

A Rapid, Nonradioactive In Situ Hybridization Technique for Use on Cryosectioned Adult Mouse Bone

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Abstract In situ hybridization (ISH) of adult bone is a difficult task that requires at least 3–5 weeks for decalcification, paraffin embedding, and sectioning. For that reason, bone ISH is often done only on embryonic or newborn animal tissue, leaving unanswered the question of gene expression in adults. Here, we report the development of an ISH system that requires only 7 days for acid-free decalcification, embedding, and sectioning, conditions that are conducive to preservation of tissue mRNA. The tissue cryosections, derived from adult mice 3–12 weeks old, were cut using the CryoJane Tape Transfer system. Paraffin-sectioned and cryosectioned tissue have comparable morphology. Examples are given of cryosections that were hybridized and stained enzymatically with digoxigenin-labeled riboprobes for mRNA found in either bone-forming osteoblasts (type I collagen, osteocalcin, Runx2) or the hypertrophic or proliferating chondrocytes (type X collagen, Runx2).

Keywords Adult bone · In situ hybridization · Type I collagen · Osteocalcin · Type X collagen · Runx2

Bone is a dynamic tissue, which undergoes continuous remodeling over the lifetime of the organism. The high

level of mineralization and the compartmentalized nature of adult bone create inherent difficulties in studying gene expression in bone cells and elucidating mechanisms of action of bone-active agents. During skeletal development, mechanical loading, bone remodeling, and aging, as well as in pathological states such as osteoporosis and osteoarthritis, differential expression of genes is evident in the osteoblasts, osteocytes, osteoclasts, and chondrocytes found within bone tissue (some examples in [1–3]).

In situ hybridization (ISH) is a powerful, commonly used technique to determine the precise spatial and temporal localization of mRNA transcripts of a particular gene of interest. Radioactive and nonradioactive ISH on paraffin-cut bone sections has been established previously for both adult and embryonic mice [4–6]; however, paraffin sectioning requires complete decalcification of bone. This takes several weeks, in addition to the time needed for dehydration, infiltration, and deparaffinization steps (3–5 weeks in total). The lack of a fast ISH procedure has limited the use of this normally powerful technique in fully calcified, adult bone tissue. Studies thorough enough to perform ISH on adult bone require weeks for each experiment and are, therefore, often not done. This can create an unrealistic portrayal of spatiotemporal gene expression.

The rate-limiting step in paraffin sectioning is complete decalcification with EDTA. Acid-based decalcification has been assessed as an alternative [7, 8]. Cryosectioning, which is a technique optimal for preservation of mRNA, is often used to produce high-quality sections of the majority of soft tissues, including embryonic bone. It is seldom used for ISH in adult mineralized bone because of the poor tissue morphology attained with these sections. Recently, the development of the commercially available CryoJane Tape Transfer System has allowed for preparation of adult bone cryosections that have similar tissue quality to

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paraffin-embedded sections [9]. We have combined the advantages of the CryoJane System with the partial decalcification in EDTA-based solution to optimize RNA preservation and save time. Here, we describe the development of a shortened, nonradioactive method for ISH that is applicable to adult mouse bone and requires only 7 days for bone tissue processing.

Materials and Methods

Animals

Female C57/Bl6 mice were kept on a 12-hour light/dark cycle with food and water provided ad libitum. Animals used in the study were between 21 days and 3 months old. Tissue was taken under Basel animal license 73.

Perfusion and Dissection

Animals were anesthetized with a cocktail of 120 mg/kg ketamine (Veterinaria, Zurich, Switzerland) and 25 mg/kg xylazine (Veterinaria) by intraperitoneal injection. Once fully under narcosis, the skin, abdominal muscle wall, and rib cage were opened with sharp scissors from the mid-abdomen to the throat. The diaphragm and ribs were further cut away from the thoracic cavity, preserving the heart, lungs, and major blood vessels. The liver was also made visible as an indicator of appropriate perfusion. The right atrium was punctured with sharp scissors, taking care not to damage the remainder of the heart, and 10 mL of 0.1 M phosphate-buffered saline (PBS, pH 7.4, 4°C; Invitrogen, Basel, Switzerland) was slowly injected into the left ventricle of the beating heart at a rate of approximately 3 mL/min to exanguinate the animal. As the blood was washed from the body, the liver turned from deep red/purple to yellowish brown, a color indicating successful perfusion. After the blood was flushed from the animal, it was further perfused with ice-cold, 4% paraformaldehyde (Sigma–Aldrich, St. Gallen, Switzerland) in 0.1 M PBS, pH 7.4. Following perfusion, the animal was rigid, another indicator of successful perfusion. Calcified tissues (femur, tibia, and calvaria) were removed and further fixed by immersion in ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Muscle and connective tissues surrounding the bone were not completely removed in order to preserve cellular structures at the periosteal surface of the bones.

Fixation and Decalcification

Tissue was immersion-fixed in ice-cold 4% paraformaldehyde, 0.1 M PBS (pH 7.4) for 16 hours with gentle agitation. Tissue was rinsed in 0.1 M PBS (pH 7.4, 4°C) for

30 min, then transferred to the following EDTA-based decalcification solution: 140 g EDTA (Sigma–Aldrich) in 750 mL double distilled (dd), diethyl pyrocarbonate (DEPC, Sigma–Aldrich)–treated H₂O (all water used in these experiments should be DEPC-treated ddH₂O) and 90 mL ammonium hydroxide (Sigma–Aldrich). The pH of the decalcification solution was adjusted to 7.4 with ammonium hydroxide, and the total volume was brought to 1 L [10]. Decalcification was continued for 3–5 days at 4°C with agitation and daily changes of decalcification solution (at least 10 times higher decalcification solution volume than tissue volume). This results in a partial decalcification of the tissue. Tissue was cryoprotected by transferring to a solution of 30% sucrose (Sigma–Aldrich) in PBS for 8 hours to 1 day. Tissue was dried of excess sucrose solution, positioned in OCT (Tissue-Tek) compound, and frozen on dry ice for cryosectioning. Frozen tissue blocks were stored in air-tight plastic bags at –20°C until sectioning.

