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ORIGINAL PAPER

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# Effects of fixation and decalcification on the immunohistochemical localization of bone matrix proteins in fresh-frozen bone sections

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**Abstract** To examine the stability of bone matrix proteins for crystal dislocation, the immunolocalization of type I collagen, bone sialoprotein, and osteopontin was investigated during different stages of fixation and decalcification. Four-week-old rat femurs were rapidly frozen, and were sectioned without fixation or decalcification. Thereafter, following or bypassing fixation in 4% paraformaldehyde, these sections were decalcified in 5% EDTA for 0-5 min. Before decalcification, marked radiopacity of bone matrix was observed in contact microradiography (CMR) images, and electron probe microanalysis (EPMA) demonstrated intense localization for phosphorus and calcium. In fixed and unfixed sections without decalcification, immunolocalization of bone matrix proteins were almost restricted to osteoid. After 1 min of decalcification, reduced radiopacity was apparent in the CMR images, and less phosphorus and calcium was observed by EPMA, which completely disappeared by 5 min decalcification. After 3-5 min of decalcification, unfixed sections showed that these proteins were immunolocalized in bone matrix, but were not detectable in osteoid. However, fixed sections demonstrated that these were found in both bone matrix and osteoid. The present findings suggest that bone matrix proteins are embedded in calcified matrix which is separated from the aqueous environment and that they hardly move, probably due to firm bonding with each other. In contrast, matrix proteins in osteoid are subject to loss after decalcification because they may be bound to scattered apatite crystals, not to each other.

**Keywords** Fresh-frozen section · Non-decalcified section · Bone matrix proteins · Immunohistochemistry · Bone

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## Introduction

Histochemical methods have progressed well to date, because the exact protein localization or gene expression can be detected using enzyme and immuno-cytohistochemistry or in situ hybridization. However, calcified hard tissue, such as bone and teeth, remains difficult to analyze due to the hardness of tissue samples. Although both fixation and decalcification are usually needed for conventional histological and histochemical observations, these procedures may cause denaturation of proteins, thus resulting in a reduction in 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 particular, decalcification may affect the degree of staining, since some materials are easily lost and/or dislocated from the original sites during the process. In order to avoid the disadvantage associated with fixation and decalcification, a number of attempts have been made to obtain unfixed and non-decalcified sections of calcified tissue (Hammarstrom 1986; Hill and Elde 1990; Fink 1992; McElroy et al. 1993; Shimada and Watanabe 1995). However, most of those previous sections could be improved, because the sections were too thick and because the sizes of the available samples were limited to

small ones. Therefore, many researchers have tried to establish new techniques that enable whole bone or tooth sectioning without fixation or decalcification.

In 2000, a new method of fresh-frozen sectioning of calcified hard tissue was reported by Kawamoto and Shimizu (2000). It provides less than 2 μm thick sections of calcified tissues without fixation and decalcification, and is widely applicable for various morphological analyses such as histochemistry, immunohistochemistry, in situ hybridization, and laser microdisectioning (Kawamoto 2003). This method has several advantages, in that sections can be prepared from their original specimens within several hours, while loss and/or dislocation of water-soluble materials is prevented, since the samples do not undergo chemical fixation and decalcification, thus preserving the immunoreactivity of each section.

This method can be used to detect the exact immunohistochemical localization of various calcified tissue proteins. Calcified hard tissue contains type I collagen, as well as several non-collagenous proteins, such as bone sialoprotein (BSP) and osteopontin (OPN). Type I collagen localized not only the calcified matrix or their formative cells, but also a wide range of connective tissues, such as skin, muscle, periosteum, and tendon (Nanci 2003). BSP, on the other hand, is confined to bone, dentin, cementum, and cartilage, as well as the cells which produce these tissues (Begue-Kirn et al. 1994; Macneil et al. 1994; Bosshardt and Nanci 1998). OPN is more widely distributed than BSP (Helder et al. 1993; Bronckers et al. 1994). In addition to areas of BSP localization, OPN is present in non-mineralized tissue, such as kidney (Mark et al. 1988) and urinary tract epithelium (Arafat et al. 2002), as well as in macrophages (Miyazaki et al. 1990), and activated T-lymphocytes (Patarca et al. 1986). All of these collagen and sialic acid-rich proteins seem to be associated with the mineralization process (Hunter et al. 1996; Hoshi et al. 2001), but the interactions among these proteins and minerals should be elucidated in more detail. Thus, in the present experiment, we used intact calcified tissue sections without fixation and decalcification, treated the sections with various degrees of fixation and decalcification, and examined the immunolocalization of calcified tissue proteins in those sections. Therefore, this method is the first technique that can reveal bonding condition among bone matrix proteins and minerals in calcified tissue sections.

