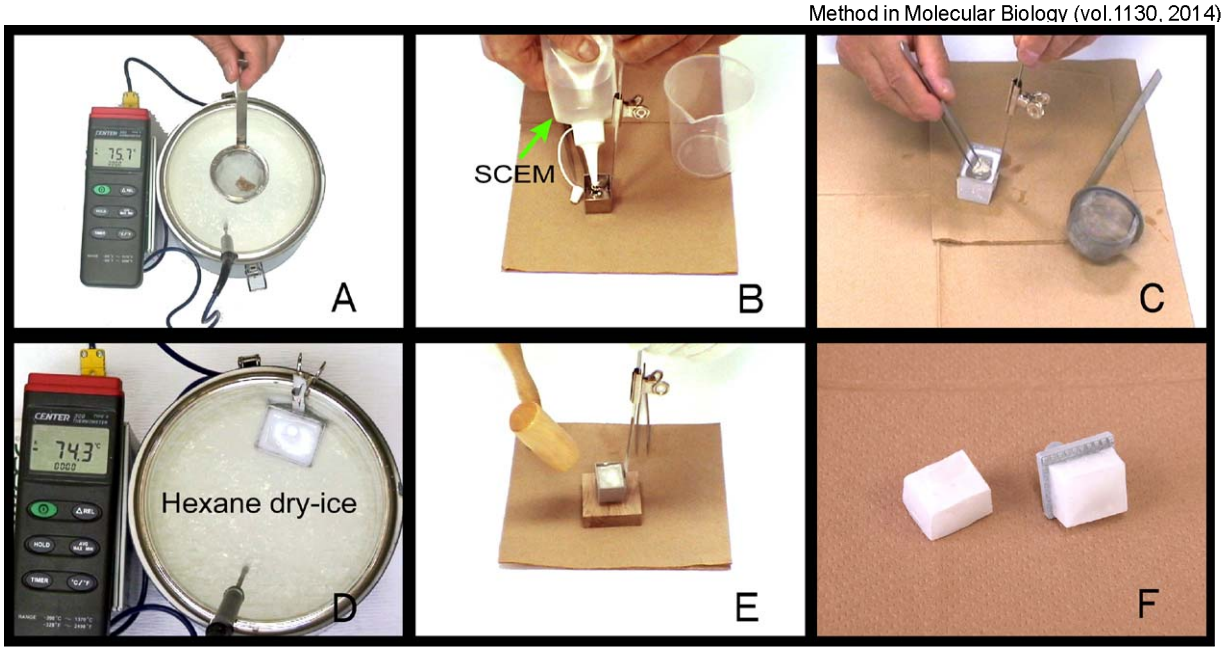

Fig. 2. The figure shows the procedures for preparing frozen sample blocks. A: freezing the sample, B: the mounting medium and stainless steel container, C and D: freeze-embedding in hexane dry-ice, E and F: fixing the frozen block to the microtome sample holder.

1. Dissect the tissues from the experimental animal.
2. Quickly freeze the sample in hexane dry-ice (Fig. 2A) (See Note 3).
3. Put the proper amount of cooled embedding medium (SCEM or SCEM-L1) in the stainless steal container (Fig. 2B).
4. Place the frozen sample in the embedding medium (Fig. 2C).
5. Quickly move the container into the coolant (hexane dry-ice, or cooled hexane) and freeze the embedding medium completely (Fig. 2D) (see Note 4).
6. Take the frozen block out of the container (Fig. 2E).
7. Fix the frozen block to the cryomicrotome sample holder (Fig. 2F).

3.2. Section preparation

Section preparation for conventional purposes (histology, histochemistry, enzyme histochemistry, immunohistochemistry, and in situ hybridization)

The temperature of the cryostat chamber is usually kept at -15 to -20℃. However, low temperature is suitable for producing good sections; the temperature of the cryostat chamber and the sample holder chuck should be adjusted to -20 to -30℃ and -25 to -35℃ respectively. We sometimes adjust the temperature of cryostat and the holder chuck to -25℃ and -35℃ respectively when cutting highly mineralized tissues. In order to cut hard tissues, a tungsten carbide blade has to be used. (see Notes 5-8).

