Video Article

Immunohistochemical Analysis in the Rat Central Nervous System and Peripheral Lymph Node Tissue Sections

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Abstract

Immunohistochemistry (IHC) provides highly specific, reliable and attractive protein visualization. Correct performance and interpretation of an IHC-based multicolor labeling is challenging, especially when utilized for assessing interrelations between target proteins in the tissue with a high fat content such as the central nervous system (CNS).

Our protocol represents a refinement of the standard immunolabeling technique particularly adjusted for detection of both structural and soluble proteins in the rat CNS and peripheral lymph nodes (LN) affected by neuroinflammation. Nonetheless, with or without further modifications, our protocol could likely be used for detection of other related protein targets, even in other organs and species than here presented.

Video Link

The video component of this article can be found at https://www.jove.com/video/50425/

Introduction

Despite utilization of advanced high-throughput analyses performed on the methylome, transcriptome or even proteome level, immunostaining remains the golden standard for protein detection directly in the tissue sample, cell culture or a cell smear. By revealing the localization/distribution pattern, immunohistochemistry (IHC) may assess relative ratios and topographical interrelations of the target proteins, and even indicate their biological activities. Therefore, IHC is widely utilized for clinical and research purposes, *e.g.*, for diagnosis, treatment evaluations, study of disease mechanisms, functional and phenotypical alterations in animal models, *etc.*

Essentially comprising histology, pathology, biochemistry and immunology, IHC has significantly advanced since 1941, when fluorescently labeled antibodies were used for the first time to identify *Pneumococcal* antigens in the infected tissue [1]. Visualization of cellular products and components by IHC is based on binding of antibodies (Abs) to their specific antigen (Ag). Besides using fluorophore tagged antibodies, immune reactions can also be visualized by using enzymes like peroxidase [2,3] or alkaline phosphatase [4]. Further, colloidal gold-tagged antibodies[5] are used for detecting specific antigen-antibody interaction by both light and electron microscopy, while radioactive labels are visualized by autoradiography.

The Ag-Ab immunoreaction can be detected via direct and indirect methods. The direct method is essentially faster and simpler, as it uses directly labeled primary Abs [6]. However, due to significant lack of sensitivity, indirect methods are preferred to the direct ones. Two-step indirect detection procedures require unlabeled primary Abs, as the first, and labeled secondary Abs directed against the primary Abs, as the second layer [7]. Signal amplification can be achieved by involving further, enzyme-coupled tertiary Ab (three-step indirect method) that binds to the secondary Ab. Commonly used indirect detection methods are avidin-biotin and peroxidase-antiperoxidase (PAP). Alternatively, alkaline phosphatase-antialkaline phosphatase (APAAP) complex can be used instead of the PAP method. Notably, alkaline phosphatase (AP) methods appear to be even more sensitive than immunoperoxidase methods [4]. Avidin-biotin complex (ABC) method uses biotinylated secondary Ab in combination with either labeled avidin-biotin complex (LAB), or labeled streptavidin-biotin complex (SLAB). Detection sensitivity can be further increased by involving avidin labeled with peroxidase or alkaline phosphatase [8]. Other detection methods in use are polymeric labeling, tyramine amplification and immuno-rolling circle [9]. Notably, different detection methods can be combined for multiple Ag detection in the same tissue sample, which was reported for the first time in 1978 [4]. Simultaneous double immunostaining presented here was performed in formalin-fixed, paraffin-embedded rat CNS and LN tissue sections using peroxidase-bound and AP-conjugated secondary antibodies, respectively. The signals were visualized using 3,3'-diaminobencidine (DAB) chromogen and the Fast Blue (FB) APAAP complex, respectively.

Protocol

Ethical Statement
Present study is performed in accordance with guidelines from the Swedish National Board for Laboratory Animals and the European Community Council Directive (86/609/EEC) under the ethical permits N338/09, N15/10 and N65/10, which were approved by the North Stockholm Animal Ethics Committee.

1. Tissue Preparation

1. **Perfusion & fixation**
    1. Anesthetize the animal with isoflurane to perform transcardial perfusion via left ventricle. Initiate the rinsing of vasculature with phosphate buffered saline (PBS) to remove the blood components, followed by 4% paraformaldehyde in 0.1 M PBS (PFA).
    2. Fixate dissected brains, spinal cords and peripheral LN tissue by immersing into PFA. After 24 hr at 4 °C, transfer the tissue from PFA into PBS and store at 4 °C until further processing. Alternatively to commercial PBS, dissolve 9 g NaCl in 250 ml the Sörensen buffer and add 750 ml deionized water (dH_2O); to prepare 0.2 M Sörensen buffer (pH 7.4) dissolve 13.8 g NaH_2PO_4 x $1H_2O$ and 71.2 g Na_2HPO_4 x $2H_2O$ in 2.5 L dH_2O.

