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Chapter 21 1

Immunostaining of Skeletal Tissues 2

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Abstract 4

Immunohistochemistry (IHC) is a routinely used technique in clinical diagnosis of pathological conditions 5
and in basic research. It combines anatomical, immunological, and biochemical methods and relies on the 6
specific binding of an antibody to an antigen. Using the technique with mineralised tissues is more com- 7
plicated than with soft tissues. This can in most cases be overcome by demineralising the samples, which 8
allows embedding in paraffin wax and a simpler work-up than for resin-embedded, or for frozen samples. 9
This chapter describes methods for IHC on paraffin-embedded formaldehyde fixed sections to detect 10
antigens in the musculoskeletal tissues. 11

Key words: Immunohistochemistry, Antigen retrieval, Mouse knee joint, Immunofluorescence, 12
Paraffin section 13

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1. Introduction 14

The use of immunohistochemistry (IHC) dates back to the early 15
1940s when Coons et al. (1) used FITC-labelled antibodies to 16
detect Pneumococcal antigens in infected tissues (1). Since then, 17
IHC has become one of the most powerful routine methods in 18
diagnostics and basic research. It combines histological, immuno- 19
logical, and biochemical techniques and is based on the principle 20
that antigens can be detected in cells or tissues using specific anti- 21
bodies. In this chapter, IHC on paraffin-embedded paraformalde- 22
hyde-fixed tissue sections using either enzyme-based or 23
fluorescence-based methods (see Fig. 1) are described. We prefer 24
the use of paraffin over frozen sections because the overall tissue 25
morphology is better preserved allowing best identification of the 26
labelled tissue components in relation to other structures. However, 27
a disadvantage of using fixed and embedded material is the fact 28
that many commercially available antibodies work better in frozen 29
sections. Increasingly though, companies screen their primary 30

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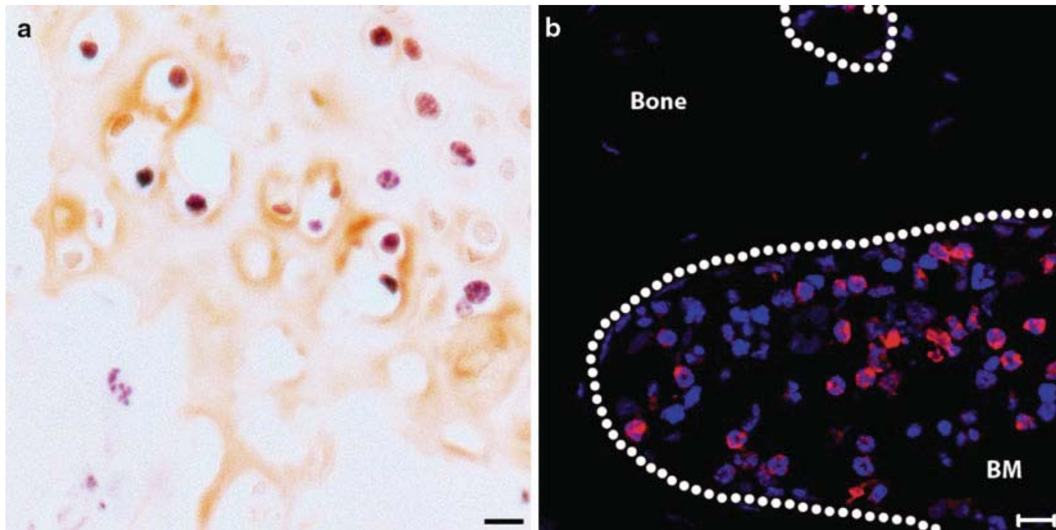

[AU1] Fig. 1. (**a**) Immunohistochemical staining for Collagen type II in the articular cartilage of a 3-month-old mouse. In this protocol, we used hyaluronidase treatment (4,000 U/ml for 60 min at 37°C) as antigen retrieval step. A peroxidase-based staining was performed using DAB which results in a brown signal of the antigen. Nuclei were counterstained with haematoxylin. Scale bar: 10 μm. (**b**) Immunofluorescence staining for the pan-haematopoietic marker CD45 in a bone marrow cavity of a 3-month-old mouse. Positive cells are demonstrated by a *red* membrane associated signal. Nuclei are counterstained with DAPI (*blue*). Scale bar: 10 μm.