Cryosectioning with the CryoJane System

CryoJane Tissue tape (Instrumedics, St. Louis, MO) and ultraviolet (UV) light-activated adhesive-coated slides (Instrumedics) were allowed to cool in the cryostat prior to sectioning (–24°C). Five-micrometer-thick sections were cut with disposable blades on a Cryostar HM 560 Cryostat (Microm, Volketswil, Switzerland). The length of the tissue in the frozen OCT block was oriented perpendicular to the disposable blade. After the block was trimmed by hand with a razor blade and cut to the required level for sectioning, a cold adhesive tape segment was adhered to the block face by slowly peeling off the removable backing, placing the adhesive side of the tape firmly on the embedded frozen sample, and firmly laminating the tape to the tissue block with the provided (cold) roller. The flywheel of the cryostat was turned by hand at a rate of approximately 90/s (one-quarter turn of the flywheel completed in 1 s) as this has been observed to give a smoother section than use of the cryostat motor. The tape was positioned on the cold adhesive-coated slide and laminated to the surface with the roller (all while remaining frozen in the cryochamber). From experience we have determined that the final sections have superior morphology and adhere better if the frozen slide with tape and tissue sections on it is gently heated on the back of the hand for 1–3 s and then rerolled immediately after. Appropriate lamination is achieved when the cortical bone tissue no longer appears bright white on the tape. The slide was then inserted into the Flash Tray and treated with a single UV flash, polymerizing the adhesive coating of the slide. The slide was left in the coldest part of the cryostat for 2–3 min. With the slide remaining in the cryostat, the adhesive backing was pulled off slowly with cold forceps, keeping

the delaminated backing parallel to the tape still adhered to the slide, to prevent the section from coming away with the tape. Slides were stored at -20°C until use.

Paraffin Embedding and Sectioning

Tissue for paraffin sectioning was prepared as for cryo-sectioning but with an extended decalcification time of 2–4 weeks. Decalcified femurs were dehydrated in graded alcohols from 50% to 100%, cleared with xylene, and impregnated in four changes of paraffin at 58°C . Bones were sectioned at 5- μm thickness and deparaffinized before staining.

Hematoxylin/Eosin Staining

Slides were rinsed in water, stained in hematoxylin for 2 min, and then washed well in tap water. After rinsing in 95% alcohol, the slides were stained with eosin for 10 s and rinsed with tap water, then dehydrated with alcohol, cleared with xylene, and mounted with polymount mounting medium (Polysciences, Warrington, PA, USA).

Gomori's Green Trichrome Staining

Gomori's Green Trichrome staining kit (Dako, Carpinteria, CA; AR166) was utilized in accordance with the manufacturer's instructions.

Riboprobe Preparation

Type I collagen and type X collagen constructs for riboprobe synthesis were provided by Markus John (Novartis Institutes for Biomedical Research, Basel, Switzerland) were originally a kind gift from E. Schipani (Massachusetts General Hospital, Boston, MA). Runx2 cDNA expression vector was a gift from Dr. Karsenty. Plasmid was linearized by Xho and reverse-transcribed using T3 RNA polymerase and digoxigenin (DIG) labeling mixture (Roche, Basel, Switzerland). The construct used to synthesize the osteocalcin riboprobe was ordered from the I.M.A.G.E. Consortium (accession BC101948). A pBluescript KS(+) plasmid containing a 293-bp fragment corresponding to region 4470–4781 bp at the C terminus of the human type I collagen gene was linearized with *EcoRI*, which cuts 5' to the fragment. T3 RNA polymerase (Roche) was used to generate antisense mRNA from the T3 promoter located 3' of the fragment. A pCR-Blunt II-TOPO plasmid containing the full-length, 438-bp cDNA of mouse osteocalcin was linearized with ERV, which cuts 5' to the fragment. SP6 RNA polymerase (Roche) was used to generate antisense mRNA from the SP6 promoter located 3' of the fragment. A

pBluescript SK(+) plasmid containing a 1,231-bp fragment of mouse type X collagen corresponding to region 1705–2936 bp of the gene was linearized with *XbaI*, which cuts 5' to the fragment. T7 RNA polymerase (Roche) was used to generate antisense mRNA from the T7 promoter located 3' of the fragment. Briefly, 10 μg of the DNA template was linearized preceding the 5' end of the molecule in a 100 μL volume with the appropriate unique restriction enzyme and buffer. To ensure linearization, 2 μL of this digested template was run on an agarose gel alongside an uncut sample. Contaminant proteins were removed from linearized DNA by extracting with an equal volume of phenol and chloroform. One hundred microliters of phenol (Sigma–Aldrich) was added to the linearized template, strongly mixed by vortex for 1 min, and centrifuged at 13,000 rpm for 2 min to separate the phases. The upper, aqueous phase was removed to a new Eppendorf tube and 100 μL of chloroform (Sigma–Aldrich) added. The sample was then mixed by vortex on high for 1 min and centrifuged at 13,000 rpm for 2 min to separate the phases. The upper, aqueous phase was removed to a new Eppendorf tube. Linearized, cleaned DNA was precipitated by addition of 0.5 volumes of 7.5 M ammonium acetate (50 μL , Sigma–Aldrich) and 2.5 volumes 100% ethanol (EtOH, 250 μL ; Merck, Darmstadt, Germany), which was mixed well by vortex and cooled at -70°C for 30 min. The sample was centrifuged at 13,000 rpm for 10 min and the supernatant discarded. The DNA precipitate pellet was washed with 1 mL 70% EtOH and centrifuged at 13,000 rpm for 10 min, and the supernatant was discarded. The pellet was air-dried (approximately 10 min) and resuspended in 10 μL RNase-free 1x transcription buffer. Concentration was checked by running 1 μL of the resuspended DNA on a 1% agarose gel alongside a marker of known concentration. To produce antisense riboprobes, the following reaction mixture was used: 1 μg clean, linearized DNA; 2 μL 10x transcription buffer (Roche); 2 μL DIG-NTP labeling mix (Roche); 0.5 μL RNase inhibitor (Roche); 1.5 μL of the appropriate RNA polymerase (T7, T3, or SP6, Roche); and DEPC ddH₂O to 20 μL . The reaction mixture was incubated at 37°C for 2–3 hours, after which the DNA template was digested by addition of 1 μL of RNase-free DNase I (Roche) and incubation for another 15 min at 37°C . After DNA digestion, the riboprobe was precipitated by addition of 2 μL 0.2 M EDTA, 2.5 μL 4 M LiCl (Merck), and 75 μL 100% EtOH. The mixture was gently mixed by vortex, chilled at -70°C for 20 min, and microcentrifuged at 13,000 rpm for 15 min. The supernatant was removed and discarded and the pellet washed in 70% EtOH and then centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and the pellet air-dried for 10–15 min. The pellet was then dissolved in 50 μL ddH₂O/RNase inhibitor (1 μL RNase inhibitor/100 μL RNase-free H₂O). One microliter of dissolved riboprobe solution was blotted

