# Chapter 15

# Immunostaining of Skeletal Tissues

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## Abstract

Immunostaining is the process of identifying proteins in tissue sections by incubating the sample with antibodies specific to the protein of interest, then visualizing the bound antibody using a chromogen (immunohistochemistry or IHC) or fluorescence (immunofluorescence or IF). Unlike in situ hybridization, which identifies gene transcripts in cells, immunostaining identifies the products themselves and provides information about their localization within cells (nuclear, cytoplasmic, or membrane) or extracellular matrix. This can be particularly important in the context of bone and cartilage because they contain many cell types as well as matrix components, each with distinct protein expression patterns. As the number of antibodies continues to grow, this technique has become vital for research laboratories studying the skeleton. Here, we describe a detailed protocol for antibody-based in situ analysis of bone and associated tissues, addressing specific issues associated with staining of hard and matrix-rich tissues.

**Key words** Immunostaining, Immunohistochemistry, Immunofluorescence, Bone, Cartilage, Decalcification, Fixation, Antibodies, Antigen retrieval

## 1 Introduction

In both clinical and research studies, histology-based methods are critical for describing phenotypes in patients and in experimental organisms. There are 3–4 main steps for immunostaining:

1. Incubation with antigen-specific primary antibody.
2. Incubation with an enzyme-, biotin-, or fluorophore-conjugated secondary antibody.
3. Detection of secondary antibody via an enzymatic reaction that produces a colored precipitate (IHC).
4. Imaging using standard light (IHC) and/or confocal fluorescence microscopy (IF).

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However, the tissue collection and processing steps that come prior to immunostaining are crucial and can greatly affect the image quality. This is particularly true for bone and cartilage where it is necessary to decalcify tissue while maintaining matrix components such as proteoglycans. Therefore careful attention must be paid to each step from tissue harvest and fixation to decalcification and antigen retrieval [1]. Mistakes and overprocessing at any of these steps can damage antigenic epitopes, tissue morphology, or adhesion of tissue to slides, making it difficult to assess morphology and obtain good staining.

### 1.1 Fixation

Fixation is the process of treating tissue with solutions that preserve gross morphology as well as molecular structures within the tissue and should be started as soon as possible after harvest [2, 3]. Penetration of fixative is determined by the size and nature of the tissue of interest. Soft tissues and small pieces of tissue will fix faster than larger or harder tissues. The standard fixative for paraffin embedding is 10% neutral buffered formalin (NBF), while the most typical for frozen sections is 4% paraformaldehyde (PFA). However, when applied properly, either fixative can be used for each embedding method. As with most fixatives, these solutions preserve tissue by cross-linking the proteins. Therefore, tissues fixed in 10% NBF and 4% PFA usually require antigen retrieval before incubation with primary antibody. Because of the cross-linking action, it is important to avoid over-fixation as this can lead to excessive cross-linking, which may mask the antigens, or dehydration, which may produce an undulation artifact during sectioning [2]. Fixation in neutral buffered zinc formalin, which prevents excessive cross-linking, should also be considered for frozen sections, as it often eliminates the need for antigen retrieval steps [4]. Although unfixed frozen sections are useful in some situations such as reporter cell lines or mice that express fluorescent proteins, fixation is almost always required when performing antibody-based detection, and results are generally better when tissue is fixed up front, rather than dipping sections into fixative.

To ensure proper preservation when working with bone or cartilage, it is necessary to clean away any unwanted soft tissue such as skin and muscle. This allows for fixative penetration in a timely manner and avoids under- or over-fixation. It also makes it easier to orient the bone during embedding. Additionally, undesired autofluorescence of blood cells can be removed by perfusing the animal with phosphate-buffered saline (PBS) and 10% NBF or 4% PFA to rinse and fix the vasculature, followed by a post-fix of immersion in 10% NBF or 4% PFA [5].

