Basics of Immunohistochemistry

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INTRODUCTION

Immunohistochemistry (IHC) is a powerful method for localizing specific antigens in formalin-fixed, paraffin-embedded (FFPE) tissues based on antigen–antibody interaction (Taylor and Burns, 1974). The technique is widely used in dermatologic diagnostics and research, and its applications continue to be extended because of its ease of use, reliability, and versatility.

In IHC an antigen–antibody construct is visualized through light microscopy by means of a color signal. The advantage of IHC over immunofluorescence techniques is the visible morphology of the tissue around the specific antigen by counterstaining, e.g., with hematoxylin (blue). Results of stained IHC markers are reported semiquantitatively and have important diagnostic and prognostic implications, particularly for skin tumors, lymphoma, and the detection of infectious microorganisms. This article presents the key steps for performing IHC and describes its current use in dermatology.

HISTORY

The term "antibody" was coined by Paul Ehrlich in 1891. Immunofluorescence staining on frozen sections based on antigen–antibody interactions was presented by Coons in 1940 (for an introduction see Odell and Cook, 2013). Taylor and Burns developed IHC on routinely processed FFPE tissues in 1974. In 1975 Köhler and Milstein presented the hybridoma technique to produce monoclonal antibodies (mAbs) by fusing an antibody-producing B cell with a myeloma cell that is selected for its ability to grow in tissue culture (Köhler and Milstein, 1975). Prior to this, polyclonal antibodies—antisera that contain molecularly different antibodies that target multiple epitopes with varying specificity—were used. These result in higher levels of nonspecific background staining than mAbs. The hybridoma technique enabled the use of mAbs in IHC, with a broad range of antigens and high staining quality.

HOW IS IMMUNOHISTOCHEMISTRY PERFORMED?

Step 1: tissue processing and epitope retrieval

For fixation, 10% neutral-buffered formalin is used for between 4 and 24 hours. This fixation preserves morphologic features but compromises antigenicity to a certain extent. It induces alterations in the tertiary and quaternary structures of proteins but does not cause irreversible reduction or total loss of antigenic determinants in paraffin sections. Therefore, the epitopes of interest remain intact (Dill and Shortle, 1991).

WHAT IMMUNOHISTOCHEMISTRY DOES

- Localizes specific antigens in formalin-fixed, paraffin-embedded skin based on antigen–antibody interaction.
- Marks antigens in three steps: processing and epitope retrieval in the skin, antigen–antibody interaction, and visualization through different detection systems.
- Illustrates the result of antigen–antibody interactions through light microscopy by means of a color signal, with the advantage of visible morphology of the cutaneous or subcutaneous surrounding tissue.
- Impacts the diagnosis and prognosis of skin tumors and lymphomas and the detection of infectious microorganisms.
- Serves numerous purposes in dermatologic research.

LIMITATIONS

- Not all antigens are equally preserved and detectable through immunohistochemistry.
- Demanding laboratory procedure with many possible variables; technical pitfalls can lead to false-negative or false-positive results.
- Nonstandardized methods vary between laboratories.
- Less sensitive and specific than PCR-based molecular diagnostic methods.

Then FFPE tissue should be cut into 3- to 4-μm thin sections and mounted on glass slides. Enzyme digestion by trypsin or protease can be used to "unmask" antigens that have been altered by formalin fixation. The most common antigen retrieval technique to restore the tertiary structure is heating tissue sections in water or buffered solutions (e.g., citrate or EDTA buffer).

Step 2: antigen–antibody interaction

For the direct method, labeled monospecific antibody is directly applied to the tissue section (Figure 1a). The antibody

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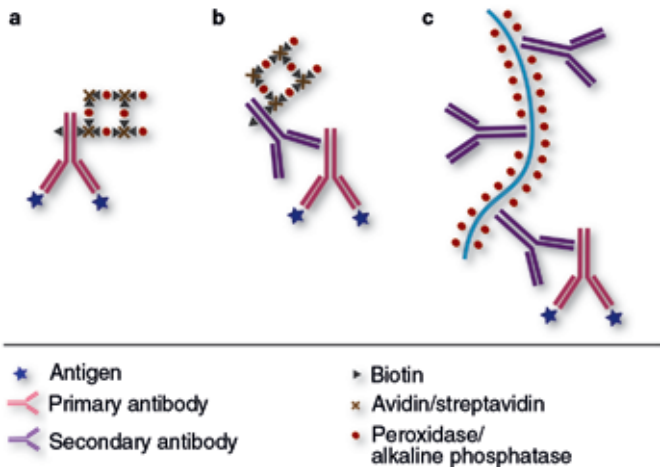

