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Chapter

Detection Systems in Immunohistochemistry

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Abstract

Immunohistochemistry (IHC) is a process of selectively imaging antigens in cells or tissue sections by exploiting antibody specificity. This technique is widely used in diagnostic pathology and research experiments for tracking specific molecular markers characteristic of a particular cell type or cellular events such as cancerous cell development, cell proliferation, or apoptosis. Visualizing the target antigen following an antibody-antigen interaction is accomplished by different detection systems. In the simplest instance, primary antibody directly conjugated to an enzyme is responsible for both specifically binding to the antigen and catalyzing a color-producing reaction. Alternatively, complex detection systems could be designed to profoundly improve minimal detection level of the antigen. During the past years, there has been a considerable improvement in designing and introduction of new and highly sensitive detection systems. The choice of an IHC detection system is a compromise of a variety of variables including desired sensitivity, cost, and the time needed for an IHC staining to be performed. This chapter covers the immunohistochemistry detection systems with emphasis on their principle, history, advantages, and limitations and delineates factors needed to be considered for choosing an appropriate detection system for IHC applications.

Keywords: immunohistochemistry, antibody, detection systems, sensitivity, background

1. Introduction

Immunohistochemistry (IHC) represents a way to build a picture of particular distribution and localization of molecular markers within cells and in the proper tissue context and is a powerful tool that provides important diagnostic, prognostic, and predictive information supplemental to the morphological assessment of the tissues. Although less sensitive quantitatively than such immunoassays as western blotting or ELISA, IHC enables observation of molecular signature in the context of intact tissue. In its very simplified method, IHC visualizes target antigens by using target-specific antibodies tagged with appropriate labels. However, lack of need for labeling of molecular marker-specific primary antibodies and higher sensitivity made indirect staining methods as the preferred staining method. The need for more sensitive detection systems in case of minimally expressed markers was a provocative factor that eventually led to the emergence of next generations of IHC detection methods with the hope to amplify staining signal. IHC

methods based on avidin-biotin interaction and polymer- and tyramide-based signal amplification are among IHC signal amplification methods that have greatly enhanced the sensitivity of IHC staining. However, when more sensitive methods are used, background signal tends to increase along with the target signal and so highly sensitive detection systems are not always desirable. Therefore, the optimal IHC method is planned as a compromise between sensitivity that allows proper and reliable visualization of a given molecular marker and at the same time avoiding background signals that impair staining index and specificity of the staining method. In an optimal IHC detection system, tissue type, level of expression of the marker of interest, localization of the marker, and cost are among important factors that should be taken into consideration. As a general rule of thumb, there is no a *bona fide* IHC detection method that is universally accepted. Although it does not rely on chemical reactions that take place in IHC, immunofluorescence (IF) staining follows almost the same rules as with IHC and so concerns on detection systems that are also applicable to IF staining methods. In this chapter, we will focus on detection methods in immunohistochemistry and immunofluorescence stainings and highlight in detail potential application, advantages, and disadvantages of each method.

2. Direct method

Direct detection methods are known as a one-step process applying a primary antibody, which is directly labeled with reporter molecules, such as biotin, colloidal gold, fluorochromes, or enzymes [1, 2]. The conjugated antibody makes a direct contact with cognate antigen in histological or cytological preparations (**Figure 1**). Direct detection methods are widely used for detecting highly expressed antigens. Furthermore, when the use of the secondary antibodies causes nonspecific and unwanted reactions, owing to the histological nature of the tissue and/or host species of the primary antibody, direct detection could be the technique of choice. For instance, in case of mouse lymph node immunostaining, labeled primary mouse monoclonal antibodies are preferred because antimouse secondary antibodies are not only bound to mouse primary antibodies bound to the tissue antigens of interest but will also react with endogenous immunoglobulins vastly found in lymph nodes. This would lead to a strong nonspecific staining. Hence, direct detection methods using mouse primary antibodies conjugated to a fluorophore or enzyme would be a better option [3]. If this approach is not practically feasible, for example, due to the low expression level of the target antigen or technical problems in primary antibody labeling, indirect methods using primary antibodies from species other than that of target tissue would be desirable.

One of the advantages of direct detection is that the incubation step with a secondary reagent is eliminated. Hence, this method is time saving and easy to

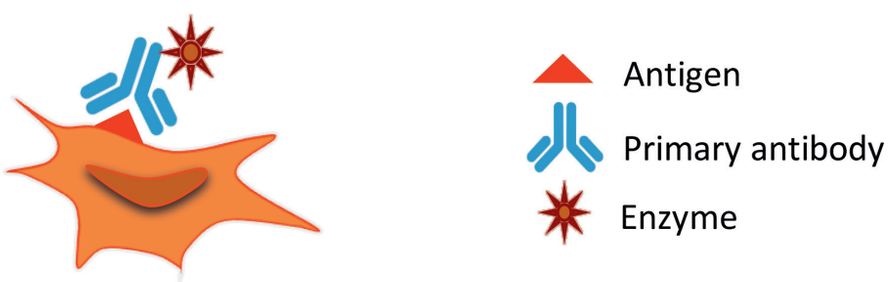


Figure 1.
Direct immunostaining method.

perform. In addition, due to the wide range of fluorochromes that are commercially available, direct detection is vastly used in multicolor experimental designs [4, 5].

It is important to note that insufficient sensitivity to detect most of the antigens found in routinely processed tissues is one of the drawbacks of using direct detection method. Furthermore, each primary antibody needs to be individually conjugated with fluorophores or enzymes, which increases considerably the cost of the whole process. Another concern with direct staining methods is the possibility of functional impairment of the antibody affinity if the process of antibody labeling is nonoptimal. This is especially case for monoclonal antibodies in which all antibody molecules in a given preparation have almost the same affinity and so are most likely to be affected all together by improper labeling. This issue is less problematic for polyclonal antibodies in which antibodies with diverse physicochemical properties are produced against an antigen [5–7].

Needless to say, direct detection methods are the method of choice in such high-sensitive protein detection systems as flow cytometry. Although this system is the simplest and the most convenient method for detection of a given marker expression, it is not routinely employed in clinical and research applications due to the limitations mentioned above.

3. Indirect method

The need for more sensitive detection systems for antigens with low expression pattern was a provoking factor that prompted Coons et al. in 1941 to develop two-step detection methods [8]. This system employs an unlabeled primary antibody as the first layer and the secondary antibody, which is raised against the primary antibody and is labeled with different fluorophores or enzymes (**Figure 2**) [6, 9–11]. In indirect methods, primary antibodies retain full avidity because they remain unlabeled. Indeed, higher number of labels per molecule of primary antibody is achieved in indirect compared to direct detection methods. The later stems from the fact that at least two labeled secondary antibodies can bind to each primary antibody molecule. These factors result in increased reaction intensity and the higher sensitivity in indirect staining methods. Accordingly, indirect methods are able to detect fewer number of antigens with less primary antibody. Moreover, indirect methods are more practical than direct methods since the same secondary antibody can be applied for detection of different sets of primary antibodies if they have been raised in the same species [12]. Another benefit of indirect method in IF stainings is possibility to select secondary antibodies with fluorophores of different colors. For example, if the tissue shows strong endogenous red autofluorescence, the secondary antibody labeled with green



Figure 2.
Indirect immunostaining method.

fluorophore could be a right choice [3]. Previously mentioned advantages of indirect detection systems eventually led to its widespread applications in research and clinical settings.

