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A method for preparing 2- to 50-µm-thick fresh-frozen sections of large samples and undecalcified hard tissues

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Abstract This article describes a method for preparing 2- to 50-µm-thick fresh-frozen sections from large samples and completely calcified tissue samples. In order to perform the more routine work involved, a tungsten carbide disposable blade was installed to a heavy-duty sledge cryomicrotome. An entire 10-day-old rat and bone and tooth samples from a 7-month-old rat were rapidly frozen. The frozen samples were attached to the cryomicrotome stage. The cutting surface of the samples was covered with a polyvinylidene chloride film coated with synthetic rubber cement and cut at –25°C. The soft tissues and the hard tissues were satisfactorily preserved and all tissue cells were easily identifiable. Enzymatic activity in the fresh sections was much stronger than that in chemically fixed and/or decalcified sections. The sections permitted histological and histochemical studies without trouble. In addition, the sections can be used for multiple experiments such as immunohistochemistry, in situ hybridization, and electron microprobe X-ray microanalysis. This method can be used with conventional cryomicrotome equipment.

Key words Fresh-frozen section · Whole-body section · Hard-tissue section · Enzyme histochemistry · Bone · Tooth

Introduction

The relationship between tissue structure and function has been studied using many different methods. These methods include histology, general histochemistry, enzyme histochemistry, immunohistochemistry, in situ hybridization, autoradiography, and electron microprobe X-ray microanalysis. When such studies are carried out with serial sections, the results are easily and accurately correlated with each other and function can be described in great detail.

The tissues are usually chemically fixed to preserve the cell structure with minimum alteration from the living stage. In calcified tissues, decalcification is performed to permit easy sectioning. Fixation and decalcification usually result in a reduction of enzyme activity and immunoreactivity (Baker et al. 1958; Takeshita et al. 1983; Mullink et al. 1985; Mukai et al. 1986; Van Noorden and Vogels 1986; Wakisaka 1986; Van Noorden et al. 1989; Van Den Munckhof et al. 1994; Fukase 1997). In addition, there is an accompanying loss and/or dislocation of water-soluble materials from the tissue.

These problems are prevented by preparing thin sections without fixation and decalcification. Many attempts to prepare such sections have been reported. Methods used are summarized as follows:

1. Coating the frozen cutting surface with a polymeric solution before each sectioning process (Fitz-William et al. 1960; Fink 1986, 1992; Aaron and Carter 1987; McElroy et al. 1993)
2. Covering the frozen cutting surface with a sheet of paper (Watanabe et al. 1978; Kihara 1984; Kawamoto and Shimizu 1986; Hill and Elde 1990; Shimada and Watanabe 1995)
3. Covering the frozen cutting surface with pressure-sensitive adhesive tape (Parmgren 1954; Ullberg 1954; Farebrother and Woods 1973; Fukuda and Shindo 1974; Watanabe et al. 1975; Deak et al. 1976; Rijntjes et al. 1979; Larsson and Ullberg 1981; Sjogren et al. 1981; Hammerstrom 1986; Kawamoto and Shimizu 1994).

Only the third method developed for whole-body autoradiography (Ullberg 1954) satisfies our requirements. However, the quality of the section is not suitable for microscopic examination. Kawamoto (1990) modified the technique and showed that the sections are very useful for histological and histochemical studies at the microscopic level. However, preparing thin sections, such as a

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2- to 5-µm-thick section, of completely calcified bone and tooth sample had not yet been achieved. We are studying the calcification of hard tissue, being especially interested in calcium transport to the mineralizing hard tissues. Therefore, we need sections thin enough to be used either for studies on water-soluble materials or for enzyme histochemistry at the microscopic level.

This paper describes a method for preparing 2- to 50-µm-thick fresh-frozen sections either from a large sample or from completely calcified tissues. Histological and histochemical demonstrations are included.

Materials and methods

Preparation of frozen sections

We modified some sectioning conditions and procedures in the method described by Kawamoto (1990) and used improved equipment. A 10-day-old rat (weighing approximately 22 g) was used as the large sample. A 7-month-old rat (weighing approximately 350 g) was used as the completely calcified tissue sample. The entire 10-day-old rat was frozen in hexane (–94°C) cooled with a cooling apparatus (Neocool Bath; Yamato, Japan). The adult rat was anesthetized with sodium pentobarbital. One milliliter 0.04% calcein (Dojindo Laboratory, Japan) in physiological saline was injected into the abdominal cavity. After 2 h, the thighbone and the lower jaw were dissected and frozen in liquid nitrogen. The frozen sample was immersed in a stainless steel container filled with a 5% carboxymethyl cellulose (CMC) gel [the container was selected to fit the size of the sample (Fig. 1A)]. The container was placed in the cooled hexane and the CMC gel was completely frozen (the upper side of the CMC gel in the container must not be immersed in the coolant to avoid cracks during freezing). The frozen CMC block was attached to the sample stage of the cryomicrotome (CM 3500; Leica Instruments, Germany) in the cryochamber (–25°C; Fig. 1B).