on an ethidium bromide/1% agarose gel to ensure that transcription occurred, and probe concentration was measured on an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Sense riboprobes were generated using a similar method but with unique restriction sites at the 3' end of the gene to linearize the DNA and at the 5' promoter to drive the transcription.

Hybridization

Slides were placed in a Wheaton (Millville, NJ) slide rack and fixed in 4% paraformaldehyde, 0.1 M PBS (pH 7.4) for 10 min, then washed three times in 0.1 M PBS (3 min/wash). Slides were treated with Proteinase K (1 mg/L, Roche) for 5 min, then transferred back to the same 4% paraformaldehyde, 0.1 M PBS (pH 7.4) for an additional 5 min, followed by three washes in 0.1 M PBS (3 min/wash). The slides were then transferred to a 1.2% solution of triethanolamine (Riedel-de Haen, Buchs, Switzerland) in double-distilled water, where they were acetylated by dropwise addition of 1.2 mL of acetic anhydride (Sigma-Aldrich) with simultaneous stirring, in order to block reactive amine groups present in the tissue from binding probes nonspecifically. Following acetylation, the slides were washed in 0.1 M PBS (3 min/wash). Slides were then removed from the Wheaton rack, laid horizontally in a humidified tray, and prehybridized with 500 μ L of a hybridization solution (50% formamide [Sigma-Aldrich], 5x SSC, 5x Denhardt's, 250 μ g/mL torula yeast RNA, 500 μ g/mL ssDNA [Roche]) for 1 hour at room temperature (RT). Dissolved riboprobe was diluted in hybridization solution to a final concentration of 1 μ g/mL, with 200 μ L of riboprobe solution prepared for each slide. Prior to hybridization, the solution was heated to 80°C for 5 min, then removed to ice for an additional 2 min. After this cooling period, the solutions were kept at RT until addition to the slides. Prehybridization solution was removed from each slide and 200 μ L of riboprobe/hybridization solution added over the entire surface of the slide. A glass coverslip was laid carefully on top of the slide covering all tissue sections, to minimize entrapment of air bubbles. A 25-slide plastic box, stood on its smallest side, served as a cassette and was humidified with a tissue soaked in 1:1 formamide/water solution placed at the bottom of the box. The slides were then placed horizontally in the cassette, which was sealed tightly with tape, and then placed in a 56°C hybridization oven overnight. A separate cassette was used for each probe.

Washes and Alkaline Phosphatase Signal Development

The following morning a water bath was used to heat covered-glass Wheaton staining dishes of 5x SSC and

0.2x SSC to 70°C. Slides were transferred with coverslips to a Wheaton rack and washed in 5x SSC for 5 min at 70°C. An empty Wheaton rack was immersed in the 0.2x SSC, and the slides were transferred from the 5x SSC one by one, leaving coverslips in 5x SSC. Slides were left at 70°C in 0.2x SSC for 1 hour to inactivate endogenous alkaline phosphatase activity, after which the staining dish was removed from the water bath and allowed to cool for 5 min at RT. Slides were transferred by Wheaton rack to B1 buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 5 min to equilibrate and then laid horizontally in a humidified chamber and covered in 500–700 μ L 2% blocking reagent/B1 buffer (blocking solution) for 1 hour at RT to block nonspecific binding. Anti-DIG antibody (Roche) conjugated to alkaline phosphatase was diluted in blocking solution (1:2,000). Blocking solution was aspirated from the slides and replaced with 500 μ L of the antibody solution. Slides were incubated either 3 hours at RT or overnight at 4°C in a humidified tray. Excess antibody solution was removed from the slides, and they were rinsed 3 x 5 min in B1 buffer at RT in a Wheaton slide rack. Slides were then laid flat in a dark, humidified chamber and covered with 500 μ L BM purple (Roche) to develop. Slides were checked for progression of development every 30 min with a dissection microscope. After the slides were developed to the desired level, they were rinsed twice in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and mounted in 250 μ L Kaiser's gelatin (Merck, Dietikon, Switzerland).