## Materials and methods

### Preparation of frozen and non-decalcified sections

All experiments were performed according to strict guidelines set forth by the Matsumoto Dental University Committee on Intramural Animal Use. Four-week-old Wistar rat (weighing 85–90 g) femurs were used. Following anesthesia (intraperitoneal injection of pentobarbital, 40 mg/kg), the femurs were removed and immediately frozen in hexane (−80°C), cooled with a cooling apparatus (PSL-1800; Tokyo Rikakikai Co. Ltd, Japan). Each sample was embedded in 5% carboxymethyl cellulose (CMC) gel and completely frozen. Each frozen CMC sample was then attached to the sample stage of a cryomicrotome (CM3500; Leica Instruments, Germany) in a cryochamber (−25°C). After sitting for 30 min, each CMC sample was covered with a polyvinylidene chloride film (10 μm thick; Asahikasei Kogyo Co., Japan), coated with synthetic rubber cement (Cryoglue Type I; FINETEC Co. Ltd, Japan), and sagittally sectioned with the film, at a thickness of 5 μm, using a disposable tungsten carbide blade (Jung TC-65; Leica Instruments, Germany).

### Contact microradiogram observations

Mineral density was examined by contact microradiography (CMR). Sections were removed from the cryochamber and immersed in 100% ethanol to remove trapped air bubbles for 5 min, after which they were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 5 min at 20°C. After this, the sections underwent decalcification with 5% EDTA (pH 7.4) for 0, 1, 3, and 5 min at 20°C. After drying at room temperature, the sections were mounted on a high-resolution film (Pelicula So-343; Kodak, Rochester, N.Y., USA) and CMR images were taken using a soft X-ray unit (SOFRON SRO-iM50; SOFRON Co., Japan) at 10.5 kVp, 2 mA, and an exposure time of 6 min.

### Electron probe microanalysis (EPMA)

In order to investigate the degree of decalcification, phosphorus and calcium were detected by EPMA (JXA-8200; JEOL Ltd, Japan). Fixed and decalcified (0 and 1 min) sections were dried, and carbon-coated (JEE-420; JEOL) to prevent charging. Cortical bone areas were measured using a beam current of 40 nA and an accelerating voltage of 15 kV.

### Histological observations

Fixed and decalcified (0, 1, 3, and 5 min) sections were used. These sections were stained with 0.1% toluidine blue (pH 7.0) or 1% alizarin red S for 3 min, after which they were washed in running water for 5 min. The sections with film were placed on the sectioned side on a glass slide coated with 30% glycerol.

### Immunohistochemical observations

For immunohistochemical localizations of type I collagen, BSP, and OPN, fixed and unfixed sections were

treated with 0.3% $H_2O_2$ in a solution of 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 30 min at room temperature to incubate endogenous peroxidase. The sections were then pretreated with 10% bovine serum albumin (Seikagaku, Tokyo, Japan) in PBS for 30 min at room temperature and incubated with polyclonal rabbit antibodies against rat type I collagen (Chemicon, Temecula, Calif., USA), human BSP (LF-120, kindly provided by Dr. L.W. Fisher), and mouse OPN (kindly provided by Dr. M. Fukae) at a 1:100 dilution for 2 h at room temperature. Sections were then rinsed in PBS and reacted with FITC-conjugated goat anti-rabbit IgG (Chemicon) at a 1:50 dilution for 1 h at room temperature, after which they were visualized using a fluorescence microscope (Axioplan 2; Carl Zeiss, Germany). Non-immune rabbit serum was diluted to the same strength for use as negative controls, instead of primary antibodies.

## Results

### CMR observations

Calcified bone matrix showed intense radiopacity without decalcification (Fig. 1a). After decalcification, radiopacity was reduced, and eventually there was no radiopaque area at all by 5 min decalcification (Fig. 1b–d).