After sitting for 2 h, the block was trimmed with a disposable tungsten carbide blade (Jung TC-65, 35° angle; Leica Instruments) having a clearance angle of 5°. The surface was covered with a polyvinylidene chloride film (10 µm thick; Asahikasei Kogyo, Japan) coated with synthetic rubber cement. The sample was cut at a speed not exceeding 4 mm/s. The sectioned side of the film section was placed on the flat metal plate cooled in the cryochamber and pressed with a rubber roller to ensure close contact. The film section was then fixed on a cooled glass slide with double-sided adhesive tape.

We recommend the 5- to 10-µm-thick sections for macroscopic examination and the 2- to 5-µm-thick sections for microscopic examination. The former application requires the following procedures:

1. Freeze-drying the frozen sections in the cryochamber (–25°C) for approximately 12 h
2. Placing the dried sections in a box containing silica gel to avoid condensation
3. Removing the sections from the cryochamber
4. Immersing the sections in 100% ethanol to remove trapped air bubbles.

The latter application requires the following procedures:

1. Removing the frozen sections from the cryochamber
2. Momentarily thawing of the sections
3. Immersing the sections in 100% ethanol at room temperature.

Histological examination

For histological staining, the sections in 100% ethanol were placed in a 3% glutaraldehyde solution buffered with 0.1 M phosphate (pH 7.2) and fixed for 5 min. The sections were then stained with 0.1% toluidine blue for 10–30 s, rinsed with running water for about 5 min, and mounted with glycerin under a cover glass. For alizarin red S staining, freeze-dried sections immersed in 100% ethanol were used. The sections were stained with 1% alizarin red S for about 3 min and mounted with glycerin under a cover glass.

When the large section is mounted, the polyvinylidene chloride film supporting the section is used as a cover. The sectioned side of the film section is turned toward the glass slide coated with 30% glycerin and thus attached to it. Excessive glycerin is removed with a sheet of filter paper and the glass slide is left for about 2 days. The section is sandwiched between the supporting film and the glass slide and is permanently preserved.

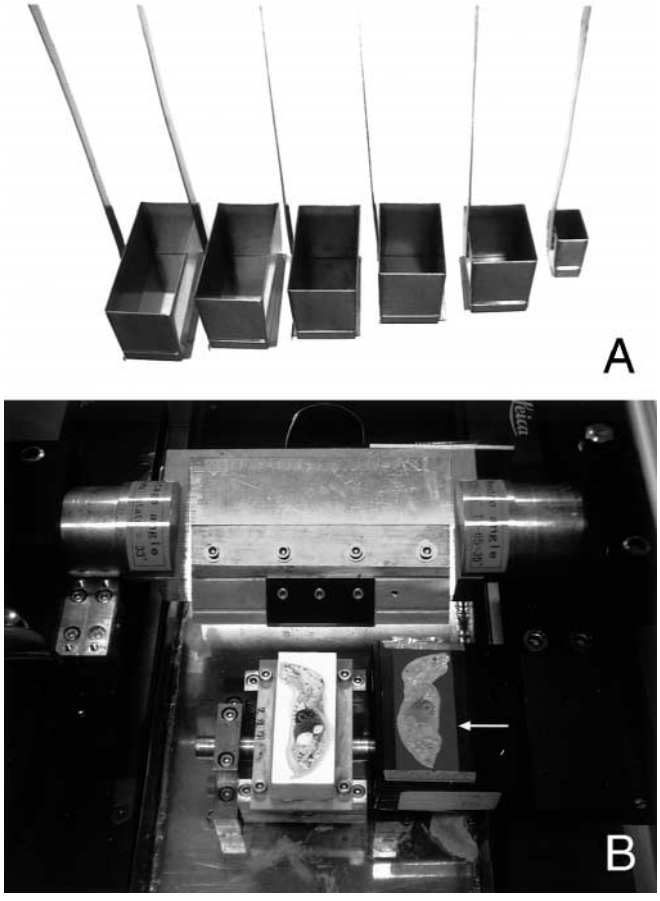

Fig. 1 **A** Stainless containers for embedding the frozen sample. **B** Cut surface of the sample, frozen section (*arrow*), and knife stage

Calcein fluorescence examination

The thighbone freeze-dried section was placed in a box containing silica gel and removed from the cryochamber. The section was placed on a glass slide and protected with a cover glass. There was no mounting medium. Calcein fluorescence was observed with a fluorescence microscope (Olympus, Japan). A blue beam, between 450 and 495 nm in wavelength, was used for calcein excitation. An emission filter, which permits the passage of wavelengths exceeding 510 nm, was used for observation.