Horseshoe Peroxidase Fluorescent Signal Development

ISH was performed according to the instructions of the TSATM Plus Cyanine 3 System (NEL744001KT; Perkin-Elmer, Beaconsfield, UK). In brief, sections were postfixed in 4% phosphonoformic acid for 10 min and quenched in 3% H₂O₂ for 30 min at room temperature. Proteinase K digestion (8 or 4 μ g/mL) was performed in either 7-week-old femur or neonatal tibias, respectively, to penetrate the tissue for better probe accession. Slides were hybridized overnight with DIG-labeled RNA probe at 55°C in hybridization solution containing 50% formamide (deionized), 10% dextran sulfate, 1% Denhardt's solution, 250 μ g/mL yeast tRNA, 0.3 M NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA, 10 mM Na₃PO₄, and 1% sarcosyl. After stringent washes, slides were blocked with 0.5% blocking reagent (Perkin-Elmer FP 1020) at room temperature for 30 min, incubated with anti-DIG-POD Fab fragment (Roche, 11 207 733 910), using 1/100 dilution at RT for 30 min, and incubated with TSA solution at room temperature for 7 min.

Table 1 Differences in ISH decalcification and tissue processing methods

	EDTA/cryosections	EDTA/paraffin
Fixation	3 days	1 day
Decalcification	3 days	14–28 days
Dehydration	–	1 day
Infiltration	–	1 day
Cutting	1 day	1 day
Drying	–	1 day
Deparaffinization	–	1 day
Hybridization	1 day	1 day
Antibody labeling	1 day	1 day
Signal development	1 day	2 days
Total time	10 days	24–38 days
Morphology	++++	++++
mRNA quality	++++	++
Speed	++++	+

Results

The presented method has been optimized to perform ISH on partially demineralized adult bone tissue. Importantly, no strong acid was used for partial demineralization, facilitating preservation of tissue mRNA. This method, described in detail in “Materials and Methods,” allows for fast, 7-day processing of bone tissue compared to the

classical paraffin sectioning that requires 3–5 weeks for this procedure (Table 1). Three to four additional days are required for hybridization and signal development by both methods. A summary of the steps required in each mode of tissue preparation (paraffin and CryoJane sectioning) and the approximate time necessary for each step is presented in Table 1, as well as the advantages of each method.

Morphology of Adult Bone Cryosections vs. Paraffin Sections

So far, complete decalcification in EDTA solution followed by paraffin embedding was standard for obtaining good morphology of bone tissue sections. To assure the quality of cryosections of partially demineralized bone, we compared the morphology of cryosections to the paraffin sections (Fig. 1). Paraffin sections (Fig. 1a, c) have a very similar, high-quality morphology compared to sections cut on the CryoJane system (Fig. 1b, d). Hematoxylin/eosin (HE) staining of both types of sections (Fig. 1a, b) revealed intact trabecular bone (pink) as well as minimally distorted bone marrow (purple). Chondrocytes of the growth plate were also well preserved (arrow). Staining intensity of HE is greater in cryosections.

Gomori’s green trichrome staining (Fig. 1c, d) also showed excellent morphology in both paraffin and cryosections. Intact trabecular bone (green) was apparent in both sectioning methods, as were the columns of

Fig. 1 Morphology of paraffin vs. cryosections. Paraffin sections (a, c) and cryosections (b, d) were stained with HE (a, b) or Gomori’s green trichrome (c, d)

