Review

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Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants

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Summary. A method for preparing thin fresh-frozen sections from large samples and hard tissues is described and the applications are shown. A new adhesive film is introduced to produce the frozen sections. The sample is frozen in a cooled hexane or liquid nitrogen, and then freeze-embedded with 4-5% carboxymethyl cellulose (CMC) in the coolant. A specially prepared adhesive film is fastened to the cut surface of the sample in order to support the section and cut slowly with a disposable tungsten carbide blade. The adhesive film is made of a thin plastic film and an adhesive before use. This method produces 2-μm thick fresh-frozen sections from a large sample, bone or tooth. The "film-section" i. e. the section attached to the adhesive film, can be used for many types of studies such as histology, general histochemistry, enzyme histochemistry, immunohistochemistry, *in situ* hybridization, elemental analysis, and autoradiography for water-soluble materials. Immunohistochemistry and *in situ* hybridization can be carried out with nonfixed and undecalcified sections. The section on the adhesive film can be transferred to a glass slide and mounted under a cover slip, and stained sections can be examined with an optical microscope at high magnification. This method is also useful for preparing frozen sections from samples of fish, insects, and plants. Furthermore, samples of particular areas can be collected from the film-section by means of a laser microdissection technique.

The multiple possible applications of the adhesive film render it highly useful for studies in biological and medico-dental fields.

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Introduction

In the biological sciences, enzyme histochemistry, immunohistochemistry, *in situ* hybridization, and autoradiography have been widely used to correlate biochemical results and histology. The reliability of these correlations depends on the quality of the section. Therefore, in addition to preserving the fine structure of the tissues, the enzyme activity, antigenicity, and tissue components (water-soluble materials) must also be maintained. Generally, tissues are treated with a fixative to preserve their structure. Hard tissues are decalcified to permit easy sectioning. Fixation and decalcification usually cause denaturation of proteins, thus resulting in a reduction in enzyme activity and immunoreactivity (Baker *et al.*, 1958; Nuki, 1967; Bosman *et al.*, 1977; Matthews, 1982; Mepham, 1982; Matthews and Mason, 1984; Mullink *et al.*, 1985; Fukase, 1997). There is also an accompanying loss and/or dislocation of water-soluble materials from the tissues. For these reasons, these procedures should be avoided whenever possible.

A type of freeze-sectioning technique can solve many of these problems (Van Noorden *et al.*, 1986; Shin *et al.*, 1993; Bratthauer, 1999). The conventional freeze-sectioning technique is remarkably successful in small soft tissues; however, there are many sample types for which the technique does not work well. These include large samples, complex samples containing different organs, and hard tissues. The biggest drawback to the application of the freeze-sectioning technique to such samples is that the tissue structure is extensively damaged during cutting. Many researchers have made great efforts to obtain sections from

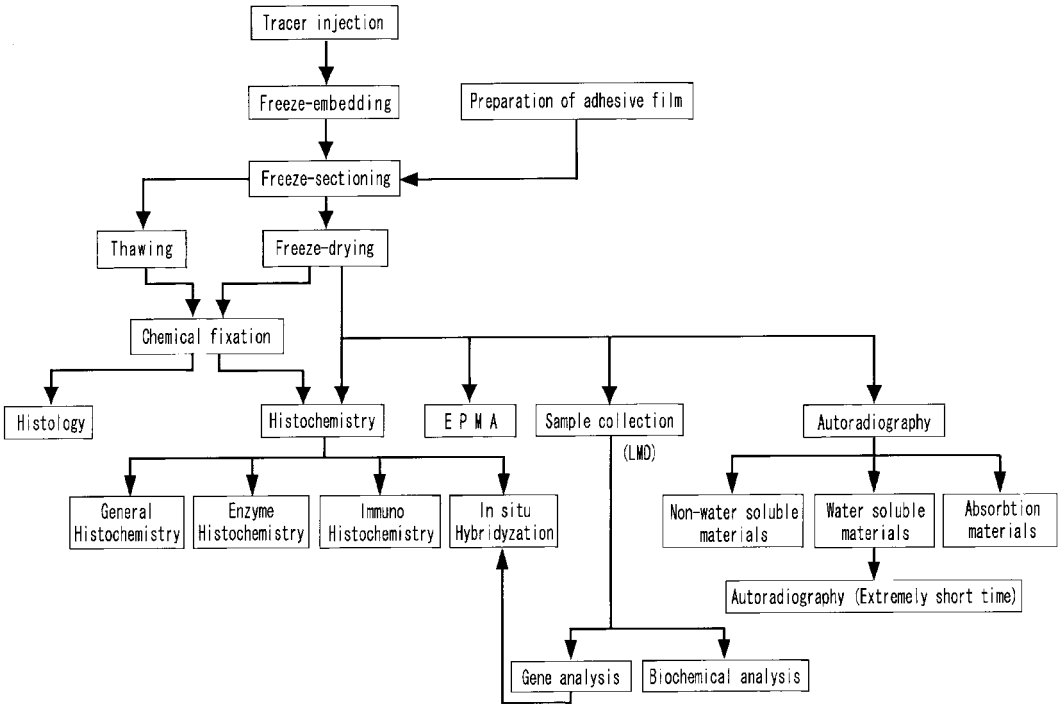

Fig. 1. Applications of the film-section. LMD: Laser microdissection, EPMA: electron probe microanalysis.

such samples without structural dislocation and histological disturbance. In all cases, materials for supporting the frozen section are used, and are summarized below:

1) Coating the cutting surface with a polymer before each sectioning process.
2) Supporting the section with a sheet of wet paper.
3) Supporting the section with a pressure-sensitive adhesive tape.

The first method produces thin frozen sections from a small bone sample (Fink, 1986, 1992; Aaron and Carter, 1987). However, the procedure is difficult and the results are often poor. The method cannot be used for studying water-soluble materials because the surface layer of the frozen sample is thawed momentarily when it is coated with the polymer.

The second method produces large sections but is not suitable for thin sections (Watanabe *et al.*, 1978; Kihara, 1984; Gillberg *et al.*, 1985; Aaron and Carter, 1987; Hill and Elde, 1990; McElroy *et al.*, 1993; Shimada and Watanabe, 1995). This method also cannot be used for water-soluble materials.

The third method, which was introduced for a whole-body autoradiography by Ullberg (1954), produces sections from large samples and highly mineralized tissues without previous fixation and demineralization. Many researchers have thus attempted to apply these sections to histological and histochemical studies (Parmgren, 1954; Farebrother and Woods, 1973; Fukuda and Shindo, 1974; Hasselgren and Hammarstrom, 1975; Watanabe *et al.*, 1975; Deak *et al.*, 1976; Rijntjes *et al.*, 1979; Ullberg and Larsson, 1981; Larsson and Ullberg, 1981; Sjogren *et al.*, 1981; Van Noorden *et al.*, 1986; Hammerstrom, 1986). Much effort has gone into these attempts, but the results have not been satisfactory. The problem is that alcohol causes the adhesive and tape to separate, making it very difficult to mount the section attached to the adhesive film.

For these reasons, these attempts have not been able to produce high quality sections which can be used for many types of biological study, from troublesome specimens such as whole animals, hard tissues (bone and tooth) and plant specimens (leaves, pollen, grains).

In order to overcome this, Kawamoto (1990) introduced a new adhesive film to replace the commercially available pressure sensitive adhesive tape. He also modified the

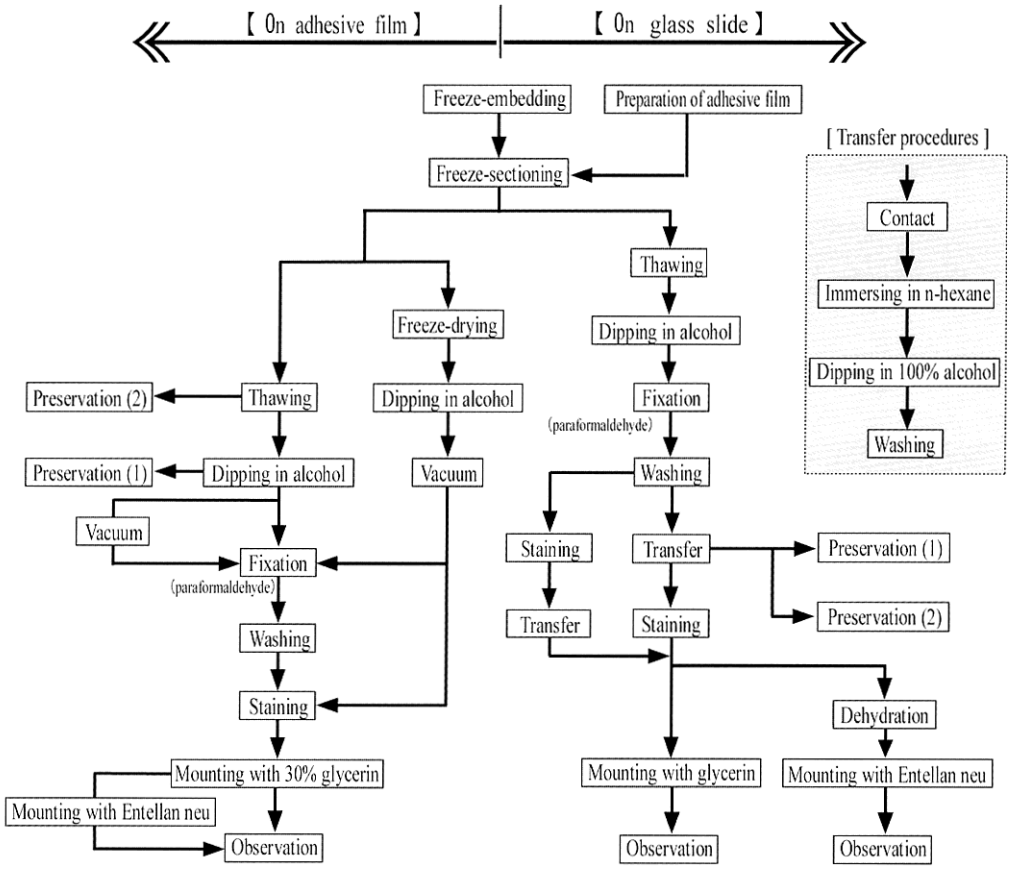

Fig. 2. Procedural flow chart. The flow chart in the rectangular area shows the transfer procedures of the section from the adhesive film to the glass slide. Preservation (1): in 100% ethanol, Preservation (2): in a deep freezer (−80℃).

standard method described by Ullberg (1954) to mount the sections. The sections produced with this new adhesive film are available for many types of biological study. To improve the quality of the sections, Kawamoto and Shimizu (2000) modified the cryomicrotome, the cryochamber, and the blade holder. They have successfully made 2-μm thick frozen sections from a 10-day-old rat whole-body, and a 7-month-old rat femur and lower incisor. The sections attached to the adhesive film can be used for conventional staining and are easily transferred to the glass slide. Possible applications include enzyme histochemistry, immunohistochemistry, *in situ* hybridization, and autoradiography for water-soluble materials as shown in Figure 1. In addition, the present freeze-dried sections can be applied to the laser micro-dissection technique. The collected samples are used for gene analysis and for measuring the radioactivity over a very small area.

This film-sectioning method has been employed for conventional cryomicrotomy as well as for heavy-duty cryomicrotomy for whole body autoradiography. In this article, the basic procedures for both types of the cryomicrotomy are described and some applications are shown.

Preparation of fresh frozen sections

The procedures for preparing, staining and mounting sections are shown in Figure 2.