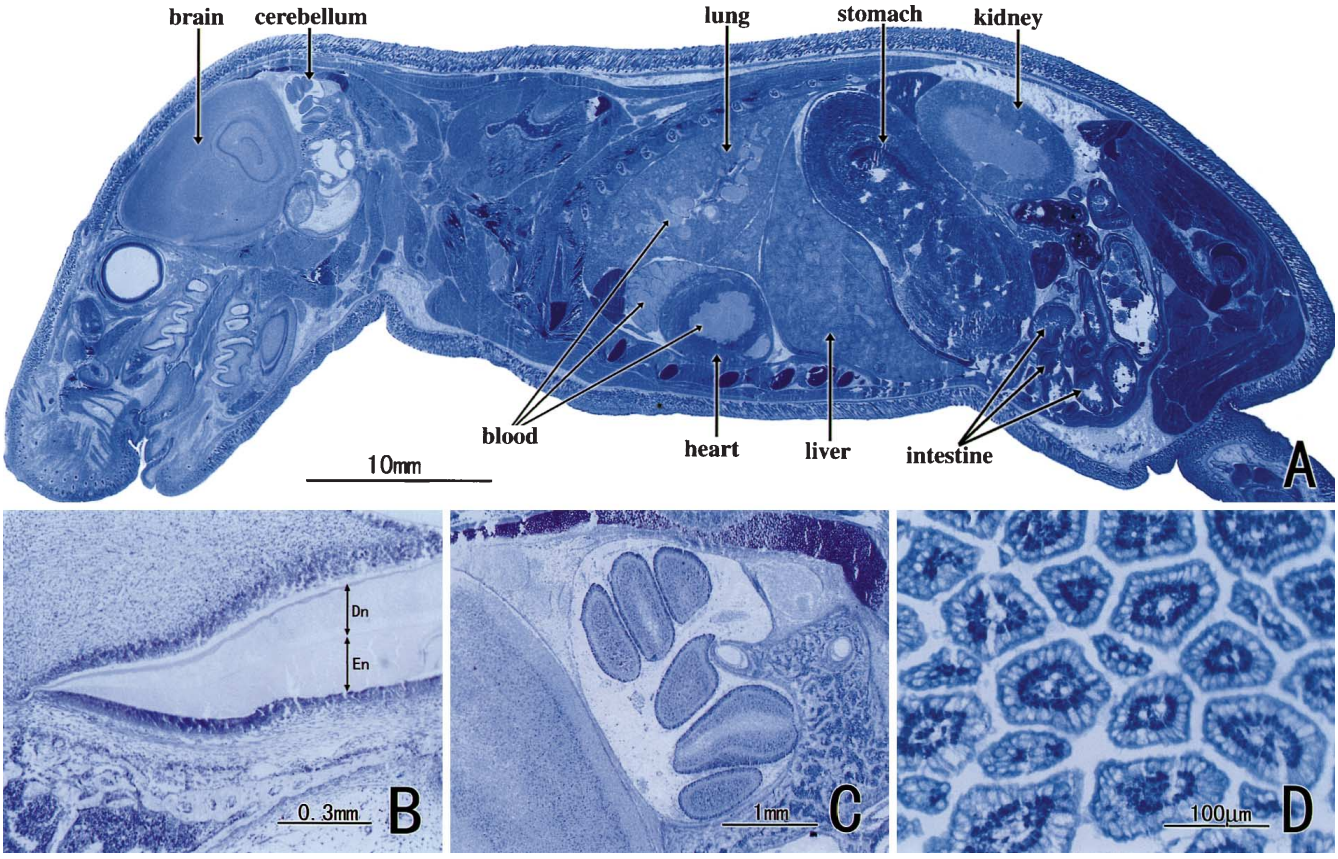

Fig. 2A–D A 2-μm-thick section of the 10-day-old rat (toluidine blue staining). **A** Whole body. **B** Molar. **C** Cerebellum. **D** Small intestine

Effects of fixation and decalcification

To study the effects of fixation and decalcification on enzyme activity, alkaline phosphatase (ALPase) was examined. The fresh sections were used as a control. To examine the fixation effect, the fresh sections were fixed with a 4% paraformaldehyde (PFA) 0.1 M phosphate buffer (pH 7.4) at room temperature for 2, 6, 12, and 24 h. To examine the decalcification effect, the fresh sections were decalcified with 5% EDTA adjusted to pH 7.0 for 6 days. For both examinations, the fresh sections were fixed with PFA for 2 h and decalcified with EDTA for 6 days. Two sections were used in each examination. The ALPase activity was demonstrated using the method described by Burstone (1958). Each section was incubated at room temperature for 2 min and either mounted with glycerin under a cover glass or sandwiched between the film and the glass slide.