### 1.2 Decalcification

Before embedding bone in paraffin or OCT, it is essential to soften the tissue by lowering the calcium content (i.e., decalcification). The duration of decalcification and degree of calcium ion removal

are influenced by the solution used. Most commercial solutions are acids, either mineral or organic, and soften bones quickly, but they can easily damage the tissue and are generally not compatible with immunostaining. Another less common method is the use of an ion-exchange resin to exchange ammonium ions for calcium ions. While this method yields the best morphology, especially in bone marrow specimens, it is also the most expensive option [2]. The most useful decalcifying method for immunostaining is treatment with 14% ethylene diamine tetraacetic acid (EDTA) [2, 3, 6]. This gentler chelating agent may decalcify hard tissues more slowly but is less likely to damage tissue or affect antigenicity. Even with EDTA, it is important to monitor and optimize the decalcification duration. Failure to do so can lead to poor morphology and weak antibody-based staining. Lastly, if decalcification is not an option for your desired antigen or preparation, specialized tape transfer methods can be used to capture calcified sections of frozen or resin-embedded tissues [7].

### 1.3 Antigen Retrieval

Due to the cross-linking action of most fixatives, it is often necessary to unmask antigens before staining [8]. The choice of retrieval method will vary according to the antigens and antibodies used. There are several methods of antigen retrieval but they fall into two main categories, enzyme digestion and heat treatment. Each retrieval method presents its own challenges and needs optimization for different specimen types. Enzyme digestion requires precision in pH and duration of treatment because different tissues will digest at different rates. The challenge in heat retrieval is in treating the tissue long enough to ensure antigen retrieval without causing it to lift off from the slide, which is a common problem when working with cartilage and bone. For frozen sections, if retrieval is necessary, enzymatic methods should be of primary consideration due to the fragility of hydrated, aqueous-treated tissues. Antigenicity in frozen sections can also be improved by use of a buffer solution containing detergent (such as Tween 0.05–0.1%).

### 1.4 Data Analysis

To accurately interpret staining, it is important to know the standard morphology and staining patterns in the tissue of interest. Textbooks on histology, pathology, and developmental biology can be a good resource for identifying the cells and structures. To interpret the staining itself, the first priority is in determining whether the signal (whether a chromogen or fluorescence) is specific or represents nonspecific background. Having both negative and positive controls is crucial in making this determination. Negative control slides can be generated in two ways: no primary antibody or isotype- and species-matched immunoglobulin or serum (which contains immunoglobulin) instead of primary antibody step [8, 9]. While the no primary negative controls are usually acceptable, the isotype control is the gold standard because it is possible to

see interactions between nonimmune immunoglobulins from one species and target tissues from another. When using fluorophores, the "no primary" control indicates native tissue autofluorescence. Another good option for negative control comparisons is the use of knockout tissue or cell lines with all the same reagents as test slides, including the primary antibody. Ideally, control slides should have no staining at all. However, some background is often unavoidable and usually related to specific structures/cells, and thus the negative control slides must be directly compared to the test slides in similar areas to demonstrate specificity.

Other important factors to consider are which tissues, cells, or organelles are stained and whether the staining pattern makes physiological sense based on known molecular pathways. If knockout/wild-type pairs of the exact site and experimental conditions are available, staining in the wild-type samples can be interpreted as positive. When such pairs are not in hand, positive control slides from other sites or conditions with previously established antigen expression are very useful here, although different tissue types may have quite different staining patterns as well as nonspecific background. Complementary techniques such as in situ RNA hybridization, which identifies gene expression in specific cells, laser capture microdissection with RNA analysis, or tissue fractionation with protein or RNA analysis can also be used to verify findings.