1. Preparation of adhesive film

In order to prepare thin fresh frozen sections of high quality, a powerful and thin adhesive film for supporting frozen-

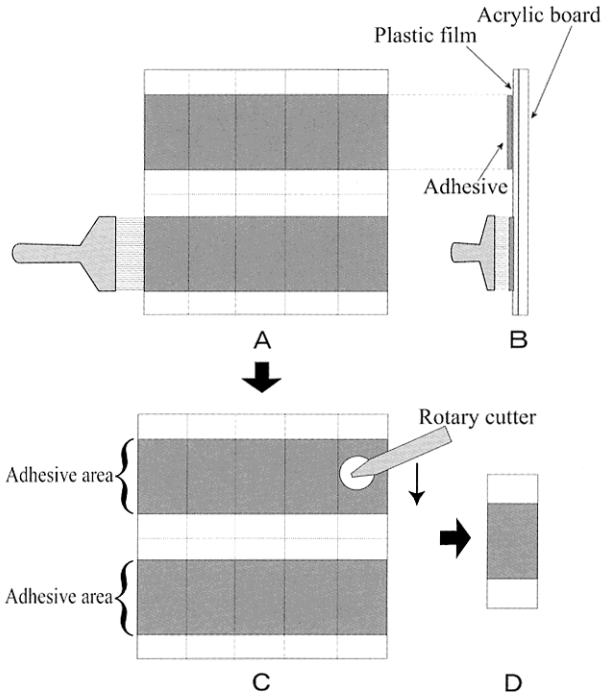

Fig. 3. Preparation of the adhesive film. **A:** Application of the adhesive to the plastic film, **B:** The cross section of Figure A, **C:** Cutting of the dried adhesive film, **D:** A sheet of the adhesive film.

sections is indispensable. Therefore, the most important aspect of this method is the production of an adhesive film using synthetic adhesive and plastic film.

The plastic film must be colorless, transparent and no-fluorescence. Furthermore it must prevent any specific staining. The thickness of the film is important in preparing thin sections. Very thin 4-μm film tightly adheres to the cut surface of the frozen sample but it is difficult to apply the film to the surface without making wrinkles. A thick 50-μm film is easier to work with but is prone to problems following mounting. A thickness of approximately 10-μm appears to be ideal.

The adhesive material also must be colorless and transparent. It must have a strong adhesion at low temperatures (−25℃). Two types of adhesive (Cryoglue Type 1 and Type 2, FINETEC Co. Ltd, Japan) were used for this study: both can be used at −25℃. The adhesion of Type 1 is stronger than that of Type 2. Type 1 can be used for immune reactions and *in situ* hybridization and is economical, and therefore, is recommended for this method. The only drawback is that the adhesive is stained with the toluidine blue. The adhesive film is prepared using special tools (Cryofilm transfer kit, FINETEC Co. Ltd.) following the procedures below.

1) Placing a plastic film (polyvinilidene chloride, 10-μm thick, Asahikasei Kogyo Co., Tokyo) on an acrylic board covered with 100% ethanol.
2) Applying a synthetic adhesive (Cryoglue Type 1 or Type 2) to the film with a brush (Fig. 3A, B).
3) Drying the adhesive for approximately 5 min.
4) Cutting the film to the size of the frozen block surface with a rotary cutter (Fig. 3C).

The most important point in preparing the film is that the adhesive area be exactly the same dimensions as the frozen block surface (Fig. 3D, 6A). The non-adhesive areas are used for handling the film-section.

2. Preparation of frozen CMC block

Ice crystal formation (freezing artifact) during freezing sample damages the tissues. This problem is especially serious in large samples. It is known that freezing with a coolant having a high cooling rate reduces the artifact (Schwabe and Terracio, 1980). The coolants for the freezing tissue sample are listed in Table 1; they are cooled with liquid nitrogen when used. Among them, cooled propane provides the best histology; however, it often causes cracks in the sample, especially in large samples. The cooled hexane rarely causes cracks and produces a relatively good frozen sample. There are no problems in the identification of each cell in most tissues although serious freezing artifacts appear in muscle and red blood cells.

To further reduce the freezing artifact, chemical fixation and cryoprotectants are available (Skaer, 1982; Rosene *et al.*, 1986). Rosene *et al.* (1986) introduced a cryoprotectant method that facilitates cutting frozen sections of brains for

Table 1. Coolant for freezing tissues.

	Boiling point	Melting point
n-Hexane	64℃	−94℃
Pentane	36℃	−130℃
Isopentane	28℃	−160℃
Propane	−45℃	−190℃

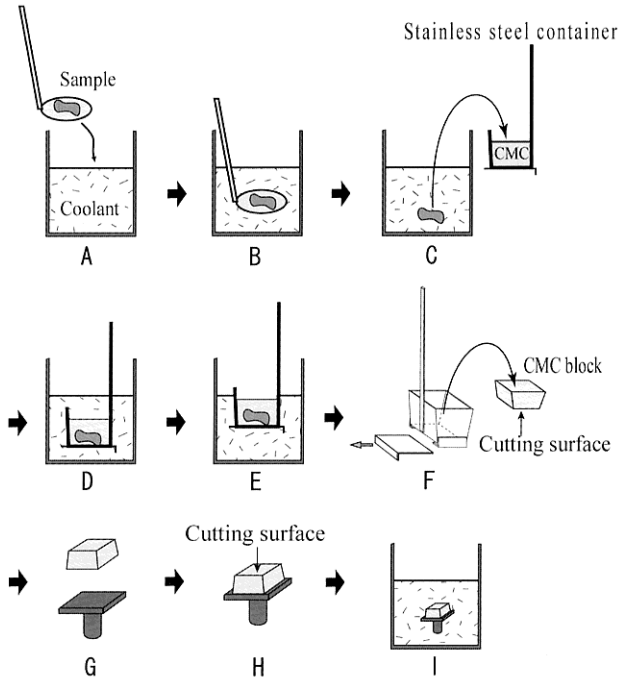

Fig. 4. Freeze-embedding of the sample. **A** to **B:** Freezing of the sample, **C** to **E:** Freeze-embedding of the frozen sample with CMC gel, and **F** to **I:** Fixing of the embedded sample to the sample holder.

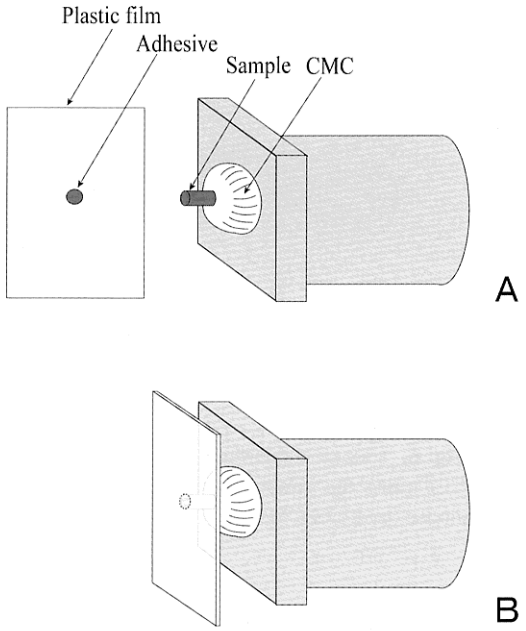

Fig. 5. Fixing and sectioning for a small sample. **A:** Adhesive film and a frozen sample fixed to the cryomicrotome stage, **B:** The sample fastened with the adhesive film.

histological and histochemical processing without freezing artifacts. They recommend mild fixation since stronger fixatives increase the freezing artifact. According to their data, the best method for eliminating freezing artifacts is graded infiltration with up to 20% glycerol and 2% DMSO (in buffer or fixative) followed by rapid freezing.

OCT compound is most often used for freeze sectioning as an embedding medium. However, in this method, CMC gel is recommended because it has superior adhesion with the film. In addition, the OCT compound provides insufficient support for fully calcified tissues. The embedding procedures using the CMC gel are as follows:

1) Rapidly freezing the sample in the coolant in order to minimize the freezing artifact (Fig. 4A, B).
2) Placing the frozen sample in the stainless steel container filled with the proper quantity of 4–5% carboxymethyl cellulose (CMC) gel (the container, which fits the size of the sample, is selected to avoid thawing of the frozen sample) (Fig. 4C).
3) Completely freezing the CMC gel in the cooled hexane (Fig. 4E), after keeping the container it in the hexane for 10–20 sec (Fig. 4D).
4) Removing the frozen CMC block from the container (Fig. 4F).
5) Fixing the frozen CMC block to the sample stage with the CMC gel and then attaching it to the cryomicrotome (Fig. 4G, H, I).
6) Storing the frozen CMC block in a deep freezer (−80℃) if the sample is not cut soon. (The frozen samples can be stored in a freezer for more than one year.)

Steps 2 and 3 are carried out quickly to avoid thawing of the frozen sample.

For small samples, careful attention must be given to the fact that the frozen sample is instantly thawed as it is embedded in the CMC gel. In order to avoid this, the frozen sample is directly fixed to the sample stage with the CMC gel as shown in Figure 5A. Then the cut section is supported with the adhesive film, which is sticky on the same size as the cutting surface (Fig. 5B).

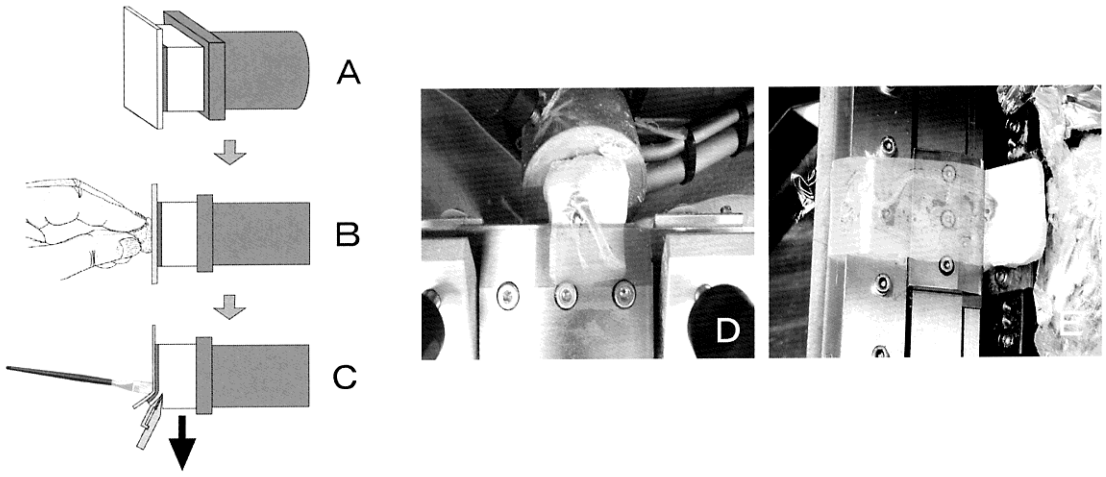

Fig. 6. Freeze-sectioning. **A:** Applying the adhesive film to the cutting surface, **B:** Adhering the film to the surface, **C:** Cutting the sample, **D:** Cutting of a 7-month-old rat femur (by Leica CM3050S), **E:** Cutting of the whole pregnant mouse (by Leica CM3500).

3. Freeze-sectioning

A sharp knife-edge is required for high quality sections, and a disposable blade is most suitable. A disposable blade made of stainless steel was successfully used for soft tissues, but not for hard tissues. A knife made of tungsten carbide allows the cutting of hard as well as soft tissues. Recently, a disposable blade (TC-65, Leica Microsystems Co. Ltd., Germany) made of tungsten carbide has been widely used for cutting hard tissue samples embedded in resin. This blade could be also used successfully in the present study.