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31 antibodies on fixed embedded material and the use of a variety of
32 antigen retrieval methods can rescue antigenicity in such tissues. So
33 in short, our method of choice is formaldehyde fixation to contrib-
34 ute to better tissue preservation, followed by antigen retrieval to
35 unblock the cross-linked amino groups of fixed proteins. We then
36 apply a primary antibody that is tagged with either a fluorochrome
37 or an enzyme (direct IHC), or we detect binding of an un-conju-
38 gated primary antibody with a secondary conjugated antibody
39 (indirect IHC). To enhance intensity, sensitivity, and specificity of
40 the signal, additional systems such as use of avidin–biotin com-
41 plexes, or use of tyramide-based signal amplification are possible.
[AU2]42 When using enzyme-conjugated antibodies, a suitable enzyme
43 substrate that precipitates at the site it is formed needs to be avail-
44 able. Many companies now produce kits for such histochemical
45 reactions that are well tested and include appropriate blockers for
46 endogenous enzyme activity in the tissue. IHC methods to detect
47 expressed antigens can be usefully combined with demonstration
48 of artificial labels deliberately integrated in the tissue, such as
49 nucleoside analogues (e.g. BrdU) for pulse chase to detect prolif-
50 erating cells. We recently used a double nucleoside analogue label-
51 ling strategy in a mouse model of articular cartilage knee joint
52 injury to identify and characterise functional mesenchymal stem
53 cells within the synovium in vivo (2). Of note, this study was
54 performed using mainly IHC methods.

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Overall, IHC is an extremely useful method to combine anatomical and biochemical information and the methods described here can be adapted for use with tissues prepared in different ways, such as tissue embedded in methyl-methacrylate, or in acrylic resins such as Lowicryl HM20 after appropriate optimisation of each step. In general, optimisation of all steps is required for each tissue type and for each primary antibody. Use of automated robotic stainers is increasingly common in diagnostic settings and in larger research units and can help standardise staining between slides by minimising inter-slide variability, and allow quantification of staining intensity. Such methods are not described here in detail as they are largely dictated by the equipment and kits used for detection. While we have found such equipment very useful, especially as the detection kits are highly optimised to give superb sensitivity and therefore save on primary antibody, autostainers remain expensive to run in a standard laboratory setting. We therefore concentrate on manual protocols here, but encourage the reader to amend and adapt the principles to their specific experimental condition.

2. Materials

Unless stated otherwise, materials can be obtained from Sigma or similar chemical suppliers.

1. Phosphate-buffered saline (PBS): use tablets and dilute with the required amount of distilled water.
2. Fixation solution, 2% paraformaldehyde (PFA) + 0.05% glutaraldehyde in PBS: to prepare 2% PFA solution place 450 ml of distilled water in a glass beaker. Heat to 60°C using a hot plate with stirring facility. While stirring, add 10 g of paraformaldehyde powder to the heated water. Cover and maintain at 60°C. Add five drops of 2N NaOH (one drop per 100 ml). The solution should clear within a short time (there will be some fine particles that will not disappear). Do not heat solution above 70°C, PFA will break down at temperatures above 70°C. Remove from heat and add 50 ml of 10× PBS. Adjust pH to 7.2; you may have to add some HCl. Final volume will be 500 ml. Filter and add 0.05% glutaraldehyde (50 μl in 100 ml). Place on ice when using it immediately or freeze aliquots at −20°C and thaw when needed (see Note 1).
3. Decalcifying Solution-Lite.
4. 4% EDTA in PBS, adjust pH to 7.2–7.4 using NaOH.
5. Superfrost^{+} slides (Menzer), 25 mm × 75 mm.
6. Citrate buffer: 10 mM citric acid, 0.05% Tween 20, pH 6.0. The solution can be stored at RT for up to 3 months or even longer when stored at 4°C.

