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Journal of Orthopaedic Translation

journal homepage: www.journals.elsevier.com/journal-of-orthopaedic-translation

Original Article

A modified tape transfer approach for rapidly preparing high-quality cryosections of undecalcified adult rodent bones

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ARTICLE INFO

Keywords:

Adhesive slides
Bone regeneration
Cryosectioning
Tape transfer
Undecalcified bone

ABSTRACT

Background/Objective: Histology-based analyses are important tools to dissect cellular and molecular mechanisms of skeletal homeostasis, diseases, and regeneration. The success of these efforts is highly dependent on rapidly obtaining high-quality sections of mineralized skeletal tissues suitable for various analyses. However, the current techniques for preparing such sections are still far from satisfactory. This study aimed to develop a new approach for preparing high-quality undecalcified bone sections applicable to various histological analyses.

Methods: Two important modifications were made to the conventional Cryojane Tape-Transfer System, including utilization of an optimized adhesive to prepare adhesive glass slides for improving the transfer efficiency, and a cheap conventional benchtop UV transilluminator for UV curing. Cryosections of undecalcified rodent bones were prepared using this modified tape transfer approach, and their tissue morphology and structural integrity were visually examined. A variety of histological analyses, including calcein labeling, Von kossa staining, immunofluorescence, and enzymatic activity staining as well as 5-Ethynyl-2'-deoxyuridine (EdU) and TUNEL assays, were performed on these sections.

Results: We developed a modified version of tape transfer approach that can prepare cryosections of undecalcified rodent adult bones within 4 days at a low cost. Bone sections prepared by this approach exhibited good tissue morphology and structural integrity. Moreover, these sections were applicable to a variety of histological analyses, including calcein labeling, Von kossa staining, immunofluorescence, and enzymatic activity staining as well as EdU and TUNEL assays.

Conclusion: The tape transfer approach we developed provides a rapid, affordable, and easy learning method for preparing high-quality undecalcified bone sections valuable for bone research.

The translational potential of this article: Our research provides a rapid, affordable, and easy learning method for preparing high-quality undecalcified bone sections that can be potentially used for accurate diagnosis of various bone disorders and evaluation of the efficacy of different therapies in the treatment of these diseases.

Introduction

Skeletal diseases, such as osteoporosis and osteoarthritis, are serious diseases that afflict millions of patients worldwide [1–3]. The pathogenesis and precise mechanism of these diseases are still under active investigation [4]. Histology-based analyses are important approaches to

dissect cellular and molecular mechanisms of skeletal homeostasis, diseases and regeneration [5]. Unfortunately, it is technically challenging to prepare histological sections of native skeletal tissues because of their highly mineralised nature [5,6]. Consequently, mineralised skeletal tissues are commonly subjected to 1–2 weeks of decalcification before being processed for embedding and sectioning [5,6]. However,

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<https://doi.org/10.1016/j.jot.2020.03.001>

Received 30 December 2019; Received in revised form 18 February 2020; Accepted 2 March 2020

Available online 31 March 2020

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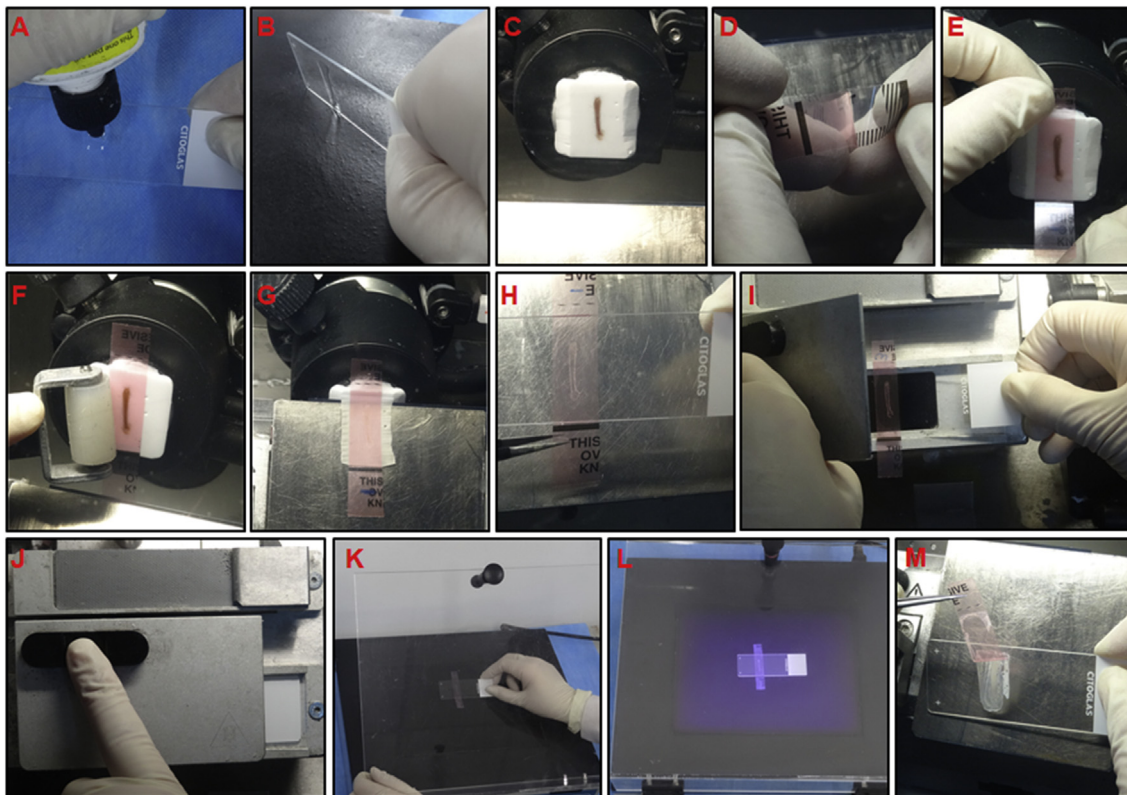


Figure 1. Illustration of procedures of the modified tape transfer approach for preparing frozen sections of undecalcified long bones from 2-month-old mice. (A–B) Preparing custom-made adhesive slide by applying a drop of Norland Optical Adhesive #63 to the glass slide (A) and then placing the slide vertically allowing the adhesive glue to spread evenly by gravity (B); (C) exposing the desired cut surface by trimming; (D) removing the protective cover from the commercial CryoJane tape; (E) attaching the exposed adhesive side of the CryoJane tape to the block surface; (F) rolling the tape firmly using a cold roller; (G) cutting the frozen sample at a thickness of 8 μm ; (H) mounting the section to the adhesive-coated glass slide with the specimen side facing down; (I–J) curing the adhesive using the curing platform of CryoJane Tape-Transfer System. The section/slide was inserted into the UV radiation tray (I) and then treated with a flash of UV light (J); (K–L) curing the adhesive using the conventional UV transilluminator. The section/slide was placed on a benchtop UV transilluminator (K) and then exposed to UV light for 4 s (L); (M) peel the CryoJane tape from the section using a cold forcep.

decalcification may impair antigenicity and enzymatic activity, therefore preventing the utility of these sections for certain immunohistochemistry and enzymatic assays [5,7]. Moreover, decalcification destroys fluorescent labels (such as calcein labels) that are used to evaluate bone formation parameters [6]. Similarly, to distinguish mineralised matrix from unmineralised osteoid for assessing various parameters of skeletal mineralisation, histological stains, such as von Kossa staining, must be performed on undecalcified bone sections [6]. Therefore, preparing the undecalcified bone sections suitable for all above purposes is of great importance for advancing bone research.