The temperature in the cryostat chamber is also an important factor in obtaining high quality sections. Cutting at a relatively high temperature (−15℃) is easy as the frozen sample is not hard, but the temperature causes the growth of the ice crystals in a frozen sample and the ice crystals damage the tissue. On the other hand, sections cut at lower temperatures show a beautiful histology, but cutting is difficult because the frozen sample hardens. Most of the samples can be cut without any difficulty at approximately −20℃ and the histology is excellent. However, in some tissues, it is better to change the temperature. For instance, many cracks may appear in a brain section when it is cut at −20℃, but this problem is solved at low temperatures (−27℃). For hard tissue samples, a temperature range of −23℃ to −25℃ is suitable. The frozen sections are cut using the following procedure.

1) Trimming the CMC block after placing it in the cryochamber for approximately 30 min (the setting time depends on the size of the block).
2) Placing the adhesive film on the exposed cutting surface and pressing it tightly with a soft cotton wad or paper (Fig. 6A, B).
3) Slowly cutting the CMC block at a constant speed (Fig. 6C, D, E). During cutting, lightly brush the film to avoid the separation of the section from the adhesive film.
4) Placing the sectioned side of the collected film-section on a metal plate cooled in the cryochamber and pressing it with a rubber roller to attach the section successfully onto the adhesive film.
5) Fastening both ends of the film-section on the pre-cooled glass slide with double-sided adhesive tape for handling the section during staining. The clips shown in Figure 7 can be used instead of the tape.

To examine the distribution of water-soluble materials, the sections are prepared using the following procedure.

1) Freeze-drying the frozen sections in a cryochamber maintained at less than −20℃ for approximately 12 h. (The temperature in the cryochamber is extremely important. Many cracks appear in the section when the section is freeze-dried at high temperature, for example −15℃.)
2) Placing the dried sections in a box containing silica gel to avoid condensation.
3) Removing the box from the cryochamber.

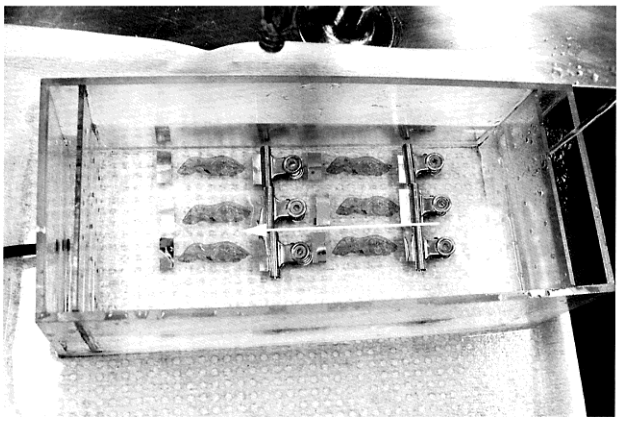

Fig. 7. Washing of the stained section. The section is fastened to the glass slide with two clips. The water gently flows in the direction of the arrow.

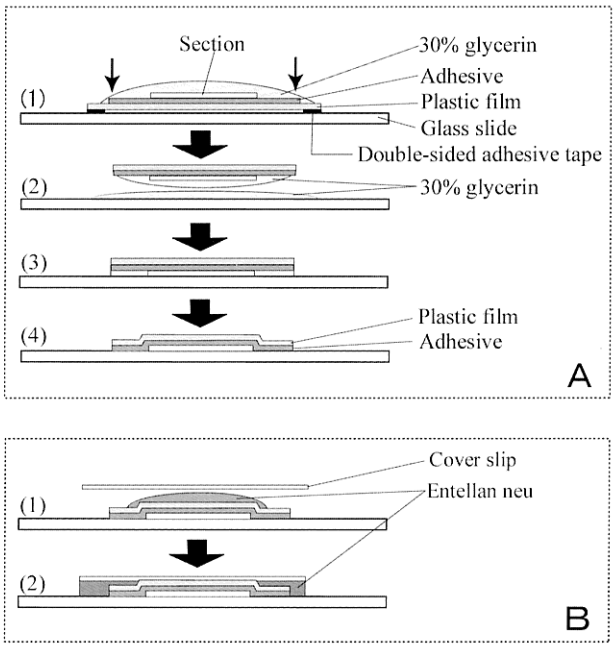

Fig. 8. Mounting procedures of the stained section. **A:** The section is mounted between the glass slide and plastic film. The film section is cut at the arrows in Figure A(1). **B:** The section of Figure A is further mounted under the cover slip with Entellan neu.

4) Allowing time for the box and its contents to reach room temperature.

For histological and histochemical applications, the frozen sections are treated using the following procedure.

1) Removing the frozen sections from the cryochamber and thawing them (15–30 sec).
2) Moving the thawed sections to a vessel or container filled with the appropriate amount of 100% ethanol.
3) Placing the vessel in a vacuum chamber to remove air bubbles trapped in the tissues.
4) Fixing the sections with 4% paraformaldehyde or 3% glutaraldehyde (pH 7.4) for more than 3 min.
5) Staining the section after washing in running water for approximately 10 min.

Thawed sections without chemical fixation sometimes detach from the adhesive film during staining in immunohistochemical study and *in situ* hybridization and are seriously damaged. In contrast, freeze-dried sections remain attached to the film. Therefore, for staining with non-fixed sections, freeze-dried sections are recommended.

Application of the sections

1. Histological staining

The present film sectioning method produces large thin sections from many types of samples and the film-sections can be used for many types of histological staining. Kawamoto and Shimizu (2000) demonstrated 2-μm thick whole-body sections from a 10-day-old rat and also from the femur and lower incisor of a 7-month-old rat. In addition to animal samples, this method is also useful for plant samples (Maekawa and Kawamoto, 2002). For example, it is known that the conventional sample preparation elutes chloroplast in a leaf. This method clearly shows chloroplasts in the green sectors of a rice leaf.

In addition to these samples, this method allows the preparation of thin fresh frozen sections from various samples: an adult whole mouse, an adult rat femur and tooth, an adult rabbit femur and tooth, an adult human bone and tooth, a fish body, an insect, a plant, cereal grains, pollen, and more.

For histological applications, chemically fixed sections are used and mounted with 30% glycerin. The plastic film

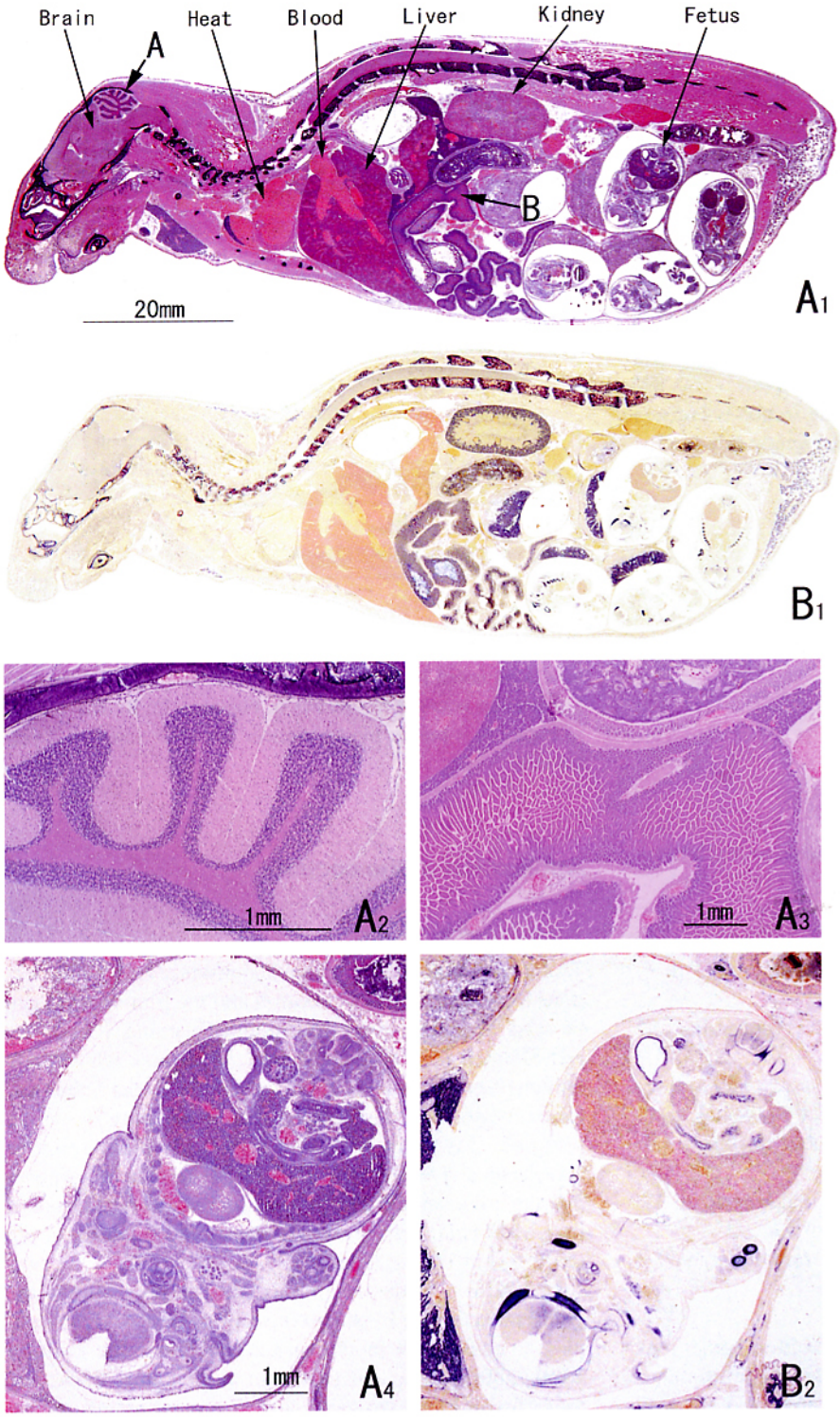

Fig. 9. Five-μm-thick serial fresh sections of the pregnant mouse. $\mathbf{A_1}$, $\mathbf{A_2}$, $\mathbf{A_3}$ and $\mathbf{A_4}$: H-E staining. $\mathbf{A_2}$ and $\mathbf{A_3}$ show the respective areas indicated by arrows A and B in Figure A_1 at high magnification. $\mathbf{B_1}$ and $\mathbf{B_2}$: Simultaneous demonstration of ACPase activity (red color) and ALPase activity (blue color) in the whole body. $\mathbf{A_4}$ and $\mathbf{B_2}$ show the fetus at high magnification. Cryoglue Type 1 is used.