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7. Tris–EDTA buffer: 10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0. The solution can be stored at RT for up to 3 months or even longer when stored at 4°C.
8. Hydrochloric acid (0.2 N): add 3 ml of fuming hydrochloric acid (37%) to 497 ml of distilled water.
9. Pepsin solution: use porcine pepsin at a concentration of 0.5–3 mg/ml diluted in 0.2N hydrochloric acid. Shake gently and keep at 37°C until the crystals have dissolved.
10. 3% H_2O_2 in distilled water.
11. Tris-buffered saline (TBS) 20× stock: add 122 g Trizma base and 180 g NaCl to 900 ml of distilled water. Stir until dissolved and adjust pH to 7.6 using concentrated HCl. Fill aliquots in 50 ml falcon tubes and freeze at −20°C. Prepare 1× TBS using one falcon tube and fill up to 1,000 ml with distilled water.
12. Washing buffer: 0.2% Triton X-100 in TBS.
13. Avidin blocking solution: ready-to-use solution (VECTOR, www.vectorlabs.com).
14. Humidified chamber: these are commercially available (e.g. staining tray from VWR) or can be made by yourself: use a box that is large enough to put in two 5 or 10 ml plastic pipettes to keep the slides raised up. Cover the bottom with some washing buffer and close the box with a lid. For immunofluorescence (IF) staining these humidified chambers must be impervious to light.
15. Biotin blocking solution: ready-to-use solution (VECTOR).
16. Blocking solution: 1% bovine serum albumin (BSA) in washing buffer.
17. MOM Ig Blocking reagent (VECTOR): add two drops of stock solution to 2.5 ml of washing buffer.
18. MOM Diluent (VECTOR): add 600 μl of protein concentrate stock solution to 7.5 ml of washing buffer.
19. DNase solution: dilute Desoxyribonuclease I from bovine pancreas to a concentration of 1,000 U/ml with 0.15 M NaCl. Store stocks of 250 μl at −20°C and dilute with 250 μl of TBS Triton X-100 to achieve working solution.
20. Parafilm: cut small pieces (depends on the size of your section; we are using approximately 20 mm × 40 mm in size for mouse knee joint samples) of parafilm and fold approximately 5 mm from one shorter end that the parafilm side forms a 90° angle. Strip of paper and cover with the parafilm side that was protected by the paper to cover the section.
21. Avidin–biotin complex (ABC) reagent (VECTOR): add two drops of reagent A to 5 ml TBS Triton X-100 and mix gently.

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Add two drops of reagent B to this solution and mix immediately. Allow to stand for 30 min. 142 143

22. DAB solution (VECTOR): add two drops of buffer stock solution to 5 ml of distilled water and mix well. Add four drops of DAB stock solution and mix well. Add two drops of hydrogen peroxide solution and mix well. Alternatively, you can add two drops of Nickel stock solution and mix well to receive a black reaction product. 144 145 146 147 148 149
23. Haematoxylin QS: ready-to-use solution (VECTOR). 150
24. DePex mounting medium. 151
25. Ammonium chloride solution: add 0.5 g of NH_4CI to 200 ml of TBS and stir until dissolved. 152 153
26. Mowiol: mix 6.0 g glycerol with 2.4 g Mowiol 4–88 and dissolve with frequent agitation for 1 h at RT. Add 6.0 ml distilled water and stir for one more hour at RT. Add 12.0 ml 0.2 M Tris–HCl (pH 8.5) and incubate for 2 h at 50°C under periodical stirring (every 20 min for 2 min). Note: in many cases Mowiol does not dissolve completely. We recommend centrifugation for 15 min at 5,000 × *g*. Continue with the supernatant. Add 25 mg/ml 1,4-diazabicyclo[2.2.2]octan (DABCO) and stir until complete dissolution. Aliquot 1 ml into 1.5 ml Eppendorf tubes. Add 1 μl DAPI stock solution (see below) and mix well. Store for long term at −20°C. Before using thaw at RT. 154 155 156 157 158 159 160 161 162 163 164 165
27. DAPI stock solution: dilute 4′,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.5 mg/ml in distilled water. Freeze 10 μl aliquots at −20°C. 166 167 168