Photographs

Photographs were taken with a digital camera (Fujix HC-300; Fuji Photo Film, Japan) connected to a desk-top computer. The resulting images were reproduced using PhotoShop (Adobe Systems, USA) and printed with a color printer (Pictrography 4000; Fuji Photo Film).

Results

The entire 10-day-old rat and the bone and tooth samples of the 7-month-old rat were easily cut into 2- to 50-μm-thick sections. All sections shown in this paper are 2 μm thick. Figure 2A shows the whole-body section stained with toluidine blue. Figure 2B–D shows the upper molar, cerebellum, and intestine at high magnification. Soft tissues such as the brain, muscles, glands, intestine, lung, liver, pancreas, kidney, and spleen were perfectly preserved. Blood remained in the heart and blood vessels. In addition, the calcifying bone, dentine, and enamel were almost perfectly preserved. All tissue cells were easily identified at high magnification (ameloblast and odontoblast in the tooth germ, epithelial cells in the small intestine, mesangial cells in the kidney, and glandular cells in the glands).

Sections prepared from the adult rat thighbone are shown in Figs. 3 and 4. Portions of the skeletal muscle were damaged during dissection, but the remaining skeletal muscle, ligament, and bone marrow were satisfactorily preserved (Fig. 3A). In the diaphysis, the outer and inner basic lamellae were easily distinguished. The alizarin red S-stained section showed that the calcified bone was almost completely preserved (Fig. 3B). Figure 4C shows the epiphysis portion of Fig. 3A (arrow) at high magnification. Some small cracks caused by cutting were observed in the bone at high magnification, but topographical and structural relationships between the calcified tissues and the soft tissues were well retained. The osteoblasts were clearly seen on the bone surface. In the bone marrow, many types of cells such as myeloblasts and eosinophilic leukocytes were identified.