Despite advantages mentioned above, indirect immunostaining methods suffer from some shortcomings. First, additional controls and blocking steps are inevitable when using secondary antibodies. Indeed, there is possibility of nonspecific staining that happens when the secondary antibody interacts with unwanted tissue targets. If nonspecific staining is noticed, blocking reagents have to be used to treat the tissue sections that could be time-consuming and cause additional costs to IHC experiment [6, 13]. The blocking agent should contain nonimmune antibody fraction from the same species in which secondary antibody has been produced. This results in competitive blocking of the nonspecific binding sites for secondary antibody in the target tissue by the unlabeled antibodies from the same species. The addition of further layers beyond their use in the two-step indirect method for increasing the sensitivity of detection can be problematic as addition of every new species of antibody considerably increases the risk of nonspecific interactions and background staining [14]. Desire for more sensitive detection systems triggered researchers to develop next-generation detection systems, especially for those antigenic markers, which are not expressed in physiological condition, and any level of their upregulation would be interpreted as a pathological condition.

4. Bridge methods

Early conjugation protocols were not efficient and did not label all antibodies leaving a fraction of antibodies unlabeled. These unlabeled antibodies were able to compete with labeled antibodies for binding to the cognate antigen and reduced the efficiency of detection. To overcome this problem, new approaches were invented that eliminated the need for chemical conjugation of antibodies. In these approaches, antigen specificity of antibodies is employed to couple antibodies to the enzymes. Taking advantage of antigen specificity of antibodies, antiperoxidase or antialkaline phosphatase antibodies are easily coupled with peroxidase or alkaline phosphatase after incubation with these enzymes without need for any chemical modifications of the antibody. These preformed soluble enzyme-antienzyme immune complexes are then used as the third layer reporter antibody for detection of the antigen-bound primary antibody in tissue section. Taking advantage of the bivalent properties of IgG binding, a second-step antibody with binding specificity to primary antibody and tertiary antienzyme antibody complexed with the enzyme bridges two layers (**Figure 3a**). The bridge antibody is usually used in excess, so that one of its two identical binding sites interacts with enzyme-coupled tertiary antibody, while the other site interacts with primary antibody. The tertiary antienzyme antibody has the same animal species of origin as the primary antibody. The bridge methods are collectively called as soluble enzyme-antienzyme methods [5, 8, 15–17].

The classical immunoenzyme bridge method [18] was rapidly replaced with an improved version in which peroxidase-antiperoxidase complex (PAP, MW: 400–430 KDa) contained three peroxidase molecules and two antiperoxidase antibodies (**Figure 3b**) [15]. In this system, antibodies against alkaline phosphatase can also be employed to form alkaline phosphatase-antialkaline phosphatase complexes (APAAP, MW: approximately 560 KDa) [17]. In contrast to PAP complexes, APAAP complexes include two molecules of alkaline phosphatase and only one antibody. The APAAP method is usually used as an alternative to PAP technique when high levels of endogenous peroxidase in such tissues as bone marrow aspirate specimens, spleen and peripheral blood, interfere with the staining or when double labeling approaches are desired [19].

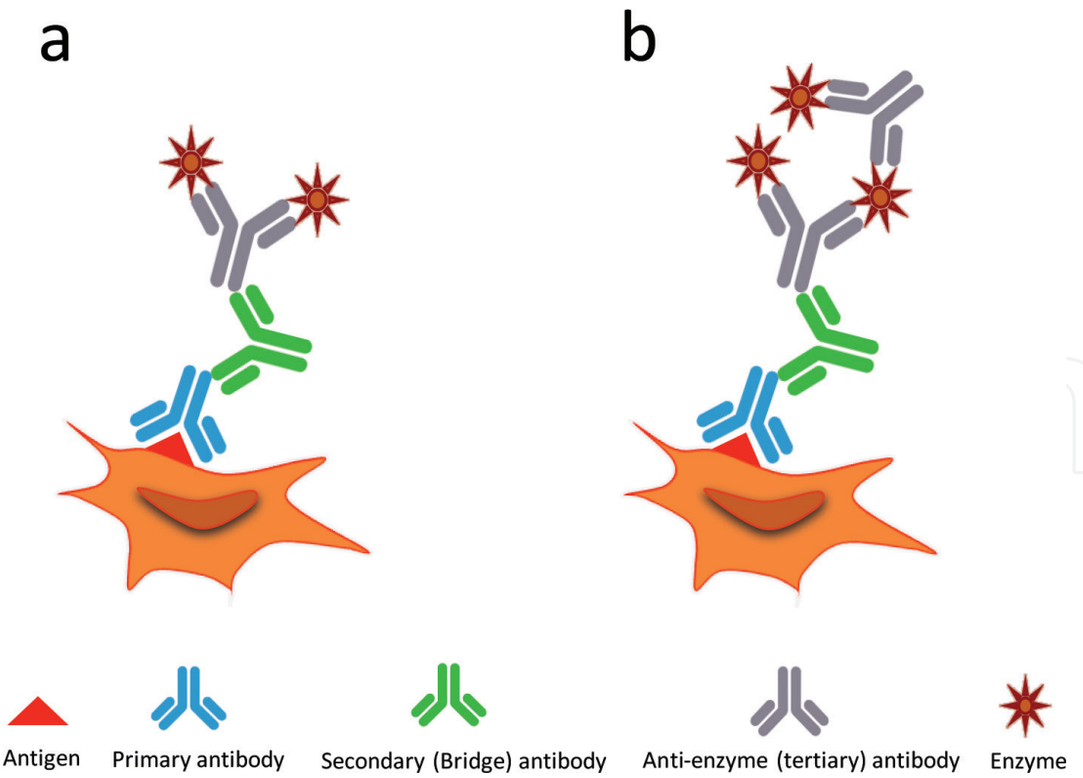


Figure 3.
Two- (a) and three-step (b) bridge immunostaining methods.