## 2 Materials

### 2.1 IHC on Paraffin Sections

1. Phosphate-buffered saline (PBS).
2. Citrate buffer, pH 6: Make 0.1 M stock solutions of citric acid and trisodium citrate. To 450 mL dd water add 9 mL of citric acid stock solution and 41 mL of sodium citrate stock solution. The pH of this final solution should be about $6.0 \pm 0.1$.
3. 10% Neutral buffered formalin (NBF).
4. 4% Paraformaldehyde (PFA) can be purchased as a 16% stock. Making your own from powder is hazardous, and respiratory precautions must be taken.
5. Peroxidase block (3% $H_2O_2$ in methanol): 25 mL 30% $H_2O_2$ to a final of 250 mL in 100% methanol chilled at −20 °C.
6. Positively charged slides such as Fisherbrand Superfrost Plus (Thermo).
7. Vectastain ABC Kit (Vector Labs).
8. DAB Chromogen (Biocare).
9. 14% Free acid EDTA, pH 7.2–7.4 (EDTA Decalcification Buffer): Mix 140 g EDTA free acid with 700 mL distilled water. While stirring, slowly add 30 mL ammonia hydroxide

at 30-min intervals (for a total of 90 mL ammonia). Check the pH. EDTA will not dissolve until pH is close to 7.2 If not up to 7.2, add the remaining 10 mL ammonia hydroxide dropwise to get to pH 7.2, while constantly stirring. Add ~300 mL distilled water for final volume of 1 L. It is critical that this solution is made properly. If you overshoot the pH, do not attempt to correct with HCl—just start over, as the excess chloride ions will prevent proper EDTA chelation of ionized calcium [2].

10. Methanol.
11. Graded ethanols (30%, 50%, 70%): It is least expensive to dilute from a purchased 70% stock, but 95% can also be used. These concentrations do not have to be very exact. Adding about 50 or 70 mL of 70% ethanol and diluting up to 100 mL in $ddH_2O$ is sufficient.
12. Xylene.
13. Coplin jars.
14. Humidity chamber: Any container with a lid can be lined with damp paper towels to make one, and several companies sell them.
15. Mounting medium, xylene compatible such as Richard-Allan Scientific™ Mounting Medium (Thermo).
16. Proteinase K in 10 mM Tris–HCl, pH 7.4–8.0. Make a 10 mg/mL Proteinase K (Roche) stock solution in $ddH_2O$. 1 M Tris–HCl pH 8.0 is commercially available. Dilute 1 M Tris–HCl stock solution at 1:100 dilution (10 μL/mL) to make a 10 mM Tris–HCl diluent solution. To 1 mL of this diluent solution, add 1 μL of the Proteinase K stock solution to get a final concentration of 10 μg/μL of Proteinase K.
17. 10% Blocking serum in PBS (*see* **Note 1**).
18. Primary antibody.
19. Secondary antibody (enzyme or biotin-conjugated).
20. Coverslips.
21. Mounting media, such as Permount (Fisher) or Cytoseal (Richard-Allan).

### 2.2 IF on Frozen Sections

1. Tris-NaCl-Tween Buffer (TNT): Combine 50 mL 1 M Tris–HCL pH 7.4, 25 mL 3 M NaCl, and 250 μL Tween-20. Bring the solution to 500 mL total using $dH_2O$ and mix. Filter the solution at .2 μm for best results. PBS with 0.1% Tween 20 (PBST) may also be used in place of TNT.
2. 10% Neutral buffered formalin (NBF), buffered aqueous zinc formalin (ZF), or 4% paraformaldehyde (PFA). PFA can be purchased as a 16% stock. Making your own PFA from powder is hazardous, and respiratory precautions must be taken.

3. 10% Blocking serum in TNT (*see* **Note 1**).
4. Primary antibody.
5. Fluorophore-conjugated secondary antibody.
6. DAPI counterstain.
7. Positively charged slides such as Fisherbrand Superfrost Plus (Thermo).
8. Coverslips.
9. PAP pen, such as SuperPAP with 4 mm tip (Biotium).
10. Aspirator vacuum pump.
11. Disposable freeze-safe cassettes for embedding.
12. 14% Free acid EDTA, pH 7.2 (EDTA Decalcification Buffer): *See* Subheading 2.1 for more details.
13. Humidity chamber.
14. Glycerol-based aqueous mounting medium, such as Fluoromount-G Mounting Medium (Affymetrix).
15. Clear nail polish.