The most commonly used approach for sectioning mineralised skeletal tissues is plastic sectioning [8,9]. This approach utilises plastics or resins (normally methyl methacrylate (MMA)) as embedding media because they have similar hardness to that of the mineralised skeletal tissues [8,9]. While this method is highly suitable for obtaining sections applicable to calcein labelling and certain bone-specific stains, it has several disadvantages [6,9]. First, it is time-consuming and usually takes several weeks. Second, it is very expensive and normally requires a special microtome equipped with a very expensive diamond knife. Third, it is technically challenging and usually requires a high degree of expertise for the operators. Finally, its tissue processing is performed in harsh conditions and involves a number of hazardous reagents, which not only destroy certain antigenicity and enzymatic activity but also cause potential detrimental effects on human health. Thus, a better approach for preparing mineralised bone sections is highly needed.

Cryosectioning, a well-established technique known for its superior capability to preserve antigenicity and enzyme activity, has been widely applied to generating high-quality sections of various soft tissues [10].

However, when used for sectioning mineralised adult bones, it often produces sections with poor tissue morphology and structural integrity. In recent years, the CryoJane Tape-Transfer System has been commercially developed to facilitate preparation of cryosections of difficult tissues [11,12]. This system employs adhesive tapes to capture cryosections as they are cut from the frozen blocks, allows these sections to be transferred to glass slides coated with ultraviolet light (UV)-curable adhesive and further assists them to be permanently bonded to adhesives on the slides after UV curing [11,12]. While this system has been successfully used to prepare high-quality cryosections of partially decalcified adult bones [13–17], its application to sectioning completely mineralised bones yielded inconsistent results, partly because of incomplete transfer of sections from CryoJane tapes to adhesive-coated glass slides. Furthermore, this system relies on a special equipment that is not routinely equipped in the histology facility and too expensive to afford for many small laboratories, further limiting its application in producing undecalcified bone sections.

In this study, we described the development of a modified version of tape transfer approach for preparing cryosections of undecalcified adult murine bones that only took 4 days at a low cost. We first optimised the adhesive on the glass slides to allow efficient transfer of sections from CryoJane tapes to these adhesive slides. We next confirmed the feasibility of using an affordable benchtop UV transilluminator for UV curing. Furthermore, we performed experiments to demonstrate that undecalcified bone sections prepared by the above modified method were suitable for various histology-based analyses. Thus, the tape transfer approach we developed provides a rapid, affordable and easy learning method for preparing high-quality undecalcified bone sections applicable

to a variety of histological analyses for bone research.

Materials and methods

Animals

All animals used in this study were kept in a specific pathogen-free (SPF) facility at Laboratory Animal Center of Soochow University, which was operated at controlled temperature and humidity conditions with a 12 h/12 h light/dark cycle. Animals were provided ad libitum access to standard chow diet and drinking water, and their health status was closely monitored on a daily basis. No adverse events occurred throughout this study. All animal experiments were performed according to the protocol approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Tissue processing and embedding

Skeletal tissues were isolated and immersed in 4% paraformaldehyde (PFA) at 4°C for 24 h with gentle agitation. Following being washed with phosphate-buffered saline (PBS), soft tissues around bones were carefully removed. Afterwards, bones were equilibrated first in 15% sucrose in PBS and then in 30% sucrose in PBS each for 24 h. Sucrose-penetrated bone samples were subsequently transferred to plastic cassettes, covered with optimal cutting temperature (OCT) compound and adjusted to the desired position. Snap-freezing of the embedded bone samples was then achieved by placing it in a Petri dish floating on liquid nitrogen. Frozen blocks were stored at –80°C before cryosectioning.

Section preparation

Before cryosectioning, CryoJane tapes (Cat# 39475214) and CryoJane adhesive slides (Cat# 39475209, Leica Biosystems, Richmond, IL, USA) were cooled in a cryostat where the temperatures of its chamber and holder chuck were set at –25°C and –30°C, respectively. Custom-made adhesive slides were freshly prepared at room temperature (RT) by applying a drop of the Norland Optical Adhesive 63 (Cat#6301, Norland Products, Cranbury, NJ, USA) to the glass slide (Fig. 1A) and then placing the slide vertically to allow the adhesive glue to spread evenly by gravity (Fig. 1B). All samples in this study were sectioned on a Leica CM3050S cryostat equipped with CryoJane Tape-Transfer System with #819 disposable steel blades (Leica Microsystems, GmbH, Nussloch, Germany). The detailed process of cryosectioning was as follows. The frozen block of the bone sample was removed from the embedding cassette and mounted onto the specimen holder following the standard procedures. Afterwards, the block was slowly trimmed at a thickness of 20 µm until the desired area appeared on the cut surface (Fig. 1C). At this point, a segment of prechilled CryoJane tape was attached to the trimmed block by slowly removing its protective cover (Fig. 1D), applying its adhesive side to the cut surface (Fig. 1E) and firmly rolling it flat using a cold roller (Fig. 1F). An 8 µm-thick section was then cut at a slow and constant speed (Fig. 1G) and subsequently placed on a commercial CryoJane slide or custom-made slide with the specimen side facing down (Fig. 1H). Every effort was made to ensure that the specimen was gently immersed into the sticky adhesive coated on the slide. Subsequently, curing of the adhesive was performed by one of two different approaches. The section/slide was inserted into the UV radiation tray (Fig. 1I) and then treated with a flash of UV light (Fig. 1J), both of which were included in the commercial CryoJane Tape-Transfer System. Alternatively, the section/slide was placed on a benchtop UV transilluminator (Model GL-3120, Kylin-Bell, Haimen, China) (Fig. 1K) and then exposed to UV light for 4–8 s (Fig. 1L). Following UV curing, the CryoJane tape was carefully peeled off with a cold forcep (Fig. 1M), leaving the specimen firmly adhered to the glass slide. Next, the slide was air-dried before being stored in a slide box at –80°C until use.