Figure 1. Schematic diagram of immunohistochemical techniques. (**a**) Direct method: the antigen-specific primary antibody is biotin labeled. Biotin binds to avidin/streptavidin. Color visualization is achieved through enzymatic reaction of horseradish peroxidase/alkaline phosphatase. (**b**) Indirect method: the antigen-specific primary antibody is unlabeled. The secondary, biotin-labeled antibody binds to primary antibody. Visualization is achieved accordingly through avidin/streptavidin and peroxidase/alkaline phosphatase complexes. The indirect method increases versatility because unlabeled primary antibodies can be used. (**c**) Indirect method with polymer chain detection system. Biotin and avidin/streptavidin are replaced by a labeled polymer chain, allowing for increased sensitivity and specificity.

is most frequently conjugated with biotin. Biotin then binds to labeled avidin or streptavidin. Through this second layer of labeling, the staining is amplified. Therefore, the development of these multiple-step detection methods resulted in greatly improved sensitivity of IHC. Thus, these multiple-step detection methods allow for detection of a wide range of antigens in routine diagnostic FFPE tissues. The indirect method uses two layers of antibodies (Figure 1b and 1c). Progression from the one-step direct conjugate method to the multiple-step indirect method greatly increased the versatility of IHC because a wide range of unlabeled primary antibodies could then be used.

Step 3: visualization through different detection systems

Antibody molecules cannot be seen—even under electron microscopy—unless they are labeled or tagged for visualization. Labeling techniques include fluorescent compounds (e.g., for direct immunofluorescence) or active enzymes (for IHC). In IHC, enzymes are added to the tissue sections, and these enzymes bind to the biotin, avidin/streptavidin labeled antibodies; the enzymes used are horseradish peroxidase or calf intestine alkaline phosphatase (Figure 1a and b). Then chromogens are added to the sections and oxidized by horseradish peroxidase or alkaline phosphatase, leading to a color reaction. The most widely used chromogens result in red or brown IHC staining. The method shown in Figure 1b is the most widely used; however, newly developed detection systems do not rely on antibody labeling through biotin and avidin/streptavidin. Instead, multiple secondary antibodies and enzymes are linked to a polymer backbone (Figure 1c). These new methods have the advantage of decreased background staining (higher specificity) and increased sensitivity. Double staining (different colors) in one tissue section can be achieved through a combination of two immunoenzymatic systems or one immunoenzymatic system with different substrates. For detailed overviews of IHC, see Dabbs and Thompson (2013).

QUALITY CONTROL

Quality control is essential to ensure that an IHC staining is sensitive and specific, reproducible, and standardized. There can be many pitfalls in IHC (Yaziji and Barry, 2006); therefore, the use of positive and negative controls in each staining run is essential. A positive control is a well-characterized sample that contains the antigen of interest and is stained the same way as the specimen to be checked. The same sample is used for the negative control as for the positive control. It is stained with the same procedure, but the primary antibody is replaced by nonbinding Ig from the same species.

Reasons for false-negative results include improper tissue fixation, processing, or pretreatment. False-positive results can occur through nonspecific background staining. The most common cause of this is ionic binding of antibodies to charged connective tissue elements, e.g., collagen fibers. To avoid this, it is recommended that the tissue be incubated with normal serum of the same species as the secondary antibody (blocking). Moreover, endogenous enzyme activity must be blocked—taking into account the fixation and retrieval method—to further avoid false-positive reactions. Undissolved precipitates of chromogen or counterstain can also be mistaken for a positive reaction.

Validation of IHC methodologies can be achieved by participation in round robin tests, by staining various tissue and tumor types to determine sensitivity and specificity, or by comparing staining results of different antibodies that recognize similar proteins.