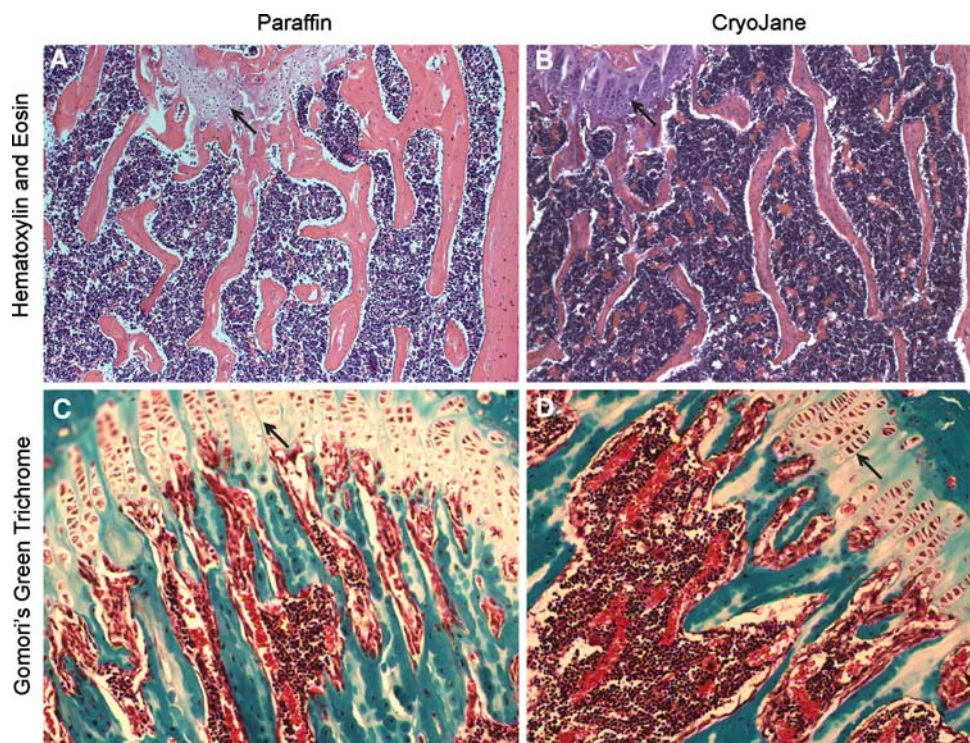
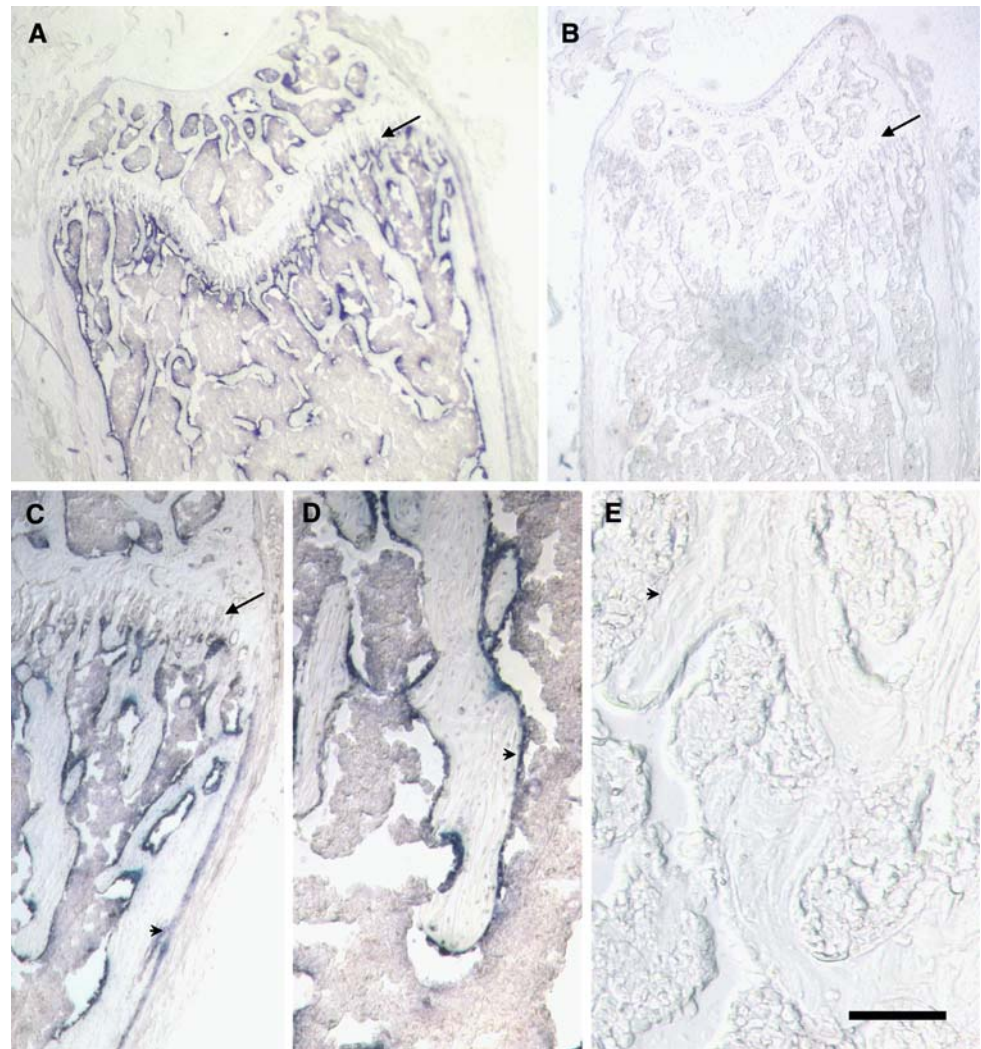


Fig. 2 Expression of type I collagen mRNA in partially decalcified adult mouse femur. Cryosections of mouse femur were probed with antisense (**a**, **c**, **d**) or control sense (**b**, **e**) riboprobes for type I collagen, as described in “Materials and Methods.” Type I collagen mRNA is expressed surrounding, but not within, the trabeculae, as well as along the endosteal and periosteal surfaces (**a**, **c**, **d**). Note the lack of expression within the growth plate. *Diagonal arrows* indicate location of hypertrophic chondrocytes of the growth plate; *horizontal arrowheads* indicate osteoblasts surrounding trabecular bone. Scale bars = (**a**) 0.75 mm, (**b**) 0.95 mm, (**c**) 0.3 mm, and (**d**, **e**) 0.15 mm



proliferating and hypertrophic chondrocytes (arrow). We conclude that morphology of cryosections is well preserved by our protocol and is comparable to paraffin sections.

ISH of Adult Bone Cryosections

To demonstrate the utility of the rapid ISH method for detection of genes expressed in bone cells, we performed ISH for several genes expressed in osteoblasts and chondrocytes. Type I collagen is specifically expressed in cells of the osteoblast lineage from precursor cells up to mature osteoblasts. ISH of adult (3-month old) mouse femur cryosections revealed strong expression of type I collagen mRNA in cells surrounding the trabeculae, as well as in the endosteal and periosteal cortex (Fig. 2a, c, d). Type I collagen expression is undetectable in the chondrocytes of the growth plate (arrow). Slides probed with sense control riboprobe for type I collagen showed no staining (Fig. 2b, e).