## 3 Methods

### 3.1 IHC on Paraffin Sections

#### 3.1.1 Tissue Preparation

1. If perfusing for rodent studies, an approved dose of anesthetic should be administered as determined by your local animal safety committee. When the animal is fully sedated, it can be perfused with an intracardial injection of 1 M PBS, followed by 10% NBF or 4% PFA (typically 10 mL to 25 mL each). Constant flow rate can be applied with a peristaltic syringe pump or other method to improve the quality of the perfusion fixation [5].
2. Immediately after dissection, fix bones in 10% neutral buffered formalin or 4% paraformaldehyde for 24–72 h at room temperature or 4 °C. Fixative volume should be 15–20 times tissue volume. To ensure complete penetration, tissue should be agitated on a shaker during fixation (*see* **Note 2**).
3. Rinse tissue in PBS or distilled deionized water ($ddH_2O$) 6 times, 15 min each.
4. Decalcify in 14% free acid EDTA, pH 7.2–7.4, with rocking, changing solution daily (on weekdays only is OK). The number of days required for decalcification of mouse bones is as follows (*see* **Note 3**):
   (a) Embryo > E17.5: 1–2 days.
   (b) Postnatal days (P) 1–4: 3 days.
   (c) P5-P10: 4–5 days.

(d) P10-P21: 7–10 days.

(e) Adults: 10–14 days.

5. Rinse tissue in PBS or $ddH_2O$ 6 times, 15 min each, to stop the decalcification process (*see* **Note 4**).
6. Dehydrate the tissue through a series of ethanol solutions, with rocking.
   (a) 30% ethanol for 15 min.
   (b) 50% ethanol for 15 min.
   (c) 70% ethanol for 15 min.
7. Place tissue in the tissue processor for dehydration, clearing, and infiltration before embedding. Typically, a 4-h processing program works well for most machines with mouse long bones (*see* **Note 5**).
8. When processing is complete, the bones are embedded in paraffin. It is important to determine the plane of interest before this point in order to orient the tissue properly.
9. Tissue sections are then cut at 5 μm using a microtome, floated onto a 45 °C water bath and placed on color frost slides (*see* **Note 6**).
10. Slides are dried at room temperature overnight before storage (*see* **Note 7**).

### 3.1.2 Antibody-Based Staining

1. Heat slides in a 55 °C oven for 1 h (*see* **Note 8**).
2. Incubate slides in Xylene 3 × 5 min.
3. Incubate slides in 100% ethanol 3 × 3 min.
4. Incubate slides in 95% ethanol 2 × 3 min.
5. Incubate slides in 70% ethanol 1 × 3 min.
6. Incubate slides in 50% ethanol 1 × 3 min.
7. Incubate slides in 30% ethanol 1 × 3 min.
8. Rinse in $ddH_2O$ 3 × 5 min.
9. DO NOT LET YOUR SLIDES DRY OUT AFTER THIS POINT. Incubate slides in Peroxidase block for 10 min (*see* **Note 9**).
10. Rinse slides once in $ddH_2O$ (2–3 dips) and then wash in PBS 3 × 5 min.
11. Start the antigen retrieval process by incubating slides in citrate buffer in a covered Coplin jar at 55 °C overnight (*see* **Note 10**).
12. Rinse slides in PBS 3 × 5 min.
13. Block endogenous biotin with avidin/biotin block according to manufacturer instructions. We find that this is important in skeletal tissues.