Calcein double labelling

Calcein double labelling was performed as we previously described [15,18]. Briefly, rodents were intraperitoneally injected with 10 mg/kg calcein dissolved in 2% sodium bicarbonate on days 7 and 2 before sacrifice. Upon sacrifice, tibias or femurs were carefully dissected and immediately transferred to 4% PFA. Following fixation at 4°C for 24 h, bones were processed for preparation of undecalcified sections according to procedures described above. After being counterstained with 4',6-diamidino-2-phenylindole (DAPI), sections were mounted with anti-fade mounting medium. Images of calcein double labelling were then acquired using a Zeiss fluorescent microscope (Carl Zeiss Microscopy, Thornwood, NY).

von Kossa staining

von Kossa staining was performed as we previously reported [18]. In brief, undecalcified bone sections were air-dried, rinsed with Milli-Q H₂O and then treated with 1% silver nitrate solution with exposure to bright light for 30 min. Afterwards, slides were rinsed three times with Milli-Q H₂O before incubation with a solution of 2.5% sodium thiosulphate for 5 min. Subsequently, slides were thoroughly washed with Milli-Q H₂O, followed by counterstaining with nuclear fast red solution. Finally, sections were dehydrated, mounted with a drop of Neutral Balsam mounting medium (Sangon, Shanghai, China) and examined under the light microscope. The mineralised areas exhibited brown/black staining on sections.

Immunofluorescence

Immunofluorescent (IF) staining of undecalcified bone sections were performed as previously described [15,16,19]. In brief, sections were air-dried, briefly rinsed with PBS and blocked with 10% normal goat serum in PBS for 1 h at RT. Antigen retrieval was then performed in 0.1 M citrate acid buffer (pH 6.0) at 65°C for 3 h for Osterix (Osx) antibody or in 10 mM Tris-ethylene diamine tetra-acetic acid (EDTA) at 65°C for 3 h for both CD31 and endomucin (Emcn) antibodies. Subsequently, sections were incubated with individual primary antibody at 4°C overnight. The primary antibodies used in this study included rabbit anti-Osx (Abcam, ab22552, 1:200), rabbit anti-perilipin (Cell Signalling Technology, #9349, 1:100), rabbit anti-CD31 (Abcam, ab28364, 1:25) and rat anti-Emcn (Santa Cruz, Sc-65495, 1:50) antibodies. Following overnight incubation with primary antibodies, sections were washed three times with PBS before incubation with appropriate Alexa Fluor-labelled secondary antibodies for 1 h. Specifically, Alexa Fluor 647 goat anti-rabbit (Life technology, A21246, 1:200) and Alexa Fluor 647 goat anti-rat (Abcam, ab150155, 1:200) were used as secondary antibodies for Osx and Emcn, respectively. Alexa Fluor 594 goat anti-rabbit (Abcam, ab150084, 1:400) was used as the secondary antibody for both perilipin and CD31. Sections were then rinsed with PBS and mounted with Vectorshield mounting medium with DAPI (Vector Laboratories), followed by imaging using a Zeiss fluorescent microscope.

EdU cell proliferation assays

For 5-Ethynyl-2'-deoxyuridine (EdU) labelling, mice were injected intraperitoneally with 10 µg of EdU (Beyotime, Shanghai, China) per gram of body weight 6 h before harvest. Bone samples were then isolated and processed for preparation of undecalcified sections as described above. EdU incorporation was detected by copper-catalysed click chemistry using an EdU staining kit (BeyoClick™ EdU-647) according to the manual accompanied with the kit. Briefly, sections were postfixed with 4% PFA for 10 min and permeabilised with 0.3% Triton X-100 in PBS for 15 min, before being incubated with freshly prepared Click-iT EdU detection cocktail for 30 min at RT. After counterstaining with DAPI, sections were mounted with anti-fade mounting medium and then

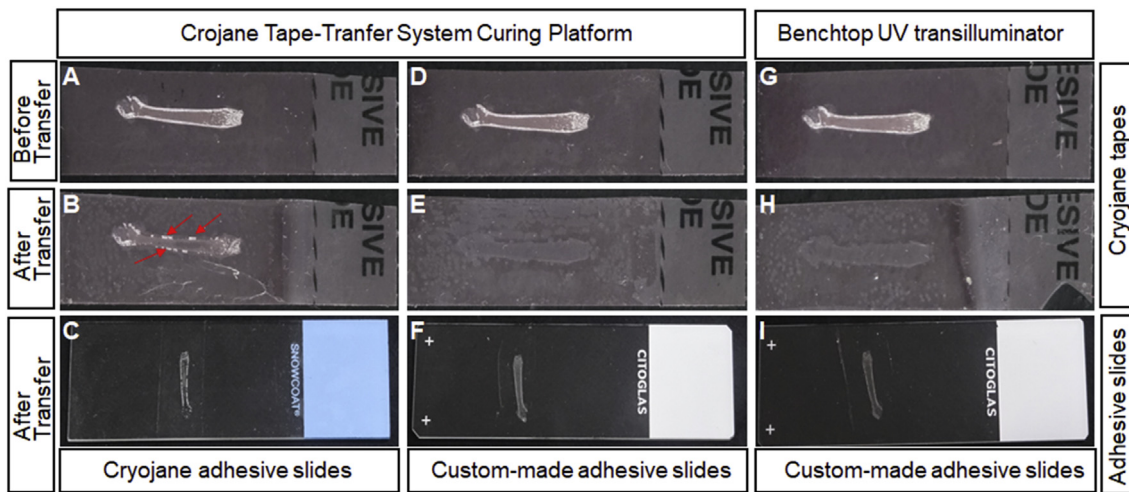


Figure 2. Comparison of three different tape transfer approaches for the efficiency of transferring bone sections from CryoJane tapes to adhesive slides. In all three approaches, commercial CryoJane tapes were used to capture bone sections when they were cut from the frozen blocks. (A–C) Representative photographs of bone sections on the CryoJane tapes (A, B) or glass slides (C) before (A) or after (B, C) being transferred to CryoJane adhesive slides using the curing platform provided in the system. Arrows indicated a portion of cortical bones retained on the CryoJane tape after transferring; (D–F) representative photographs of bone sections on the CryoJane tapes (D, E) or glass slides (F) before (D) or after (E, F) being transferred to the custom-made adhesive slides using the curing platform provided in the CryoJane system. Note no residual bone tissue on the CryoJane tape after transferring in E; (G–I) representative photographs of bone sections on the CryoJane tapes (G, H) or glass slides (I) before (G) or after (H, I) being transferred to custom-made adhesive slides using the conventional UV transilluminator as the curing platform. Note no residual bone tissue on the CryoJane tape after transferring in I.