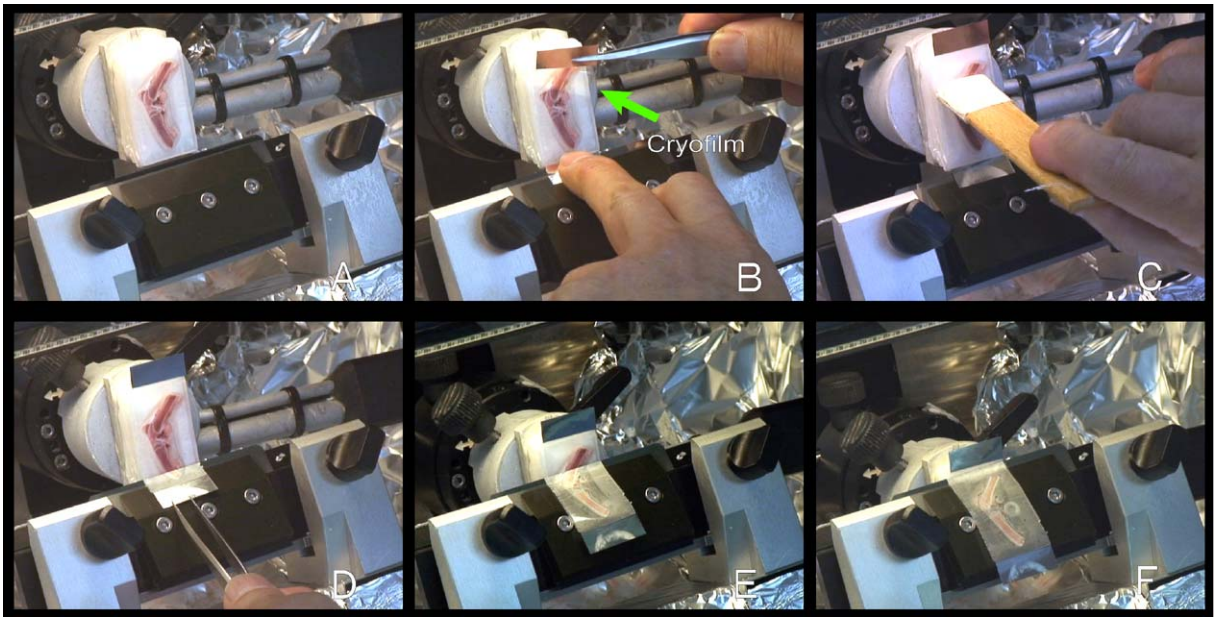

Fig. 3. The figure shows the procedures for preparing frozen sections. The sample is an adult rat hindlimb. The arrow in panel B indicates the Cryofilm. A: the cut surface, B and C: applying the Cryofilm to the cut surface, D, E and F: cutting the frozen sample. The thickness: 2μm.

1. Leave the frozen sample block in the cryochamber to acclimatize for approximately 10 min after fixing it to the sample holder chuck. The temperature of the cryostat is adjusted to -20 to -30℃. The temperature of sample holder chuck is adjusted to -25 to -35℃ (see Note 5).
2. Trim the block with a disposable tungsten carbide blade (SL-T25 or -T30) until the area of interest appears on the cut surface (Fig. 3A). After trimming, move the blade and adjust the sharp blade edge to the cut position. If the sharpness of the blade is lost, use a blade with a large cutting angle such as the SL-T35 (see Notes 6 and 7).
3. Mount the adhesive film (Cryofilm) onto the cut surface (Fig. 3B). For gene analysis, use the LMD film instead of the Cryofilm (see Note 8).
4. Tightly adhere the Cryofilm to the surface with a fitting tool (Fig. 3C).
5. Cut the specimen slowly at a constant speed (Fig. 3D,3E).
6. Take the section out of the cryochamber and thaw it.
7. Leave it for 10-60 seconds in order to dry lightly the section. (The optimum drying time depends on tissues.)
8. Turn the 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℃ (See Note 9).

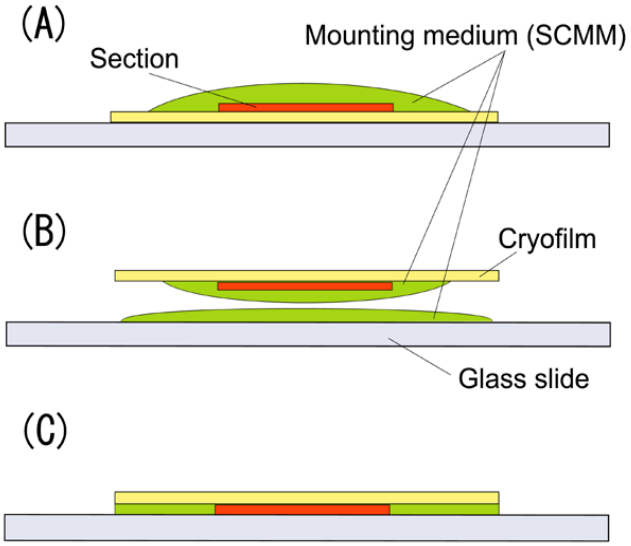

Fig. 4. The figure shows the procedures for mounting the sections. A: applying the mounting medium on the section, B: changing the section side upside down, C: closely contacting the section to the glass slide. The section is preserved between the Cryofilm and the glass slide.