Soluble enzyme-antienzyme methods offer several advantages over direct and indirect detection methods. The drawback of chemical conjugation process, which could potentially lead to impairment of antibody activity, is entirely avoided in enzyme-antienzyme methods. Due to a greater number of enzyme molecules localized per antigenic site, enzyme-antienzyme process shows the higher sensitivity compared to previously described methods. It is reported that PAP method exhibits nearly 100- to 1000-fold higher sensitivity than two-step indirect method [7]. Although multilayering of detection antibodies could potentially increase the risk for nonspecific interaction with the tissue antigens, PAP and APAAP methods that offer triple level detection are among the exceptions. These methods are suitable for cell and cryosection IHC [14].

In comparison to two-step indirect methods, PAP and APAAP are more time-consuming. Indeed, these methods may not have sensitivity enough required for use in formalin-fixed paraffin-embedded (FFPE) preparations, especially when used in combination with monoclonal antibodies [14]. Although PAP and APAAP methods have been known as the highly sensitive, reliable, and popular techniques in pathology laboratories for a long time, they have gradually been replaced by more improved methods such as streptavidin-biotin- and polymer-based systems.

5. Biotin-avidin/streptavidin-based methods

5.1 Labeled avidin/streptavidin-biotin (LAB/LSAB)

Labeled avidin/streptavidin-biotin (LAB/LSAB) are among very sensitive IHC detection methods, which take advantage of high-affinity binding of avidin/streptavidin to a water-soluble vitamin, biotin (vitamin H or B_7) [5, 20–22]. The affinity constant of avidin binding to biotin (10^{15} M^{-1}) is nearly 10^3–10^6 times more than the binding affinity of antibody-antigen interaction [22].

The potential of avidin-biotin system to be used in immunoassays was inspired for the first time from a study in 1972 in which it has been shown that avidin could inactivate the biotinylated bacteriophages [23]. Avidin-biotin-based system was used for the first time in an immunological experiment in 1976 when an erythrocyte surface antigen was localized by using biotin-labeled antibody and ferritin-avidin conjugate [24]. In 1979, Guesdon et al. showed that avidin-biotin complex could be effectively used for immunoassays. Using avidin-biotin system, they suggested different related methods for enhancing the specificity and sensitivity of solid-phase immunoassays. They also used avidin-biotin-based immunohistochemistry for localization of intracellular immunoglobulins [25].

The principle of labeled avidin-biotin (LAB) technique is based on sequential interaction of biotin-labeled antibody with tissue antigen and enzyme-labeled avidin with biotinylated primary antibody (**Figure 4a**). In the bridged avidin-biotin (BRAB) technique, however, avidin bridges biotin-labeled primary antibody and biotin-labeled enzyme (**Figure 4b**). BRAB is particularly suitable in cases where intracellular penetration and/or sensitivity of the staining reaction are the major concerns. An indirect approach of BRAB technique (IBRAB) can also be applied for identification of antigens in formalin-fixed paraffin-embedded tissues in which avidin and biotin-labeled peroxidase are added sequentially to the system after primary antibody and biotin-labeled secondary antibody. The superiority of BRAB over LAB method is that there is no need to prepare protein-protein conjugate [20, 25].

Avidin extracted from egg white is a large tetrameric glycoprotein with molecular weight of about 66 KDa. Each subunit (MW 16,400 Da) contains one high-affinity binding site for biotin [26] and one oligosaccharide modification (Asn-linked). Tryptophan and lysine residues in each subunit are believed to be involved in forming the binding pocket with high affinity for biotin molecule [27–29]. Biotin with molecular weight of about 244.31 Da is a small molecule, which has only one binding site specific for avidin. Biotin can be easily conjugated with an antibody or other macromolecules such as fluorochrome and enzymes through other sites [6, 22].

Due to some limitations mentioned below, avidin has been mostly substituted by streptavidin in IHC applications. In this regard, labeled streptavidin-biotin methods (LSAB) are now more popular than LAB methods in both diagnostic and research IHC laboratories [30–34]. The sequence of streptavidin from *Streptomyces avidinii* shows only 30% similarity to avidin, while it has nearly identical secondary, tertiary, and quaternary structure [35]. It is a nonglycosylated protein with a molecular weight of 60 KDa [36]. Avidin is a basic glycoprotein (pI ~ 10.5) that contains nearly 7% carbohydrates, which gives avidin a natural tendency to nonspecifically bind to lectin-like substances found in kidney, liver, brain, and mast cells [7, 36–38]. Avidin can also bind electrostatically to negatively charged tissue elements at physiological pH. Sterptavidin (pI ~ 6.5), however, remains uncharged at neutral pH and does not contain carbohydrate in its structure eliminating its nonspecific binding to tissue lectins. In addition, streptavidin shows less propensity for aggregation [7, 39–41]. Although avidin shows higher binding affinity to free, unconjugated biotin, streptavidin has more tendency to bind biotin-protein conjugates [42]. Indeed, lot to lot variations in binding affinity of biotin and avidin have been reported, which negatively affect sensitivity and reproducibility of the procedure [7].

LAB/LSAB methods offer several advantages for IHC applications. Biological activities of macromolecules (e.g., enzymatic catalysis or antibody binding) are not affected when they are conjugated with biotin. On the other hand, the affinity of avidin/streptavidin to biotin is quite high enough that ensures the biotin-avidin/

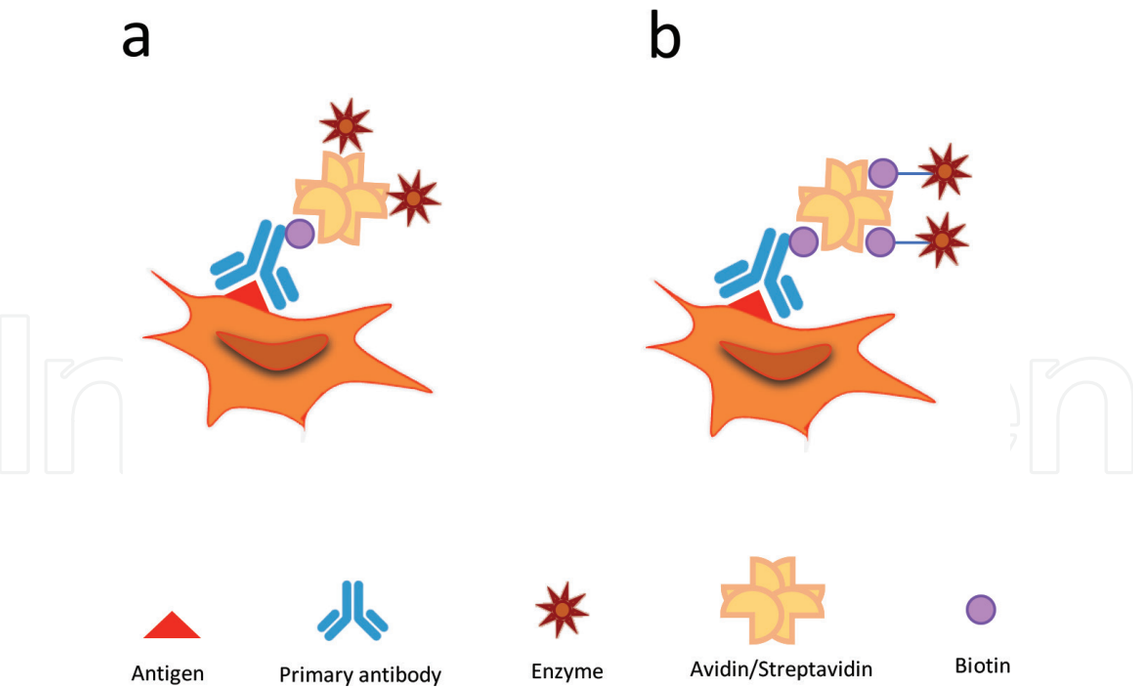


Figure 4.
LAB/LSAB (a) and BRAB (b) immunostaining methods.