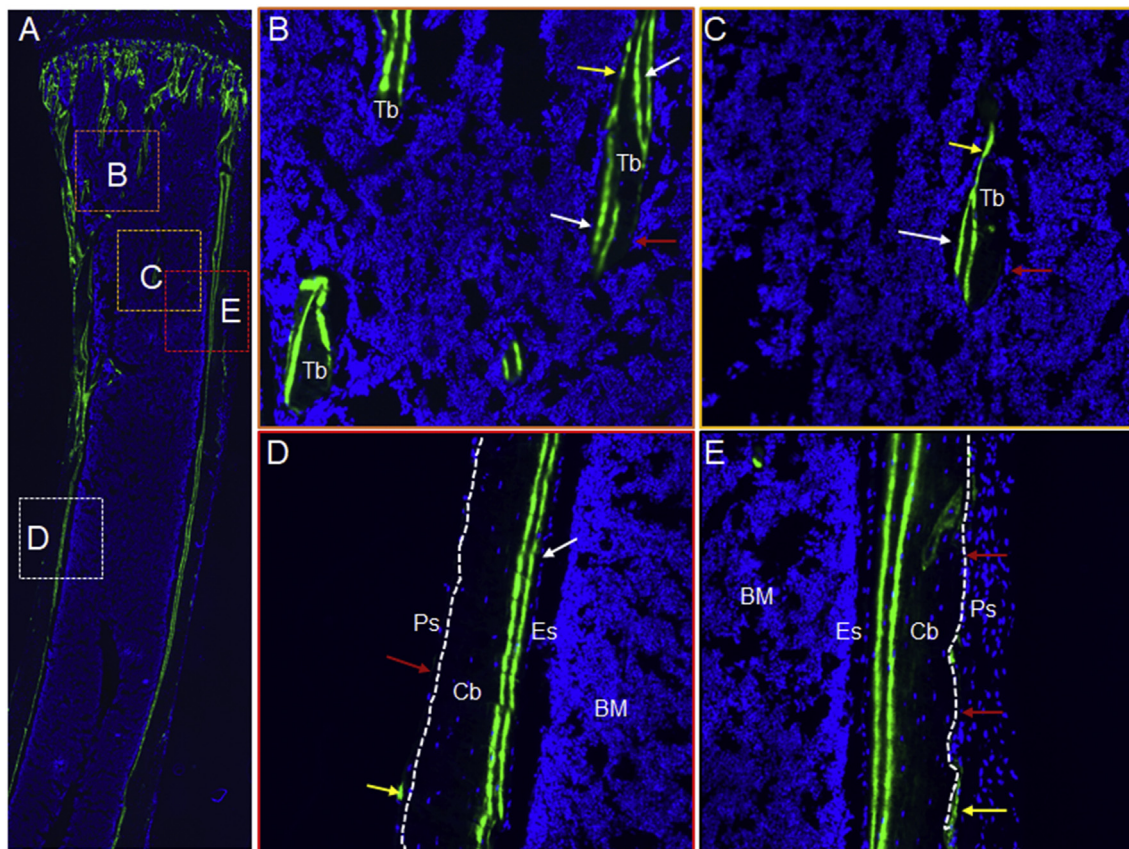


Figure 3. Undecalcified bone sections prepared by our approach are suitable for dynamic histomorphometry. Two-month-old mice were injected with calcein at 7 and 2 days before harvest. Undecalcified cryosections of tibiae from these mice were prepared by the modified tape transfer approach, stained with DAPI and then observed under a fluorescent microscope. (A) Representative fluorescent images of the longitudinal tibial sections from calcein-injected mice; (B–E) higher magnification images of the boxed areas in A, showing single-labelled (yellow arrows), double-labelled (white arrows) and unlabelled (red arrows) surfaces of trabecular (B, C) and cortical bones (D, E). BM = bone marrow; Cb, cortical bones; Es = endosteal surface; Ps = periosteal surface; Tb = trabecular bone. Green, calcein labels; Blue, DAPI staining.

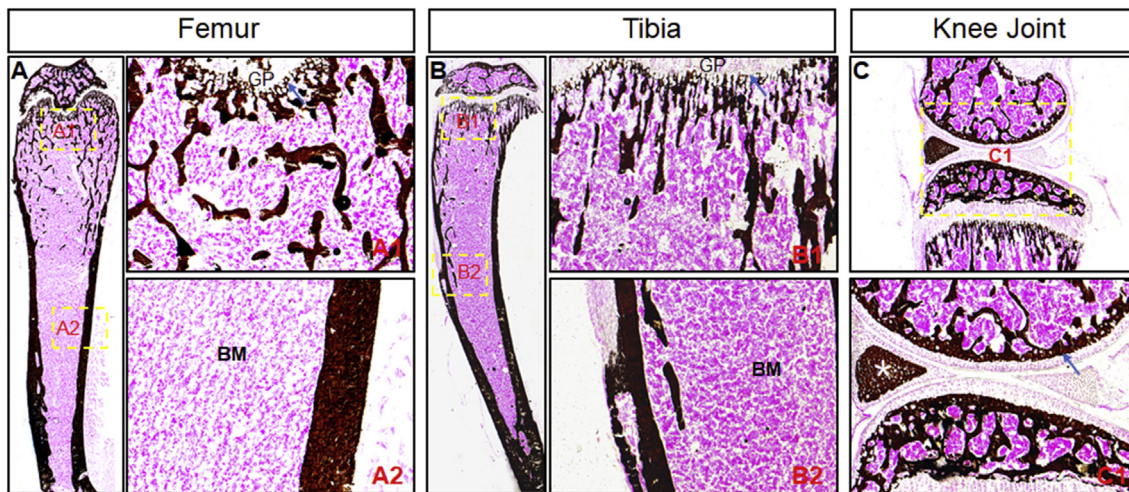


Figure 4. Undecalcified bone sections prepared by our approach are suitable for von Kossa staining. Undecalcified cryosections of femurs, tibias or knee joints from two-month-old mice were prepared by the modified tape transfer approach and subsequently stained with Von kossa. (A) Representative images of Von kossa-stained femoral section. Boxed areas were shown at higher magnification to the right, representing the metaphyseal (A1) and diaphyseal (A2) regions. The blue arrow in A1 indicated positive Von kossa staining (black) in the hypertrophic zone of the growth plate; (B) representative images of von Kossa-stained tibial section. Boxed areas were shown at higher magnification to the right, showing the metaphyseal (B1) and diaphyseal (B2) regions. The blue arrow in B1 pointed to von Kossa staining (black) in the hypertrophic zone of the growth plate; (C) representative images of von Kossa-stained cryosection of the knee joint. The magnified image of the boxed area in C was shown in C1. The blue arrow and asterisk denoted positive von Kossa staining (black) in the hypertrophic zone of the articular cartilage and the core of the meniscus, respectively. Black, von Kossa staining; Red, nuclear fast red staining. BM = bone marrow; GP = growth plate.

analysed by fluorescence microscopy.

Terminal deoxynucleotidyl transferase-catalysed dUTP nick end labelling assays

For detection of apoptotic cells, terminal deoxynucleotidyl transferase-catalysed 2'-Deoxyuridine 5'-triphosphate (dUTP) nick end labelling (TUNEL) assays were performed on undecalcified bone sections using an *In Situ* Cell Death Detection Kit TMR Red (Roche), following the manufacturer's instructions. In brief, sections were washed with PBS and then permeabilised with a solution of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min, followed by incubation with freshly prepared TUNEL reaction mixture for 60 min at 37°C in a humidified chamber in the dark. After staining nuclei with DAPI, sections were applied with a drop of antifade mounting medium and then covered with glass coverslips before being analysed by a fluorescence microscopy.