3.3 Histological and Histochemical Staining

In this method, the section is preserved between the adhesive film (Cryofilm) and the glass slide (Fig. 4). The water- soluble mounting medium is used instead of a conventional mounting medium as xylene (used in a conventional method), which causes serious shrinkage and cracking of the tissues. (see Note 10).

1. Stain the section with Hematoxylin for approximately 1 min (Fig. 5A).
2. Carefully wash the section in running water for approximately 4 minutes.
3. Stain the section with water soluble eosin for approximately10 seconds (Fig. 5B).
4. Rinse the section with water for a few seconds.
5. Then rinse it with 100% ethanol for 10 seconds.
6. Drop the mounting medium (SCMM) on the section (Fig. 11.4a and Fig. 5C).
7. Turn the section side of the Cryofilm down (Fig. 4B and Fig. 5D).
8. Remove the excess SCMM from the glass slide with filter paper (Fig. 4C and 5E).
9. Place the glass slide to polymerize the SCMM under the UV light when the SCMM-R2 is used (Fig. 11.5f).
10. After polymerization, wash the glass slide and Cryofilm to remove the un-polymerized mounting medium on the Cryofilm and glass slide.

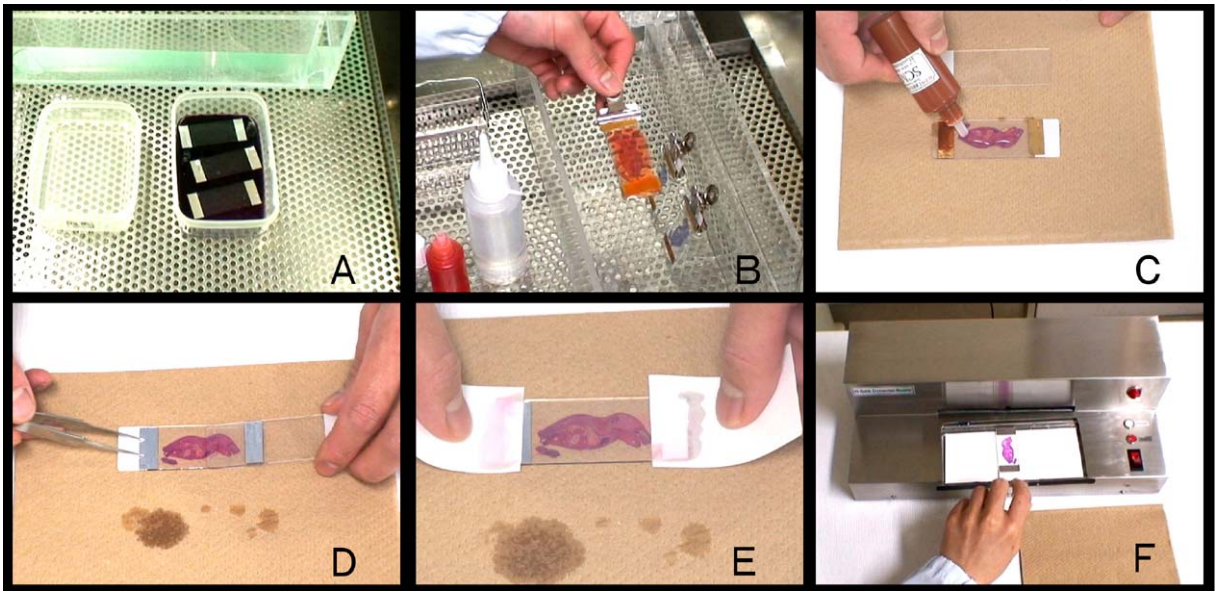

Fig. 5. The figure shows the procedures for staining and mounting the sections. A: Hematoxylin staining, B: Eosin staining, C: applying the mounting medium (SCMM-R2), D: mounting the section to the glass slide, E: removing the excess mounting medium, F: polymerizing the mounting medium.

3.4 Enzyme Histochemistry and Immunmohistochemistry

A few air bubbles are sometimes trapped in the section. To remove the bubbles, see the Note 11.

1. Place the section (see Subheading 3.2) on the glass slide with the section facing up.
2. Drop the staining solution on the section according to standard IHC protocols.
3. After staining, preserve the section between the Cryofilm and glass slide according to the above procedures (see Subheading 3.3).

3.5 Enzyme Histochemistry and Immunmohistochemistry Using Nonfixed and Undecalcified Sections

For the enzyme histochemical staining and immunohistochemical staining with nonfixed and undecalcified section, freeze-dried sections are used.