2. **Dehydration & embedding**
    1. Cut the tissue into approximately 5 mm thick portions by using a razor blade.
    2. Initiate the tissue hardening process (dehydration) by using the Tissue-Tek$^{V.I.P}$ Vacuum Infiltration Processor (standard procedure based on submersion into ascending concentrations of ethanol, followed by xylene and finally paraffin (**Table 1**).
    3. Place the tissue in a mold and pour in the liquid paraffin around the sample to form a "paraffin block". Long-term storage requires room temperature (RT). However, prior to sectioning, it is recommendable to cool down the blocks, *e.g.,* overnight (o/n) at 4 °C.

2. Sectioning

1. Place the paraffin block into a fixed holder of the sledge microtome, which can move backwards and forwards across the knife. Adjust the optimal angle between the block and the microtome knife (that depends on knife geometry, but also on the cutting speed and technique).
2. Cut 3 - 5 µm thick cross-sections from the paraffin block.
3. Transfer just cut section into a water container and mount it subsequently from the water onto the glass slide.
4. Press the mounted section carefully against a paper towel to remove residual water and potential air bubbles. Commercially pre-coated adhesive glass slides are recommendable.
5. Dry mounted slides for a couple of hours in a stove on 50 - 60 °C.

3. Deparaffinization (Rehydration & Blocking the Endogenous Peroxidase)

1. Immerse the slides 2x into xylene (alternatively xylene substitute XEM-200), each time 15 - 20'.
2. Rinse in 99% ethanol.
3. To block the endogenous peroxidase activity, incubate the sections for 30' in methanol solution containing 0.25% hydrogen peroxide.
4. Continue rehydration by using ethanol with increasing water content (99%, 70%, ending up in distilled water.
5. From this point until mounting of the cover slips sections have to be kept moist.

4. Antigen Retrieval

1. Use a food steamer for boiling the slides in an antigen retrieval solution (in this case EDTA pH 8.5 buffer) for 60' (EDTA buffer stock solution contains 1.21 g Tris and 0.37 g EDTA dissolved in 50 ml dH_2O; to prepare the working solution dilute 2.5ml EDTA stock solution in 47.5 ml dH_2O).
2. Cool down the slides on RT for approx. 1 hr and then rinse 3 - 5x with Tris buffered saline (TBS, use alternatively PBS) consisting of 0.05 M Tris and 0.15 M NaCl; pH 7.5 adjusted with HCl. Alternatively use commercial TBS.

5. Blocking the Unspecific Binding Sites

1. To avoid unspecific background reactions, incubate the sections on RT for 30' in the blocking solution containing 10% fetal calf serum (FCS) and 90% DAKO buffer.

6. Double Immunolabeling: Simultaneous Incubation with the Primary Abs

1. Dilute required amount of primary antibodies in the blocking solution and incubate o/n at 4 °C. For the double immunostaining combine α-eotaxin (Ccl11; 1:300) with α-Cd68 (Ed1; 1:1,000) or α-Iba1 (Aif1, 1:1,000) or α-Cd8α (Ox-8; 1:200).
2. Rinse the slides 3-5x with TBS buffer (use alternatively 10x diluted DAKO buffer).
3. Dilute required amount of secondary antibodies (biotinylated anti-goat and AP-conjugated anti-mouse, both 1:200) in the blocking solution and incubate 1 hr on RT.
4. Rinse the slides 3 - 5x with TBS buffer (use alternatively 10x diluted DAKO buffer).
5. Incubate the slides with avidin-horseradish peroxidase complex (HRP) diluted in the blocking solution for 1 hr on RT.