### Alizarin red S staining

In the non-decalcified section, almost all bone matrix was stained strongly, although some areas demonstrated weaker staining. Osteoid was negative for this staining (Fig. 2a). After decalcification, a gradual reduction in staining of the bone matrix was observed, and staining was not evident after 5 min (Fig. 2b-d). Staining of bone marrow and skeletal muscle was not observed in non-decalcified or decalcified sections.

The CMR images and alizarin red S staining results suggest that the bone matrix of the cortical area was decalcified by about 50% by 3 min, and completely within 5 min. Trabecular bone showed a similar time course of decalcification (data not shown).

### EPMA observations

Phosphorus was distributed extensively throughout mineralized cortical bone, and slightly in non-mineralized areas of bone marrow and skeletal muscle (Fig. 3a). After 1 min of decalcification, almost no phosphorus was observed in bone and non-mineralized areas (Fig. 3c). Calcium was observed in calcified cortical bone and reduced levels were observed after decalcification (Fig. 3b, d).

### Toluidine blue staining

Prior to decalcification, the bone matrix of cortical bone did not stain with toluidine blue (Fig. 4a). After decalcification, however, a gradual increase in metachromagia with toluidine blue staining was observed. The intensity of staining of bone marrow and skeletal muscle did not change with decalcification (Fig. 4b–d). In trabecular bone, bone matrix was hardly stained without decalcification (Fig. 4e). After 1 min of decalcification, the cartilage matrix embedded in the central part of trabecular bone was metachromatic, while osteoid in the surface of trabecular bone did not show the metachromagia (Fig. 4f). After 3 min, the metachromagia of the

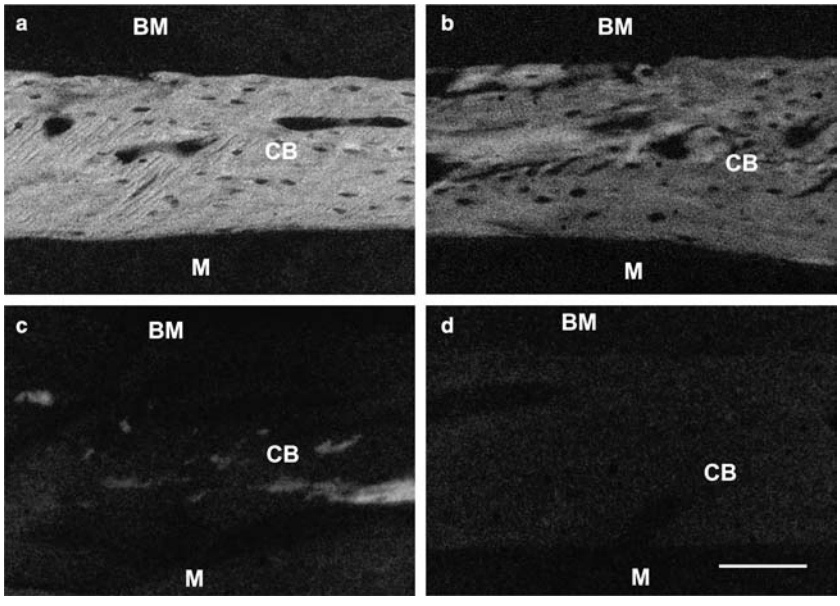


**Fig. 1** CMR analysis of cortical bone. Sections decalcified for 0 (**a**), 1 (**b**), 3 (**c**), and 5 (**d**) min. **a** Calcified bone matrix demonstrates intense radiopacity. **b** Reduced radiopacity is observed after decalcification. **c** Radiopaque area is still partially observed. **d** There is no radiopaque area at all. *BM* bone marrow; *M* skeletal muscle. *Bar* = 200 μm