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3. Methods 169

3.1. Preparation of Mouse Knee Joint Paraffin Blocks

1. Dissect knee joints from the mouse, remove skin, and strip muscle as far as possible (do not cut into the joint!). 170 171
2. Wash 3 × 10 min in PBS by gently shaking at room temperature (RT). 172 173
3. Fix the samples in 2% PFA and 0.05% glutaraldehyde in PBS at RT for 1 h. 174 175
4. Wash 3 × 10 min in PBS by gently shaking at RT. 176
5. Decalcify samples: 177
   (a) In Decalcifying Solution-Lite (20:1 ratio for solution:tissue) at RT for 1 h; rinse extensively in tap water (this method is rapid, but destructive for a number of antigens). 178 179 180
   (b) In 4% EDTA in PBS at 4°C by gently shaking for 2 weeks (change solution every 2–3 days). 181 182

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6. Wash 3 × 10 min in PBS by gently shaking at RT.
7. Put the samples in 70% ethanol at 4°C and embed in paraffin wax using a tissue processor and standard wax protocol (see Note 2).
8. Cut 5-μm thick sections on a rotary microtome (Leica), float to stretch in a warmed water bath (45°C) and collect on Superfrost⁺ slides; allow the sections to dry overnight (this is important!) before starting with the staining protocol.

3.2. Immunohistochemical Staining Using Enzyme-Conjugated Antibodies

1. De-wax and rehydrate 5-μm thick paraffin sections using the following protocol:
2. 2 × 5 min xylene, 2 × 2 min ethanol 100%, 2 min ethanol 95%, 2 min ethanol 70%, and 5 min H_2O.
3. Perform antigen retrieval using heat-mediated epitope retrieval (HIER) and/or proteolytic-induced epitope retrieval (PIER) (see Note 3).
4. Rinse 2 × 5 min in H_2O.
5. Quench endogenous peroxidase with 3% H_2O_2 in H_2O for 10 min (see Note 4).
6. Rinse 2 × 5 min in H_2O.
7. Rinse in TBS for 5 min.
8. Rinse in washing buffer for 5 min.
9. Put one drop of avidin blocking solution on the section and incubate in a humidified chamber for 15 min (see Note 5).
10. Rinse in washing buffer for 5 min.
11. Block with one drop of Biotin blocking solution in a humidified chamber for 15 min.
12. Block with blocking solution for 45 min (see Note 6).
13. Blot the excess blocking solution off, but do not allow to dry and do not wash.
14. Incubate with primary antibody diluted in blocking solution at RT for 1 h or at 4°C overnight (or in case of IdU staining in DNase solution at RT for 1 h); cover section with a small piece of parafilm to prevent evaporation (see Note 7).
15. Rinse 3 × 5 min in washing buffer.
16. Incubate with biotinylated secondary antibody at RT for 30 min (see Note 8).
17. Rinse 3 × 5 min in washing buffer.
18. Incubate with ABC reagent for 30 min (see Note 9).
19. Rinse 3 × 5 min in washing buffer.