streptavidin complex is not disrupted by manipulations like multiple washing when the complex is immobilized in the tissue sections or by changes in pH and presence of chaotropes [22]. LAB/LSAB techniques considerably improve the sensitivity and efficiency of the immunohistochemical detections and allow researchers to use even more diluted primary antibodies. An immunohistochemical staining that employs a single layer of biotin-labeled monoclonal antibody provides sensitivity equivalent or much greater than PAP methods [43]. The increased sensitivity of avidin-biotin methods stems from larger numbers of biotin molecules that is conjugated to a primary antibody [20, 25, 44]. Due to very high sensitivity, IHC stainings using LAB/LSAB techniques are rapid [45, 46]. LAB and LSAB technique can also be applied in an indirect manner, where biotinylated secondary antibodies are used in conjunction with unlabeled primary antibody [47].

The main challenge of LAB/LSAB techniques is the nonspecific (false-positive) staining, which occurs when the tissue of target contains endogenous biotin [6, 7]. Endogenous avidin biotin activity (EABA) or tissue affinity for avidin/streptavidin is especially common in tissues and cells that contain high amount of biotin, such as placenta, mammary glands, kidney, adrenal cortex, brain, liver, fat, and mast cells [3, 6, 48]. EABA is much highlighted by heat-induced epitope retrieval (HIER) but also develops in tissues subjected to other types of antigen retrieval [49–51]. The level of endogenous biotin activity is especially higher in frozen compared to FFPE tissue sections, which leads to unwanted nonspecific reaction [52, 53]. EABA is typically found in cytoplasm, but it has been reported in the nucleus as well [51, 54–56]. Although paraffin embedding and formalin fixation have been found to significantly decrease the level of endogenous biotin, it is highly recommended to use a biotin blocking step when using avidin/streptavidin-biotin-based detection systems to decrease endogenous biotin activity. Since the commercially available EABA blocking reagents (pure avidin and biotin solutions) are very expensive, many researchers prefer to use homemade blocking reagent containing egg white and 5% powdered milk as sources of avidin and biotin, respectively [57–60].

5.2 Avidin-biotin complex (ABC) method

For signal amplification, another biotin-based IHC detection method was developed, namely avidin-biotin complex (ABC) method, in which a preformed avidin-biotin-peroxidase complex is used as the detection layer [20, 61]. This technique induces three different layers; an unconjugated primary antibody, a biotinylated secondary antibody, and finally a large complex of enzyme-labeled biotin and avidin, which is attached to the biotin molecules conjugated to the secondary antibodies (**Figure 5**). Two biotins from adjacent biotinylated enzyme molecules can be joined via an avidin molecule [62]. Four biotin binding site of avidin molecule could result in formation of lattice complexes in which avidins are attached together by biotinylated enzyme molecules creating a large complex, which is attached to the biotinylated secondary antibody [20, 63]. In normal circumstances, not all the four avidin's capacity for biotin are taken up by biotinylated enzyme. This allows the complexes to attach to biotin of primary or secondary antibodies [64].

Before the advent of biotin-avidin-based methods, the PAP method was considered the most sensitive detection technique. The ABC method was then found to be nearly 40 times more sensitive compared to PAP method [65, 66].

By applying biotinylated primary antibodies, the ABC protocol can be shortened to a two-step method [3]. It is reported that application of biotin-labeled primary antibody in the two-step ABC method creates an equal sensitivity to the unconjugated antibody in the three-step ABC method. This finding proposes that biotinylation does not impair antibody activity and that application of a secondary antibody to intensify the reaction would not be necessary, if a suitable biotin-labeled primary antibody is used [43].

Although formation of lattice complexes of avidin and biotinylated enzyme seems to increase the sensitivity of ABC method compared to LAB/LSAB, it was found that

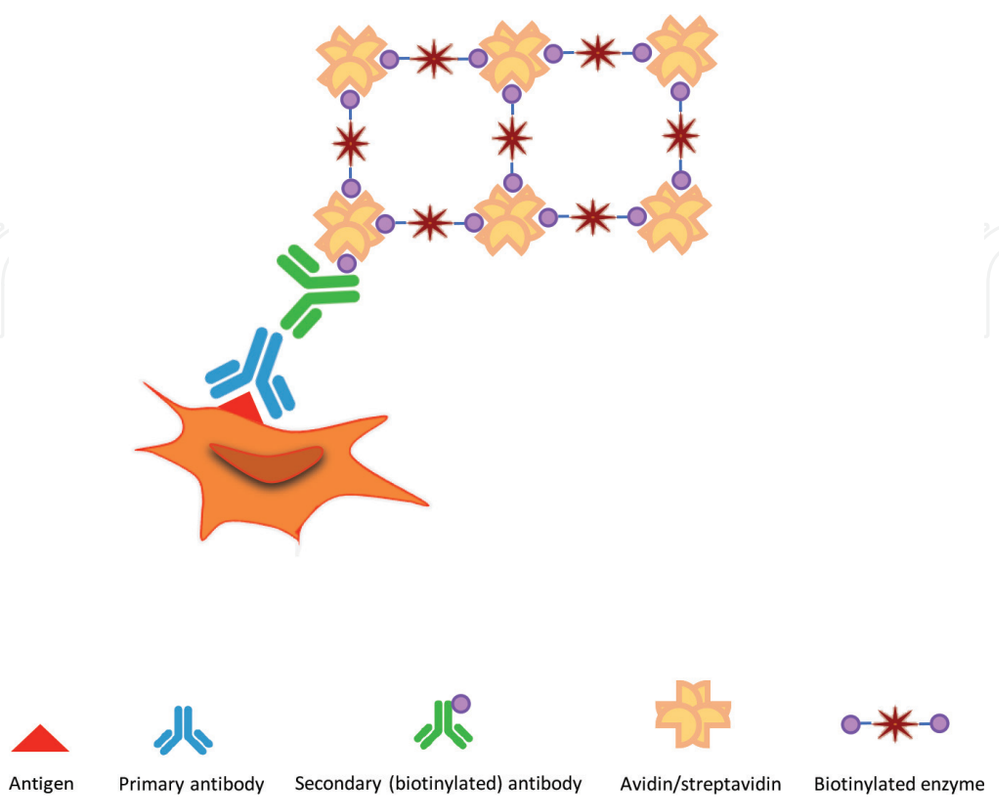


Figure 5.
Avidin/streptavidin immunostaining method.

the sensitivity of ABC method is 5–10 times less than the LSAB method [67]. This disadvantage of ABC method is due to the large size of lattice complexes that hinder their penetration into the cells. Indeed, as with LAB/LSAB methods, the background in ABC method cannot be removed due to the irreversibility of the avidin-biotin reaction [21, 68]. As with LAB/LSAB methods, tissue endogenous biotin is one of the concerns in ABC-based IHC staining methods that results in nonspecific staining.