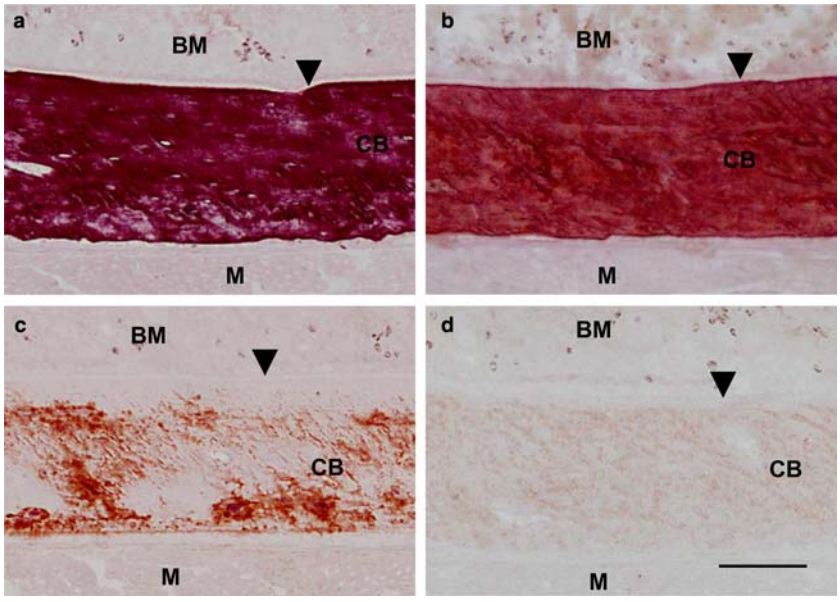


**Fig. 2** Alizarin red S staining of cortical bone. Sections decalcified for 0 (**a**), 1(**b**), 3 (**c**), and 5 (**d**) min. **a** Intense staining is apparent throughout the bone matrix, although none was observed in osteoid (*arrow head*). **b–d** Following decalcification, a gradual reduction in intensity of staining of bone matrix is observed, which disappeared after 5 min of decalcification (b–d). *BM* bone marrow; *CB* cortical bone; *M* skeletal muscle. *Bar* = 200 μm

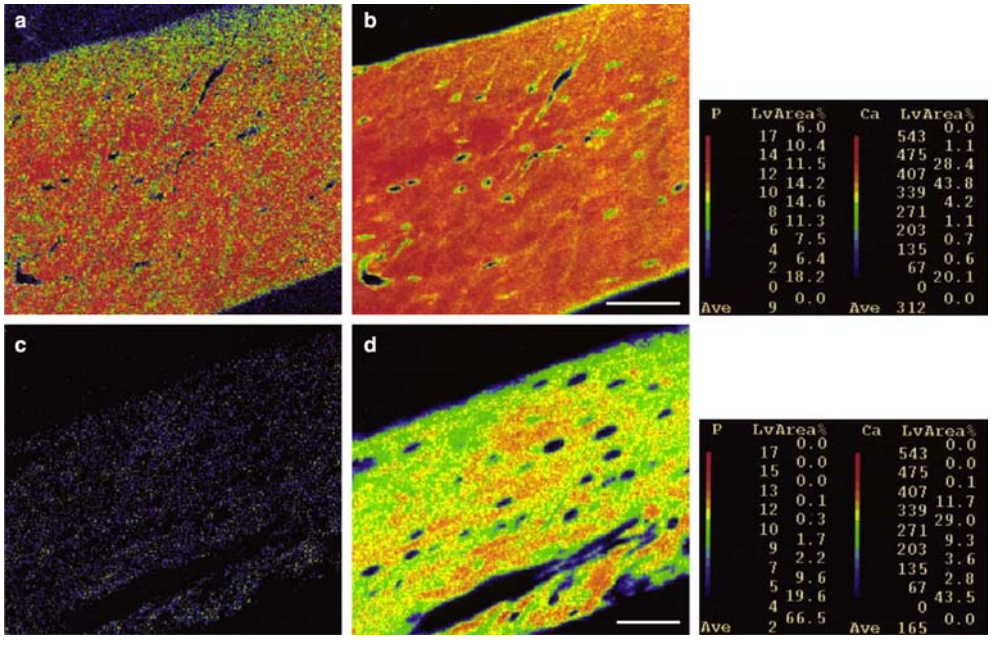


**Fig. 3** Phosphorus (**a, c**) and calcium (**b, d**) mapping images using EPMA after 0 (**a, b**) and 1 (**c, d**) min of decalcification. **a** Phosphorus is localized intensively in cortical bone, and slightly in non-mineralized areas. **b** Calcium is detected in calcified bone. **c-d** Following decalcification, phosphorus and calcium localizations are clearly decreased. *Bar* = 100 μm

calcified cartilage had faded, and it completely disappeared after 5 min (Fig. 4g–h).

## Immunohistochemical observations

The patterns of localization of type 1 collagen and two non-collagenous proteins (BSP and OPN) are summarized in Tables 1 and 2, respectively.