14. Incubate in 10% serum diluted in PBS for 60 min at room temperature in humidified chamber (*see* **Note 1**).
15. Drain off serum solution. Do not rinse slides or let the slides dry completely.
16. Incubate in primary antibody diluted in PBS with 1.5% serum (*see* **Note 11**) overnight at 4 °C or for 1 h at room temperature in humidified chamber (*see* **Note 12**).
17. Rinse slides 3 × 5 min in PBS.
18. Incubate sections in biotinylated secondary antibody for 30 min, following data sheet from manufacturer for dilutions (*see* **Note 13**).
19. Rinse slides 3 × 5 min in PBS.
20. Prepare ABC solution as per manufacturer's instructions and incubate with slides for 30 min.
21. Prepare DAB substrate. Also prepare an extra Coplin jar with water to stop the substrate reaction (*see* **Note 14**).
22. For developing the slides you will need a light microscope. Lay out all your reagents—the substrate solution, several Coplin jars with water (*see* **Note 15**), and slides should all be easy to reach. Some reactions take as little as 30 s before developing background so there is little margin for error at this point.
23. Place your positive (+ve) and negative (−ve) control slides on the microscope stage and add a drop of substrate solution to each. The ideal time interval will give you the most intense staining in your +ve control without giving any staining in the −ve. The maximum staining time should be 5 min or less. Place the slides in the extra Coplin jars of $ddH_2O$ to stop the reaction. Do not return them to jars with undeveloped slides since this will start the substrate reaction prematurely.
24. Develop the rest of the slides one at a time at the optimal time determined in **step 23** Once a slide has developed put it in the extra Coplin Jar with water.
25. Rinse slides well in $ddH_2O$.
26. Counter stain the slides in Harris hematoxylin for 30 s to 1 min.
27. Wash in running tap distilled water for 10 min.
28. Dehydrate and clear through 2 changes of 95% ethanol, 3 changes of 100% ethanol, and then xylene.
29. Add coverslip with mounting medium. In a fume hood, place a drop or thin line of mounting medium on the edge of the coverslip on the benchtop and touch it with the edge of an inverted slide at a 45° angle, and gently bring it down onto coverslip. Avoid bubbles under the coverslip (*see* **Note 16**).

Wipe the excess xylene and mounting medium with gauze or Kimwipe prior to viewing under microscope. Allow slides to dry and xylene to evaporate in a fume hood.

## 3.2 IF on Frozen Sections

### 3.2.1 Tissue Preparation

1. Perfusion, fixation, decalcification, and washing are the same as Subheading 3.1.1, **steps 1–5** above.
2. Incubate tissue in 30% sucrose solution at 4 °C for 3–5 days. This helps to prevent formation of damaging ice crystals during freezing/embedding. Tissue should sink to the bottom of the container when ready for embedding.
3. Embed sucrose-permeated tissue in OCT Compound (Tissue-Tek), paying close attention to orientation (*see* **Note 17**). Store frozen blocks at –80 °C until sectioning.
4. Tissue sections are then cut at the desired thickness (generally from 10–100 μm) using a cryostat and placed on color frost slides (*see* **Note 18**).
5. Store frozen slides at –80 °C for long-term storage.

### 3.2.2 Antibody-Based Staining

NOTE: All steps should be performed in a humidified chamber.

1. Remove slides from freezer and allow to thaw in a humidifying chamber for 10 min.
2. Draw a PAP pen barrier around tissue prior to rinsing for maximum adherence of hydrophobic barrier to slide (*see* **Note 19**).
3. Pipette enough PBS to create surface tension over the tissue inside the hydrophobic barrier (generally 200 μL or more) onto the slide to wash OCT from the tissue for 5 min. Then aspirate the liquid.
4. Pipette PBST buffer to rinse for 5 min. Then aspirate the liquid.
5. Incubate in 10% serum diluted in TNT for 60 min at room temperature in humidified chamber (*see* **Note 1**). Then aspirate the liquid.
6. Incubate in primary antibody diluted in TNT (*see* **Notes 11** and **12**). Close lid and wrap in plastic wrap, taking care not to disturb the slides. Incubate slides at 4 °C. For incubation time suggestions, *see* **Note 20**.
7. After incubation, aspirate the antibody and rinse with PBST 3 × 10 min.
8. Incubate in secondary antibody diluted in TNT (*see* **Notes 11** and **12**). Close lid and wrap in plastic wrap, taking care not to disturb the slides. Incubate slides at 4 °C. For incubation time and temperature suggestions, *see* **Note 21**.
9. After incubation, aspirate the antibody and rinse with PBST 3 × 10 min.