Alkaline phosphatase staining

Staining for alkaline phosphatase (ALP) activity was performed as reported previously [20,21]. In brief, sections of undecalcified bones were air-dried, briefly rinsed in PBS and then incubated with a staining solution consisting of 0.1 M Tris-HCl (pH8.5), 0.1 mg/ml naphthol AS-MX phosphate, 0.5% N, N-dimethylformamide, 2 mM MgCl₂ and 0.6 mg/ml fast blue 4-benzamido-2,5-diethoxybenzenediazonium (BB) salt for 30 min at RT. All of these staining reagents were purchased from Sigma. At the end of incubation, slides were washed with PBS, dehydrated with ethanol gradients and mounted with mounting medium before being imaged with the light microscope. Cells with ALP activity appeared blue on stained sections.

Results

The modified tape transfer approach we developed can prepare undecalcified bone sections within 4 days at a low cost

To accelerate the process of sectioning undecalcified bones, we initially tried commercial CryoJane Tape-Transfer System, using the transfer tapes and adhesive-coated slides provided by the manufacture.

However, we continuously failed to get sections with intact bone architecture (Fig. 2). After careful analysis, we found that bone sections were properly captured by CryoJane transfer tapes but unable to be completely transferred to adhesive slides (Fig. 2A–C). In particular, a portion of cortical bones tended to retain in transfer tapes after transferring (Fig. 2B). We reasoned that the adhesive on these commercial slides may be not strong enough to keep undecalcified bone sections on the slides after UV curing. To solve this problem, we tested several UV-curable adhesives for their capability to keep bone sections on the slides after UV curing. We found that the CryoJane Tape-Transfer System can constantly produce bone sections with good tissue morphology when the optical adhesive 63 (Norland) was used to coat glass slides (Figs. 1 and 2D–F). We therefore used these custom-made glass slides in all subsequent experiments.

While the commercial CryoJane Tape-Transfer System when combined with the above custom-made adhesive slides can prepare high-quality undecalcified bone sections (Fig. 2D–F), this system is not normally available in the histology facility and unaffordable to many small laboratories, especially those in developing countries. We therefore tested the feasibility of utilising a benchtop UV transilluminator as an alternative equipment for UV curing during section preparation. This type of UV transilluminator only costs about \$200 and is clearly much cheaper than the CryoJane system. Specifically, after bone sections were captured by CryoJane transfer tapes and placed on freshly prepared adhesive slides as described above, section/slides were exposed to UV light produced by a benchtop UV transilluminator (Kylin-Bell) for 4–8 s and then immediately transferred into the cryostat chamber for subsequent procedures. We found that sectioned bone tissues can be completely transferred to custom-made adhesive slides using this alternative UV curing equipment (Fig. 2G–I). The entire process of the above approach takes less than 4 days, including 1 day of sample fixation, 2 days of sample processing and embedding and less than 1 day of sectioning. Thus, the modified tape transfer approach we developed can rapidly prepare undecalcified bone sections at a low cost.

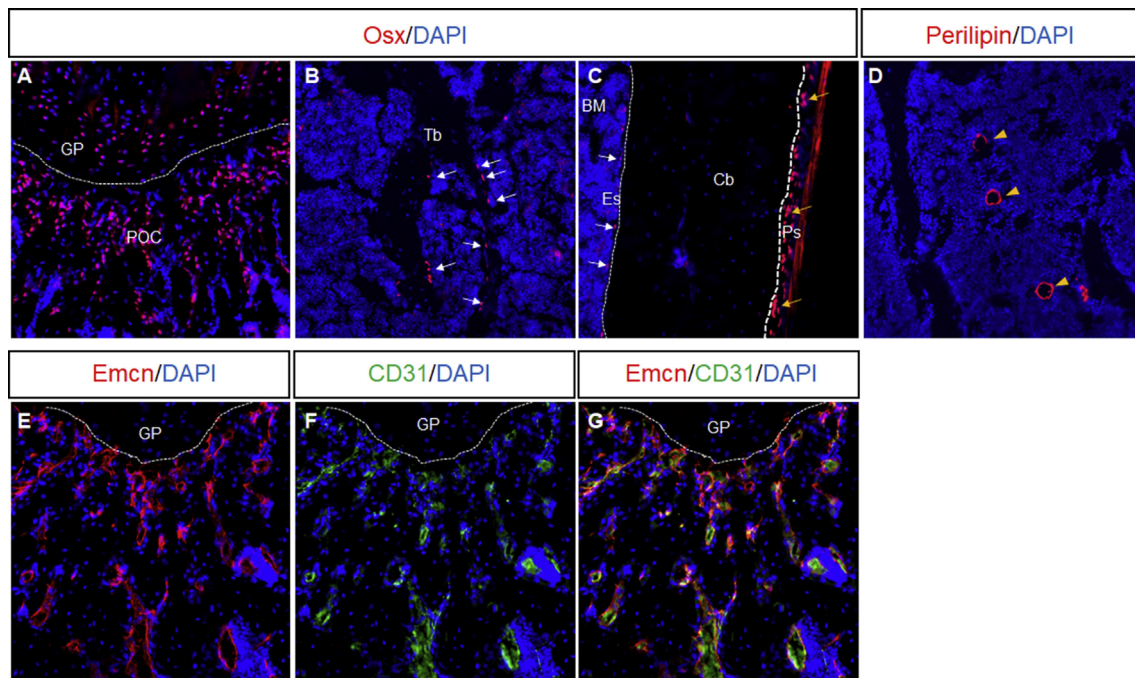


Figure 5. Undecalcified bone sections prepared by our approach are suitable for immunofluorescent staining of osteoblastic, adipocytic and endothelial markers. Undecalcified cryosections of femurs from 2-month-old mice were prepared by the modified tape transfer approach and subsequently subjected to immunofluorescence staining. (A–C) Representative fluorescent images of undecalcified cryosections stained with Osx antibody. Osx-positive cells were detected in the growth plate and the primary ossification centre (A) as well as surfaces of the trabecular (B) and cortical (C) bones. Arrows in B and C indicated Osx-expressing preosteoblasts; (D) representative image of immunofluorescence staining for perilipin. Arrowheads pointed to perilipin-positive cells; (E–F) representative images of undecalcified femoral sections subjected to double immunofluorescence for Emcn (E, red) and CD31 (F, green); (G) merged view of E and F. Yellow colours indicated co-expression of Emcn and CD31 in the type-H vessels. BM = bone marrow; Cb = cortical bone; Es = endosteal surface; GP = growth plate; Ps = periosteal surface; POC = primary ossification centre; Tb = trabecular bone; Blue, DAPI.

Undecalcified bone sections prepared by our approach are suitable for dynamic histomorphometry and von Kossa staining

One of the most important applications of undecalcified bone sections is used for dynamic histomorphometry, which evaluates bone formation-related parameters such as mineral acquisition rates and bone formation rates and is therefore extremely useful for studying bone physiology [15]. To test whether cryosections prepared by our method can be applied to dynamic histomorphometry, we performed calcein labelling. Specifically, we administered two intraperitoneal injections of calcein 7 days and 2 days before harvest and then prepared the cryosections using the method described above. After being counterstained with DAPI, sections were observed under a fluorescence microscope. As shown in Fig. 3, bone architecture was well preserved on the sections, as evidenced by intact trabecular and cortical bones as well as minimally damaged bone marrow. Moreover, numerous green calcein labels were detected throughout the bone sections and specifically associated with the surfaces of trabecular bones and cortical bones. In the magnified pictures (Fig. 3B–E), unlabelled, single-labelled or double-labelled surfaces were clearly visible for quantification and the distance between these two newly formed fluorescent lines could be easily measured (Fig. 3). These measurements allowed to determine various bone formation parameters. Thus, undecalcified bone sections prepared by our approach are suitable for dynamic histomorphometric analyses.