## Unfixed sections

Prior to decalcification, immunoreactivity for type 1 collagen was observed at the periphery of trabecular bone corresponding to osteoid, and reticulately in bone marrow. In contrast, bone matrix was hardly stained (Fig. 5a). Following decalcification, increased immunostaining for type I collagen in the bone matrix was observed, although its localization diminished at the periphery of bone (Fig. 5b, c). The stainings in bone marrow were observed reticulately in decalcified as well as non-decalcified sections (Fig. 5 a–c).

Intense immunoreactivity for BSP and OPN was observed in the periphery of trabecular bone, but not in bone matrix of non-decalcified sections. (Fig. 5d, g). After decalcification, immunoreaction of BSP and OPN was strongly observed lineally in bone matrix, around the cement line. The central part of trabecular bone, corresponding to the calcified cartilage matrix, was not immunoreactive for BSP or OPN. Immunoreactivity for BSP and OPN appeared in bone matrix and disappeared

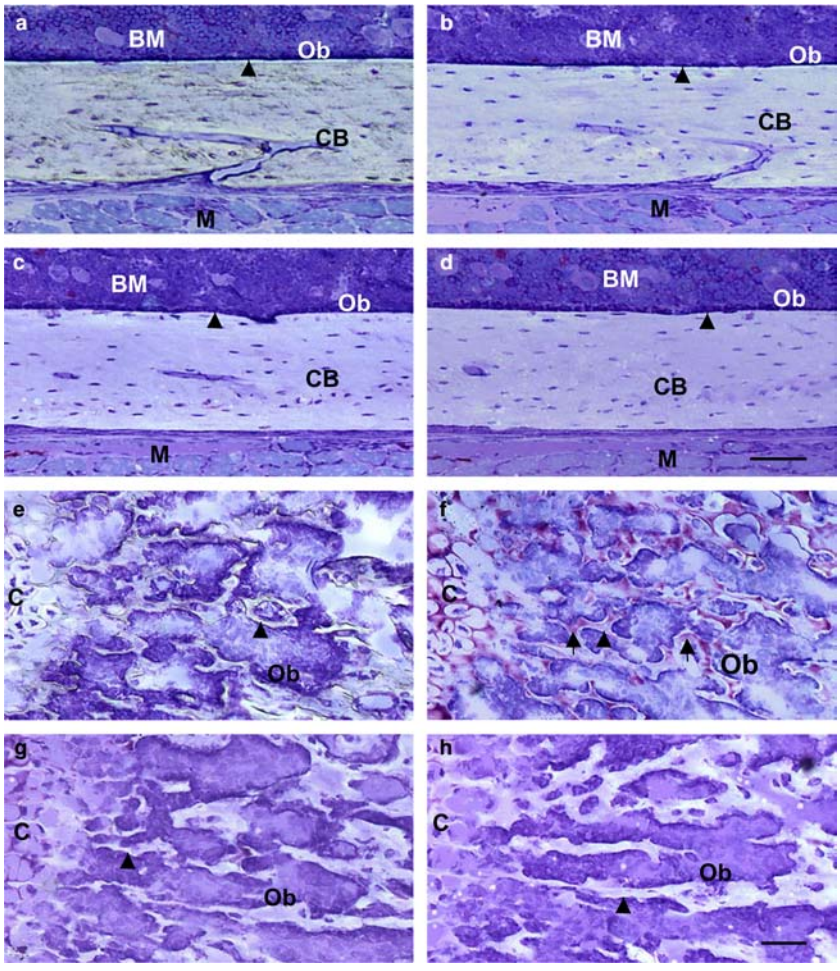


**Fig. 4** Toluidine blue staining of cortical (**a–d**) and trabecular bone (**e–i**). Sections decalcified for 0 (**a, e**), 1(**b, f**), 3 (**c, g**), and 5 (**d, h**) min. **a, e** Toluidine blue staining is faint in cortical (CB) and trabecular bone prior to decalcification. **b–d** A gradual increase in metachromagia of calcified cortical bone matrix was observed as decalcification progressed. **f–h** Intense metachromagia of calcified cartilage matrix of trabecular bone (*arrows* in f) is observed after 1 min of decalcification. Osteoid (*arrow head*) is hardly stained regardless of the degree of decalcification. As decalcification progressed, a reduction in metachromagia of calcified cartilage matrix was seen. *BM* bone marrow; *C* cartilage; *CB* cortical bone; *M* skeletal muscle; *Ob* odontoblast. *Bar* = 200 μm (**a–d**), 100 μm (**e–h**)

from the periphery of bone after decalcification (Fig. 5e, f, h, i). OPN demonstrated a similar pattern of immunoreactivity as BSP; however, weaker staining of the bone matrix was observed (Fig. 5h).