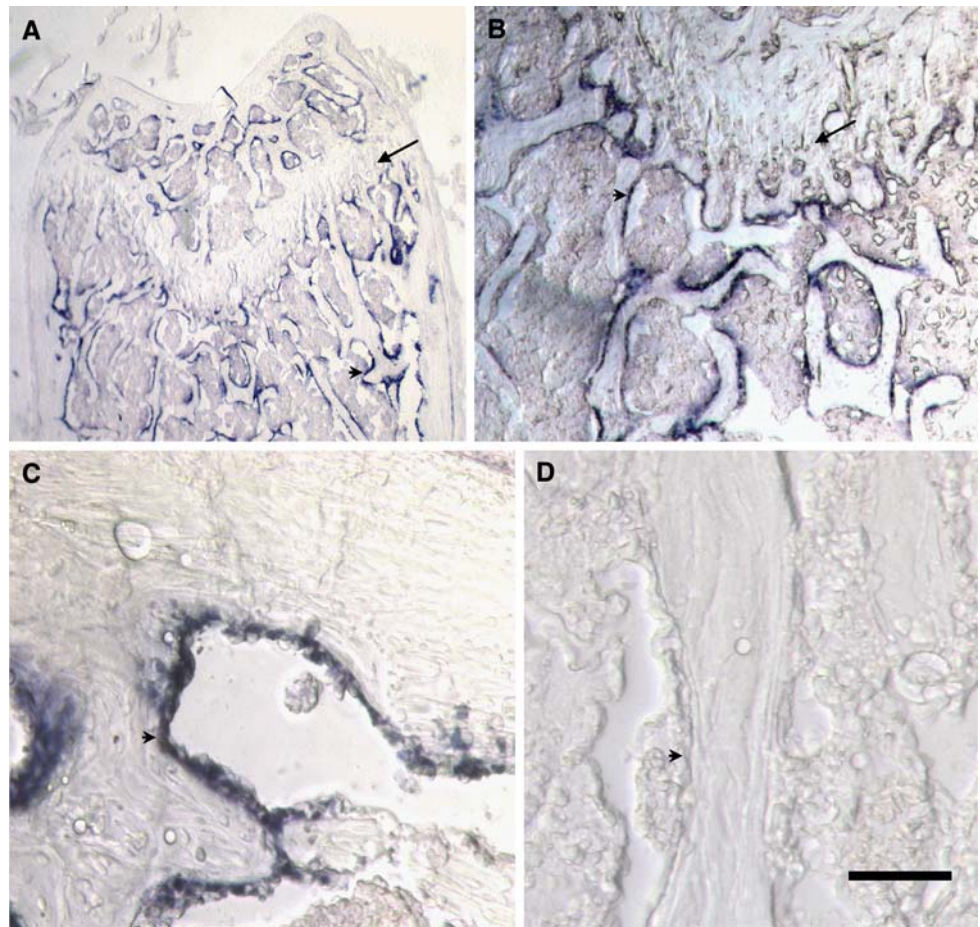
Osteocalcin is a gene that is very specifically expressed in mature later-stage osteoblasts. Using anti-osteocalcin

riboprobe on cryosectioned adult (3-month old) mouse bone, osteocalcin mRNA was detectable in osteoblasts on the trabecular surface (Fig. 3a–c). Similar to type I collagen, osteocalcin is not found in the growth plate chondrocytes. Slides probed with sense control riboprobe for osteocalcin showed no staining (Fig. 3d).

Type X collagen is specifically expressed in hypertrophic chondrocytes. In the growth plate of the skeletally immature, 3-week-old mouse, strong and specific expression of type X collagen was observed, exclusively localized to the hypertrophic chondrocytes of the growth plate undergoing endochondral ossification (Fig. 4a–c). In the adult femur (3-month old), expression occurs in the same region of the growth plate but at a significantly reduced level (Fig. 4e, f). Type X collagen is not expressed in the regions around the trabeculae, where osteoblasts predominate. Slides probed with sense control riboprobe for type X collagen showed no staining (Fig. 4d).

We have also performed ISH with probes against osteonectin and osteopontin of wild-type and *Hey1* transgenic

Fig. 3 Expression of osteocalcin mRNA in partially decalcified adult mouse femur. Cryosections of mouse femur were probed with antisense (a–c) or control sense (d) riboprobes for osteocalcin, as described in “Materials and Methods.” Osteocalcin is expressed around, but not within, the trabeculae, as well as along the endosteal surfaces (a–c). Note the lack of expression within the chondrocytes of the growth plate. *Diagonal arrows* indicate location of hypertrophic chondrocytes of the growth plate; *horizontal arrowheads* indicate osteoblasts surrounding trabecular bone. Scale bars = (a) 0.75 mm, (b) 0.35 mm, (c) 0.07 mm, (d) 0.06 mm



mice, which have also shown expected expression (data not shown). We conclude that both osteoblast- and chondrocyte-specific genes can be reliably detected in adult bone cryosections using our rapid ISH protocol.

In order to ensure that a low-abundance mRNA can also be detected with this method, we performed ISH with the probe against transcription factor Runx2. To enhance the sensitivity of detection, we used horseradish peroxidase fluorescence instead of the alkaline phosphatase visual detection method. Since Runx2 mRNA expression declines as animals age, we performed ISH both on neonatal 7-day-old tissue and on bone tissue from 7-week-old mice (Fig. 5). As expected, expression was clearly detected in the neonatal tissue (Fig. 5e–h') and much weaker in 7-week old bone (Fig. 5a–c'). The location of expressed Runx2 was as expected in proliferating chondrocytes and osteoblasts. The sense control riboprobes were negative.