1. Prepare the sections from a fresh frozen sample using the Cryofilm according to the above procedures (see Subheading 3.2).
2. Freeze-dry completely the section in the cryochamber kept at lower than -20℃. (Frozen sections of 10mm thickness will be freeze-dried for approximately 4 h.)
3. Place the sections in an airtight box to avoid condensation on the section.
4. Take the box out of the cryochamber.
5. Stain the section according to the procedures described above (see Subheading 3.4) after immersing in 100% ethanol or acetone.

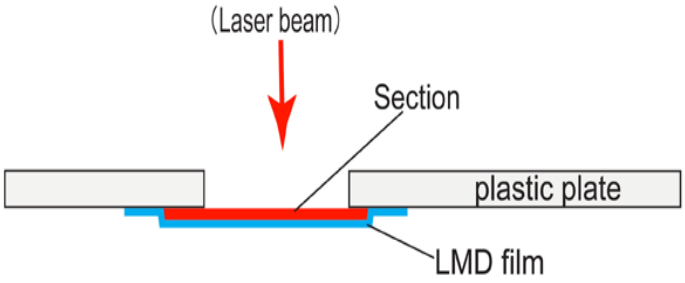

Fig. 6. The figure shows a schematic diagram of sample collection with the LMD.

3.6 Sample Sample Collection for Gene Analysis Using LMD Technique

The freeze-dried sections are also used for this purpose.

1. Prepare the sections with the LMD film and freeze-dry according to the procedures described above (see Subheading 3.5).
2. Take it out of the cryochamber.
3. Fix the section on the plastic slide using the stickiness of the LMD film. A hole is made in the plastic slide (Fig. 6).
4. Place the slide on the sample stage of LMD (Leica LMD 7400, Leica microsystems KK, Germany) and then cut the region of interest with a laser beam. The cut sample falls into the sample collecting tube under the slide after cutting (Fig. 7D, E, F).
5. Analyze the collected sample by conventional protocol for gene analysis.

3.7 Observation of Fluorescence (Calcein, Alizarin red, GFP, etc)

For studying the distribution of fluorescent tracers such as calcein and alizarin red the sections are preserved between the glass slide and the Cryofilm without any staining. For GFP fluorescence the sections are prepared from chemically fixed sample.

1. Prepare the sections from the frozen sample with the Cryofilm type 2C(9) 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 Studying the Distribution of Water- Soluble Substances (Autoradiography, Water-Soluble subtances, Water-soluble Tracers, etc.)

For this purpose, the sections are prepared from fresh samples and the sections are freeze-dried. See refs. (4,9,10,32,33).

1. Prepare the frozen sections with the Cryofilm type 2C(9) according to the above procedures (see Subheading 3.2).
2. Freeze-dry the section in the cryochamber and then take it out of