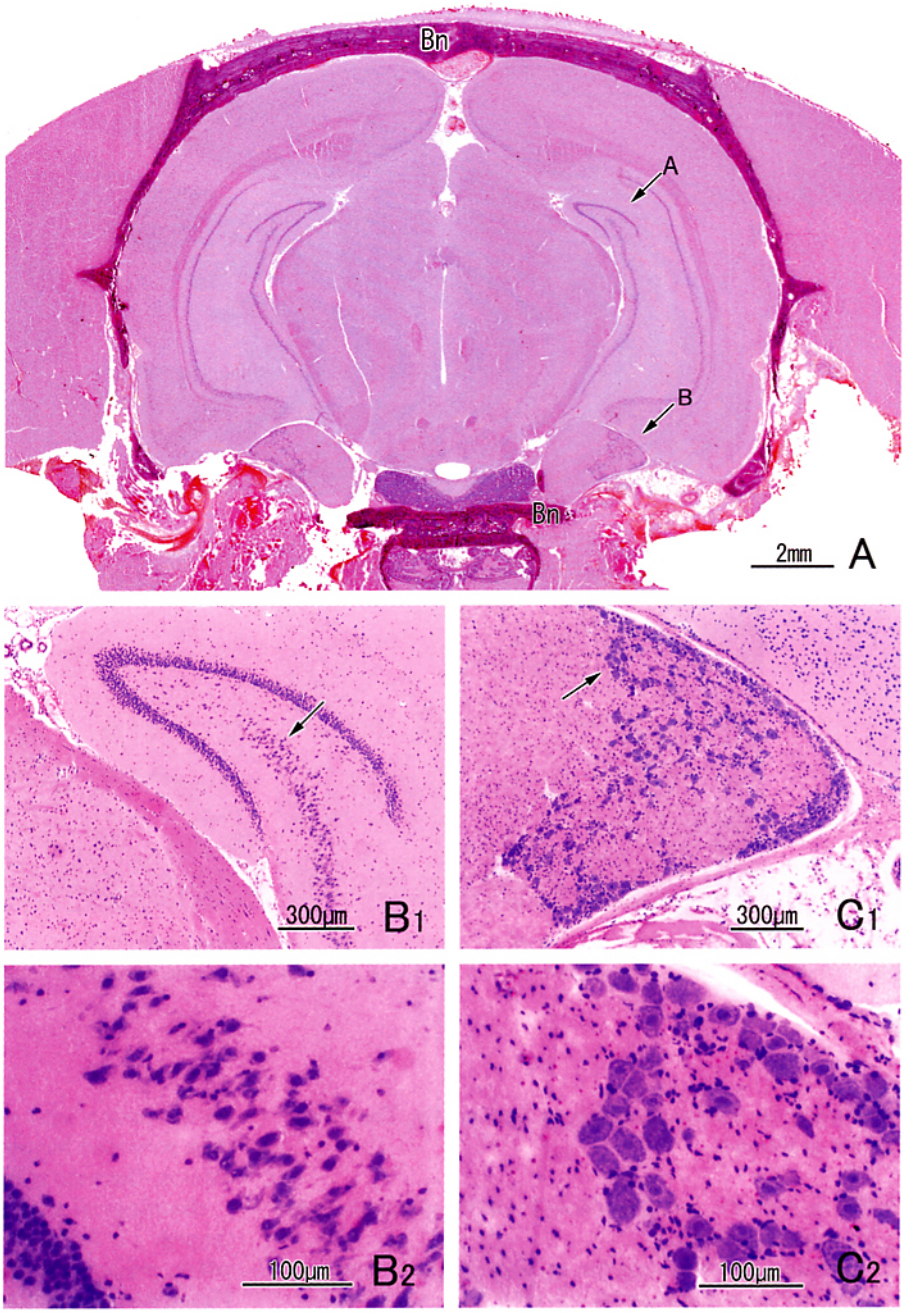

Fig. 10. A 5-μm-thick fresh section of a 7-month-old rat brain stained with H-E. $\mathbf{B_1}$ and $\mathbf{C_1}$ show the respective areas indicated by arrows A and B in Figure A at high magnification. $\mathbf{B_2}$ and $\mathbf{C_2}$ show the respective areas indicated by the arrows in Figures B_1 and C_1 at high magnification. Bn: bone. Cryoglue Type 1 is used.

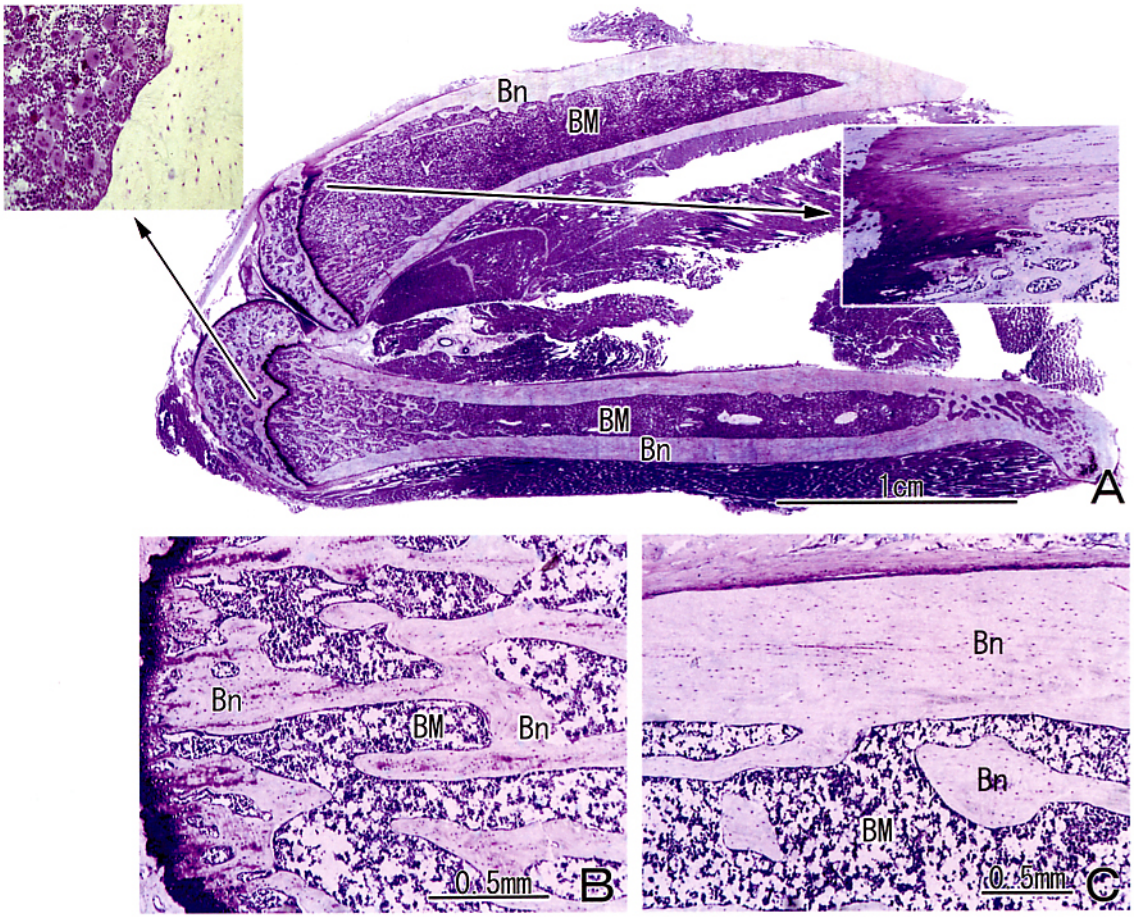

Fig. 11. A 2-μm-thick fresh section of a 7-month-old rat femur stained with 0.5% toluidine blue. **B** and **C:** High power respective views of the metaphysis and diaphysis. Bn: bone, BM: bone marrow. Cryoglue Type 2 is used.

supporting the section functions as a cover slip. The procedure is as follows.

1) Fixing with 4% paraformaldehyde or 3% glutaraldehyde for more than 3 min.
2) Staining after washing the section in gently running water for approximately 10 min (Fig. 7).
3) Placing the sectioned side on the glass slide coated with 30% glycerin (Fig. 8A).
4) Removing the excessive glycerin with a sheet of filter paper.
5) Allowing the section to dry (removal of the water containing in the glycerin). Then the section is mounted between the plastic film and the glass slide.

For examining the sections at high magnification with an oil-immersion lens, the section mounted with glycerin is then mounted beneath a cover slip using Entellan neu (Merck, Germany) (Fig 8B).

Histological demonstrations are shown in Figures 9 through 12. As an example of a large complex sample, a whole pregnant mouse (50 mm × 110 mm) was selected. Figure $9A_1$ shows a 5-μm thick whole-body section stained with hematoxilin and eosin. The section is free of distortion and clearly shows each organ (brain, heart, liver, intestine, and kidney). Furthermore, the blood in the blood vessels and heart is preserved. The fetus can be examined in detail without removing it from the mother's body (Fig. $9A_4$). The section can also be examined with a light microscope at high magnification.

Figure 10 shows a fresh section of an adult rat brain with the cranium. There is no serious damage to the tissues; the

relative position of the tissues in the cranium, including the cerebrum, hippocampus and pitiritary, is maintained. Brain cells can be identified under high magnification (Fig. $10B_2$, C_2).

As a demonstration of a hard tissue sample, the fresh femur and lower incisor of a 7-month-old rat and an extracted adult human molar were selected. Figure 11 shows a 2-μm thick section of the fresh femur. The calcified tissue and the bone marrow are well preserved. The osteocytes and the osteoblasts are easily identified. Figure 12 shows a 2-μm thick section of the lower incisor. Ameloblasts and odontoblasts are maintained on the enamel and dentine surface, respectively.

The adult human tooth presented most difficulties among the materials treated in the present study; the enamel is hard to cut even when using a tungsten carbide blade. In such a case, it is recommended that the enamel be ground away from the tooth using a dental burr. Figure 13 shows a 5-μm thick section of the tooth with the enamel removed. The section clearly shows calcification lines in the cementum.

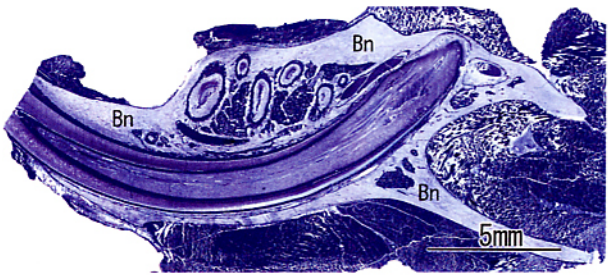

Fig. 12. A 2-μm-thick fresh section of a 7-month-old rat lower incisor stained with 0.5% toluidine blue. Bn: bone. Cryoglue Type 2 is used.

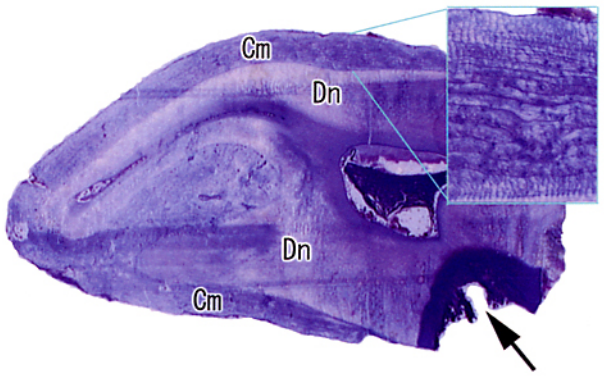

Fig. 13. A 5-μm-thick undecalcified section of the adult human molar stained with 0.5% toluidine blue. Arrow shows a cavity in the tooth. Cm: cementum, Dn: dentin. Cryoglue Type 2 is used.

2. Enzyme histochemistry and immunohistochemistry

It is obvious that fresh sections (non-fixed and undecalcified) are superior to chemically fixed sections in immune and enzyme reactivity. The present film sections can be used for enzyme histochemistry and immunohistochemistry (Kawamoto, 1990; Nakano *et al.*, 2002; Hiraoka *et al.*, 2002). However, when thawed sections are used, they are sometimes seriously damaged during treatment with solutions. This damage is minimized by fixation, even when the section is treated with a fixative (such as paraformaldehyde) for only a few minutes after thawing.

When histochemical examination on non-fixed sections is required, the freeze-dried sections are easily available. The freeze-dried sections could tolerate the treatment with solution for 4 days.

For enzyme histochemical demonstrations, alkaline phosphatase (ALPase), acid phosphatase (ACPase) and Tartrate-resistant acid phosphatase (TRAPase) activity was detected with a fresh section. Enzymatic activities were all demonstrated using the method described by Burstone (1958).

For the detection of ALPase activity, the fresh freeze-dried section was incubated in a solution of naphtol AS-MX phosphate (manufactured by Sigma Chemical, USA) and fast blue BB salt (manufactured by Sigma Chemical, USA) in a 0.1 M Tris-HCL buffer (pH 8.5). For ACPase activity, the section was incubated in a solution of naphtol AS-MX phosphate (manufactured by Sigma Chemical, USA) and fast red violet LB salt (manufactured by Sigma Chemical, USA) in a 0.1M sodium acetate buffer (pH 5.1). For TRAPase activity, the section was incubated in a solution of naphtol AS-MX phosphate (manufactured by Sigma Chemical, USA) and fast red violet LB salt (manufactured by Sigma Chemical, USA) in a 0.1M acetate buffer (pH 5.1) containing 50mM (+) tartaric acid (manufactured by Sigma Chemical, USA).