10. If the mounting media used contains DAPI, skip to **step 12**. If not, add DAPI counterstain cocktail to slide and incubate for 5–7 min. Aspirate the liquid immediately to avoid overstaining. For DAPI concentration suggestions, *see* **Note 22**.
11. Aspirate the liquid immediately to avoid overstaining. Rinse with PBST 3 × 5 min.
12. Place 2–3 drops or a thin line of glycerol-based aqueous mounting media along the edge of a coverslip. Carefully place the slide (tissue down) on top, beginning on the edge with the mounting media. This helps to prevent bubbles.
13. Seal slide edges with clear nail polish. This prevents the slides from drying out and media from escaping, as aqueous media does not fully set.
14. Keep slides at 4 °C. Room temperature slides may dry out, mold, or lose fluorescence more quickly (*see* **Note 23**).

## 4 Notes

1. Blocking and primary antibody incubation serum should be from the same species as the secondary antibody (i.e., if the secondary antibody is goat anti-rabbit then goat serum should be used to block slides).
2. For example, 1–2 mouse femurs and/or tibiae should be placed in a 15 mL tube with at least 10 mL fixative. Most tissues will be properly fixed in 24 h, but large bones, such as from rabbits, might require longer fixation. However, antigenicity can be reduced with longer fixation so optimization of fix time may be needed.
3. The first time you do this, include a test bone of the same type as you will analyze in the decalcification and use this one to bend and compress. A fully decalcified bone should bend easily and not break.
4. Total rinse time should be about 2 h.
5. First step in processor should be 70% ethanol. Whole bone specimens from larger animals may require 6–8 h processing times.
6. Slides can be checked using light, darkfield, or phase microscopy at this point for proper plane of section.
7. Do not skip this step and go straight to heating slides at 55 °C. The tissue is likely to fall of the slides during staining if you do this.
8. Alternatively, slides may be baked overnight at 55 °C.
9. Start with 10 min and extend if background is high on negative controls. The 30% hydrogen peroxide can also be diluted in

PBS instead of cold methanol. In addition, there are commercial peroxidase block alternatives such as Biocare 1, which may be preferable because they have shorter incubation times.

10. Slides can also be heated to 95 °C in citrate buffer for 10 min, followed by cooling in hot buffer for 15 min. However, overnight citrate buffer treatment is preferable to high heat because it preserves tissue morphology better. If the high heat method is used, make sure the buffer does not come to a full boil as this will cause the tissue to fall off the slide. Other alternative retrieval methods include enzymatic digestion at 37 °C with proteinase K (10 μg/mL in 10 mM Tris–HCl pH 7.4–8.0 for 20 min) or hyaluronidase (1% in PBS for 30 min). Avoid using reagents generated in donkeys (serum or antibodies) when using hyaluronidase since it increases background staining. In addition, some antigens do not require retrieval. This is a step that must be optimized for each antigen.
11. Data from the antibody manufacturer are good sources for determining the primary and secondary antibody concentrations to use. However, users may have to run serial dilution experiments to determine the optimal concentration for specific tissue/antibody combinations.
12. Depending on the size of your tissue, you will need 50–200 μL of antibody solution. Thick frozen sections may require up to 300 μL. Cut a piece of parafilm the same size as the slide and float this on top to retain the antibody over the tissue, taking care not to have any bubbles. Do not use a glass or plastic coverslip. You do not need to use the parafilm for shorter incubation, but make sure the tissue is covered with the solution. Do not allow the tissue dry or you will get very high background.
13. Alternatively, incubate in secondary antibody conjugated to horseradish peroxidase, diluted with 1.5% serum in PBS for 30 min at room temperature. If you do this, then skip **step 20** and go straight to **step 21**.
14. We usually use DAB solution from Biocare, although other chromagens are available. DAB generates a brown precipitate and is very carcinogenic. Take care to use gloves and follow manufacturer's instructions.
15. Deionized water is sufficient in most cases, but milliQ or $ddH_2O$ is fine to use if available.
16. You can use gentle pressure to push small bubbles to the edge of the coverslip. If the bubbles are very large, you probably did not use enough mounting medium. Put the whole slide with the coverslip back into xylene to float off the coverslip and start over. If you try to pry off the coverslip, you are likely to damage the tissue.