6. Polymer-based immunohistochemistry

Desire for IHC detection systems with improved sensitivity led to the development of chain polymer-conjugate technology in the last decade of the former century [69, 70]. Improved sensitivity of this technology is based on using synthetic or natural polymers that increase the capacity for incorporating ligands or enzymes to be coupled to linker antibodies [71–78]. Using this technology, much higher antigen detectability could be obtained in comparison to standard ABC and LSAB methods or in enzyme-antienzyme immune complex techniques (PAP and APAAP) [69, 70]. The chain polymer-conjugate technology normally utilizes a backbone of an inert polymer molecule of dextran [71–73], polypeptides [74], dendrimers [75, 77], or DNA branches [78]. The backbone is able to carry both antibodies and multiple enzymes. Hence, nearly 11 antibodies and up to 40 HRP molecules could be anchor to one 500 KDa dextran molecule [79].

In 1993, a one-step direct polymer immunohistochemical staining method, namely enhanced polymer one-step staining (EPOS) system, was introduced by Bisgaard and Pluzed [80]. In this method, up to 10 monoclonal primary antibodies and 70 enzyme molecules are attached to a dextran backbone with a high molecular weight. This would enable the whole immunohistochemical staining process (from primary antibody to enzyme) to be completed in a single step (**Figure 6a**) [81]. The whole process can be performed in nearly 7 min for frozen sections and to less than 3 h for regularly processed, paraffin-embedded specimens. Hence, when a quick and reproducible IHC-based diagnostic approach is demanded in emergency circumstances, for example, during surgeries, this method should be taken into consideration [82]. However, applicability of this method is restricted to primary antibodies provided by the manufacturer and was not suitable for user supplied primary antibodies.

To overcome this limitation, a polymer-enhanced two-step IHC detection system (EnVision, EV) was introduced in 1995. EV system contains secondary anti-mouse and antirabbit Ig antibodies and could be applied to localize tissue-bound primary antibodies of mouse and rabbit origins (**Figure 6b**) [83–85]. The EnVision complex is composed of up to 20 secondary antibodies and nearly 100 molecules of peroxidase molecules, which all are directly attached to an activated dextran polymer backbone [86].

EnVision is a user-friendly technique and provides the users a rapid visualization in only 45 min. This method offers a very high sensitivity and does not lead to false-positive reaction due to the endogenous biotin [87]. Although the EV system is a very expensive method, it can be applied with higher dilutions of primary antibodies. Indeed, because endogenous biotin is not a problem anymore, EV permits more efficient heat-induced epitope retrieval (HIER) [69, 88]. The detection systems based on polymers could also be a choice for quick immunostaining of frozen sections when tumor margin and micrometastasis is to be identified. Furthermore, polymer-based detection systems are sensitive enough to be applied as an alternative detection system in western blotting [89] and in chromogenic *in situ* hybridization (CISH) [90].

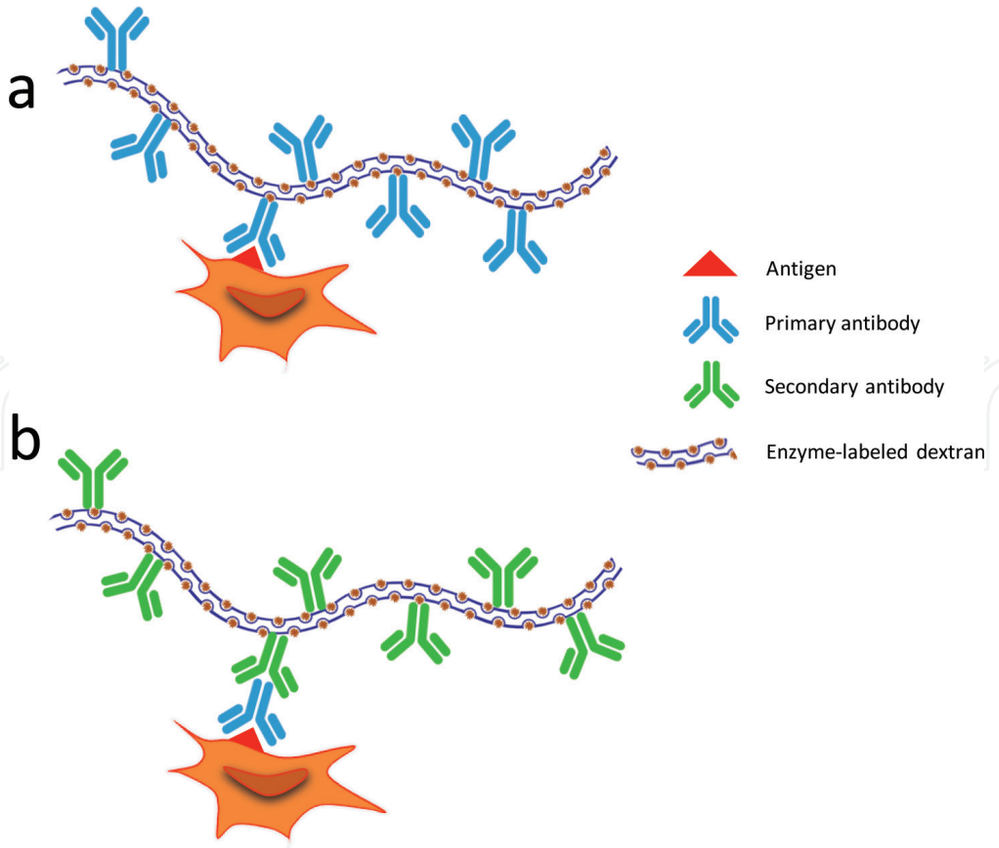


Figure 6.
Polymer-based immunostaining method: (a) EPOS and (b) EnVision.