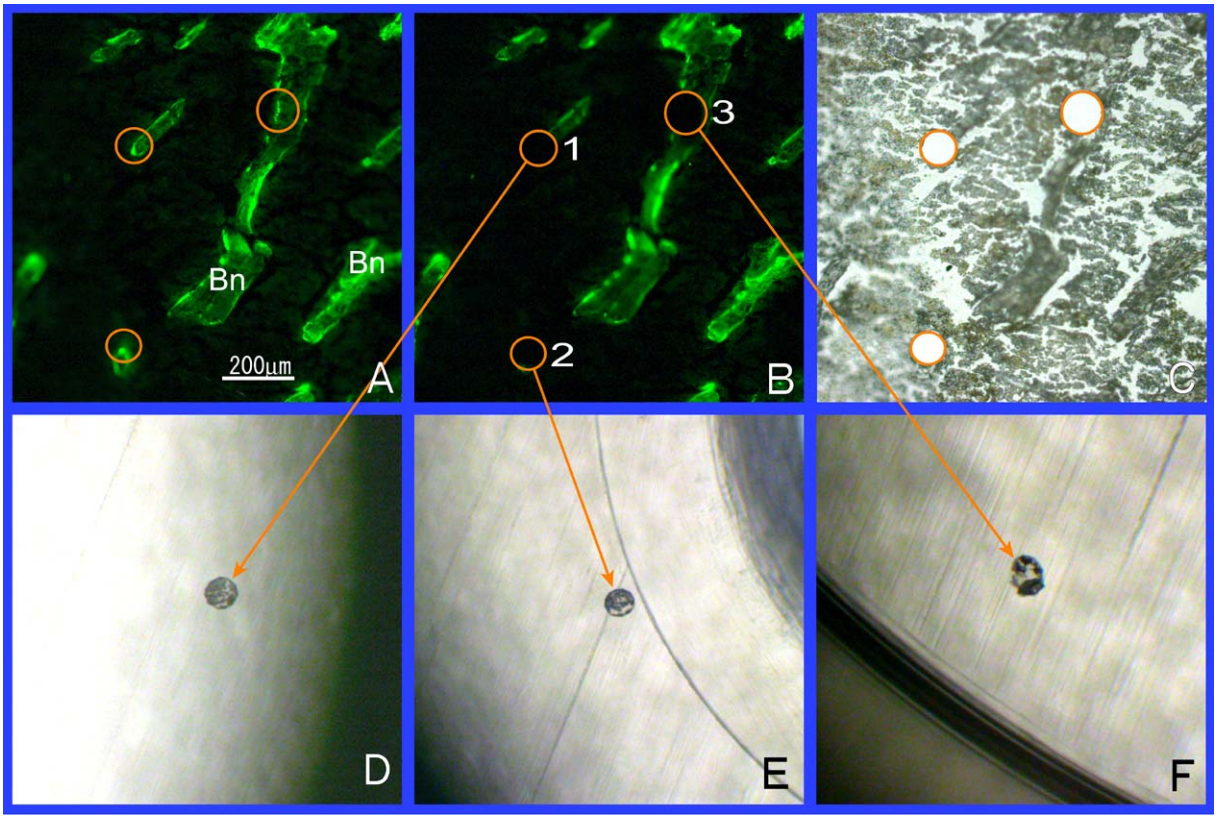

Fig. 7. The figure shows samples collected using LMD. The section is prepared with LMD film. The bone surface is labeled with calcein. The samples for gene analysis (indicated with the circles 1, 2, and 3 in the panel B) are successfully collected in the sample collecting tubes. A: before cutting, B and C: after cutting, A and B: calcein fluorescence images, C: bright-field images, D, E and F: the cut samples. Bn: bone.

the cryochamber according to the above procedures (see Subheading 3.3).

3. For autoradiography, contact the section to the imaging plate.
4. For elemental analysis, fix the section on the sample holder of an EPMA and analyze the section with EPMA.
5. For observing fluorescence tracers, preserve the section between the glass slide and Cryofilm without any mounting medium, and then observe the section with a fluorescent microscope or CLSM.

Results: Figs. 8, 9, 10, 11, 12, 13, and 14 show representative sections and applications of the Kawamoto technique for generating thin frozen sections from nonfixed and undecalcified hard tissues.

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. We have not been able to produce good sections with conventional mounting medium. OCT is most widely used for freeze-embedding tissues. However, this mounting medium is not suitable for cutting at low temperature (for instance a temperature lower than -30℃) and the Cryofilm does not stick strongly to the cut surface. The SCEM is a different type of mounting medium than the conventional mounting medium, and it has been optimized to this method. The SCEM allows smooth cutting of the frozen sample at -35℃ and the Cryofilm tightly adheres to the frozen cut surface.

3. Pentane and isopentane cooled with liquid nitrogen are also useful for freezing the tissue sample.

4. Don't use liquid nitrogen to freeze the embedding media within the mold. Use of liquid nitrogen to freeze the embedding media will result in cracks within the frozen block.

5. The blade is a very important item in preparing good sections. The SL-T series blades were specially designed for this method with a fine tungsten carbide. The blade of low angle (SL-T20) produces excellent sections but the sharpness is easily lost when cutting hard tissues such as bone. Therefore the blade suitable for the tissue must be used, for example SL-T20 or T25 for soft tissues, SL-T25 or T30 for bone and cartilage, SL-T30 or SL-T35 for normally calcified bone, SL-T35 or T40 for highly mineralized bone and tooth. The cutting angle of each blade is as follows;
   SL-T20: 20 degree
   SL-T25: 25 degree
   SL-T30: 30 degree
   SL-T35: 35 degree
   SL-T40: 40 degree

6. The disposable blade is fastened to the holder for the SL series of disposable blades 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.

7. Some types of adhesive films are used to support frozen sections. The Cryofilm type 2C(9) is typically used to support frozen sectioning as the film can be immersed in acetone and xylene. This film is compatible with many types of staining such as histology, histochemistry, enzyme histochemistry, and in situ hybridization. The Cryofilm type 2C(10) is compatible with studying the tissues

with a polarized optical microscope, although Cryofilm type 2C(9) is not since it has polarity. The LMD film is used for the LMD technique. The LMD film thickness is thinner than that of Cryofilm and allows for easy cutting using a laser beam.

8. The temperatures of the cryochamber and sample holder chuck are important for generating quality bone sections, especially important is the temperature of the chuck holder. High-quality sections are made by cutting at low temperatures, such as -30℃ or lower. We occasionally adjust the temperature to -35℃ when cutting bone tissues.