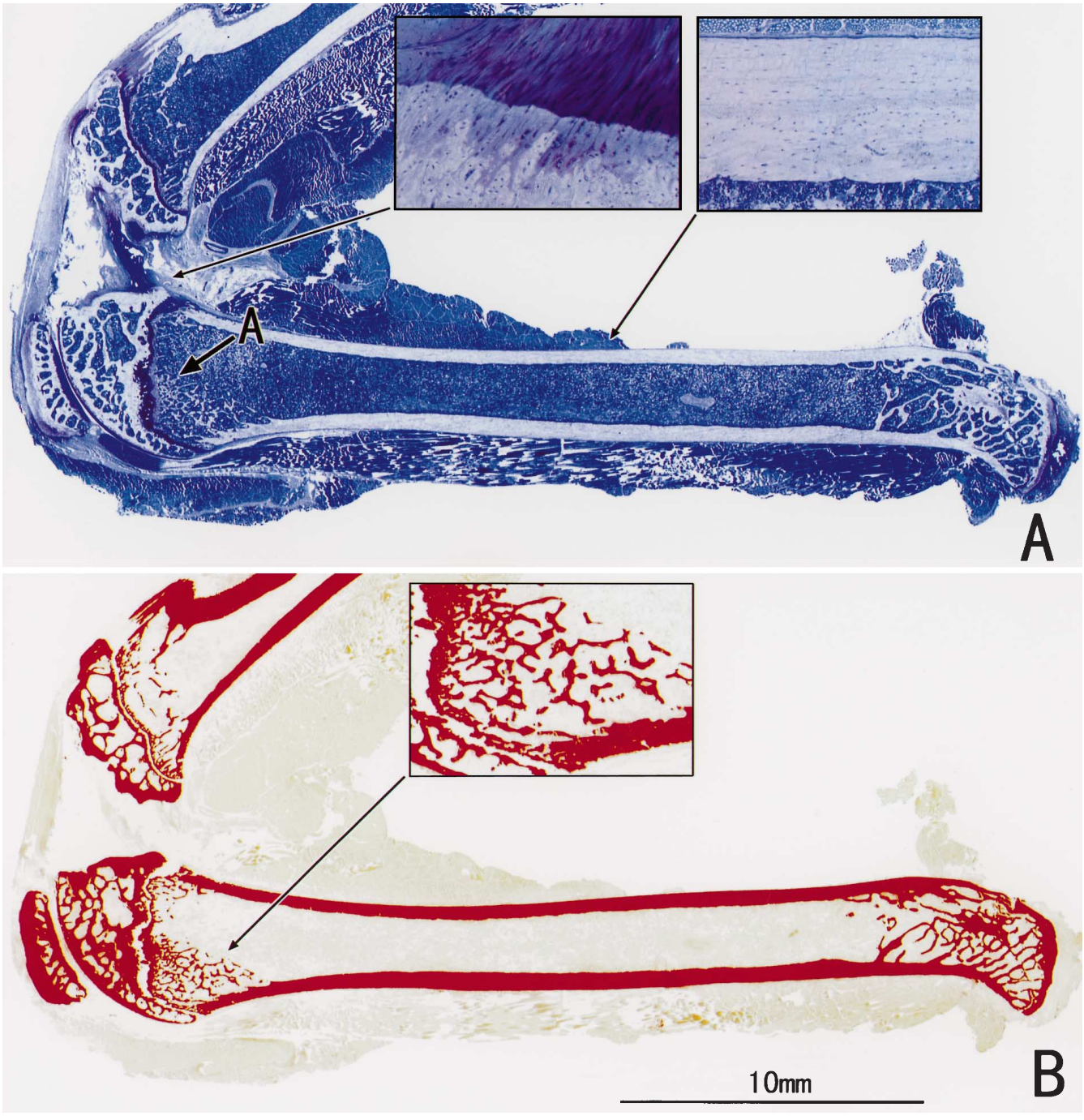

Fig. 3A,B A 2-µm-thick serial sections of the 7-month-old rat thighbone. *Insets* of the micrograph show magnified views of the areas indicated with each *arrow*. **A** Toluidine blue staining. **B** Alizarin red S staining. *A* Area of epiphysis shown at high magnification in Fig. 4C

Calcein fluorescence in the thighbone serial sections is shown in Fig. 4A,B,D. Fluorescence appeared on most of the bone surface in the freeze-dried sections (Fig. 4A). Double fluorescent lines were noticed on some parts of the surface layer (Fig. 4D). Very intense fluorescence was observed in spots on the bone surface layer. In the fixed stained section, fluorescence was significantly reduced (Fig. 4B).

Figure 5 shows the lower jaw serial sections of the adult rat. The soft tissues and the hard tissues were satisfactorily preserved (Fig. 5A,B). In the molar, the dentine and the cementum were perfectly preserved and the noncalcified areas in the dentine and cementum were clearly shown (Fig. 5C). The topographical relationship between the periodontal fibers and the fibroblasts in the periodontium was maintained. In the lower incisor, the enamel and the dentine were almost perfectly preserved. The ameloblasts and odontoblasts remained attached to the enamel and dentine surfaces (Fig. 5D).