Figure $9B_1$ shows simultaneous ALPase and ACPase activity in the fresh whole-body section of a pregnant mouse. The enzymatic activity in the section is extremely intense compared with chemically fixed sections. The reaction products in Figure $9B_1$ appeared in only 4 min for ALPase and in 20 min for ACPase. The enzymatic activity in the fetus is also clearly shown (Fig. $9B_2$). The localization of the enzymatic activity can be demonstrated under high magnification.

The TRAPase activity is shown in Figure 14. It is well known that TRAPase is localized in the osteoclasts and ALPase is localized in the osteoblasts. In addition to the cells, the section shows the strong TRAPase activity in the

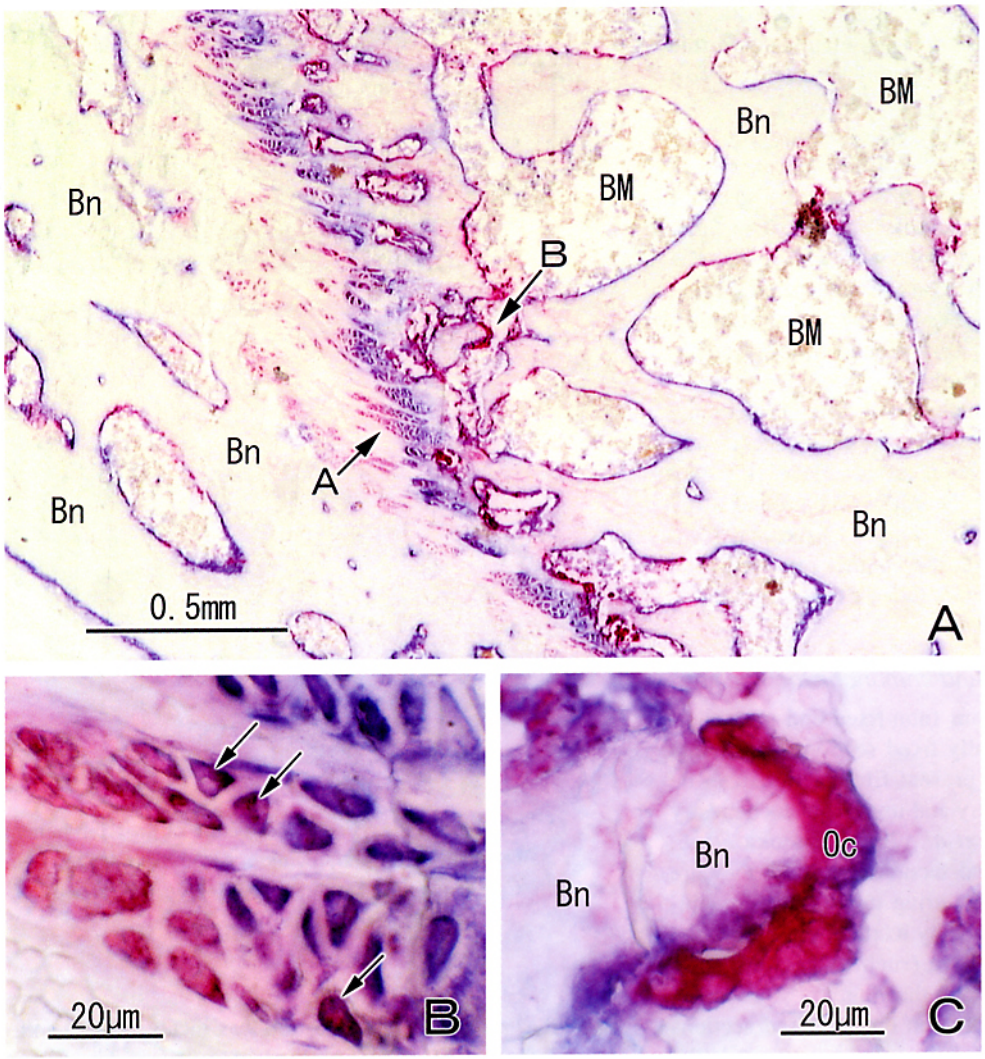

Fig. 14. A 2-μm-thick fresh section of the 7-month-old rat femur. Alkaline phosphatase activity and TRAPase activity simultaneously are demonstrated in the section. **A:** Epiphysis. **B:** Area indicated by arrow A in Figure A. Arrows indicate chondrocytes. **C:** The area indicated by arrow B in Figure A. Red and blue colors indicate TRAPase activity and alkaline phosphatase activity respectively. Bn: bone, BM: bone marrow, Oc: osteoclasts. Cryoglue Type 1 is used.

chondrocytes in the resting cell zone, the hypertrophy zone, and the proliferation zone. In the calcification zone, very faint TRAPase activity is localized but intense ALPase activity is evident (Fig. 14A, B).

As an immunohistochemical application, the distribution of PGP9.5 was examined with the fresh freeze-dried section (non-fixed and undecalcified). Figure 15A shows the distribution of PGP9.5 in the whole body. Such a whole-body section allows examination of the localization at high magnification (Fig. 15B, C). The structures are well preserved, and the localization of the substance is clearly observed. Figure 16 shows fluorescent immunohistochemical application. There is no fluorescence in the adhesive film; thus, the localization of fibronectine in the small intestinal villi is clearly shown.

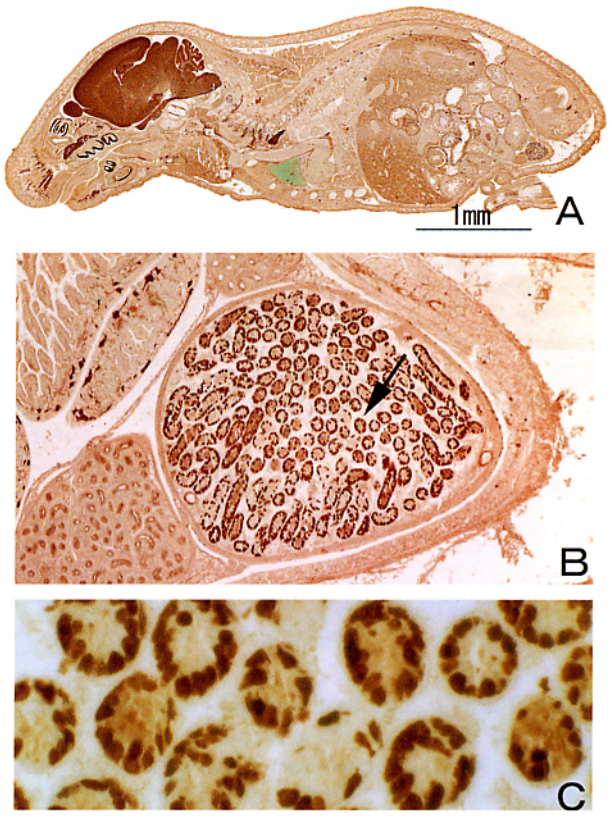

Fig. 15. Immunohistochemical localization of PGP9.5 in a 6-day-old mouse. **A:** The immunohistochemical localization in a 10-μm-thick fresh whole-body section. **B:** The localization in the testis. **C:** High power view of the area indicated by the arrow in Figure B. Cryoglue Type 1 is used.(Provided by Dr. Yukiko Nakano)

3. In situ hybridization (ISH)

ISH is a powerful tool for investigating and localizing gene expression at the single cell level within intact tissues. The aims of tissue preparation for the ISH are to preventing loss of nucleic acids from the tissue, to preserve the tissue morphology, and to allow the penetration of a probe to a target. Walash *et al.* (1993) showed that the mRNA signal within undecalcified tissues is stronger than that within decalcified tissues. The present film-sections are useful for ISH. The problem of performing a probe hybridization when the section is supported by adhesive film arises from heat treatment. The adhesive film, made of polyvinilidene chloride, can be used at temperatures up to 45℃. In other words, the section supported with the adhesive film can be used for hybridizing the probe at temperatures lower than 45℃. Adhesive film made of polyester allows the hybridization at

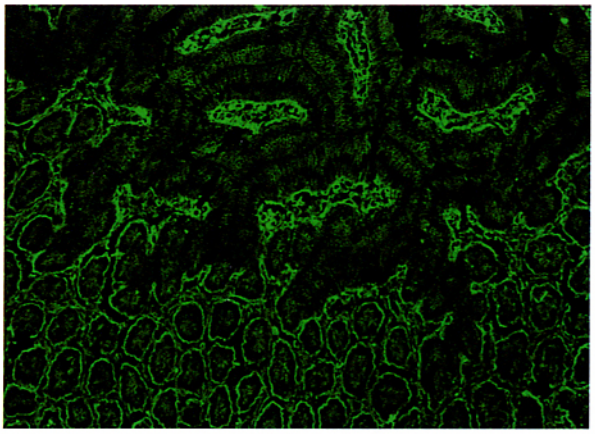

Fig. 16. Fluorescent immunohistochemical localization of fibronectine in the small intestinal villi of a 9-day-old rat. Cryoglue Type 1 is used.

higher temperatures than one made of polyvinilidene chloride. A section placed in hot water kept at 75℃ for 2 h is shown in Figure 18C. Each organ remains on the adhesive film and the structure is preserved without distortion.

Ohno *et al.* (2001) applied this film-section to ISH for HAS2 and HAS3, and showed the expression of HAS2 mRNA in the articular cartilage of rabbit knee joints. In this article, as a demonstration of ISH, a probe of rat 28S rRNA cDNA was selected. Figure 17-A shows the localization in the fresh freeze-dried section of a 6-day-old mouse. The section is supported with adhesive film made of polyvinilidene chloride. The hybridization is carried out at 42℃. The expression of the probe on the whole-body section can be observed and examined in detail (Fig.17B).