9. The frozen sample blocks are preserved in a deep freezer (approx. -80℃) 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.

10. The mounting medium is also important feature in order to generate beautiful histology. 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. So, the mounting mediums suitable for stains are as follows:
    a) SCMM-G1 (drying type): Hematoxylin and eosin staining
    b) SCMM-R1 (drying type): Enzyme histochemistry, immunohistochemistry (ABC method).
    c) SCMM-R2 (polymerizing type): Hematoxylin and eosin staining, Frozen Immunohistochemistry (depends on the antibody), et al..
    d) SCMM-R3 (polymerizing type): Toluidine blue staining

11. Air bubbles that may develop in the sections can be removed by the following procedure.
    a) Place the section in 100% ethanol after fixing it with 4%PFA.
    b) Move it to a vacuum chamber and evacuate the chamber.
    c) Keep it in the vacuum chamber for approximately 1 min.
    d) Move the section into water.
    e) Stain the section.

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I would like to thank Dr Chikara Ushiku (The Jikei University School of Medicine) for providing the beautiful picture of PGF fluorescence (Figure 11).

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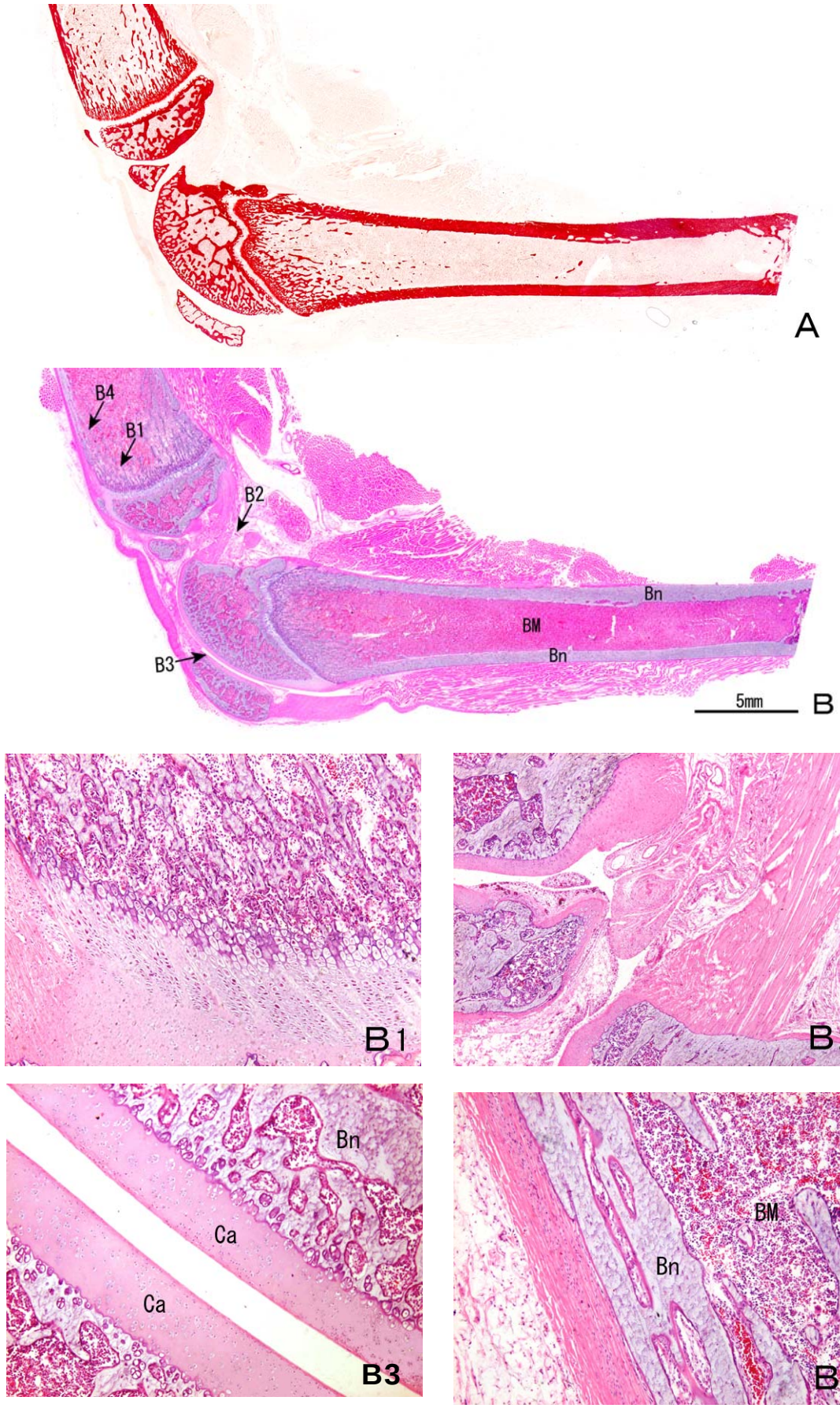

Fig. 8. The figure shows 3μm thick serial sections prepared from an undecalcified hindlimb of an adult rat. A: alizarin red S staining, B, B1, B2, B3, B4: Hematoxylin and eosin staining. B1, B2, B3, and B4 panels are indicated by the B1, B2, B3, and B4 arrows in panel B. Bn: bone, BM: bone marrow, Ca: cartilage.