Dextran carriers with a high molecular weight, however, appear to compromise the penetrative ability of the detection reagent due to spatial hindrance. Accordingly, the sensitivity of polymer-enhanced systems is profoundly affected by antigen localization. For instance, remarkably low sensitivity has been noticed in nuclear antigens [88, 91]. Indeed, in thick tissue sections, where the antigens are located beneath the surface area, only a part of antigens are amplified. This happens because of the large size of dextran-enzyme complex, which could not disperse into the deeper layers making quantitative results unreliable [87]. Subsequently, EnVision+ was developed, which was a modified version of EV system with higher sensitivity. EnVision+ contains a mixture of dextran polymers with two different secondary antibodies (goat antirabbit and goat antimouse IgG) anchored to it [86, 88, 92]. Nonetheless, EnVision systems were reported to give less sensitivity in case of some antibodies especially those that require proteolytic digestion, which was believed to stem from problems of tissue penetration of the labeled polymer.

Although the application of polymer gives a chance of increasing the number of enzymes coupled to the carrier backbone, it also profoundly increases the size of complex. Therefore, enzyme density per unit surface may not be increased to the degree that would be expected. Hence, it would be a desirable approach to design a compact polymer-enzyme-linker antibody conjugates with optimal number of enzyme molecules. Based on this goal, Shi et al. [91] suggested to use small linear molecules that have a capacity to polymerize with enzymes and linker antibodies in a tightly packed size. The IHC results with this newly designed detection system (Power Vision) showed that it possesses compact size and, compared to conjugates containing polymer linkers, shows higher detection efficiency for antigens located on the cell surface or in the nucleus [91]. Compared to EnVision+, this "second-generation" polymer-based conjugate was found to

be less expensive and fast and showed better reproducibility and capacity to be standardized [93, 94]. From clinical point of view, these methods are extremely useful when emergency results (for example, assessing the intraoperatively surgical margins of tumor specimens) are needed [95].

7. Tyramide-based signal amplification

7.1 Biotinylated tyramide signal amplification

In 1989, a novel signal amplification method for immunoassays was introduced by Bobrow et al. called catalyzed reporter deposition (CARD). The CARD was first used in western blots and immunodots [96–98] and was then adapted for IHC by Adams [99].

The signal amplification in this system is based on an analyte-dependent reporter enzyme (ADRE), which catalyzes the deposition of additional reporter molecules. The first step of this system relies on the same principle as LAB/LSAB detection system. Accordingly, primary antibody is first added to the tissue section followed by biotinylated secondary antibody and either HRP-labeled streptavidin (in tyramine signal amplification (TSA)) or streptavidin-biotin-HRP complex (in catalyzed signal amplification (CSA)). The amplification process happens when the peroxidase enzyme (ADRE) oxidizes the phenolic components to produce extremely unstable and reactive intermediate radicals, which are then bound to a tissue section [96, 100]. Tyramine, a biogen amine derived from aromatic amino acid tyrosine, is a substrate commonly used in this technique. It contains an amine at one end and a phenol at another end, which is used by peroxidase enzyme. The amine group is employed to conjugate the molecule with biotin or any other target molecules via an amide bound [101]. In the presence of HRP and H_2O_2, biotinylated tyramine is oxidized and resulting highly reactive radicals will react with electron-rich aromatic components, such as tyrosine-rich moieties of proteins in the vicinity of the HRP binding sites in tissues. This binding occurs very rapidly within 10 min. Due to a very short half-life of tyramide radicals, they are deposited at the same location where they are generated [102]. This reaction is then followed by incubation of the tissues with streptavidin-peroxidase complex. This complex is attached to the biotin sites of the tyramine, which are remained free. This reaction is restricted to the sites of primary antibody binding site where HRP had previously accumulated (**Figure 7**).

Because of the high sensitivity of this method, biotinylated tyramide amplification enabled many antigens to be traced, which had previously been unreactive in formalin-fixed paraffin-embedded tissues [101]. In comparison to the avidin-biotin-based methods, biotinylated tyramide signal amplification exhibits 5- to 10-fold more sensitivity. Some researchers believed in even more sensitivity [103]. It was reported by Sanno et al. that staining of pituitary hormones with CSA showed nearly 100-fold higher sensitivity compared to standard ABC method [104]. It is recommended to use this method when (1) antigen expression in target tissue is extremely low or the amount of antibody available is limited and (2) primary antibodies possess low affinity or are not compatible with paraffin-embedded tissue sections [104, 105]. Repeating the biotinyl-tyramide reaction can further increase the signal intensity. However, this circuit is restricted to only two or three rounds before the background noise becomes an issue [106]. CSA and/or TSA methods are found to be cheaper than EnVision system but with the same effectiveness [86].

These methods, however, are laborious because they involve an initial avidin-biotin procedure followed by the tyramine reaction. Background can also be considered a serious problem, particularly with HIER. In this case, more prolonged

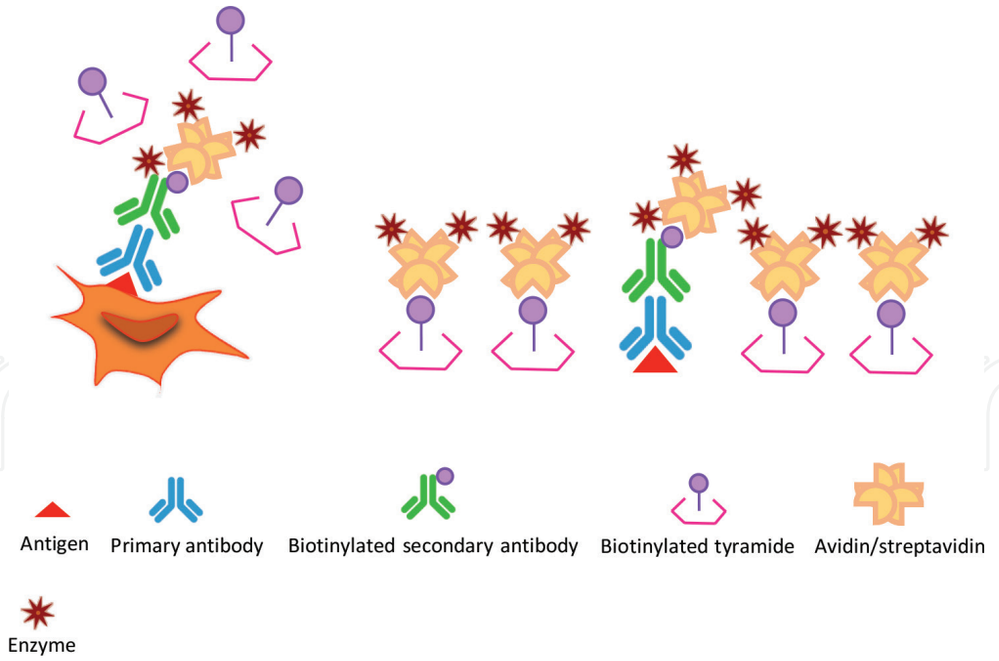


Figure 7.
Tyramide-based immunostaining method.

treatment of tissues to quench endogenous peroxidase or endogenous avidin-biotin activities (EABA) is usually necessary [105, 107–109]. Although TSA/CSA detection methods have resulted in satisfactory results in terms of significantly increased sensitivity in IHC and *in situ* hybridization (ISH), they are not widely employed in diagnostic pathology. The reasons include: additional steps that make the method more time-consuming, nonspecific background staining, and that optimal AR treatment with existing methods may achieve equivalent results and that second-generation polymer-based methods are simpler and equally sensitive [14, 110, 111].