6. Rinse the slides 3 - 5x with TBS buffer (use alternatively 10x diluted DAKO buffer).

7. Visualization

1. **Visualization of the bound AP-labeled secondary antibody**
    1. Prepare 0.1 M Tris-HCl buffer by dissolving 12.1 g Tris in 1 L dH_2O and adjust pH to by using HCl. Use the same Tris-HCl buffer to prepare 1 M levamisole solution. Prepare freshly 4% $NaNO_2$ solution in dH_2O.
    2. To obtain 50 ml of the Fast Blue (FB) substrate (volume required for one standard glass cuvette) dissolve 6.25 mg Naphtol-AS-MX-Phosphate in 312.5 µl DMF in the glass tube and stir into 50 ml of pre-warmed (37 °C) Tris-HCl buffer. To dissolve 12.5 mg FB RR Salt in 312.5 µl 2 N HCl add 312.5 µl of previously prepared 4% $NaNO_2$ solution and stir the mixture into the same 50 ml of pre-warmed Tris-HCl buffer. Shake lightly until the yellow liquid becomes clear and finally add 77 µl of previously prepared 1 M Levamisole solution. Filtrate obtained mixture and pour onto slides placed in the glass cuvette.
    3. Initiate the incubation at 37 °C and control developing process under the light microscope approx. every 15 - 30 min. If turning fuzzy, replace the FB solution with the fresh mixture.
    4. Rinse the slides 3 - 5x with TBS buffer and transfer subsequently into PBS buffer.

2. **Visualization of the bound biotinylated secondary antibody**
    1. Prepare DAB/H_2O_2 developing solution by diluting 1 ml DAB stock solution (25 mg DAB per 1 ml PBS) in 49 ml PBS. Add 16.5 µl H_2O_2 and filtrate prior pouring onto sections.
    2. Conversion of the chromogen DAB into precipitating brown pigment can be immediate. Control the developing process under the light microscope (even couple of seconds longer incubation may raise a high background and mask the specific signal).
    3. The intensity of the brown pigment precipitate can be alternatively enhanced by incubation in the solution consisting of 2% copper sulphate and 0.9 % NaCl for 5'.
    4. Rinse the slides 3 - 5x with PBS and finally with dH_2O, use alternatively tap water.
    5. Mount the slides with cover slips directly from the water by using aqueous GelTol mounting medium. Avoid creating air bubbles.
    6. Allow complete drying of the mounting medium, *e.g.,* o/n at 4 °C. Store dried slides on RT.

1.	% Ethanol	20'	40 °C
2.	% Ethanol	60'	40 °C
3.	% Ethanol	90'	40 °C
4.	% Ethanol	60'	40 °C
5.	% Ethanol	90'	40 °C
6.	% Ethanol	60'	40 °C
7.	% Ethanol	90'	40 °C
8.	% Ethanol	120'	40 °C
9.	Xylol	30'	40 °C
10.	Xylol	60'	40 °C
11.	Paraffin	60'	60 °C
12.	Paraffin	60'	60 °C
13.	Paraffin	60'	60 °C
14.	Paraffin	120'	60 °C

Table 1. Tissue Processing by Tissue-Tek$^{V.I.P}$ Vacuum Infiltration Processor.

Representative Results

Double immunostainings (co-stainings) were performed in formalin-fixed, paraffin-embedded rat CNS and LN sections. 3-5 µm thick tissue slices were cut using a sledge microtome, mounted subsequently onto pre-coated adhesive glass slides and treated as previously described [10,11,12]. Briefly, after deparaffinizing, tissue rehydration and endogenous peroxidase inactivation, sections were subjected to the antigen retrieval process, followed by a blocking step to eliminate unspecific binding sites. The co-stainings were performed in the following combinations: i) Ccl11/Iba-1, ii) Ccl11/Ed1 and iii) Ccl11/Cd8. The primary Abs used for each co-staining were raised in two different species (goat and mouse, respectively), which enabled their simultaneous incubation. Ccl11 was visualized by the brown peroxidase reaction product, while Iba-1, Ed1 and Cd8, were visualized by the blue AP signal.

Results presented here have already been described in more detail and in a larger context in our recent study about the role of the Ccl11 chemokine in multiple sclerosis (MS)-like neuroinflammation[10]. According to the IHC analyses performed in the rat CNS, Ccl11 was present in pericarya, dendrites and axons of the neurons located in the ventral horns of the spinal cord grey matter (**Figure 1A and B**). Moreover, single $Ccl11^+$ plasma cells were detectable in the same tissue sections (**Figure 1A**), as well as single $Ccl11^+/Ed1^+$ macrophages and brain resident microglia observed at the inflammation site (data not shown; Ed1 is a marker of lysosomal protein in activated macrophages and microglia[13]). Notably, Ccl11 chemokine was not found co-localizing with Iba-1^+ macrophages and brain resident microglia (1A; Iba-1 is a marker for detecting ionized Ca^{2+}-binding protein[14]).