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20. Incubate with peroxidase substrate solution for 2–12 min; monitor development of the staining under a microscope (see Note 10). 222 223 224
21. Rinse in tap water for 5 min. 225
22. Counterstain with Haematoxylin QS for 5 s. 226
23. Rinse in tap water until water is colourless. 227
24. Dehydrate using the following protocol: 3 min ethanol 70%, 2×3 min ethanol 100%, and 2×3 min xylene. 228 229
25. Mount with DePex and apply coverslip. 230
26. Sections can be analysed after polymerisation of the mounting medium (usually overnight) using a brightfield microscope and can be stored long term at room temperature. 231 232 233

3.3. Immunofluorescence Staining Using Fluorochrome-Conjugated Antibodies

1. De-wax and rehydrate 5-μm thick paraffin sections as described in Subheading 3.2 step 1. 234 235
2. Perform antigen retrieval as described in Subheading 3.2 step 2. 236
3. Rinse in H_2O for 5 min. 237
4. Rinse in TBS for 5 min. 238
5. Quench autofluorescence 2×5 min with TBS containing 50 mM NH_4CI (see Note 11). 239 240
6. Wash 2×5 min in washing buffer. 241
7. Block with blocking solution at room temperature for 45 min. 242 243
8. Blot the excess blocking solution off but do not allow to dry and do not wash. 244 245
9. Incubate with primary antibody diluted in blocking solution for 1 h at RT or at 4°C overnight (or in case of IdU staining in DNase solution for 1 h at RT). Apply a small piece of parafilm over the section to prevent evaporation. 246 247 248 249
10. Wash 3×5 min in washing buffer. 250
11. Incubate with fluorochrome-conjugated secondary antibody at RT for 30 min. From here on incubation should be performed in the dark; avoid exposing the sections too long to light sources as this might affect the fluorescence intensity of the secondary antibody. 251 252 253 254 255
12. Wash 3×5 min in washing buffer. 256
13. Mount with Mowiol containing DAPI. 257
14. Sections can be analysed after polymerisation of the mounting medium (usually overnight) under a fluorescent microscope and can be stored long term at −20°C. 258 259 260

A series of website that list useful information about reagents and procedures for IHC and IF is given in Note 12. 261 262

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263 ## 4. Notes

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1. The process of fixation prevents the decay of the tissue that might happen through intrinsic components like endogenous proteolytic enzymes or extrinsic factors like bacteria. Frozen samples are usually not fixed. However, a post-fixation step on the sections is performed by using rather short times of incubation in formaldehyde solutions, acetone, or methanol. Whereas formaldehyde preserves and strengthens structure within the tissue, it can result in tissue shrinkage and alcoholic fixatives may destroy morphological details like nuclei. Among the many fixation agents that are described in detail in histology textbooks (3), the mostly commonly used is aldehyde-based crosslinking. Usually samples are immersed in a 3.7% formaldehyde solution (also called 10% Neutral Buffered Formalin) for at least 1 h. The often stated risk of potential over-fixation of tissue leading to compromised immunohistochemical staining was recently tested and found to be small when tissue showed good immunoreactivity even after 7 weeks of fixation (4). The advantages of formaldehyde are its fast penetration and the possibility to store samples for long term. By contrast glutaraldehyde penetrates more slowly, but preserves cellular morphology better. For immunostaining formaldehyde generally gives better results, but for both reagents antigen retrieval has to be considered (see Note 3). We prefer a mixture of both aldehyde fixation agents as a compromise between preservation of tissue and cellular details, penetration speed, and the quality of immunohistochemical signals.
2. Embedding samples in paraffin and sectioning paraffin blocks is best done using tissue processors which eliminate exposure to solvents. This equipment can be found in all Pathology Departments or in most Histology facilities. You may of course use manual methods, but beware of solvents and use a chemical fume hood. Methods for embedding can be found in all Histology text books, for example in "Theory and Practice of Histological Techniques" (3).
3. Antigen retrieval: a detailed overview of antigen retrieval methods was recently published by D'Amico et al. (5). Two general principles are HIER and PIER. The choice between these methods depends on the fixation status of the antigen, the primary antibody to be used and the tissue of interest. For example, detection of antigens in synovial tissue might be facilitated by PIER as this tissue is rich in fibrous extracellular matrix which has to be opened by the enzyme. The same antigen in bone marrow, however, might be destroyed by PIER and HIER should be the method of choice in this case. In HIER,