### Fixed sections

Type I collagen was localized at the periphery of trabecular bone and in bone marrow as in unfixed and non-decalcified sections (Fig. 6a). After decalcification, degree of immunostaining for type I collagen increased in calcified bone matrix, and staining at the periphery of bone remained (Fig. 6b–c).

In non-decalcified sections, intense immunostaining for BSP and OPN was observed at the periphery of bone, while almost none was observed in calcified bone matrix (Fig. 6d, g). After decalcification, intense staining for BSP and OPN was observed in areas corresponding to osteoid and cement lines, as well as in bone matrix (Fig. 6e, f, h, i). OPN showed the same localization as BSP, but the staining in bone matrix was weaker than that of BSP (Fig. 6h–i). Calcified cartilage matrix showed no immunoreactivity for BSP or OPN (Fig. 6a–i).

None of the specific localizations was identified in the negative controls for type I collagen, BSP or OPN (data not shown).

## Discussion

A number of strategies have been devised to enable researchers to examine calcified hard tissue without decalcification. The method of fresh-frozen sectioning, first described by Kawamoto and Shimizu (2000), is useful for the preparation of the serious unfixed and non-decalcified sections. In the present study, we examined 5 μm thick fresh-frozen bone sections. These sections were decalcified by about 50% within 3 min, and completely by 5 min using 5% EDTA, in CMR images and alizarin red S staining results. Further, EPMA results showed a reduction in phosphorus and calcium content after 1 min of decalcification. Using these sections, we analyzed immunohistochemically the relation between the stability of bone matrix proteins and crystal dislocation.

In unfixed sections without decalcification, immunoreactivity for type I collagen, BSP, and OPN was restricted to osteoid. After decalcification, however,

**Table 1** Immunolocalization of type I collagen in trabecular bone

| Sections | | Bone | Osteoid | Cement line | Bone marrow |
|---|---|---|---|---|---|
| Non-fixed | Non-decalcified | – | + | – | + |
| | Decalcified | + | – | – | + |
| Fixed | Non-decalcified | – | + | – | + |
| | Decalcified | – | + | – | + |

+ Immunoreactive, – Negative

**Table 2** Immunolocalization of BSP and OPN in trabecular bone

| Sections | | Bone | Osteoid | Cement line | Bone marrow |
|---|---|---|---|---|---|
| Non-fixed | Non-decalcified | – | + | – | – |
| | Decalcified | + | – | + | – |
| Fixed | Non-decalcified | – | + | – | – |
| | Decalcified | + | + | + | – |

+ Immunoreactive, – Negative

immunoreactivity for these proteins was detected in bone matrix, and not in osteoid. It was considered that the immunoreactivity of bone matrix proteins might be masked by minerals, and that after decalcification, they become exposed and immunodetectable. Furthermore, the present findings suggest that bone matrix proteins embedded in calcified matrix which is separated from aqueous environment are relatively immobile, perhaps because of strong bonds between them. In contrast, matrix proteins embedded in osteoid might be bound to scattered apatite crystals, rather than each other, and may be lost during decalcification.

Some bone matrix proteins may be cross-linked to each other, playing roles in bone matrix maturation and calcification. OPN forms covalent cross-links with fibronectin (Beninati et al. 1994), thereby enhancing its cell–matrix interfacial adhesion properties and providing increased physical dimensions. Furthermore, BSP and OPN in mineralized bone matrix form large protein aggregates in the presence of tissue transglutaminase (Kaartinen et al. 2002). This further supports our assertion that BSP and OPN are relatively immobile in mineralized bone following decalcification. Meanwhile, some proteins, including OPN and BSP, are localized in calcified nodules in osteoid (McKee et al. 1993, Hoshi et al. 2001). They are thought to associate with hydroxyapatite, and thus to regulate crystal growth and formation. As demonstrated in the present study, since they may not bind to each other or to other organic substances, they might be lost or drift when hydroxyapatite disappear after decalcification. Although BSP possesses nucleation activity of hydroxyapatite in vitro, OPN plays a negative role in crystal growth (Hunter et al. 1996). The ability of those matrix proteins to move easily may rather be advantageous for hydroxyapatite growth and maturation of calcified matrix.