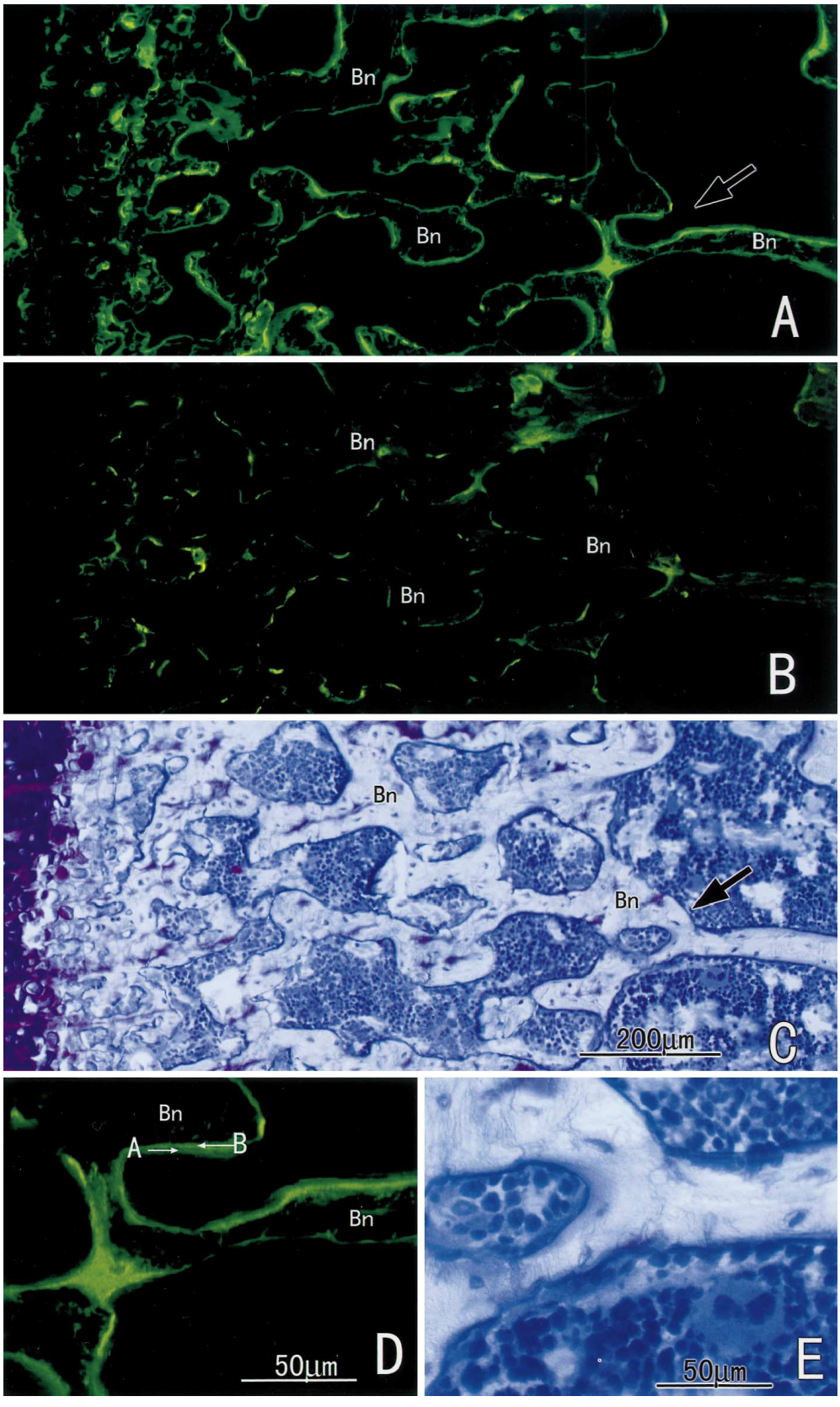

Fig. 4A–E Calcein fluorescence in the epiphysis of the 2-μm-thick sections prepared from the 7-month-old rat thighbone.**A** Fluorescence in the freeze-dried section. **B,C** Fluorescence and light micrograph in the toluidine blue-stained section. **D,E** Highly magnified views of the portions indicated with the *arrows* in **A** and **C**, respectively. *Arrows* in **D** show the double fluorescence lines. *Bn* Bone