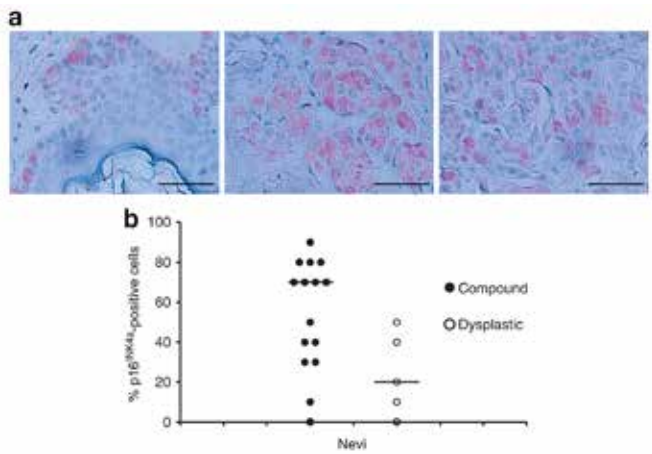

Figure 2. p16INK4a expression in human melanocytic tumors. p16INK4a expression was determined using immunohistochemical analysis of 20 benign nevi. (**a**) Representative examples of compound nevi stained with p16INK4a antibody (N20; Santa Cruz Biotechnology, Santa Cruz, CA) and positive cells detected using Permanent Red (Dako, Glostrup, Denmark). Bar = 50 μm. (**b**) The results for 15 compound and 5 dysplastic nevi are represented graphically. Horizontal bars indicate the median p16INK4a expression values. Reprinted from Scurr *et al.*, 2011.

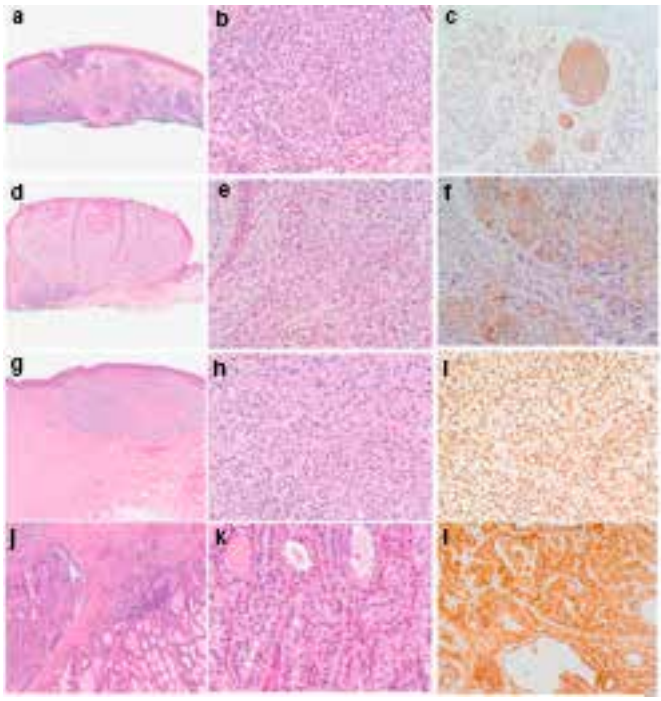

Figure 3. Immunohistochemical results in melanoma for selected BRAF V600E mutation–positive cases by DNA analyses. Case 9, **a**–**c**; case 10, **d**–**f**; case 20, **g**–**i**; papillary thyroid carcinoma control, **j**–**l**. (**a**, **d**, **g**, and **j**) Scanning magnification, hematoxylin and eosin (H&E). (**b**, **e**, **h**, and **k**) High power, H&E. (**c**, **f**, **i**, and **l**) Immunohistochemical stain with the anti-B-Raf (V600E) antibody. Reprinted with permission from Feller *et al.*, 2013.

IMMUNOHISTOCHEMISTRY IN DERMATOLOGY

IHC is possibly the most widely used technique at the protein level in dermatologic diagnostics. It complements morphologic histopathology, especially for the precise diagnosis of skin tumors and skin lymphoma and for the detection of infectious microorganisms. Protein expression profiles detected through IHC—on the cell surface, intracellularly, and in the nucleus—enable the characterization of cell lineage, tumor, lymphoma, and inflammatory cell infiltrate. Intra- and extracellular pathogens—bacteria, parasites, and viruses (e.g., *Mycobacterium tuberculosis*, leishmaniasis, and human herpesviruses)—can be directly detected. IHC also plays an important role in dermatologic research. The following two examples demonstrate how IHC is used in melanoma research.