In fixed sections, bone matrix proteins were observed in area of bone matrix and osteoid after decalcification. These findings suggest that fixation with paraformaldehyde, followed by decalcification with EDTA, prevents loss of bone matrix proteins from osteoid. However, it is worth noting that fixation and decalcification usually reduce enzyme activity and immunoreactivity (Baker et al. 1958; Takeshita et al. 1983; Mullink et al. 1985;

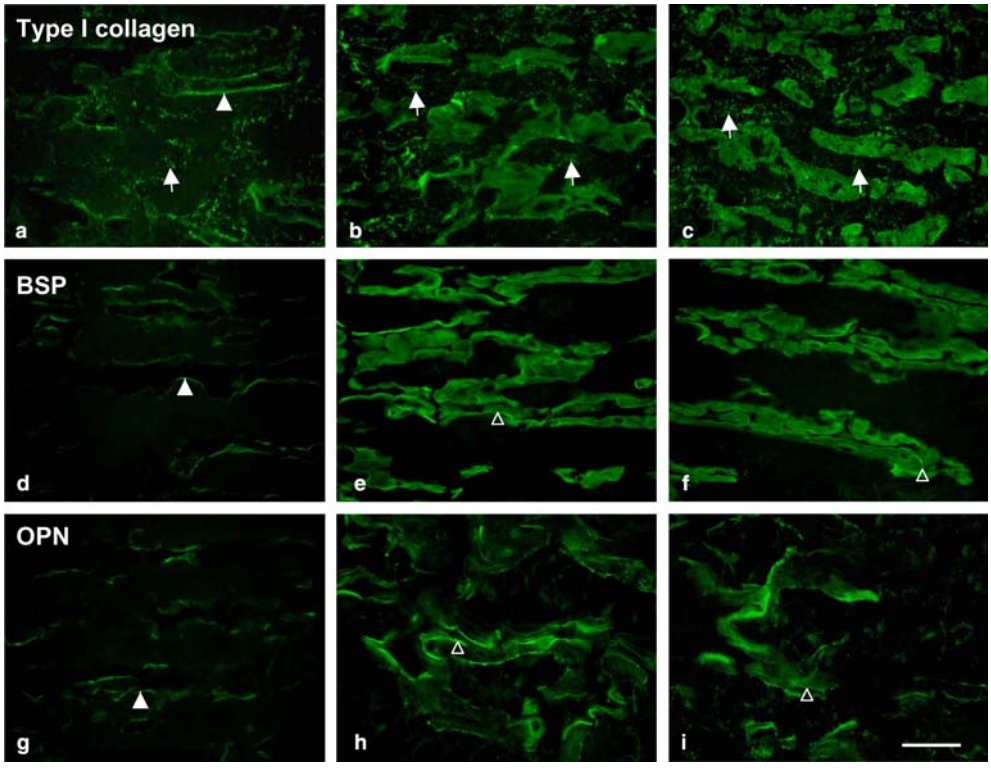


**Fig. 5** Immunolocalization of type I collagen (**a–c**), BSP (**d–f**), and OPN (**g–i**), in trabecular bone. Unfixed sections decalcified for 0 (**a, d, g**), 3 (**b, e, h**), and 5 (**c, f, i**) min. **a** Immunolocalization of type I collagen is observed in the periphery of trabecular bone according to osteoid area (*arrow head*), as well as in bone marrow (*arrow*), although bone matrix is hardly stained. **b–c** Type I collagen is found in bone matrix and bone marrow, but not in osteoid. **d, g** Staining for BSP and OPN is restricted to the osteoid area. **e–f, h–i** After decalcification, increased immunostaining for BSP and OPN is observed in the bone matrix, especially the lineal bone matrix (*open arrow*), while diminished staining of osteoid is observed. *Bar* = 100 μm