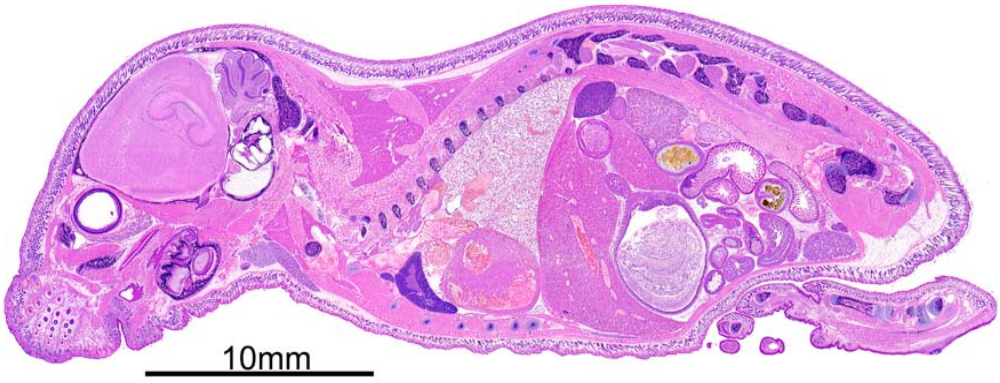

Fig. 9 The figure shows a 4μm thick section prepared from a fresh 9-day old mouse. Hematoxylin and eosin staining.

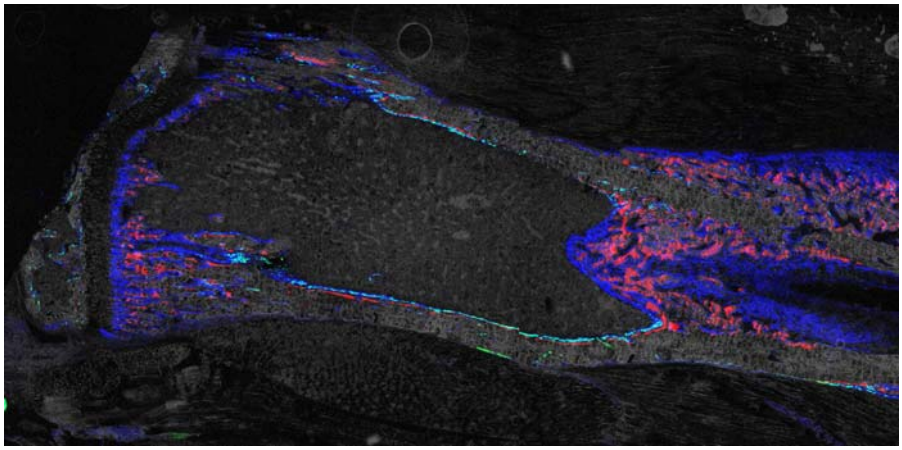

Fig. 10. The figure shows the GFP fluorescence (Col3[2].6: Cyan, xSMAA: cherry) on the section prepared from an adult mouse tibia. (Presented by Dr. Chikara Ushiku, see reference 25.)

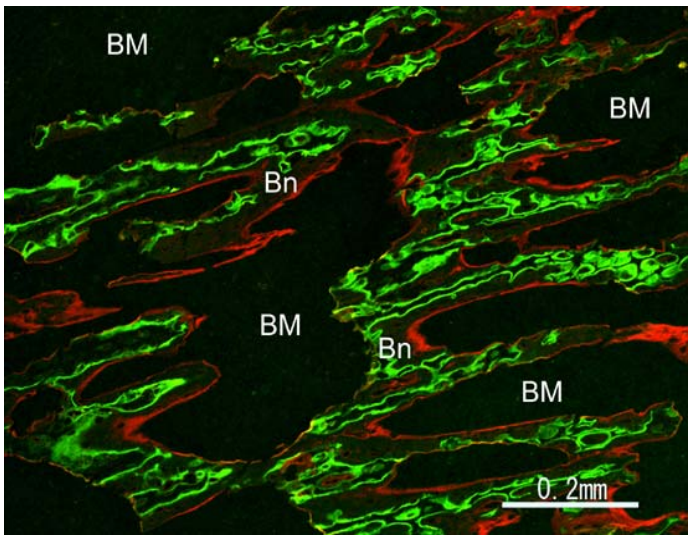

Fig. 11. The figure shows fluorescence of calcein (green color) and alizarin red S (red color) in a 2μm thick section prepared from a fresh adult rat hindlimb. Bn: bone, BM: bone marrow