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sections are boiled in a specific buffer using various devices such as microwave oven, steamer, pressure cooker, autoclave, or water bath. The length and the temperature of the boiling step are crucial and should be evaluated for each antibody used. Also the choice of buffer is important and may vary between antibodies. Two mostly used buffers are citrate buffer of pH 6 and Tris–EDTA buffer of pH 9 (these buffers are available in consistent quality from a broad range of suppliers like DAKO or Vector, but can also be prepared fresh as detailed in Subheading 2). In our laboratory, we used an autostainer (Bone Max, Leica) to perform HIER. This staining robot performs automated de-waxing and rehydration steps and can be programmed to heat sections at 99°C for 10–30 min using either the buffer of pH 6 or 9. The advantage of this staining robot is that the temperature is consistent over the whole section and that the section stays in a horizontal position which prevents it from floating off the slide. However, remember that the process of boiling is destructive to joint sections. Especially, articular cartilage experiences a high degree of shrinkage and sections tend to detach from the slide. Even with all these artefacts, HIER is less destructive to the antigens than PIER and results in good signal quality. Enzymes used in PIER include pepsin, trypsin, Proteinase K or pronase, or hyaluronidase (see Fig. 1a). As mentioned above the destructive nature of the enzyme can affect antigens and even tissue morphology. A careful evaluation of incubation time and concentration of the protease is therefore crucial. Also the solution in which the enzyme is diluted is of importance. Pepsin, for example works only in a high acidic environment that might irreversibly denature antigens. However, in our laboratory we used 15- and 45-min incubation with porcine pepsin (0.5–3 mg/ml) successfully to detect a range of antigens. Antigen retrieval for nucleoside analogues such as IdU requires harsh antigen retrieval. We obtained very good signals when we used a pepsin solution; the low pH and the proteolytic activity combined, helps to denature the DNA and to remove nuclear proteins, to result in single-stranded DNA. Additionally, we diluted the primary antibody in a DNase solution (see Subheading 2, item 19) and incubated for 1 h at RT. The enzyme cuts the DNA strands into small pieces and the access of the primary antibody to the nucleoside is greatly facilitated resulting in excellent staining with very good signal to noise ratio.

4. If the enzyme peroxidase is used in the detection system, the tissue of interest should be tested for endogenous peroxidase expression. This is done by de-waxing and re-hydrating your section and then applying a drop of peroxidase substrate solution like DAB (see Note 10). If endogenous peroxidase activity

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is detected this can be blocked by incubating the section in 3% hydrogen peroxide in water for 10–30 min. Some people use 0.3% hydrogen peroxide in methanol, but methanol affects nuclear morphology and therefore we would not recommend this method. We use this blocking step after de-waxing and re-hydration and wash 2 × 5 min with water to remove hydrogen peroxide residues. Blocking of endogenous peroxide can be done later in the protocol as long as it is done before the peroxidase-conjugated reagent is introduced.

5. The use of the avidin–biotin system greatly enhances signals, but endogenous biotin can cause problems with false-positive signals. VECTOR offers a special kit to prevent such problems by blocking the endogenous biotin (www.vector.com).

6. Blocking solutions are used to inhibit specific or non-specific background staining. These false-positive signals occur generally through binding of the antibody as a consequence of electrostatic forces within the tissue. To prevent this, a blocking solution with high protein content can be applied to the section to cover non-specific binding sites for antibodies. Frequently used is a blocking solution that contains up to 20% of serum from the species in which the secondary antibody is produced. In our laboratory, we successfully block with a 1% BSA solution. A special case arises when you use an antibody that is raised in the species as the test tissue. This happens most frequently when using mouse monoclonal antibodies on mouse tissues. The use of the Mouse-On-Mouse-Kit (VECTOR) can be helpful in this case. In the first step, sections are incubated with a mouse Ig blocking reagent for 1 h and then, after a quick wash, sections are additionally incubated in a mouse protein cocktail for 5 min. This treatment, in our experience, results in highly specific staining.