IHC-based protein targeting performed in the peripheral rat LN exhibited Ccl11 protein co-localization with Ed1 (**Figure 2B**). However, no Ccl11-secreting $Cd8^+$ T lymphocytes were detectable in the same tissue sections (**Figure 2A**; $Cd8^+$ cells were detected using anti-Cd8α, marker for predominantly cytotoxic T cells that bind to MHC class I molecules[15]). Except for omitting primary Abs during o/n incubation in the blocking solution, control sections were treated as described in the protocol. No detection signals of the target proteins (as presented in the **Figures 1A**, **B** and **2A, B**) were observed in the CNS and LN control sections (**Figure 1C** and **2C**, respectively).

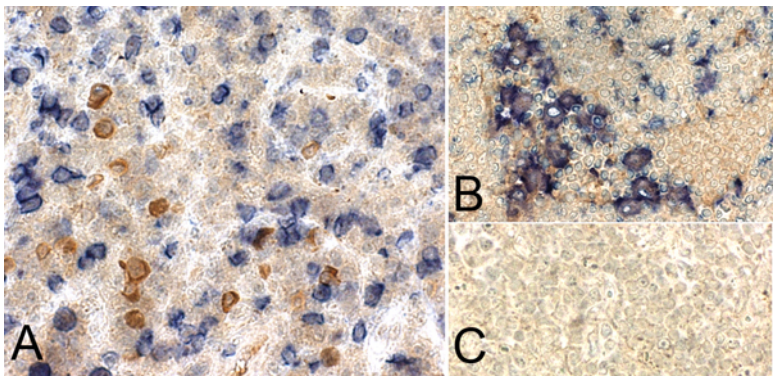

Figure 1. A, B: Double Immunostaining in the Rat LN. Primary antibodies directed against the Ccl11 chemokine in combination with either α-Cd8 **(A)** or Ed1 **(B)**. No co-localization within the same cell was observed for $Ccl11^+$ (brown) and $Cd8^+$ cells (blue). **B:** Ccl11 detection signal (brown) co-localizing with the marker for a lysosomal protein in macrophages (Ed1, blue). **C:** Negative control; a detail showing unlabeled resident cells in the peripheral LN. Please click here to view a larger version of this figure.

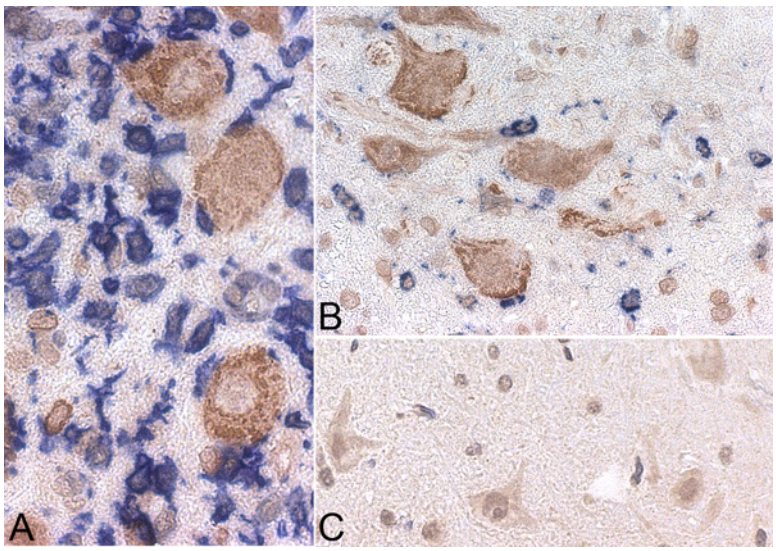

Figure 2. A, B: Double Immunostaining in the Rat Spinal Cord. Ventral horn of the grey matter exhibiting Ccl11 in neuronal pericarya, dendrites and axons (brown) co-stained with either Iba-1^+ (A; blue) or $ED1^+$ (B; blue) macrophages/microglia cells. **C:** Negative control; a detail showing unlabeled resident cells in the ventral horn of the spinal cord grey matter. Please click here to view a larger version of this figure.