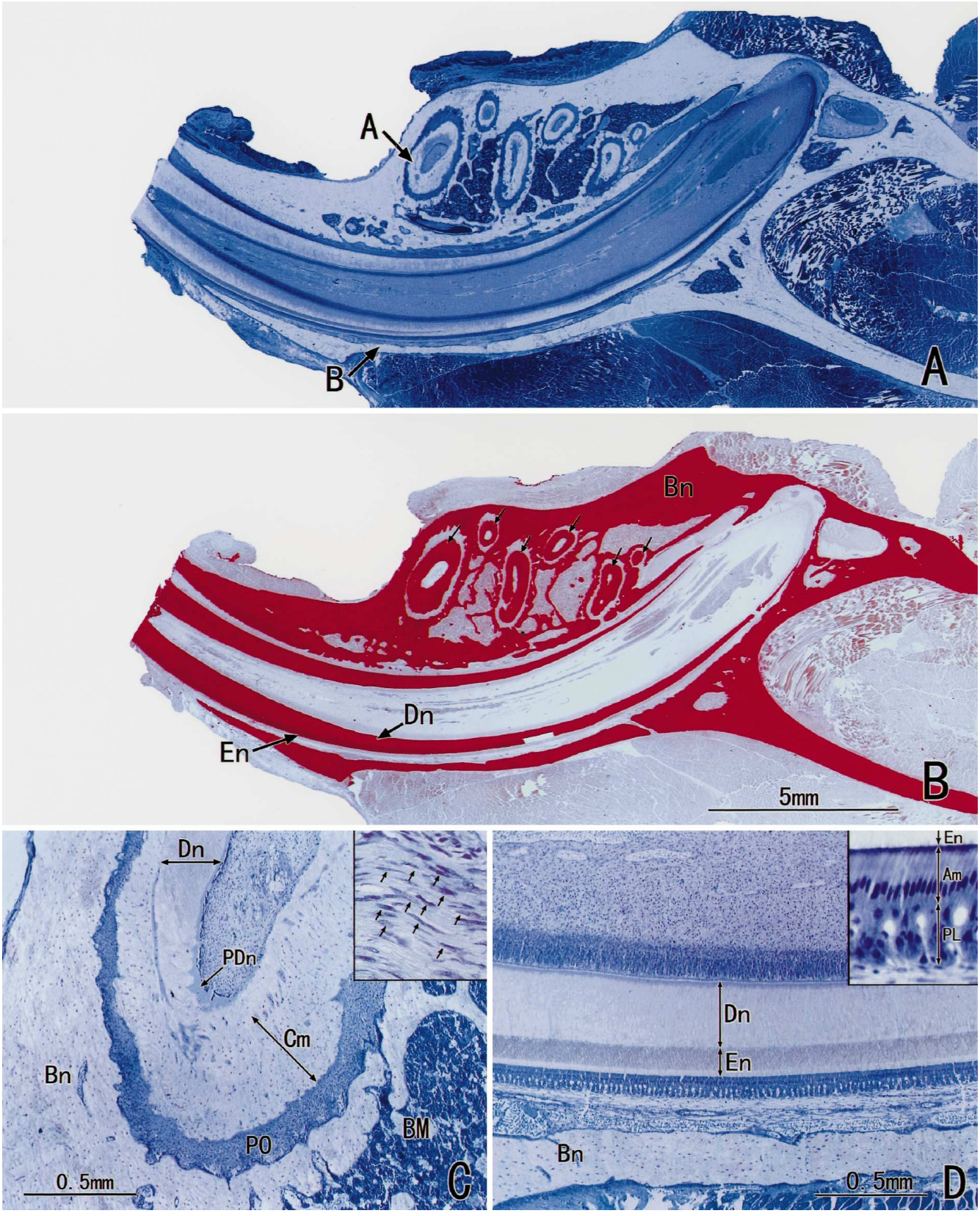

Fig. 5A–D A 2-μm-thick serial sections of the 7-month-old rat lower jaw. **A** Toluidine blue staining. **B** Alizarin red S staining. **C,D** Magnified views of the portion indicated respectively by the *arrows A* and *B* in **A**. *Insets* of **C** and **D** show periodontium and ameloblast layer. *Arrows* in **B** and **C** indicate the root of the molar and fibroblasts, respectively. *Bn* Bone, *Dn* dentine, *PDn* predentine, *Cm* cementum, *PO* periodontium, *En* enamel, *Am* ameloblast, *PL* papillary layer