7. The choice of a good primary antibody is sometimes a challenge. We usually start by checking published data on antibodies used for the antigen we wish to detect. Another source of information are the datasheets (helpfully available online for most suppliers) in which suppliers may state whether the antibody can be used on frozen or paraffin embedded, formalin-fixed sections. Comparison websites can help source the companies that make antibodies to the antigen of your choice (e.g. www.biocompare.com). Monoclonal antibodies give highly specific signals and are low in background staining, but are often sensitive to fixation and paraffin embedding which might change conformation of the antigen. By contrast, polyclonal antibodies are more robust, but can give false-positive signals due to presence of irrelevant antibodies in the immunised animal. An appropriate control in this case would be pre-immune serum, but this is not always available. The antibody

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concentration has to be determined empirically using a range of concentrations spanning the optimal dilution recommended by the supplier. This is necessary because the antigen of interest may, in your test situation, be located in different tissue compartments, or you may wish to use different antigen retrieval methods, or the abundance of antigen is quite different than in the supplier's test situation. Although antibodies can be used for years if stored correctly (*do* read the instruction from the supplier and abide by them), their affinity may decline over time. If you expect this is the case, do not throw out the antibody just yet, but re-optimise the optimal dilution.

For each staining a positive and a negative control should be used. The positive control should be a section similarly prepared as the experimental sample, i.e. identical fixation and embedding protocol. This could be a different tissue, or even better, a different organ/tissue compartment inside the same section which is known to express the antigen of interest (internal positive control). However, remember that different tissues may require different antigen retrieval methods or primary antibody dilutions to reveal the antigen so as to optimise the conditions for the positive control properly. The negative control sections of your experimental sample should ideally be incubated with an Ig control antibody raised in the same species and containing the same Ig subclass as your primary antibody (isotype negative control). For polyclonal antibodies, pre-immune serum should be used as negative control. Concentration of Ig (NOT dilution of antibody) should be the same as for the optimised primary antibody. If the concentration of the primary antibody is unknown, an option is to omit the first antibody entirely and replace with incubation in wash buffer alone.

Non-specific binding of an antibody to proteins other than the antigen can sometimes occur. To determine whether the staining is specific, a blocking experiment with an immunising peptide (usually available from the commercial supplier of the antibody) can be performed. Prior to staining, the primary antibody is neutralised by incubation with an excess of peptide that corresponds to the epitope recognised by the antibody. The antibody that is bound to the blocking peptide is no longer available to bind to its epitope. Therefore, specific staining will be absent in the immunostaining performed with the neutralised antibody.

8. The secondary antibody is raised against the species from which the primary antibody is generated. Secondary antibodies are usually tagged by conjugation to a fluorochrome or an enzyme. Secondary antibodies can also be tagged with biotin and then be used with signal enhancement systems such as the

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ABC-complex. If double staining is performed, a mix of the secondary antibodies can only be used if none of the secondaries is raised in the same species than one of the primary antibodies. For example, if one of the primary antibodies is raised in goat, a mix of two secondary antibodies that includes one that is raised in goat would lead to cross-reactivity between the secondaries. In this case, secondary antibodies should be applied sequentially with an intermediate blocking step using goat serum: first antibodies → wash → anti-goat secondary antibody → wash → blocking with goat serum → secondary antibody raised in goat.

In the same way as for the primary antibody, optimal concentrations for secondary antibodies should be determined empirically using datasheets for guidance.