4. Transferring the section from the adhesive film to the glass slide

Fink (1987) showed that frozen sections can be fastened tightly to the glass slide coated with hydrolyzed vinyltriethoxysilane (silane). Silane is very useful when the section on the adhesive film is transferred to the glass slide. The section attached to the adhesive film is transferred to the glass slide by the following steps:

1) Tightly placing the chemically fixed section on the silane-coated glass slide.
2) Removing the excessive water between the glass slide and adhesive film with filter paper or a rubber roller.
3) Leaving the glass slide with the section on a hot plate (approximately 36℃) for approximately 30 min or at room temperature for about 3 h.
4) Placing the glass slide in n-hexane till the adhesive on

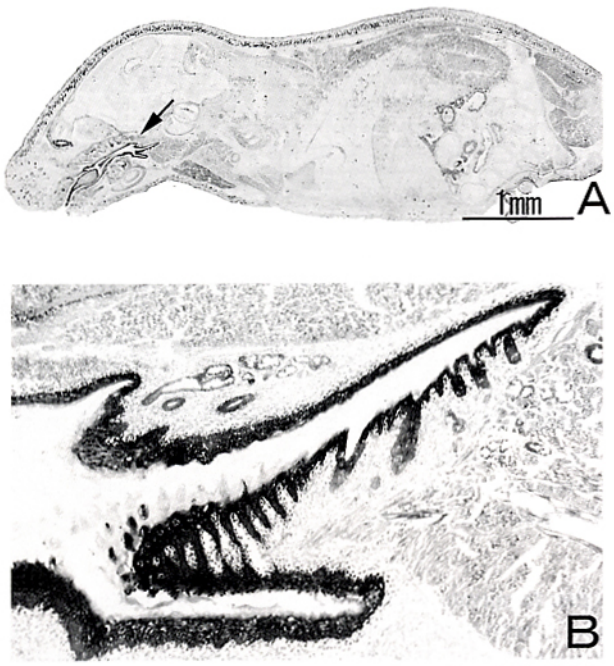

Fig. 17. *In situ* hybridization (expression of rat 28S rRNA, cDNA). **A:** The expression in a 5-μm-thick fresh whole body section of the 6-day-old mouse. **B:** High power photograph indicated by the arrow in Figure A. About the histology, see Figure 18A. Cryoglue Type 1 is used.
(Provided by Dr. Yukiko Nakano)

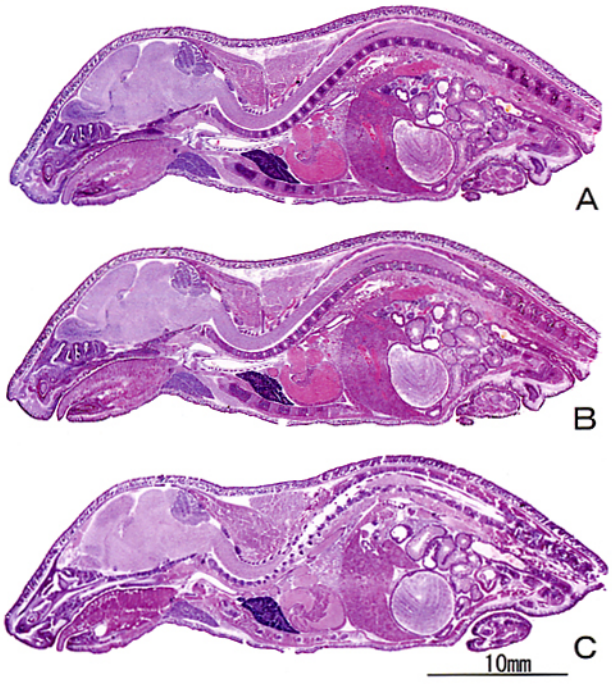

Fig. 18. Three-μm-thick fresh sections of the 6-day-old mouse stained with H-E. **A:** A section supported with the adhesive film. **B:** A section transferred from the adhesive film to a glass slide after staining. **C:** A section left in hot water at 75℃ for 2 h. Cryoglue Type 1 is used.

the film is solved completely; thus the section is left on the glass slide after the plastic film is removed.

5) Moving the glass slide with the section into water via 100% ethanol.
6) Staining the sections.
7) Mounting the section under a cover slip using conventional procedures.

In some tissues, especially those containing the intestine, autolysis occurs when the fresh sections are transferred. In such a case, transfer of the section from the adhesive film to the glass slide after staining is recommended.

Figure 18B shows a 3-μm thick section transferred to a glass slide after staining. All the tissues have been transferred almost perfectly and detailed structures can be examined under a light microscope.

5. Laser microdissection (LMD)

With the rapid development of gene research, it has become very important to collect precise samples from a very small area of tissue (Fend *et al.*, 1999). The LMD technique allows the precise collection of such a sample; the latest model LMD has been successfully used to isolate single cells. Samples collected with the LMD can be used in many kinds of research including gene analysis and protein analysis (Rivet *et al.*, 2000; Willenberg *et al.*, 2002; Shibutani and Uneyama, 2002; Casciola-Rosen and Nagraju, 2002; Westphal *et al.*, 2002; Fink and Bohle, 2002). The efficiency of extraction and quality of extracted RNA, protein, and genomic DNA is limited with a cross-linking fixative. Therefore, frozen sections of unfixed tissues have now become the standard for the analysis of microdissected cells.

The film sections can be applied to the LMD technique. The adhesive film is made with very thin plastic (2-μm thick) and a diluted adhesive (the synthetic adhesive diluted with hexane 8 times) to permit cutting of the section with a laser beam. In the case indicated in Figure 19, the bone and soft tissues in the film-section were cut with the beam; then the cut sample was successfully collected (LMD-2, Leica Microsystems Co. Ltd., Germany).

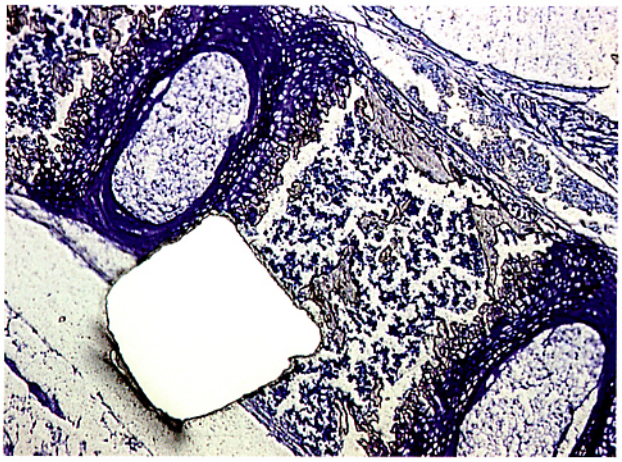

Fig. 19. Application of a laser microdissection technique. The photograph shows vertebrae of a 6-day-old mouse stained with toluidine blue. Arrow indicates the area cut with a LMD. Cryoglue Type 1 is used.

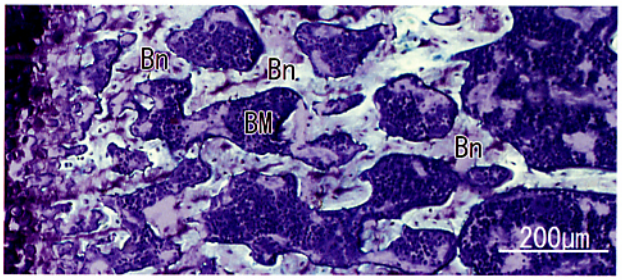

Fig. 20. Calcein fluorescence in a 2-μm-thick fresh section of a 7-month-old rat femur stained with 0.5% toluidine blue. Bn: bone, BM: bone marrow. Cryoglue Type 2 is used.

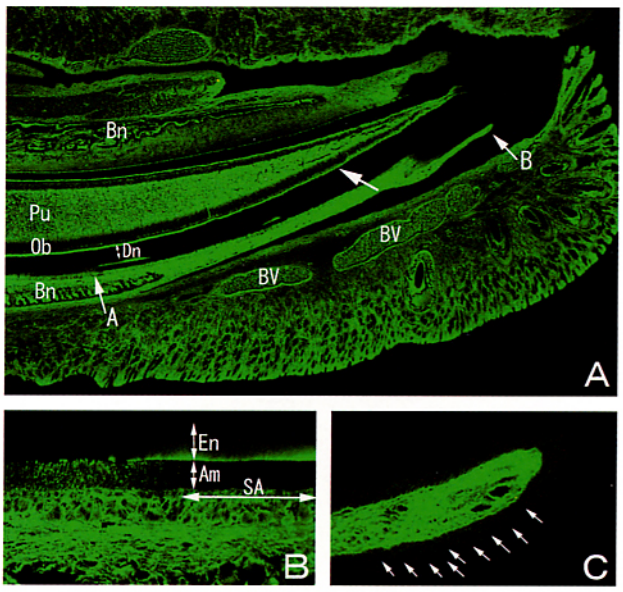

Fig. 21. Calcein fluorescence in a lower incisor of a 10 day-old rat. **A:** Distribution of the fluorescence in the apical aria of the lower incisor 30 sec after an i.v. injection. Large arrow indicates predentine. **B** and **C:** High power viewings of the respective areas indicated with arrows A and B in Figure A respectively. Arrows in Figure C indicate the fluorescence in the intercellular spaces. Am: ameloblast layer, Bn: bone, BV: blood vessel, Dn: dentine, En: enamel, Ob: osteoblast layer. Pu: pulpa, SA: smooth-ended ameloblast region. Cryoglue type 1 is used.

6. Visualization of fluorescent bone label and examination of water-soluble fluorescence tracer

To examine the distribution of a fluorescent tracer in hard tissues. They then are usually embedded with acrylic resin and then cut. However, the method is time-consuming and expensive in terms of the reagent cost. The present method is very simple compared with the method using resin. The sections made by the present method can be also used to demonstrate the distribution of fluorescent labels such as calcein and tetracycline (used for determining bone formation rates). The use of frozen sections has distinct advantages: 1) the tissue processing time is reduced as dehydration and resin embedding are not needed; 2) the intensity of the fluorescence in the freeze-dried sections is stronger than that in the resin embedded samples; 3) possible toxicity is avoided as no label loss means reduced doses of label given to the animal.

The present freeze-dried sections also allow a study of the distribution of a fluorescent tracer in the soft tissues (Paschoud *et al.*, 1985; Kawamoto and Shimizu, 1990, 1994). For these purposes, the frozen section is freeze-dried in a cryochamber maintained at less than −20℃. The dried section is placed in a box containing silica gel and removed from the cryochamber. The section is placed on a glass slide and protected with a cover glass. There is no mounting medium so as to avoid dislocation of the tracer.

Figure 20 shows the calcein fluorescence in a 2-μm thick section of an adult rat femur. The distribution and histology can be simultaneously observed.

Figure 21 shows the distribution of calcein fluorescence in an 11-day-old rat lower jaw 30 sec after intravenous injection. Intense fluorescence is observed on the predentine. In the soft tissues, it is clearly demonstrated that the calcein is localized in the intercellular spaces (Fig. 21B, C). Figure 21B clearly shows that the calcein is incorporated into the enamel through intercellular spaces.

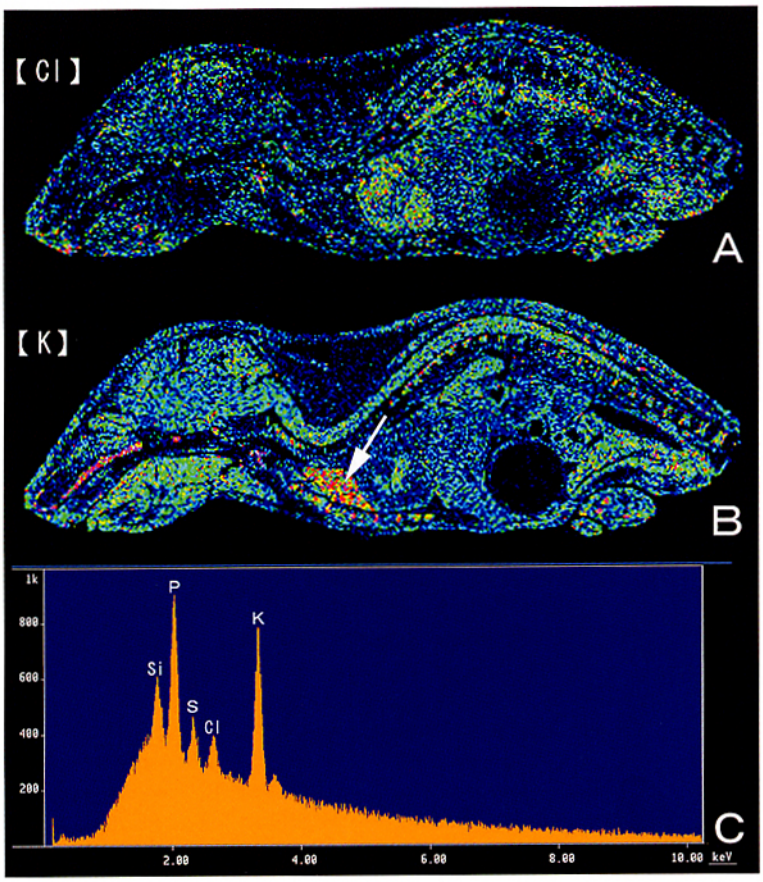

Fig. 22. Electron probe microanalysis of a 20-μm-thick fresh whole-body section adjacent to the section shown in Figure 18A. **A:** Mapping image (Cl), **B:** Mapping image (K), **C:** Energy-dispersive X-ray microanalysis spectra of the area indicated with arrow in Figure B. Cryoglue Type 1 is used.