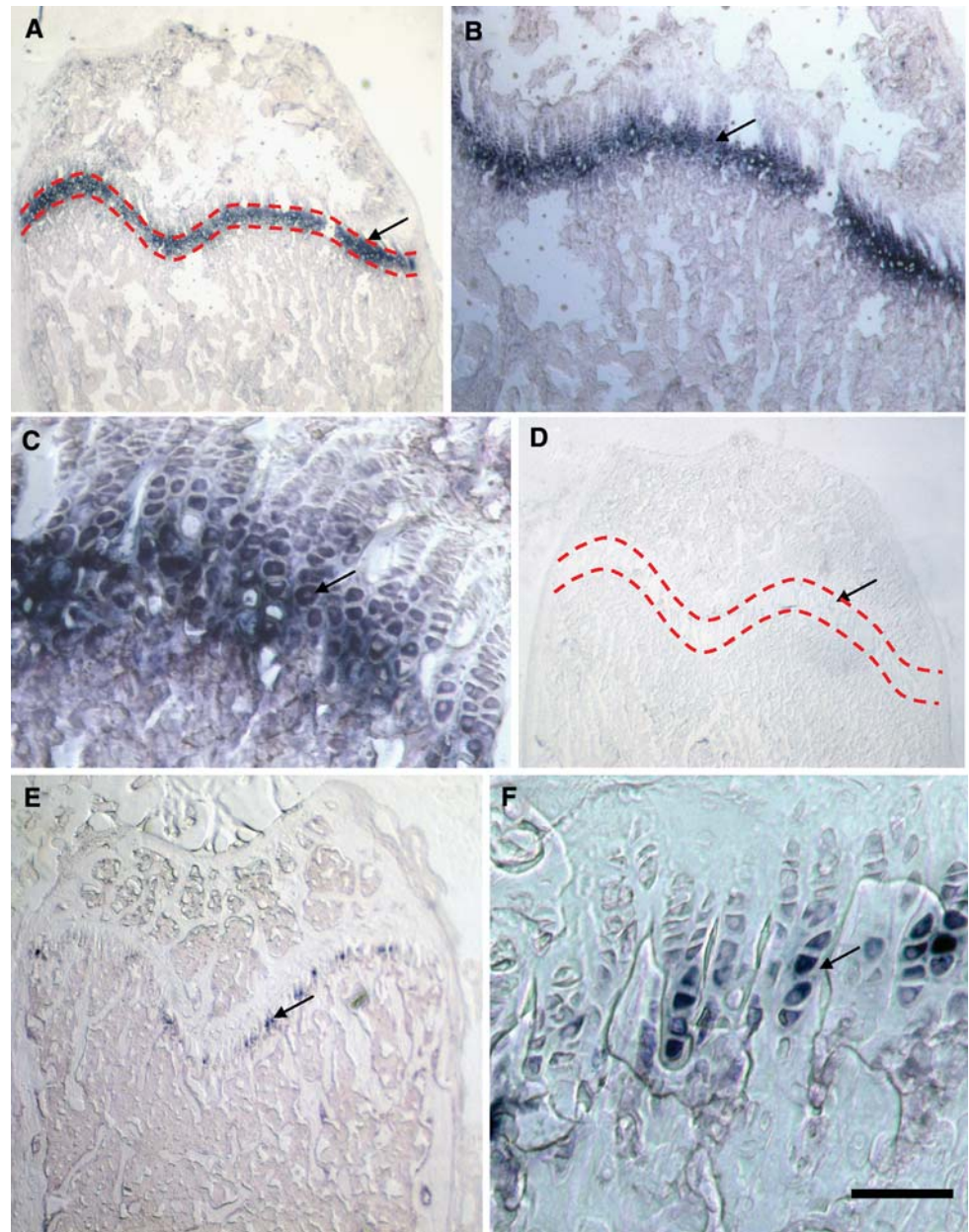
Discussion

Here, we describe in detail a modified technique to perform rapid, nonradioactive ISH on partially decalcified adult mouse bone cut with the CryoJane Tape Transfer system.

The morphology of these sections is comparable to that of paraffin sections and has the advantage of much quicker preparation and of the use of EDTA-based decalcification solution, which preserves tissue mRNA. The reduced preparation time also results in a decreased chance of mRNA degradation occurring by accidental RNase contamination of the samples.

The major obstacles in bone histology are the mineralization and heterogeneity of the tissue. Embedding in plastic allows for cutting of the tissue without decalcification; however, it is not applicable for ISH. Decalcification removes the mineralization, while leaving behind the other components of the tissue. Acid-decalcifying solutions containing HCl, nitric acid, and formic acid, while decalcifying quickly, have been shown to significantly reduce RNA levels [11, 12]. Others have argued that Morse's solution, which contains formic acid, preserves mRNA [7, 8]. Since RNA is generally sensitive to acid and is degraded by hydrolysis, any protocol that is free of harsh acids has a potential to be more sensitive and reproducible. EDTA, a divalent metal chelator, strips Ca^{2+} ions out of the bone matrix and is often used for decalcification of bone. This method is slower than acid decalcification but results in intact mRNA in the tissue. However, the extended time

Fig. 4 Expression of type X collagen mRNA in decalcified 3-week-old and adult mouse femur. Cryosections of mouse femur were probed with antisense (a–f) or control sense (d) riboprobes for type X collagen, as described in “Materials and Methods.” Type X collagen is expressed specifically in hypertrophic chondrocytes located in the growth plate. Expression is significantly stronger in growing bone (a–d, 3 weeks old) than in adult bone (e, f, 3 months old). *Diagonal arrows* indicate location of hypertrophic chondrocytes of the growth plate. *Red dashed lines* indicate zone of hypertrophy. Scale bars = (a) 0.7 mm, (b) 0.4 mm, (c) 0.1 mm, (d) 0.7 mm, (e) 0.8 mm, (f) 0.1 mm



period for complete EDTA decalcification (2–4 weeks) puts the mRNA at risk for degradation, and reducing the decalcification time is important for protecting the mRNA. The advent of the CryoJane system allows for easy sectioning of only partially decalcified tissue, reducing the time frame of each experiment by several weeks. While the CryoJane system requires an initial equipment investment, the time costs saved by its use quickly compensate for the money spent. Crucial factors for successful application of our method are (1) proper fixation and decalcification, as described in “Materials and Methods”; (2) use of DEPC-treated solutions; (3) good mastering of CryoJane cryosectioning, which requires practice and possibly advice of experienced experimenters; (4) use of good-quality probes

that are ideally checked on neonatal tissue; and (5) use of fluorescent detection for greatest sensitivity.