Besides calcein labelling, bone-specific histological stains are also important techniques for bone research [9]. These stains can distinguish mineralised matrix from unmineralised osteoid and therefore allow to assess degree of mineralisation in bones. To test whether cryosections obtained by our method can be used for these assays, we performed von Kossa staining, a commonly used approach for staining mineralised hard tissues, on these sections. Specifically, we treated sections with a silver nitrate solution. The silver is deposited by replacing the calcium in the

presence of the strong light and can be visualised as black/brown stains on the sections. As shown in Fig. 4, black signals were clearly detected in trabecular bones and cortical bones, both of which were well preserved after staining. Moreover, black signals also appeared in other regions, including hypertrophic zones of articular cartilage and growth plates, as well as the core of meniscus (Fig. 4). All of these regions were known to be calcified. In contrast, areas that were known to be calcium-free, such as bone marrows and surfaces of articular cartilage did not exhibit any black staining. Thus, these results indicated that cryosections are suitable for von Kossa staining to reliably detect and evaluate mineralisation in the skeleton.

Undecalcified bone sections prepared by our approach are suitable for immunofluorescent staining of osteoblastic, adipocytic and endothelial markers

IF staining is an invaluable technique that utilises both antibodies and fluorescence imaging to reveal relative expression, spatial distribution and even activity states of target proteins. Furthermore, IF staining is used for identifying different cell types based on expression of their specific protein markers. To demonstrate that undecalcified bone sections we prepared are compatible with IF staining, we stained these sections with primary antibodies against specific markers of osteoblastic, adipocytic and endothelial cells, respectively, followed by detection with fluorescently labelled secondary antibodies.

Osx, a marker for preosteoblasts, is a zinc finger transcription factor critical for osteoblast differentiation [14]. To demonstrate the utility of cryosections for detection of osteoblast-specific markers, we performed IF staining with Osx antibody. As a result, we observed abundant Osx⁺ cells in the metaphyseal region that are known to be rich in Osx-expressing preosteoblasts (Fig. 5A–C). In addition, expression of Osx was detected in a small percentage of cells on the trabecular and cortical

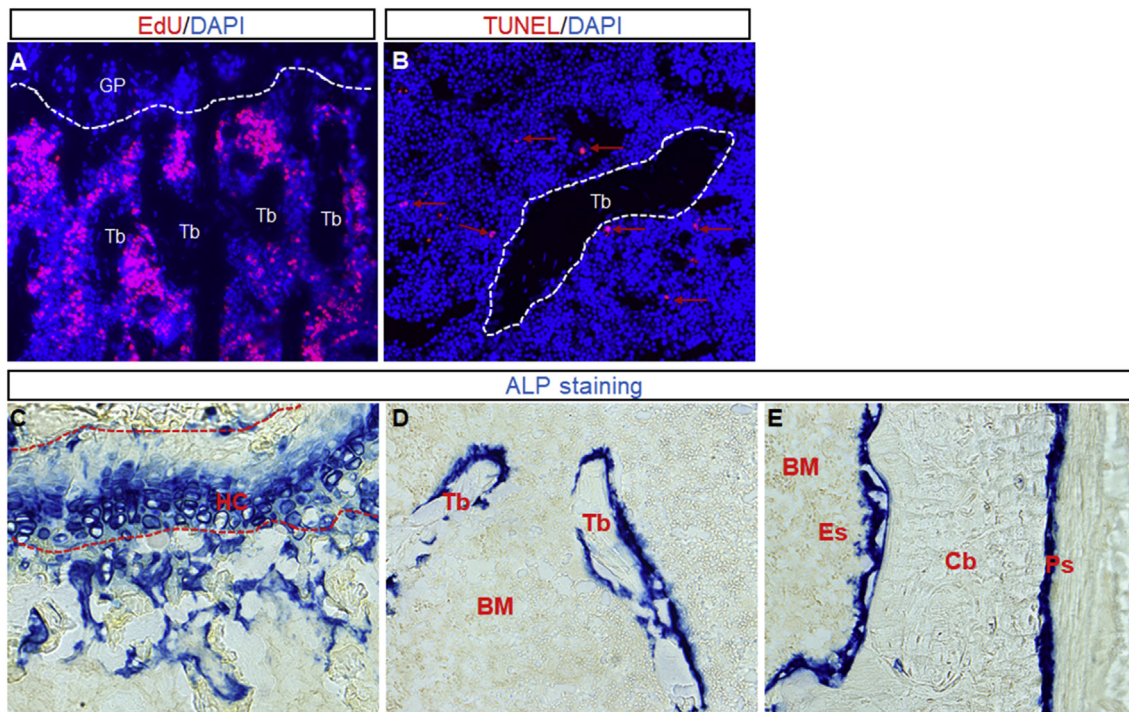


Figure 6. Undecalcified bone sections prepared by our approach are suitable for detection of cell proliferation, apoptosis and ALP enzymatic activity. Undecalcified cryosections of femurs from 2-month-old mice were prepared by the modified tape transfer approach and subsequently subjected to EdU, TUNEL and ALP staining, respectively. (A) Representative fluorescent images of EdU staining showing numerous EdU-labelled proliferating cells (red) in the marrow between trabecular bones in the metaphyseal region. Nuclei were stained with DAPI (blue); (B) representative fluorescent images of TUNEL staining. Arrows pointed to apoptotic cells (red) in the bone marrow. Nuclei were visualised with DAPI (blue); (C–E) representative bright-field photographs of ALP staining showing the positive staining (blue) in the hypertrophic chondrocytes and osteoblastic lineage cells in the metaphyseal region (C) as well as the cells covering trabecular bones (D) and cortical bones (E). BM, bone marrow; Cb = cortical bone; Es = endosteal surface; GP = growth plate; HC = hypertrophic chondrocytes; Ps = periosteal surface; Tb = trabecular bone.

bone surfaces where some preosteoblasts reside (Fig. 5B and C). Similarly, *Osx*-positive cells were also observed in the prehypertrophic zone of the growth plate (Fig. 5A), consistent with its known expression in prehypertrophic chondrocytes [22].