7. Electron probe microanalysis (EPMA)

It is known that freeze-dried sections made from fresh frozen tissues are suitable for EPMA (Masters *et al.*, 1979; Engel, 1981; Rick *et al.*, 1982; Timms and Chandler, 1984). It is generally accepted that physical fixation by rapid freezing of the tissue is the only possible way to keep readily diffusible elements such as sodium, potassium, chlorine, and calcium in place. The present freeze-dried sections can be applied for examining the distribution of those water-soluble elements in tissues. For this purpose, an adhesive film made of polyester is used because the polyvinilidene chloride film contains chloride. Kawamoto and Shimizu (1987) have applied freeze-dried sections supported with adhesive film to EPMA, and examined the diffusion of the electron beam in the tissue with a lower incisor tooth as the test sample. They showed that the electron beam at 15Kv did not excite the enamel to give false-positive results for Ca when the beam position being examined was situated farther than 2-μm from the enamel surface. The sample for the EPMA is prepared using the following procedure.

1) Freeze-drying the frozen section in a cryochamber for more than 12 h.
2) Placing the section in a desiccator containing silica gel within the cryostat to prevent condensation on the sections when they are removed from the cryostat.
3) Allowing the sections to reach room temperature.
4) Fixing the freeze-dried section to the aluminum stub with double-sided adhesive tape.
5) Coating the section with carbon.

As an EPMA demonstration, Figure 22 shows mapping images in a whole-body section from a 6-day-old mouse and the energy-dispersive X-ray spectra obtained from a selected area of the thymus gland of the section. Pro-

nounced X-ray peaks of Cl and K, which are soluble elements, are obtained (Fig. 22C). The distribution of chlorine and potassium in the whole-body is clearly shown in Figures 22A and 22B. The mapping images indicate the large quantity of chlorine within the blood and potassium in the thymus gland.

8. Whole-body autoradiography

In addition to histochemical uses, the present film-sections can be used for whole-body autoradiography. The basic procedures are the same as that described by Ullberg (1954). Kawamoto and Shimizu (1986) modified the procedures to improve the resolution of the autoradiogram; only the section side of the exposed X-ray film is developed. The autoradiography is carried out using the following procedure.

1) Fastening 5–20μm thick sections to a pre-cooled glass slide with a double-sided adhesive tape.
2) Freeze-drying the sections in a cryochamber for approximately 12 h.
3) Placing the sections in a desiccator containing silica gel within the cryostat.
4) Allowing the sections to reach room temperature.
5) Covering the freeze-dried sections with a thin plastic film (Diafoil, 4-μm thick, Mitsubishi Plastic Co., Japan) to prevent chemography and to prevent tissue damage.
6) Placing an X-ray film (IX-100, Fuji Photo Co. Ltd., Japan) on the sections.
7) Exposing the film in the refrigerator.
8) Developing the section side of the film to improve the resolution of the autoradiogram and then fixing both emulsion layers with a photographic fixative.

The resolution of the ^{45}Ca autoradiogram obtained with the above procedure is approximately 25-μm. Recently, an imaging plate has been widely used instead of X-ray film. The resolution of the imaging plate is less than that of the autoradiogram using X-ray film; however, the sensitivity and linearity to the radiation is far superior (Motoji *et al.*, 1995a–d). Thus, the imaging plate is usually used for quantifying the autoradiogram. The film-sections also can be applied to the imaging plate.

An application of the present film-section to autoradiography is shown the Figure 23. Figure 23 shows a stained section and autoradiogram of 15-day-old rat frozen 60 sec after an i.v. injection of $^{45}CaCl_2$. The high radioactivity appeared on the feces in the intestinal canal in addition to the blood and hard tissues (Fig. 23B). After washing the section in running water for 30 min, the high radioactivity remained in only the hard tissues (Fig. 23C). The autoradiogram clearly showed that ^{45}Ca is incorporated in the insoluble calcium deposits within 60 sec.

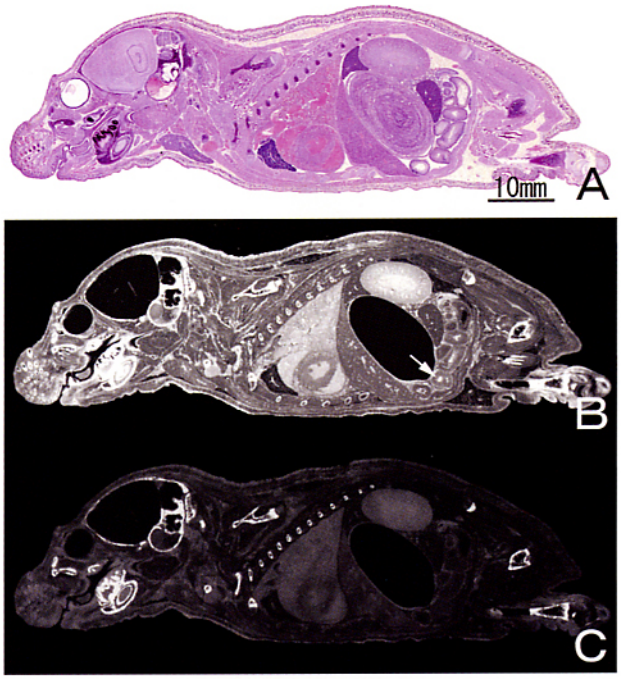

Fig. 23. Contact autoradiogram of whole-body sections of a 15-day-old rat frozen 60 sec after an i.v. injection of $^{45}CaCl_2$. **A:** Neighboring section stained with hematoxilin and eosin. **B:** Autoradiograph of the freeze-dried section. **C:** Autoradiograph of the section after washing in running water for 30 min. White areas correspond to the high radioactive regions. Arrow in Figure B indicates the feces in the intestinal canal.

9. Light microscopical autoradiography (LMA) for water-soluble materials

The film section can be used for LMA as well as whole-body autoradiography. The film section is covered with a thin emulsion film approximately 1-μm thick made of an emulsion bubble. The LMA using the film-sections is described in previous reports (Kawamoto and Shimizu, 1986; Kawamoto, 1990). The greatest advantage of the LMA is that it can be carried out with whole-body sections and allows examination of the distribution of tracers over a very short time exposure time. Kawamoto and Shimizu (1997) have reported the distribution of radiocalcium in the lower incisor enamel of a 9-day-old rat only 4.3 sec after

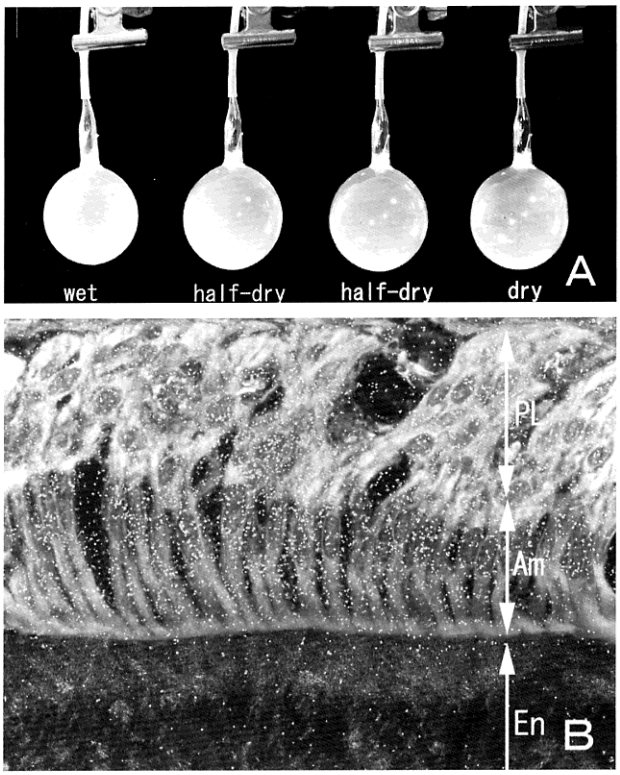

Fig. 24. Emulsion film used for light microscopical autoradiography and the autoradiograph. **A:** Bubbles (approximately 6 cm in diameter) made of autoradiographic emulsion. The bubbles are more completely dried toward the right hand side. **B:** Light microscopical autoradiograph of a section of the rat lower incisor 7.8 sec after an i.v. injection of $^{45}CaCl_2$. The white spots indicate silver grains. Am: ameloblast, En: enamel, PL: papillary layer.

the injection. They clearly demonstrated that calcium is transported to the enamel by ameloblasts at the region of ruffled-ended ameloblasts. In addition to this advantage, the distribution of the tracer can be examined at macroscopical and microscopical levels because both WBA and LMA are carried out using neighboring sections. The LMA is performed using the following procedure.

1) Freeze-drying 2–5 μm thick sections
2) Covering the dried sections with a thin dried-emulsion film (approximately 1-μm) made with autoradiographic emulsion according to the previously reported method (Kawamoto and Shimizu, 1986, 1990) (Fig. 24A).
3) Placing the sections in a light-tight box containing silica gel.
4) Exposing the sections in a freezer (−80℃) to prevent chemography.
5) Developing and fixing the emulsion film on the sections with a developing solution and a fixing solution.
6) Staining the sections with hematoxiline and eosin.
7) Mounting the sections with glycerin between the plastic film and the glass slide.
8) Examining the sections with a confocal laser-scanning microscope or a light microscope.

Figure 24A shows bubbles made of autoradiographic emulsion. The diffusion of the tracer is checked with blood and gelatin containing a radiotracer; the emulsion film of half-dried and dried bubbles does not cause significant diffusion at the microscopical level (Kawamoto and Shimizu, 1986). The ^{45}Ca light microscopical autoradiogram, carried out using a half-dried emulsion film, clearly shows many grains on the ameloblasts but only a few grains on the enamel (Fig. 24B).

10. Storing of the sections

The present film sections can be stored in 100% ethanol or a deep freezer (−80℃). For short-term storage (several days), keeping the section in 100% ethanol is convenient. For long-term storage, keeping the section in a deep freezer (−80℃) is best. The thawed film sections, fixed on a glass slide with double-sided adhesive tape, are placed in a box, which is kept in a deep freezer. The freeze-dried sections are also kept in a deep freezer.

11. Photographic processing

Difficulties arise when photographs of large thin sections are taken with ordinary cameras. The section contrast is insufficient in most cases. Digital cameras with high resolution now available are recommended for recording the images. The contrast, brightness, and color balance of digital images are adjusted easily on a computer; furthermore, the digital image can be printed directly with a color printer connected to a computer. An image scanner is also useful for recording the image; especially, it is recommended for a large section. All the photographs in this article were recorded digitally, processed using PhotoShop (Adobe Systems, USA), and printed with a color printer (Pictrography, Fuji Photo Co. Ltd., Japan).

Conclusions

The present film-sectioning method allows the preparation of large and very thin sections (2-μm) from nearly any kind

of animal sample—including hard tissues—in addition to fish bodies, insects, and plants. There is significantly less distortion and dislocation of sections made with the adhesive film than those made without any supporting materials. The sections are easily transferred to glass slides and examined at both the macroscopical and microscopical levels. The sections preserve tissue information better than chemically fixed sections and yield useful information concerning cell and tissue functions.

In addition to these applications, the film section allows a sample collection from a particular area employing the LMD technique together with pH mapping (pH-imaging microscope) using a semiconductor silicon sensor (Nakano *et al.*, 2002).

The various possible applications of the film section make it a very versatile and useful tool for biological research.

Acknowledgments

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References

Aaron JE, Carter DH: Rapid preparation of fresh-frozen undecalcified bone for histological and histochemical analysis. *J Histochem Cytochem* 35: 361-369 (1987).