We studied the expression of type I collagen and osteocalcin, two genes known to be expressed in osteoblasts, as well as that of type X collagen, which is specific to hypertrophic chondrocytes. Type I collagen mRNA is highly expressed by all osteoblasts during both development and adulthood but is strongly reduced in the chondrocytes of the growth plate [13, 14]. Osteocalcin is a bone-specific, non-collagenous protein found only in the mature osteoblast [1, 15]. Type X collagen belongs to the family of short-chained collagens and is synthesized by the terminally differentiated, hypertrophic chondrocytes (reviewed in [16]). Its expression in the growth plate decreases as the bone finishes elongating

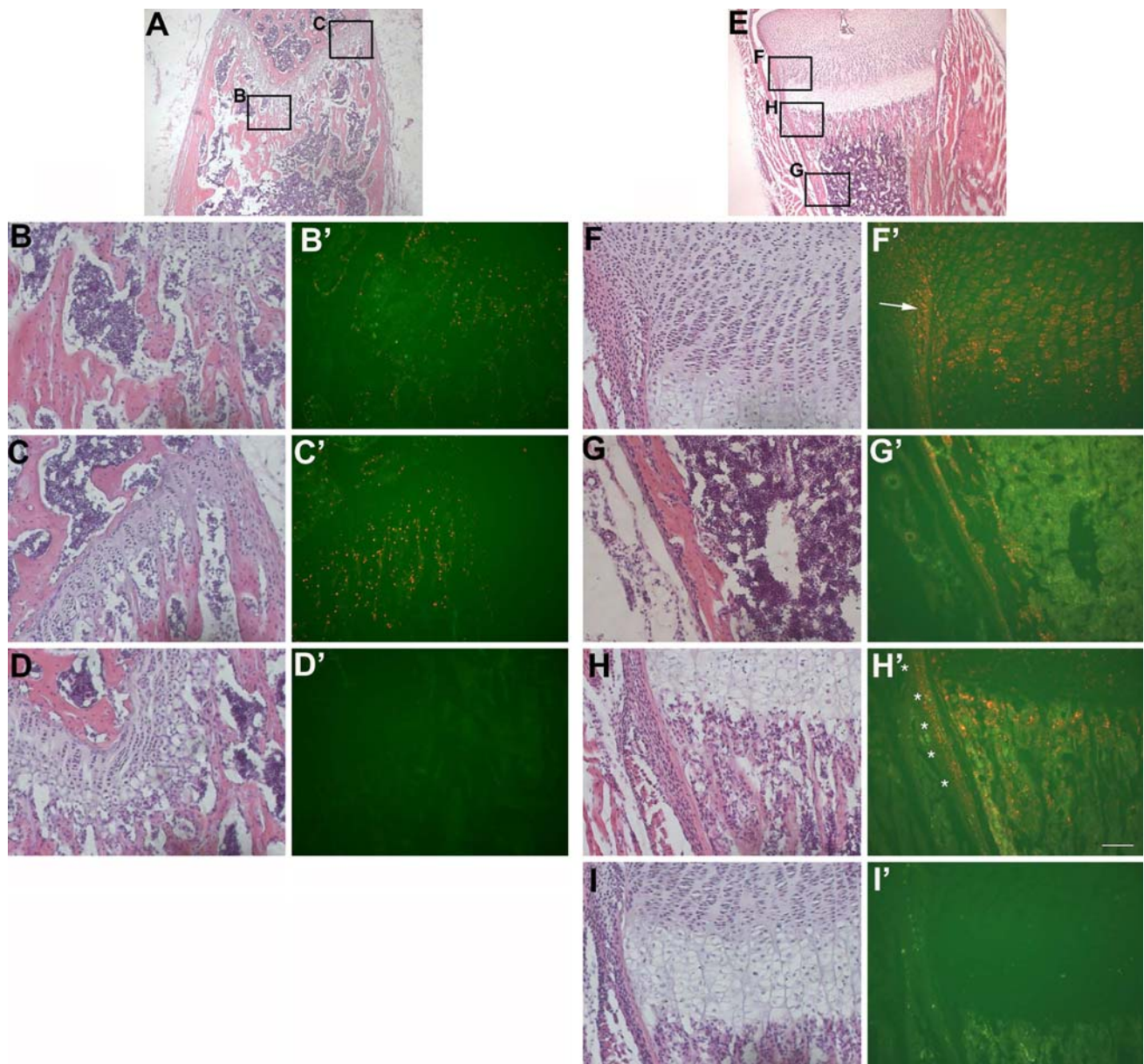


Fig. 5 Expression of Runx2 mRNA in decalcified 7-week-old mouse femur and 7-day-old mouse tibia. Cryosections of mouse tibia and femur were probed with antisense (**a-c'**, **e-h'**) or control sense (**d, d'** and **i, i'**) riboprobes for Runx2, as described in "Materials and Methods." Fluorescent signal detection was used on 7-week-old murine femur (**a-c'**) or 7-day-old murine tibia (**e-h'**). Expression is

stronger in the neonatal long bone compared to 7-week-old bone. Runx2 was expressed in the proliferating chondrocytes (**f'**), osteoblasts on periosteal and endocortical bone surfaces (**g'**), osteoblasts on trabecular bone surface in spongiosa (**h', b', c'**), fibroblasts in perichondrium (**f'**, indicated by *arrow*), and osteoblasts in the bony collar region (**h'**, indicated by *). Scale bar = 0.05 mm

and reaches maturity [17]. In agreement with this, we detected massive reduction in type X collagen mRNA expression between 3- and 12-week-old animals. Runx2 is a low-abundance mRNA that is expressed in chondrocytes and osteoblasts. Using sensitive fluorescence detection, we obtained a signal not only in neonatal but also in 7-week-old bone. As with collagen X, the expression declines as mice age. Therefore, even with sensitive detection methods, it may be necessary for some mRNAs to perform ISH in

neonatal or growing bone, as well as in adult bone, to ensure positive signals. Such analyses also provide better insight into the relevance of gene expression in adult bone, which is often necessary when deciding on novel drug targets in bone.

We have demonstrated robust, specific detection of mRNA for four bone-related molecules in cryosections of adult bone. To our knowledge, this is the first report of rapid, nonradioactive ISH on adult bone cryosections minimally decalcified with EDTA. We believe that this technique has

the potential to be adopted as the gold standard for speed, signal strength, specificity, and morphology for in situ studies of mRNA expression within calcified adult bone tissue.

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