9. Amplification systems greatly enhance signal strength and can be used in enzyme-based IHC. Avidin–biotin conjugates that are coupled to peroxidase molecules exist as large polymers and can bind to biotinylated secondary antibodies. As a consequence multiple enzyme reaction sites are offered in contrast to only single sites when the enzyme is directly conjugated to the secondary antibody. A similar signal amplification can be obtained in IF staining, when the tyramide amplification system from Perkin Elmer is used (6). Here, a secondary antibody conjugated with peroxidase is used to react with a substrate that deposits fluorophore-labelled tyramide. This method has the potential problem of giving a high specific background if the method is not carefully optimised. Therefore, always include a negative control (by omitting primary antibody) and a control without TSA. The tyramide reaction itself is very quick (less than 10 min).

10. If peroxidase is used as a detection enzyme in IHC, 3,3′-diaminobenzidine (DAB) is the most widely used substrate which gives a brown or by adding a nickel solution a black reaction product. It is very sensitive, the reaction is very fast (sometimes within seconds; recommended incubation times are 2–12 min; monitor the staining under a light microscope to prevent overstaining), gives good contrast and easy to use as many suppliers offer the solution in kit format. However, handling of DAB must be done with caution as it is known to be a carcinogen. VECTOR now offers other substrates that are also very good in contrast like NovaRed (red reaction product) or VIP (violet reaction product) or can be used like AEC (red reaction product) with aqueous mounting media. Whereas the counterstain with haematoxylin gives a good contrast to DAB, these other substrates might benefit from being used with different nuclear counterstains such as Methyl Green. Vector provides a useful chart which substrate is best combined with which counterstain (www.vector.com).

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11. Autofluorescence is an artefact that is not easy to deal with. If the experiment consists of an immunohistochemical staining for a single antigen we recommend non-fluorescent methods as an option. However, some experiments require double or triple staining and co-localisation of these signals is important to show that two antigens are simultaneously expressed by the same cell or in the same compartment of that cell. Some autofluorescent signals can be spotted readily in the microscope and distinguished from real signals. For example, using a long-pass green emission filter, green fluorescent tags like GFP or Alexa Fluor 488 appear in bright green colour and not yellowish like autofluorescent structures. It is, however, challenging when it comes to take pictures.

    Autofluorescence might be caused by fixation agents which contain aldehydes that react with amines and proteins and therefore create autofluorescent structures. Proper antigen retrieval can reduce these artefacts, but cannot avoid them. Other sources of autofluorescene are biochemical molecules like lipofuscin, a break-down product of red blood cells, collagen, or elastin. A strong source of autofluorescence which is seen in bone marrow or sites of bleeding, are red blood cells due to the porphyrin structure of haemoglobin. They give strong signals in the excitation spectrum of blue and green lasers. Red blood cells can be easily spotted after nuclear counterstain (e.g. DAPI) as they are devoid of a nucleus. We observed that erythrocyte autofluorescence disappeared after pepsin treatment for antigen retrieval while it persisted after HIER. Other methods that have been used to block autofluorescence are treating sections with agents like Sudan Black in 70% ethanol or copper sulphate in ammonium acetate buffer (7). However, it has to be noted that such treatments may also reduce the fluorescent signal of the staining. Other approaches to reduce autofluorescence are the incubation in ammonium chloride or pretreatment of sections for some hours by irradiation with UV light to photobleach autofluorescent structures (8).

12. The following websites provide useful information about IHC and IF and reagents:

    http://jhc.sagepub.com/ – Official Journal of the Histochemical Society.

    www.ihcworld.com – Webpage around IHC with background information, technical support, and also a store that offers a range of products including antibodies.

    www.proteinatlas.org/ – Database showing staining for a wide range of antibodies directed against human antigens in different tissues.

    http://www.biocompare.com/ProductCategories/2045/Antibodies.html – Good webpage that lists antibodies of different suppliers.

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546 http://dshb.biology.uiowa.edu/ – Developmental Studies
547 Hybridioma Bank (University of Iowa); offers monoclonal
548 antibodies for use in research at reasonable prices.
549 www.vector.com – Supplier specialised in IHC.
550 www.dako.com – Supplier with a range of IHC products
551 including antibodies used in diagnostics

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Uncorrected Proof