Baker JR, Hew H, Fishman WH: The use of a chloral hydrate formaldehyde fixative solution in enzyme histochemistry. *J Histochem Cytochem* 6: 244-250 (1958).

Bratthauer GL: Preparation of frozen sections for analysis. *Meth Molecul Biol* 115: 57-62 (1999).

Bosman FT, Lindeman J, Kuiper G, Van Der Wal A, Kreunig J: The influence of fixation on immunoperoxidase staining of plasma cells in paraffin sections of intestinal biopsy specimens. *Histochemistry* 53:57-62 (1977).

Burstone MS: The relationship between fixation and techniques for the histochemical localization of hydrolytic enzymes. *J Histochem Cytochem* 6: 322-339 (1958).

Casciola-Rosen L, Nagaraju K: Immunoblotting of single cell types isolated from frozen sections by laser microdissection. *Methods Enzymol* 356: 70-79 (2002).

Deak ST, Csaky KG, Waddell WJ: Localization and histochemical correlation of ^{73}As by whole-body autoradiography in mice. *J Toxicol Environ Health* 1: 981-984 (1976)!

Engel MB: Microprobe analysis of calcifying matrices and formative cells in developing mouse molars. *Histochemistry* 72: 443-452 (1981).

Farebrother D, Woods N: Permanent preparations of stained whole-body sections using 'Trycolac', *J Micros* 97: 373-375 (1973).

Fend F, Emmert-Buck MR, Chuaqui R, Cole K, Lee J, Liotta LA, Raffeld M: Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. *Am J Pathol* 154: 61-6 (1999).

Fink L, Bohle RM: Internal standards for laser microdissection. *Methods Enzymol* 356: 99-113 (2002).

Fink S: A new integrated concept for the improved preparation of sections of fresh or frozen tissue for light microscope histochemistry. *Histochemistry* 86: 43-52 (1986).

Fink S: Some new methods for affixing sections to glass slides. 1: aqueous adhesives. *Stain Technol* 62: 27-33 (1987).

Fink S: A solvent-free coating-procedure for the improved preparation of cryostat sections in light microscope histochemistry. *Histochemistry* 97: 243-246 (1992).

Fukuda K, Shindo H: Histochemical demonstration of nonspecific esterase in whole-body section of rat. *Acta Histochem Cytochem* 7: 181-183 (1974).

Fukase Y: Immunohistochemical staining of human teeth, and production of monoclonal antibodies against cementum. *J Osaka Dent Univ* 31: 1-9 (1997).

Gillberg P-G, Jossan SS, Askmark H, Aquilnonius S-M: Large-section cryomicrotomy for in vitro receptor autoradiography. *J Pharmacol Methods* 15: 169-180 (1985).

Hammarstrom L: Autoradiography and histochemistry of mineralized tissues by means of the Ullberg freeze-sectioning technique. *Ups J Med Sci* 91: 239-243 (1986).

Hasselgren G, Hammarstrom LE: Histochemical studies on beta-glucuromidase activity in developing teeth and bone of rat and macaque monkey. *Acta Odontol Scand* 33: 161-167 (1975).

Hill EL, Elde R: An improved method for preparing cryostat sections of undecalcified bone for multiple uses. *J Histochem Cytochem* 38: 443-448 (1990).

Kawamoto T, Shimizu M: A method for preparing whole-body sections suitable for autoradiographic, histological and histochemical studies. *Stain Technol* 61: 169-183 (1986).

Kawamoto T, Shimizu M: Distribution of calcium and phosphate in cells of the enamel organ in the rat lower incisor. *Adv Dent Res* 1: 236-244 (1987).

Kawamoto T: Light microscopic autoradiography for study of early changes in the distribution of water-soluble materials. *J Histochem Cytochem* 38: 1805-1814 (1990).

Kawamoto T, Shimizu M: Changes in the mode of calcium and phosphate transport during rat incisal enamel formation. *Calcif Tiss Int* 46: 406-414 (1990).

Kawamoto T, Shimizu M: Changes of the ratio of calcium to phosphate transported into the mineralizing enamel, dentin and bone. *Jap J Oral Biol* 36:365-382 (1994).

Kawamoto T, Shimizu M: Pathway and speed of calcium movement from blood to mineralizing enamel. *J Histochem Cytochem* 45: 213-230 (1997).

Kawamoto T, Shimizu M: A method for preparing 2- to 50-μm-thick fresh-frozen sections of large samples and undecalcified hard tissues. *Histochem Cell Biol* 113: 331-339 (2000).

Kihara T: Whole-body autoradiography and utilization of its cryosections. *Bull Osaka Med Sch Suppl* 14: 36-55 (1984).

Larsson B, Ullberg S: Whole-body autoradiography. *J Histochem Cytochem* 29: 216-225 (1981).

Maekawa M, Kawamoto T: Intact sectioning of plant tissues with a cryomicrotome. *Breed Sci* 52:57-60 (2002).

Masters SK, Bell SW, Ingram P, Adams DO, Shelburne JD: Preparative techniques for freezing and freeze-sectioning macrophages for energy dispersive x-ray microanalysis. *Scanning Electron Microsc* 3: 97-110 (1979).

Matthews JB, Mason GI: Influence of decalcifying agents on immunoreactivity of formalin-fixed, paraffin-embedded tissue. *Histochem J* 16: 771-787 (1984)

Matthews JB: Influence of decalcification on immunohistochemical staining of formalin-fixed paraffin-embedded tissue. *J Clin Pathol* 35: 1392-4 (1982).

McElroy HH, Shih M-S, Parfitt AM: Producing frozen sections of calcified bone. *Biotech Histochem* 68: 50-55 (1993).

Mepham BL: Influence of fixatives on the immunoreactivity of paraffin sections. *Histochem J* 14: 731-737 (1982).

Motoji N, Hayama E, Shigematsu A: Studies on the quantitative autoradiography. Ⅰ. radioluminography for quantitative autoradiography of ^{14}C. *Biol Pharm Bull* 18: 89-93 (1995a).

Motoji N, Hayama E, Shigematsu A, Tazaki S, Mori N, Miyahara J: Studies on the quantitative autoradiography. Ⅱ radioluminography for quantitative autoradiography of ^{3}H. *Biol Pharm Bull* 18: 94-99 (1995b).

Motoji N, Hamai Y, Nikura Y, Shigematsu A: Studies on the quantitative autoradiography. Ⅲ. quantitative comparison of a nove tissue-mold measurement technique "Paste-Mold Method," to the semiquantitative whole-body autoradiography (WBA), using the same animals. *Biol Pharm Bull* 18: 100-107 (1995c).

Motoji N, Kuroiwa H, Shigematsu A: Studies on the quantitative autoradiography. Ⅳ. quantitative reliability of TLC-autioradoigraphy. *Biol Pharm Bull* 18: 108-114 (1995d).

Mullink H, Henzen-Logmans SC, Tadema TM, Mol JJ, Meijer CJ: Influence of fixation and decalcification on the immunohistochemical staining of cell-specific markers in paraffin-embedded human bone biopsies. *J Histochem Cytochem* 33: 1103-9 (1985).

Nakano U, Kawamoto T, Takano Y: Phosphatase activities of rat intestinal enterocytes and its relation to diverse luminal pH, with special references to possible localization of phytase along the brush border membrane. *Arch Histol Cytol* 61: 483-492 (2001).

Nakano U, Nomura S, Kawamoto T, Tagami J, Horiba A, Takano Y: An attempt of pH mapping on biological specimens by pH-imaging microscope using a semiconductor silicon sensor. *Bunseki Kagaku* 51: 473-476 (2002). (in Japanese, summary in English)

Nuki K: Some histochemical aspects of the periodontium. *Dent Practit* 17: 245-250 (1967).

Ohno S, Tanimoto K, Fujimoto K, Ijuin C, Honda K, Tanaka N, Doi T, Nakahara M, Tanne K: Molecular cloning of rabbit hyaluronic acid synthases and their expression patterns in synovial membrane and articular cartilage. *Biochim Biophys Acta* 1520: 71-78 (2001).

Parmgren A: Tape for microsectioning of very large, hard or brittle specimens. *Nature* 174: 46-46 (1954).

Paschoud N, Bignell C, Reinhold HS: A double fluorescence method for determining doxorubicin distribution and vascular supply in the mouse kidney. *J Histochem Cytochem* 33: 73-76 (1985).

Rick R, Dorge A, Thurau K: Quantitative analysis of electronlytes in frozen dried sections. *J Microsc* 125: 239-247 (1982).

Rijntjes NV, Van de Putte LB, Van der Pol M, Guelen PJ: Cryosectioning of undecalcified tissues for immunofluorescence. *J Immunol Methods* 30: 263-268 (1979).

Rivet J, Bertheau P, Daneshpouy M, Plassa F, Grolet L, Meignin V, Janin A: Microdissection of frozen sections. *J Mol Med* 78: B24 (2000).

Rosene DL, Roy NJ, Davis BJ: A cryoprotection method that facilitates cutting frozen sections of whole monkey brains for histological and histochemical processing without freezing artifact. *J Histochem Cytochem* 34: 1301-15 (1986).

Schwabe KG, Terracio L: Ultrastructural and thermocouple evaluation of rapid freezing techniques. *Cryobiology* 17: 571-584 (1980).

Shibutani M, Uneyama C: [10] Methacarn, A fixation tool for multipurpose genetic analysis from paraffin-embedded tissues. *Methods Enzymol* 356: 114-125 (2002)

Shimada M, Watanabe M: Recent progress in whole-body radioautography. *Cell Mol Biol* 41: 39-48 (1995).

Shin SS, Sheibani K, Kezirian J, Winberg CD: Immunohistologic studies of bone marrow biopsies on frozen sections: an analysis of 42 cases. *Human Pathol* 24: 30-36 (1993).

Sjogren S, Hammarstrom L, Larsson A: Enzyme histochemistry of developing rat oral mucosa. *J Histochem Cytochem* 29: 57-64 (1981).

Skaer H: Chemical cryoprotection for structural studies. *J Microsc* 125:137-147 (1982).

Timms BG, Chandler JA: Endogenous elements in the prostate. An X-ray microanalytical study of freeze-dried frozen sections and histochemical localization of zinc by potassium pyroantimonate. *Histochem J* 16: 733-54 (1984).

Ullberg S: Studies on distribution and fate of S35-labelled benzylpenicillin in body. *Acta Radiol (Suppl)* 118: 1-100 (1954).

Ullberg S, Larsson B: whole-body autoradiography. *Meth Enzymol* 77: 64-80 (1981).

Van Noorden CJ, Vogels IM: Enzyme histochemical reactions in unfixed and undecalcified cryostat sections of mouse knee joints with special reference to arthritic lesions. *Histochemistry* 86: 127-133 (1986).

Walsh L, Freemont AJ, Hoyland JA: The effect of tissue decalcification on mRNA retention within bone for in-situ hybridization studies. *Int J Exp Path* 74: 237-241 (1993).

Watanabe M, Kihara T, Shimada M, Kurimoto K: Preparation and stain of whole-body sections. *Cell Mol Biol* 23: 311-315 (1978).

Watanabe M, Shimada M, Kurimoto K: Staining method for whole-body autoradiography. *Stain Technol* 50: 239-243 (1975).

Westphal G, Burgemeister R, Fridemann G, Wellmann A, Wernert N, Wollscheid V, Becker B, Vogt T, Knuchel R, Stolz W, Schutze K: Noncontact Laser Catapulting: A basic procedure for functional genomics and proteomices. *Methods Enzymol* 356: 80-99 (2002).

Willenberg HS, Walters R, Bornstein SR: Use of laser microdissection in complex tissue. *Methods Enzymol* 356: 216-23, (2002).