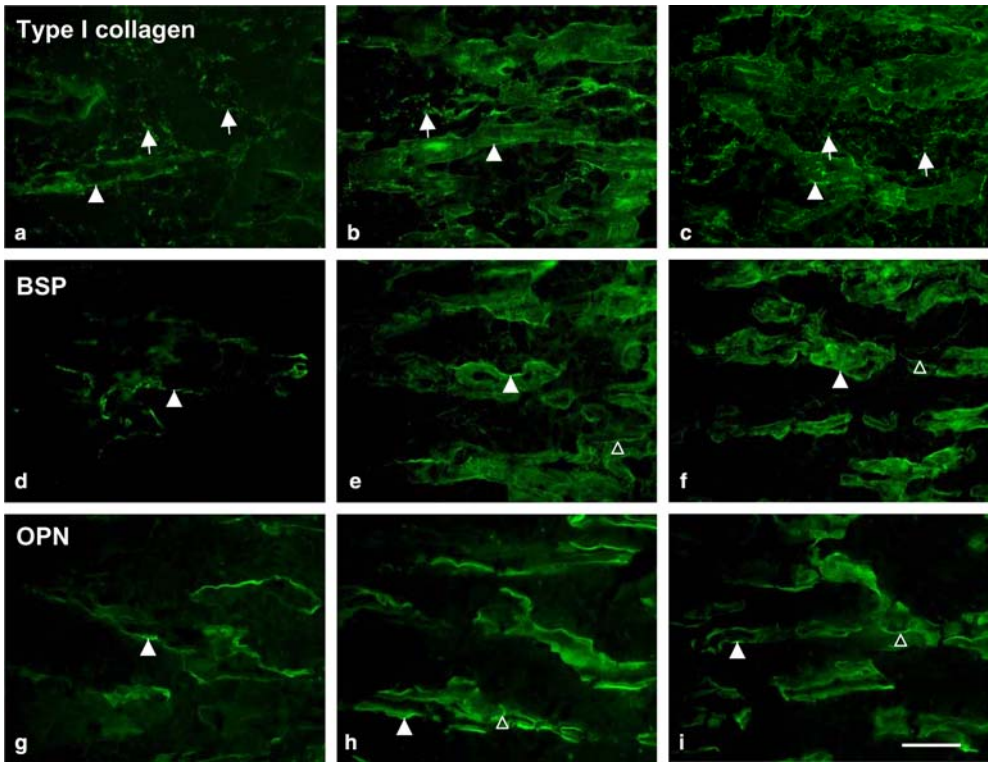


**Fig. 6** Immunolocalization of type I collagen (**a–c**), BSP (**d–f**), and OPN (**g–i**), in trabecular bone. Fixed sections decalcified for 0 (**a, d, g**), 3 (**b, e, h**) and 5 (**c, f, i**) min. **a** Immunoreaction of type I collagen is observed in the area of osteoid (*arrow head*) and bone marrow (*arrow*). **b-c** Type I collagen localizes in bone matrix, and staining in osteoid area (*arrow*) remains. **d, g** Immunolocalizations of BSP and OPN are intensively found in the osteoid area. **e–f, h–i** After decalcification, these proteins localize in the area of osteoid, cement line (*open arrow*), and bone matrix. *Bar* = 100 μm.

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, reduced ALP activity is noted in fresh-frozen sections, such as those used in the present experiment, following long-term fixation (24 h) and decalcification (6 days) (Kawamoto and Shimizu 2000). Because the immunoreactivity of bone matrix proteins, analyzed in the present study, was rarely affected by paraformaldehyde fixation, it could be still observed in the fixed section after decalcification.

In non-decalcified sections, immunostaining for bone matrix proteins was observed in osteoid, but hardly in mineralized bone. A number of methods exist for osteoid staining (Tripp and Mackay 1972; Yoshiki 1973; Villanueva and Mehr 1977); however, they all involve difficult procedures. Our staining methods using non-decalcified fresh-frozen sections can stain to osteoid in serial sections, and can combine with bone labels such as tetracycline and calcein labeling. Thus, it is thought to be the useful method for bone morphometry observation.

This study describes a method for fresh-frozen sections, and these observations provided additional insight for interaction between calcification and bone matrix. This method can be prepared by unfixed and non-decalcified hard tissue sections, and analyzed for many types of research including water-soluble molecule distribution and localizations of proteins, which immunoreactivity may reduce by chemical fixation. We believe that this procedure will be become the method of choice for calcified hard tissue research.

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