Osteoblasts is closely related to bone marrow adipocytes, both of which are differentiated from bone marrow mesenchymal stem/stromal cells [23,24]. Moreover, recent studies showed that the number of bone marrow adipocytes was inversely correlated with bone mass [23,24]. Therefore, it is of great importance to analyse bone marrow adipocytes for bone research. We next performed IF staining to test the feasibility of identifying bone marrow adipocytes in our cryosections. Specifically, we examined expression and localisation of perilipin, an adipocyte-specific marker that is localised on surfaces of lipid droplet in adipocytes. IF staining of femoral sections from 2-month-old mice with perilipin antibody only detected a few perilipin + cells in the bone marrow (Fig. 5D). However, in these few perilipin + cells, strong expression of perilipin was indeed restricted to the periphery of cells, which exhibited typical round and large morphology of mature adipocytes (Fig. 5D). These results were consistent with the fact that we used young mice in our experiments and that mature adipocytes were rare in the bone marrow of young mice.

While osteogenesis is inversely related to adipogenesis, it is tightly coupled with angiogenesis. In particular, the H-type vessel, a special subtype of blood vessels that express high level of endothelial markers CD31 and *Emcn* (shown as CD31^{hi}*Emcn*^{hi}) was recently found to play important roles in promoting osteogenesis and bone formation [25,26]. To further test whether cryosections we prepared can be used for detection of H-type vessels, we performed double IF staining with CD31 and *Emcn* antibodies. Consistent with previous observation, we detected a large number of vessels in the metaphysis of the femur, which expressed high level of *Emcn* (*Emcn*^{hi}) (Fig. 5E). Moreover, some of these *Emcn*^{hi} vessels also exhibited strong CD31 staining (Fig. 5F and G), therefore

representing H-type vessels by the definition.

Collectively, our data demonstrated that cryosections we prepared are compatible with IF staining, in particular for detection of markers for osteoblasts, adipocytes and H-type vessels.

Undecalcified bone sections prepared by our approach are suitable for analysis of cell proliferation and apoptosis

Both cell proliferation and apoptosis are known to play important physiological roles in maintaining skeletal homeostasis, and their deregulation is implicated in a number of skeletal diseases [27,28]. We next conducted experiments to demonstrate the utility of cryosections for identification of proliferating or apoptotic cells.

We first performed EdU assay to detect cell proliferation. Specifically, mice were intraperitoneally injected with EdU before being sacrificed. EdU is a thymidine analogue with an alkyne and incorporated into DNA during active DNA synthesis [27,29]. As a result, proliferating cells are labelled with EdU. EdU labelling is then detected by a click reaction catalysed by a copper, which occurs between the alkyne group in EdU and a fluorescent azide [27,29]. In our EdU assays performed on undecalcified bone sections, we consistently detected a large number of EdU-labelled cells in the marrow cavity of femurs (Fig. 6A). These results are similar to those obtained from EdU assays performed on decalcified paraffin sections. Thus, our cryosections can applied to analysis of cell proliferation.

We next performed TUNEL assay, a widely used method for identifying apoptotic cells. In TUNEL assays, free 3'-OH termini of DNA strand breaks generated during apoptosis are labelled with a fluorescently modified nucleotide (such as fluorescein-dUTP) in an enzymatic reaction catalysed by terminal deoxynucleotidyl transferase [28,30]. TUNEL-labelled cells are then visualised by fluorescence microscopy. As

shown in Fig. 6B, TUNEL-positive cells were readily detected in the bone marrow. As a negative control, TUNEL-positive cells were rarely observed in the osteoblast lineage cells on the bone surfaces, consistent with the findings of previous studies. Thus, TUNEL assays performed on our cryosections reliably detected apoptotic cells.

Cryosectioning by our approach preserved enzymatic activity of alkaline phosphatase

ALP is an important enzyme required for matrix mineralisation of bone and hypertrophic cartilage and specifically expressed in early osteoblastic lineage cells and hypertrophic chondrocytes [31,32]. Accordingly, ALP staining has been an important method to assess status of differentiation as well as function of both osteoblast and chondrocyte. To test whether enzymatic activity of ALP was still preserved in cryosections, we performed ALP staining on these sections. As shown in Fig. 6, strong blue staining, indicative of high ALP activity, were detected in cells localised in the chondro-osseous junction (Fig. 6C), an area that contains abundant osteoblast progenitors. Similarly, high ALP activity was observed in cells lining the trabeculae as well as in the endosteal and periosteal surfaces of cortical bones (Fig. 6D and E). In addition to osteoblastic lineage cells, strong ALP staining was also observed specifically in hypertrophic chondrocytes of the growth plate (Fig. 6C). In contrast, cells in the bone marrow and osteocytes within the bone matrix exhibited no to little ALP activity (Fig. 6C–E). Thus, ALP activity was specifically detected in all cell types with known expression of ALP in the long bones, therefore verifying preservation of enzymatic activity of ALP in these cryosections.

Discussion

Although many efforts have been made, it is still a tough job to rapidly prepare undecalcified bone sections that can be used for assessing status of mineralisation as well as other histology-based analyses. The tape transfer technology in combination with cryosectioning provides an easy solution to preparing high-quality cryosections of difficult tissues, including partially decalcified bones. However, its application in sectioning completely mineralised bones gave rise to mixed results. In this study, we optimised the tape transfer approach to allow rapid preparation of undecalcified bone sections with a low cost. Importantly, sections prepared by our approach constantly exhibited good tissue morphology and could be used for a wide spectrum of histological analyses. Thus, this modified version of tape transfer approach will be a valuable tool for bone research.

Compared with traditional plastic sectioning that is widely used for preparing sections of mineralised tissues, our approach holds the following advantages. First, we can rapidly obtain the specimen sections usually within 4 days, including 1 day of sample fixation and 2 days of sample cryoprotection and embedding, before samples are sectioned on the fourth day. Second, our method has a relatively lower cost than traditional plastic sectioning because we use custom-made adhesive slides (about \$0.2/slide), regular disposable blades and a cheap benchtop UV transilluminator instead of expensive CryoJane Tape-Transfer system. Third, cryosectioning nature of our approach better suits preservation of antigenicity and enzymatic activity.

We should acknowledge that enormous efforts have been made to adapt cryosectioning for the preparation of undecalcified sections before this study [5,10,33–35]. In particular, a film-based method was developed by Kawamoto and Shimizu [5,10]. In this method, an adhesive film was used to capture and support sections when it was cut from the frozen block. After sectioning, sections were preserved on the films, instead of being transferred to adhesive glass slides. Sections generated by this approach were reported to exhibit superior tissue morphology and integrity [5]. However, it is uncertain whether sections preserved on the films are suitable for all of histological analyses described above because most of these assays are normally performed on sections supported by

glass slides. In addition, the Kawamoto film method requires a special Cryofilm transfer kit and tungsten carbide disposable blades [5], which are unavailable in some regions or unaffordable to some laboratories. Our modified tape transfer approach can produce high-quality undecalcified bone sections with a lower cost and thus provides an alternative approach to the Kawamoto film method, especially for the assays that require sections preserved on glass slides.

While our approach works reasonably well for sectioning adult bone samples from rodents, it is still to be determined whether this approach can be applied to sectioning undecalcified bones from large animals or even humans. Similarly, it is still undetermined whether this approach is suitable for preparing cryosections of other mineralised tissues (such as teeth) or tissues implanted with titanium or other hard materials. Clearly, future efforts will be necessary to develop the approach for sectioning all types of hard tissues from both small and large animals.