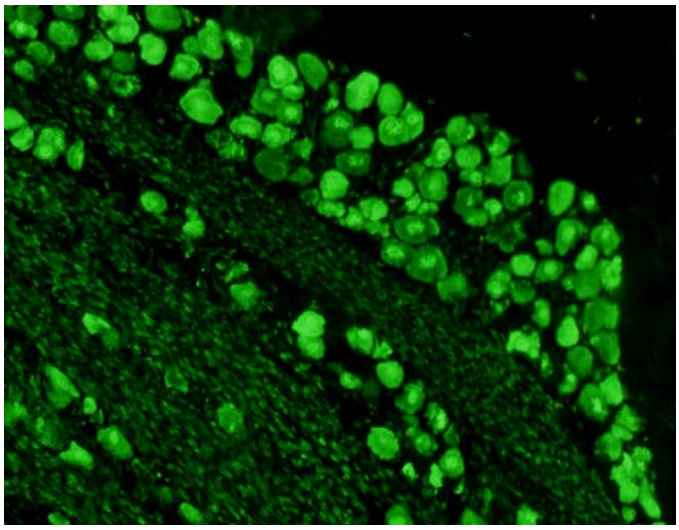

Fig. 12. The figure shows Immunohistochemical localization (FIHC) of PGP9.5 in a section prepared from a 9-day-old mouse.

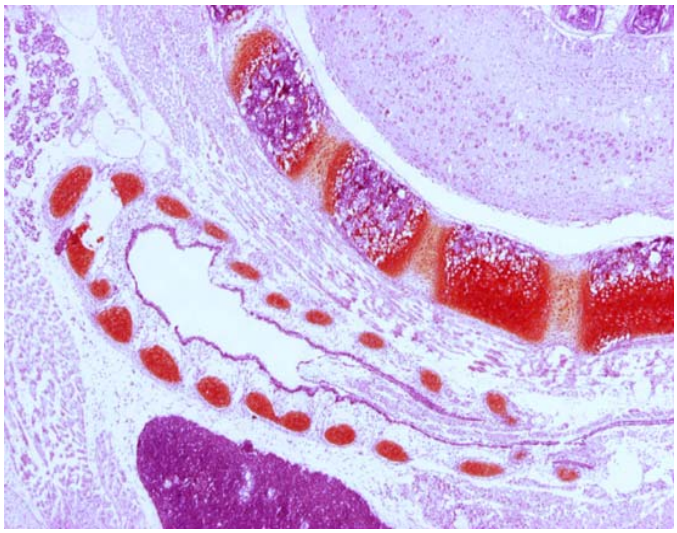

Fig. 13. The figure shows Safranin-O staining (Red), 8-day-old mouse 5µm thick section. Counterstaining: Hematoxylin.

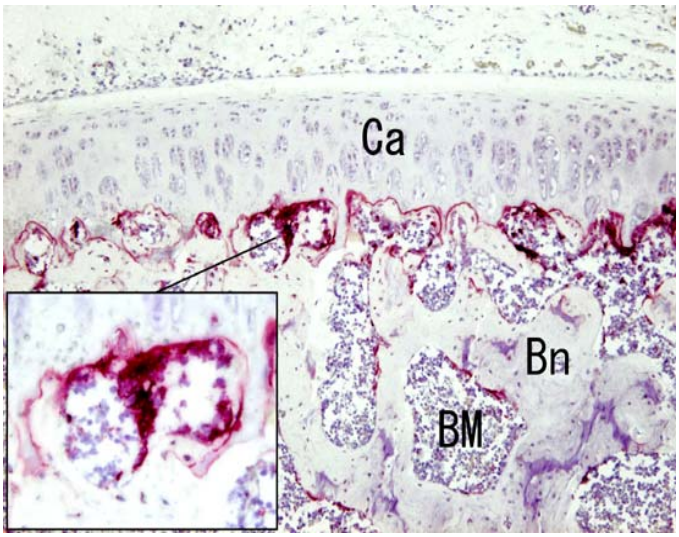

Fig. 14. The figure shows the distriution of TRAPase activity (red color) in a 3µm thick section prepared from an undecalcified adult rat hindlimb. Counterstaining: Hematoxylin, Ca: cartilage, Bn: bone, BM: bone marrow