7.2 Biotin-free TSA/CSA

In an attempt to reduce the problems associated with endogenous biotin in conventional tyramide signal amplification, a biotin-free system, fluorescyl-tyramide amplification system (FT-CSA or CSAII), was introduced. Rather than biotinyl-tyramide, this system uses fluorescyl-tyramide and does not contain avidin/biotin reagents avoiding the problem associated with endogenous biotin. In this method, addition of primary antibody is followed by a peroxidase-labeled secondary antibody. Peroxidase enzyme is responsible to catalyze the transformation and deposition of fluorescyl-tyramide in the tissue section. When the reaction terminates, it could be inspected by fluorescence microscopy. The produced signals could even be converted to a colorimetric reaction by using peroxidase-conjugated antifluorescein antibody and a diaminobenzidine-hydrogen peroxide substrate.

This method is highly sensitive enabling researchers to detect and localize antigens with low expression level and to use primary antibodies with very low affinities [105, 106]. Alternative reporter includes dinitrophenol, which also results in marked reduction of background from endogenous biotin. Absence of nonspecific staining is due to no endogenous tissue distribution of dinitrophenol [14].

In the latest improvement of the biotin-free CSA method, fluorescein is conserved in the substrate, while the tyramine is substituted with ferulic acid, which is a much better peroxidase substrate and increases signal-to-noise ratio. In this system, the incubation time in each step can be significantly reduced, making it possible to stain a tissue in less than 1 h [112].

8. Rolling circle amplification

Rolling circle amplification (RCA) reaction was first developed for the purpose of nucleic acid detection [106], but it was then adapted for amplification of signals from antibodies bound to antigens [113–118]. RCA is an enzymatic process in which a short DNA or RNA primer is amplified using a circular DNA template and special DNA or RNA polymerases to form a long single-stranded DNA or RNA [119, 120]. The end product of RCA is a long continuous sequence of DNA containing several tandem repeats complementary to the circular template. Unlike PCR, RCA could be performed at a constant temperature (room temperature to 37°C). A RCA reaction contains five different components: (i) a short DNA or RNA primer, (ii) a polymerase enzyme (e.g., Phi29 DNA polymerase for DNA, and T7 RNA polymerase for RNA), (iii) a suitable buffer compatible with polymerase enzyme, (iv) a circular DNA template, and (v) deoxy nucleotide triphosphates (dNTPs) [121].

RCA reaction has three different steps: (1) the circular DNA template with typically ~15–200 nt in length is synthesized through the intramolecular ligation of phosphate and hydroxyl end groups of a linear probe with the use of the target DNA or RNA as a ligation template [121–123], (2) the polymerase enzyme continuously adds dNTPs to a circular template-annealed primer to form a long ssDNA with tens to hundreds of tandem repeats, and (3) the RCA end products could be detected and even monitored by different signal readout methods (**Figure 8**) [121]. Different methods are available to visualize and also analyze the RCA process including (a) labeling the RCA products directly during the amplification process by using

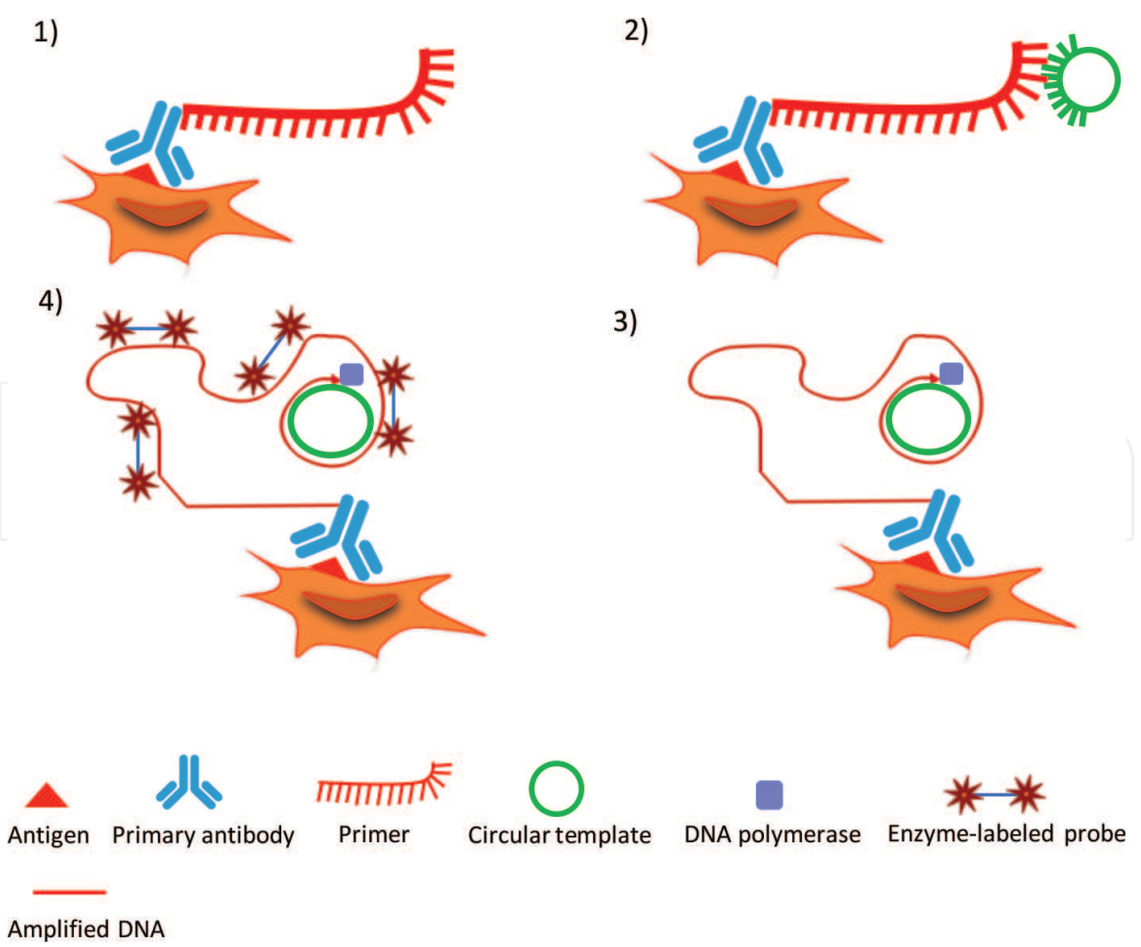


Figure 8.
Rolling circle amplification immunostaining method. (1) Immunoconjugate bound to target antigen. (2) RCA primer hybridized to circle template (3) Synthesis of new DNA strand by DNA polymerase (4) Detection of amplified DNA by enzyme-labeled probe at the site of bound antibody.