Discussion

Standard IHC procedures often require specific adjustments to obtain an optimal result, which commonly implies extensive experience but also "trial and error" approach. From tissue preparation until target visualization, almost each step in the protocol may be subjected to individually designed modifications in order to improve the final outcome. Double staining protocol presented here exemplifies IHC-based protein targeting particularly adjusted for assessing interrelations between the target proteins of our interest in formalin-fixed, paraffin-embedded rat CNS and LN tissue sections affected by neuroinflammation. As such, it may be used as a tutorial for learning the technique of IHC, but also as a source of inspiration for more advanced trials. However, it should be kept in mind that a mere reproduction of this protocol for targeting other proteins and/or in other tissues than here presented may generate suboptimal results.

In situ hybridization using locked nucleic acid (LNA) riboprobe against *Ccl11* exhibited upregulation of the respective mRNA levels in CNS of the congenic rat strain[10]. This finding was confirmed by qPCR analyses. Further, qPCR revealed *Ccl11* upregulation also in the congenic peripheral LN compared with the wild type (wt) strain. Finally, Western Blot (WB) showed significant upregulation of the Ccl11 protein in both target tissues of the disease-protected congenic rat strain[10]. Therefore, we aimed to investigate the origin of this chemokine directly in the target tissues, and for that purpose we developed the here described double staining protocol.

Besides avoiding mechanical damaging of the tissue during post-mortem dissection and post-fixation decalcification, tissue sectioning might appear quite challenging. The sectioning usually requires skill and practice. Quality of slice is dependent on many aspects such as adjusting a right angle between the tissue block and the knife in order to reduce the pressure applied on sample during cutting, cutting speed, *etc.* Thickness of the paraffin-embedded tissue slices produced by sledge microtome may vary from 2 to 50 µm. Cut paraffin tissue slices are mounted from a warm water bath onto glass slides preferably commercially coated with an adhesive such as poly-L-lysine or aminopropyltriethoxysilane (APTS) to ensure a better hold during the demanding staining procedure. In contrast to cryo tissue sections which are cut at approximately -20 °C using cryomicrotome and dried at RT prior to the staining, cooled paraffin blocks are sliced at RT, and subsequently dried at 50- 60 °C prior to deparaffinization.

Highly preserved tissue architecture, cell morphology and target epitopes are essential for assessing localization and interrelation of the target proteins. In order to achieve optimal tissue preservation, samples have to be properly fixed, using either coagulating or cross-linking fixatives. Coagulating fixatives, such as ethanol, are more often used for cryopreservation. Protein denaturation using ethanol is achieved through tissue dehydration[9]; which may result in inadequate cellular preservation[16]. In contrast to coagulating fixatives, formaldehyde is a cross-linking fixative which is most frequently used in routine histology and IHC[9] for both cryo- and paraffin tissue preservation. We have achieved an optimal fixation for the rat CNS and LN by immersing into formaldehyde solution for 24 hr at 4 °C prior to decalcification and subsequent paraffin embedding. Despite causing profound changes in the conformation of macromolecules (formaldehyde namely forms methylene bridges that crosslink proteins and thereby mask antigens, which may prevent specific binding of antibodies), this semi-reversible, covalent reagent provides high quality tissue preservation. Notably, temperature and duration of fixation with cross-linking reagents may impact several aspects of IHC such as sectioning, epitope detection and even cross-reactivity. Thus both under-fixation and over-fixation using aldehyde-based reagents such as formaldehyde may cause artefacts that are primarily due to autolysis or excessive cross-links[17], respectively. Nevertheless, an unspecific background staining due to hydrophobic protein binding observed by overfixation can be reduced using low salted buffers containing nonionic detergents, or by raising the pH of the dilution buffer when using polyclonal Abs[18]. However, an unspecific background staining (noise) caused by ionic and electrostatic protein interactions could be reduced using diluent buffers with high ionic strength, which at the same time may aggravate the noise caused by hydrophobic protein binding[18]. When it comes to detection of the target epitopes, formaldehyde-induced alteration of the tertiary structure of proteins can be, although not entirely, reversed by antigen retrieval[19]: i) by heating in buffers with various pH values (heat-induced epitope retrieval (HIER), ii) by enzymatic degradation (protease-induced epitope retrieval (PIER;[20])), iii) by incubation in strong alkaline or acid solution, or sucrose[21,22] or iiii) by treating with concentrated formic acid[23]. HIER appears convenient for majority of the target epitopes[19]. Besides heating duration, pH value of the solutions such as EDTA (pH 5.5, 8.5 or 9.0) or sodium citrate buffer (pH 6.0- 6.2) may be essential for the outcome of HIER. Notably, the most satisfying result in our study was achieved by steaming the samples for 1h in the EDTA solution with the pH value 8.5.