IDENTIFICATION OF A NEW MARKER FOR MELANOMA

In addition to the identification of cell lineages, IHC can be used to find markers that allow for discrimination of benign versus malignant lesions, e.g., nevi versus malignant melanoma. Ideally, those markers are of prognostic value. Some antigens show a specific IHC staining pattern, e.g., HMB45/MART1 expression is lost in deeper dermal parts of many benign nevi as a sign of cell maturation. Other markers, such as certain oncogenes, are overexpressed in malignant lesions. The $p16^{INK4a}$ cyclin–dependent kinase plays an important role in cell cycle regulation. Mutations in the coding gene are found in families affected by multiple melanomas. In

QUESTIONS

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For each question, more than one answer may be correct.

1. What does IHC detect?

A. Tissue-bound autoantibodies in fresh tissues.
B. DNA aberrations in FFPE tissues.
C. Specific antigens in FFPE tissues unmasked by different epitope-retrieval techniques.
D. The presence of pathogenic RNA in fresh and FFPE tissues.

2. Which of the following is a true description of the indirect method of IHC?

A. Can be used only with biotin-labeled primary antibodies.
B. Uses two layers of antibodies; the primary antibody is unlabeled and can be visualized through different secondary antibody–based detection systems.
C. Is performed with circulating autoantibodies from patient serum.
D. Is not very versatile because all primary antibodies must be labeled.

3. What is a major advantage of antigen detection with IHC?

A. Antigen–antibody constructs are visualized through light microscopy by means of a color signal; the morphology of the surrounding tissue can be made visible by a counterstaining.
B. It is the most sensitive method for detection of all antigens.
C. FFPE tissue does not need to be prepared for antigen detection.
D. It is a highly standardized and simple technique; there are almost no technical pitfalls.

4. Which of the following techniques is more sensitive than IHC for the detection of some antigens in the skin?

A. Light microscopy: hematoxylin and eosin staining.
B. Light microscopy: special stainings.
C. Dermatoscope.
D. PCR-based methods.

5. What thickness of FFPE tissue sections should be used for IHC?

A. 10 μm.
B. 10 nm.
C. 3–4 μm.
D. 3–4 nm.

their recent investigation, Scurr *et al*. (2011) found that p16 expression was significantly decreased in dysplastic nevi compared to benign melanocytic nevi in IHC (Figure 2). It has been shown that loss of p16 is common in melanomas and might be an independent adverse prognostic marker in melanoma (Lade-Keller *et al*., 2014). By contrast, expression of p16 did not help to differentiate between Spitz nevi and spitzoid melanomas in another study (Mason *et al*., 2012). Therefore, IHC staining of p16 in melanocytic lesions can be valuable for the dermatopathologist, but its full potential role in melanocytic lesions warrants further investigation.

IHC FOR DETERMINING SUITABILITY OF TARGETED THERAPIES IN MELANOMA

Another important marker in melanoma is the protooncogene BRAF that is involved in regulating cell growth. Certain mutations in the BRAF gene are associated with shorter progression-free survival. The advent of new drugs specifically targeting cells harboring a V600E mutation in the BRAF gene has drastically changed the treatment of end-stage melanoma patients. To identify melanomas that harbor V600E mutations in the BRAF gene, PCR-based technologies and direct sequencing are used, which are often time- and work-intensive. In their recent work, Feller *et al*. (2013) tested a mutation-specific antibody against BRAFV600E in IHC and demonstrated that it is sensitive and specific (Figure 3), indicating that IHC can be used as a simple screening tool for BRAFV600E in melanoma. IHC could also complement PCR-based technologies because it has the major advantage of a visible morphology. Therefore, parts of a tumor that are BRAFV600E-positive could be identified, or contamination by a large number of BRAFV600E-negative cells (e.g., lymphocytes in a lymph node metastasis) can be excluded.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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CME ACCREDITATION

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of the Duke University School of Medicine and Society for Investigative Dermatology. The Duke University School of Medicine is accredited by the ACCME to provide continuing medical education for physicians. To participate in the CME activity, follow the link provided. Physicians should only claim credit commensurate with the extent of their participation in the activity.

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SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2014.541.

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