Conflict of Interest

The authors have no conflicts of interest to disclose in relation to this article.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (81974344, 81772294), the Natural Science Foundation of Huaian (HAB201848), and the project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

- [1] Reginster JY, Burlet N. Osteoporosis: a still increasing prevalence. *Bone* 2006;38(2 Suppl 1):S4–9.
- [2] Hunter DJ, Schofield D, Callander E. The individual and socioeconomic impact of osteoarthritis. *Nat Rev Rheumatol* 2014;10(7):437–41.
- [3] Hootman JM, Helmick CG. Projections of US prevalence of arthritis and associated activity limitations. *Arthritis Rheum* 2006;54(1):226–9.
- [4] Chen J, Long F. mTOR signaling in skeletal development and disease. *Bone Res* 2018;6:1.
- [5] Kawamoto T, Kawamoto K. Preparation of thin frozen sections from nonfixed and undecalcified hard tissues using Kawamoto's film method (2012). *Methods Mol Biol* 2014;1130:149–64.
- [6] Mack SA, Maltby KM, Hilton MJ. Demineralized murine skeletal histology. *Methods Mol Biol* 2014;1130:165–83.
- [7] Hosoya A, Hoshi K, Sahara N, Ninomiya T, Akahane S, Kawamoto T, et al. Effects of fixation and decalcification on the immunohistochemical localization of bone matrix proteins in fresh-frozen bone sections. *Histochem Cell Biol* 2005;123(6): 639–46.
- [8] Erben RG. Embedding of bone samples in methylmethacrylate: an improved method suitable for bone histomorphometry, histochemistry, and immunohistochemistry. *J Histochem Cytochem* 1997;45(2):307–13.
- [9] Bemenderfer TB, Harris JS, Condon KW, Kacena MA. Tips and techniques for processing and sectioning undecalcified murine bone specimens. *Methods Mol Biol* 2014;1130:123–47.
- [10] Kawamoto T. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Arch Histol Cytol* 2003;66(2):123–43.
- [11] Golubeva YG, Smith RM, Sternberg LR. Optimizing frozen sample preparation for laser microdissection: assessment of CryoJane tape-transfer system(R). *PLoS One* 2013;8(6):e66854.
- [12] Pinskiy V, Jones J, Tolpygo AS, Franciotti N, Weber K, Mitra PP. High-throughput method of whole-brain sectioning, using the tape-transfer technique. *PLoS One* 2015;10(7):e0102363.
- [13] Jiang X, Kalajzic Z, Maye P, Braut A, Bellizzi J, Mina M, et al. Histological analysis of GFP expression in murine bone. *J Histochem Cytochem* 2005;53(5):593–602.
- [14] Long F. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* 2011;13(1):27–38.
- [15] Chen J, Long F. beta-catenin promotes bone formation and suppresses bone resorption in postnatal growing mice. *J Bone Miner Res* 2013;28(5):1160–9.
- [16] Chen J, Shi Y, Regan J, Karuppaiah K, Ornitz DM, Long F. Osx-Cre targets multiple cell types besides osteoblast lineage in postnatal mice. *PLoS One* 2014;9(1):e85161.
- [17] Salie R, Li H, Jiang X, Rowe DW, Kalajzic I, Susa M. A rapid, nonradioactive in situ hybridization technique for use on cryosectioned adult mouse bone. *Calcif Tissue Int* 2008;83(3):212–21.
- [18] Liu Q, Liu C, Yang Y, Yang H, Chen J. Osteocyte-intrinsic mTORC1 signaling restrains trabecular bone accrual in mice. *J Cell Biochem* 2018;119(11):8743–9.
- [19] Chen J, Long F. mTORC1 signaling controls mammalian skeletal growth through stimulation of protein synthesis. *Development* 2014;141(14):2848–54.

- [20] Gu X, Fu X, Lu J, Saijilafu, Li B, Luo ZP, et al. Pharmacological inhibition of S6K1 impairs self-renewal and osteogenic differentiation of bone marrow stromal cells. *J Cell Biochem* 2018;119(1):1041–9.
- [21] Li K, Xiu C, Zhou Q, Ni L, Du J, Gong T, et al. A dual role of cholesterol in osteogenic differentiation of bone marrow stromal cells. *J Cell Physiol* 2019;234(3):2058–66.
- [22] Xing W, Godwin C, Pourteymoor S, Mohan S. Conditional disruption of the osterix gene in chondrocytes during early postnatal growth impairs secondary ossification in the mouse tibial epiphysis. *Bone Res* 2019;7:24.
- [23] Yu B, Huo L, Liu Y, Deng P, Szymanski J, Li J, et al. PGC-1alpha controls skeletal stem cell fate and bone-fat balance in osteoporosis and skeletal aging by inducing TAZ. *Cell Stem Cell* 2018;23(4):615–23.
- [24] Sadie-Van Gijzen H, Crowther NJ, Hough FS, Ferris WF. The interrelationship between bone and fat: from cellular see-saw to endocrine reciprocity. *Cell Mol Life Sci* 2013;70(13):2331–49.
- [25] Xie H, Cui Z, Wang L, Xia Z, Hu Y, Xian L, et al. PDGF-BB secreted by preosteoclasts induces angiogenesis during coupling with osteogenesis. *Nat Med* 2014;20(11):1270–8.
- [26] Huang J, Yin H, Rao SS, Xie PL, Cao X, Rao T, et al. Harmine enhances type H vessel formation and prevents bone loss in ovariectomized mice. *Theranostics* 2018;8(9):2435–46.
- [27] Mead TJ, Lefebvre V. Proliferation assays (BrdU and EdU) on skeletal tissue sections. *Methods Mol Biol* 2014;1130:233–43.
- [28] Mangiavini L, Schipani E. TUNEL assay on skeletal tissue sections to detect cell death. *Methods Mol Biol* 2014;1130:245–8.
- [29] Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc Natl Acad Sci U S A* 2008;105(7):2415–20.
- [30] Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119(3):493–501.
- [31] Genge BR, Sauer GR, Wu LN, McLean FM, Wuthier RE. Correlation between loss of alkaline phosphatase activity and accumulation of calcium during matrix vesicle-mediated mineralization. *J Biol Chem* 1988;263(34):18513–9.
- [32] Mirando AJ, Dong Y, Kim J, Hilton MJ. Isolation and culture of murine primary chondrocytes. *Methods Mol Biol* 2014;1130:267–77.
- [33] Hill EL, Elde R. An improved method for preparing cryostat sections of undecalcified bone for multiple uses. *J Histochem Cytochem* 1990;38(3):443–8.
- [34] Hammarstrom L. Autoradiography and histochemistry of mineralized tissues by means of the Ullberg freeze-sectioning technique. *Ups J Med Sci* 1986;91(3):239–43.
- [35] Aaron JE, Carter DH. Rapid preparation of fresh-frozen undecalcified bone for histological and histochemical analysis. *J Histochem Cytochem* 1987;35(3):361–9.