fluorescent dyes-conjugated dNTPs; (b) detecting the RCA product with hybridization of fluorophore-tethered complementary strands; quantum dots or gold nanoparticles can be attached to RCA products via a complimentary strand to visualize RCA product; (c) using molecular beacon for fluorescent detection of RCA products; (d) using DNA binding dyes such as SYBR green; (e) using biotinylated decorator and streptavidin-HRP conjugate or by DNA-peptide nucleic acid (PNA) intercalating dye for colorimetric detection of RCA product; and (f) using luciferase to generate light for bioluminescence detection of RCA products [121].

One of the important advantages of RCA is that circular templates can be customized so that the signals of a single binding event are amplified in an exponential manner (e.g., multiprimed RCA) [124–126]. In this approach, signal amplification more than 10^9-fold is feasible, while a linear mode of RCA has a capacity to amplify signals to nearly 10^5-fold [127]. RCA reactions could be accomplished on a solid surface and also in a solution environment. In solid-phase RCA, reaction is conducted on a solid surface such as glass, microwell plates, microbead or nanobead particles, paper strips, or microfluidic devices. This system gives researchers an advantage of high-throughput analysis and potential for easy detection of target from complex sample matrices [121].

RCA is appeared to be a powerful method in immunoassays. The combination of RCA method with ELISA is found to grant more sensitivity and decrease the lower detection limit. In this regard, there is an approach called immuno-RCA in which, a RCA primer-conjugated antibody is applied on a target antigen that has been coated on a solid surface followed by a RCA reaction. The first immuno-RCA test was introduced by Schweitzer B et al. (2000) on a glass slide for IgE quantification [128]. From that time, solid-phase RCA has become popular as signal amplification method in antibody microarray analysis of multiplexed proteins [129–132]. A sandwich immuno-RCA has been adapted to detect the target with high sensitivity. In this technique, the target antigen in biological media is first captured on a solid surface using coated antibody. In the next step, a RCA primer-conjugated secondary antibody is applied to conduct the RCA reaction [133–135].

Konry et al. [136], by combining the capacity of RCA reaction to detect a single-molecule and microfluidic technology, demonstrated the feasibility of identification of specific protein markers on tumor cell surfaces in miniaturized nanoliter reaction droplets. This approach of signal amplification in a microfluidic format could improve the applicability of existing methods by reducing consumption of sample and reagent and increasing the specificity and sensitivity for various applications such as early diagnosis of cancer [136]. Specific immunocytochemical and immunohistochemial identification of a wide range of intracellular molecules (prostate-specific antigen and vimentin) and cell surface antigens (epithelial membrane antigen, CD3 and CD20) in a variety of tissues (tonsil and breast) and cell lines (U266, Jurkat) has successfully been accomplished using RCA-mediated signal amplification. Indeed, immuno-RCA was reported to give more uniform staining pattern compared to the conventional methods [129]. *In situ* proximity ligation assays (*in situ* PLA) are an important adaptation of the RCA method in which primary antibodies against two distinct antigens are applied. Having two antigens in close proximity to each other, RCA reaction will occur and the proximity of two distinct antigens can be visualized [137].

It has been shown that attachment of a RCA primer to primary or secondary antibody does not impair affinity or avidity of the conjugate. Nonetheless, RCA reaction adds 60–90 min to the conventional IHC protocol. Although RCA is able to generate amplification of DNA up to 10^9-fold, immuno-RCA in LSAB-based IHC applications is able to increase the signal to only about fourfold [129]. Simultaneous evaluation of TSA and RCA detection techniques by Warford et al. revealed that both methods are capable to produce results with a high signal-to-noise ratio. However, they found TSA detection system to be more sensitive than the RCA method [14].

9. Choice of detection system and concluding remarks

The sensitivity of an IHC staining is a function of detection method for signal amplification [138].

The choice of a detection system is mainly determined by laboratories based on the nature of the specimen, expression level of the antigen, cost, desired sensitivity, and possible automation [53]. Choosing an appropriate detection system enables maximum sensitivity and optimum visibility of the immune reaction with the fewest steps and in the shortest time [139]. As a general rule, the more complex an IHC method, the more sensitive it is. One- or two-step IHC procedures are usually less sensitive than more complex, multistep procedures. In addition, the detection system must be accurate, reproducible, and results in a high signal-to-noise ratio [140]. When choosing a desirable detection system, several factors are needed to be taken into consideration: (1) the expertise/experience of the technician; (2) type of the antigen to be identified; for example, some antigens are widely expressed and do not need a sensitive method to be visualized; (3) number of tests and the amount of antibody that is available; (4) the affinity of the antibody: each antibody has its own affinity that requires a specific detection system, antibodies with less affinity usually need more sensitive detection systems; (5) species idiosyncrasies (does the tissue contain endogenous biotin), (6) budget; (7) localization of the antigen of interest (some detection systems do not have high cell penetration capacity due to the large size and regardless of having high sensitivity for detection of surface antigens, do not yield a high sensitivity for intracellular or nuclear antigens), (8) the need for or type of antigen retrieval; typically, a non-biotin-labeled detection system is recommended if HIER is used to avoid background from endogenous avidin-biotin activity (EABA) [141].

A detection system should be compatible with animal species as well. A detection system with an outstanding performance in human is not always suitable for animal models [6, 142]. The sensitivity of commercially available detection kits, some optimized for particular animal species, should be validated in-house before use. The secondary and tertiary reagents of some kits may contain antibodies or other compounds that potentially nonspecifically react with tissue antigens, leading to a background or staining. This is one justification for negative controls in IHC [141].

As a further general rule, one should always try to use the simplest detection method with sensitivity enough for detection of the antigen. The multilayering of detection antibodies beyond this threshold can be problematic as with the addition of every new step, the risk of nonspecific interaction with the preparation increases. There are some exceptions to this rule. For example, in tumor-specific antigens, which are not expressed in normal condition, the use of more sensitive methods might decrease detection level cutoff and increase the likelihood for early detection of cancer [143, 144].

In emergency conditions when results are needed in a short amount of time (such as evaluating intraoperatively surgical margins of tumor specimens), applying a detection method with high sensitivity will definitely improve accuracy of the procedure and help surgeon to obtain wider surgical margins if needed.

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Conflict of interest

The authors declare no conflict of interest.

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