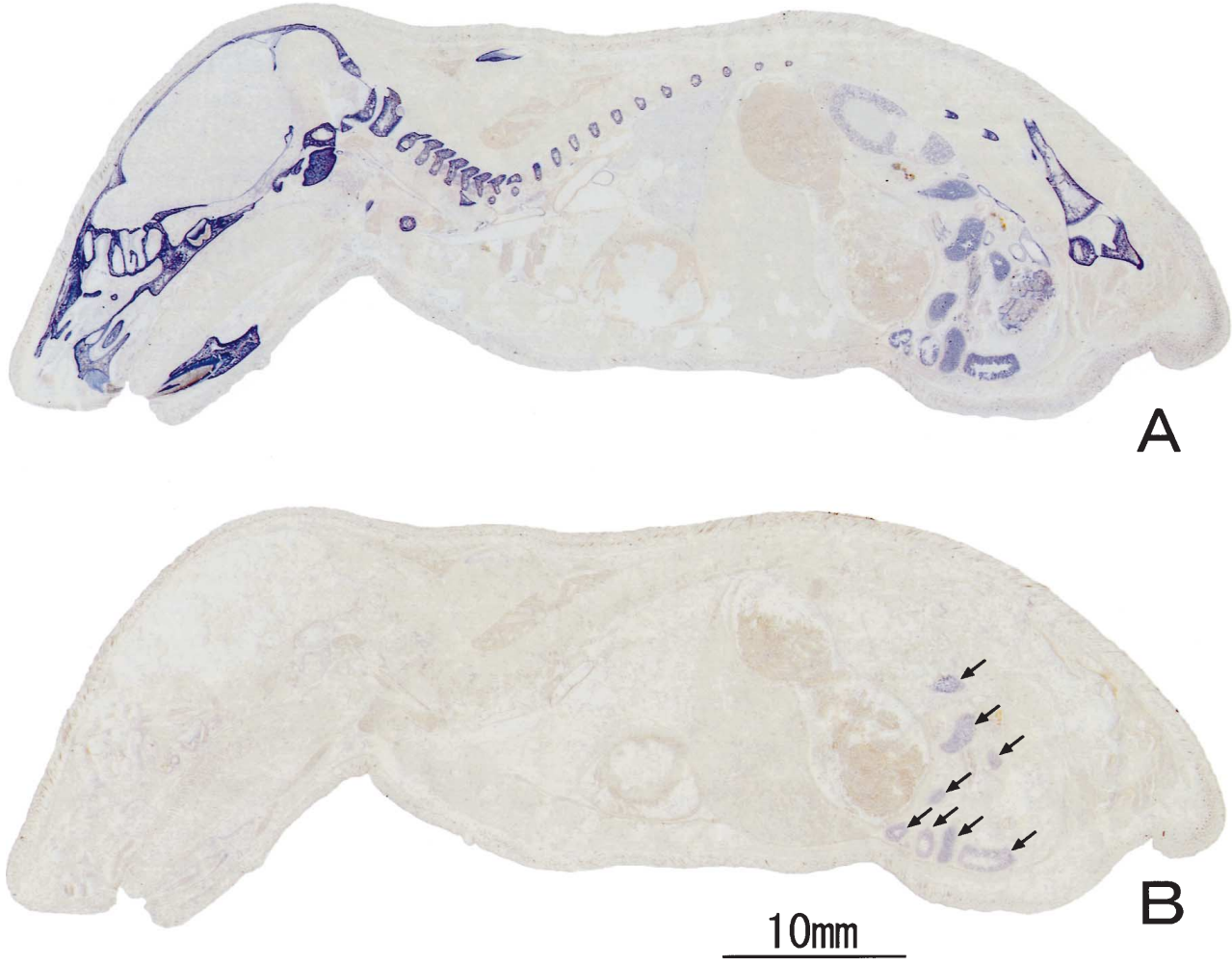

Fig. 6A,B Alkaline phosphatase activity (shown in *blue*) in the 10-day-old rat molar tooth. **A** Fresh section. **B** Section fixed with 4% paraformaldehyde for 2 h and decalcified with 5% EDTA for 6 days. *Arrows* in **B** indicate the small intestine

ALPase activity in the fresh section was very strong and the reaction products shown in Fig. 6A were produced within 2 min. We confirmed the observation of many other researchers that ALPase activity is distributed through many tissues (kidney, intestine, bone, tooth germ, and glands) and that it is localized on the cell membrane. The 4% PFA fixation and the 5% EDTA decalcification reduced ALPase activity. ALPase activity on the kidney, the bone, the tooth germ, and the gland was reduced conspicuously, as shown in Fig. 6B, when the sections were fixed with the PFA for 2 h and decalcified with EDTA for 6 days. But the activity on the small intestine remained.

Discussion

The 2- to 50-µm-thick sections were prepared routinely from large samples and from completely calcified bone and tooth samples. In order to achieve this, some refinements were made in the equipment. A disposable tungsten carbide blade was used and steps were taken to avoid temperature increases in the chamber. A superhard reusable knife made of a hardened tungsten steel permits cutting of hard tissues such as bone, dentine, and enamel (Van Noorden and Vogels 1986; Hill and Elde 1990; Haines 1992; McElroy et al. 1993; Nakamura et al. 1994). However, even a knife made of such a hard material loses sharpness when cutting completely calcified hard tissues. A disposable blade made from special high-grade tungsten carbide is also able to cut hard tissue. A sharp knife is indispensable to a high quality section, so we designed a holder to use a disposable knife. The blade can be moved from side to side in the holder so that the samples are always cut with a sharp knife-edge. Another advantage of the disposable blade is the ability to change blades without changing the conditions of an experiment. A disposable blade is also economical.

Doing handwork in a cryochamber with a large lid causes an undesirable temperature increase, particularly in the upper half of the cryochamber where the handwork is done. To prevent temperature increase, we installed a new lid with a small access window to the cryochamber. A small electric fan mounted inside the cryo-

chamber was used to gently circulate cool air in the cryochamber. These steps minimized temperature increase.

Sections prepared at lower temperatures show excellent histology. However, we were unable to make sections using commercially available adhesive tape such as Scotch type 688, 800, or 810 at such low temperatures because the adhesion of the tape deteriorated rapidly with a drop in temperature. The polyvinylidene chloride film coated with the synthetic rubber cement maintains its adhesion at low temperatures and holds and supports the sections tightly during cutting

Using these improvements, large samples and completely calcified bone, dentin, and enamel samples were cut without special technical skills and the tissues were satisfactorily preserved as shown in Figs. 2, 3, 4, and 5. Almost all 3-μm-thick sections were satisfactorily preserved. These sections withstood immunohistochemical treatment for 4 days. There was no deterioration even after leaving the sections in 100% ethanol for more than 1 month.

Ice crystal formation damages tissue and appears conspicuously in the central portion of the large samples. This damage can be minimized by using hexane containing solid hexane (–94°C) or liquid nitrogen instead of dry-ice hexane. This is especially true when dissected and trimmed samples are used. Damage can be further minimized by using liquid propane. However, there appears to be no way to avoid it completely. We found that the ice crystals could be eliminated by thawing the frozen section immediately after cutting and that this technique considerably improves histological views. We recommend this technique when the section is examined at high magnification.

Either fixation or dehydration as shown in Fig. 4 results in fluorescent dye loss in the bone. It also decreases enzyme activity (Van Noorden and Vogels 1986; Wakisaka 1986; Van Den Munckhof et al. 1994). Our technique (shown in Figs. 4, 6) eliminates both these problems. Though the dates are not shown, the immunoreactivity of the fresh section is better than that of fixed and/or decalcified sections.

This technique can be used with conventional cryomicrotome equipment. Sections can be used for many types of research including characterizing enzyme, antigen, mRNA, and water-soluble molecule (or element) distribution during growth and development of general body tissue. When such research is done using serial sections, the technique is a very powerful tool.

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