Although antibodies preferentially bind to their specific epitopes, attraction to nonspecific, similar to the cognate Ag binding sites, cannot be entirely excluded. Notably, probably the most efficient method for reducing the background staining is incubation in blocking proteins (such as FCS used in our study) prior to application of primary Abs [9]. Monoclonal primary Abs are anyhow preferred over polyclonal Abs due to higher specificity and thereby reduced unspecific background signal. Nevertheless, cross-reactivity still might appear, as monoclonal Abs are directed against epitopes commonly consisting of a small number of amino acids, which may be a part of some other (unspecific) protein[24]. Unlike monoclonal, polyclonal Abs have a higher chance of recognizing multiple isoforms of the target protein. We initially used two different primary Abs to detect Ccl11: i) a polyclonal raised in goat and ii) a monoclonal raised in mouse. In our hands, both products exhibited the same staining pattern for the target chemokine. In order to perform simultaneous co-staining, we used goat-anti rat Ccl11 Ab in combination with the antibodies against Iba-1, Ed1 and Cd8, respectively, which were all produced in mouse. Nevertheless, besides simultaneously, double immunostaining can also be performed sequentially[25], as required for co-labeling of the primary Abs produced in the same species.

The optimal working concentration of the Abs used in our study was estimated through test-dilution series as an optimal ratio between intensity of the specific signal and the background noise. However, it should be kept in mind that an optimal working concentration of the same antibody may sometimes vary between organs, species, background pathology, *etc.* Notably, permeabilization was not required in our staining procedure performed in the paraffin embedded, 3-5 µm thick tissue sections, however, detergents like Triton X-100 could still have been used to reduce surface tension and thus to facilitate the binding between antigen and antibody. On the contrary, permeabilization- mediated improvement of Ab penetration is commonly required for protein detection in cultured cells or cell suspensions (immunocytochemistry (ICC)), for immunofluorescence (IF) in the cryo sections and IHC/IF in fresh (thick, vibratome-cut) free- floating tissue slices.

Multiple controls are essentially important for generating specific/reliable immunotargeting. As a positive control, we used lungs from a rat model of asthma, where we could detect $Ccl11^+$ eosinophils. The negative controls were incubated only in the blocking solution o/n at 4 °C in absence of the primary Abs.

We have already mentioned some causes and potential remedies for reduction of a background signal. An unspecific immunostaining product may also occur as reaction between DAB and pseudoperoxidase of the red blood cells and peroxidase of the myeloid cells[26]. Activity of these enzymes, just as the noise caused by endogenous biotin or AP has already been reduced during formalin-fixation. However, the pretreatment with methanol/H_2O_2 is necessary for their further or complete inactivation [27, 28]. Background staining caused by AP activity in mammalian tissues can be further inhibited by levamisole[29], or by acetic acid [29]. Unspecific binding as the result of ionic attraction between avidin and oppositely charged cellular molecules [30] can be avoided by substituting avidin from egg white with streptavidin from *Streptomyces avidinii* [31].

DAB is probably the most frequently used chromogen in IHC. Besides DAB (brown reaction product), other peroxidase chromogens in use are 3-amino-9-ethylcarbazole (AEC; red), 4-Chlor-1-Naphthol (CN, blue) and tetramethylbenzidine (TMB; blue). Commonly chosen AP chromogens are FB, Fast Red (FR), new fuchsin and 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium chloride (BCIP/NBT). The choice of enzymes and chromogens is basically a matter of individual preference, however, it may be steered by the target features such as localization, expression

pattern, but also by the presence of endogenous pigments (melanin, hemosiderin)[32]. Counterstain is the last, facultative step in the IHC procedure usually performed using hematoxylin, nuclear fast red or methyl green[18]. Generally more suitable for the single labeling, counterstain facilitates the interpretation of tissue morphology and it should be subtly developed to avoid any interference with the chromogen precipitate.

Upon completion of the staining procedure tissue sections should be carefully mounted with a coverslip using a mounting medium. Formation of air bubbles should be avoided. Beside physical protection, mounting medium improves visualization and quality of imaging. Either organic/hydrophobic or aqueous/hydrophilic, the choice of the mounting medium is depending on solubility of the end product in the organic solvent. While a toluene- based mounting medium would be suitable, *e.g.,* for DAB, aqueous mounting medium can be applied to all enzymatic chromogens, including fluorescence labeling.

Disclosures

The authors declare no conflict of interest.

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