17. Embedding frozen tissue is best accomplished using a cryoplate for even freezing of the sample.
18. Section thickness depends on structure or antigen of interest. Thicker sections can be used for larger structures, such as vasculature or nerves. Thinner sections may be used for molecular or cellular antigens of interest. Of note, stronger detergents may be needed to facilitate antibody penetration into thick sections. Section thickness is also limited by decreasing optical transparency and subsequent image quality.
19. The PAP pen may need to be vortexed prior to use. Take care to only press the pen tip gently on the slide, as the liquid may rush and pool. If greater adherence to the slide is needed, the PAP pen outline may be re-enforced by spreading with a cotton tip applicator. This barrier allows for use of smaller aliquots of antibody and also prevents drying out of tissue over long periods of time.
20. Thin sections (10–20 μm) may be incubated in primary antibody overnight. Thick sections (20 μm+) should be incubated for 48-h to allow maximum penetration and equilibration.
21. Secondary antibody incubation times may vary. Thin sections (10–20 μm) may be incubated at room temperature for 1–3 h. Thick sections (20 μm+) should be incubated for 24 h at 4 °C.
22. DAPI concentration may range from 0.1 μg/mL to 10 μg/mL. For best results, troubleshoot concentrations when a new batch is received for your desired application. For example, high concentrations will result in densely stained, more uniform nuclei, while low concentrations will reveal differences between chromocenters and chromatin compaction if imaging of nuclear architecture is needed.
23. The quality of the fluorescence will begin to degrade after the first few months of storage. Use of mounting media containing DAPI may require imaging within the first few days of staining. For best results, specimens should be imaged as soon as possible to ensure the most accurate representation of the antigens.

## References

1. Bord S (2003) Protein localization in wax-embedded and frozen sections of bone using immunohistochemistry. Methods Mol Med 80:237–247
2. Freida CL, Hladik C. Histotechnology.; A self instructional text , 2009
3. Sheehan DC, Hrapchak BB (1980) Theory and Practice of Histotechnology. 2nd ed. Bancroft JD, Stevens J, editors. St. Louis, MO, The CV Mosby Company
4. L'Hoste RJ Jr, Tourres MA (1995) Using Zinc Formalin as a Routine Fixative in the Histology Laboratory. Lab Med 26(3):210–214
5. Gage GJ, Kipke DR, Shain W (2012) Whole animal perfusion fixation for rodents. J Vis Exp 65
6. Hao Z, Kalscheur VL, Muir P (2002) Decalcification of bone for histochemistry and immunohistochemistry procedures. J Histotechnol 25 (1):33–37

7. Serowoky MA, Patel DD, Hsieh JW, Mariani FV (2018) The use of commercially available adhesive tapes to preserve cartilage and bone tissue integrity during cryosectioning. BioTechniques 65(4):191–196
8. Battifora H (1999) Quality assurance issues in immunohistochemistry. J Histotechnol 22 (3):169–175
9. Watkins S (2003) Immunohistochemistry. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA et al (eds) Current protocols in molecular biology. John Wiley & Sons Inc., Hoboken, NJ. p. Supplement 7 Unit 14.6