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Antigen retrieval, blocking, detection and visualisation systems in immunohistochemistry: A review and practical evaluation of tyramide and rolling circle amplification systems

Anthony Warford *, Hameed Akbar, Deise Riberio

University of Westminster, 115 New Cavendish Street, London W1W 6UW, United Kingdom

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ABSTRACT

To achieve specificity and sensitivity using immunohistochemistry it is necessary to combine the application of validated primary antibodies with optimised pre-treatment, detection and visualisation steps. The influence of these surrounding procedures is reviewed. A practical evaluation of tyramide signal amplification and rolling circle amplification detection methods is provided in which formalin fixed paraffin embedded sections of adenocarcinomas of breast, colon and lung together with squamous metaplasia of lung were immunostained with CD20 and CK19 primary antibodies. The results indicate that the detection systems are of comparable sensitivity and specificity.

1. Introduction

If the primary antibody in an immunohistochemical (IHC) system confers specificity, see Howat et al., this issue [1], then sensitivity is mainly driven by the pre-treatment, detection and visualisation systems that wrap around this central procedural step. In the majority of diagnostic laboratories, and in many research institutions, IHC is automated and these 'wrap around' steps are optimised through the use of reagent kits under controlled conditions. However, when these fail to provide expected staining, or in situations where manual IHC is undertaken it is necessary to understand the influence of these components and to be able to adjust conditions to ensure that optimal staining is achieved.

The purpose of this paper is twofold:

To review the underlying principles of how pre-treatment, detection and visualisation systems influence IHC at light microscope level.

Provide a practical exploration of tryramide amplification [2] and rolling circle amplification [3] detection methods that have the claimed potential of demonstrating low copy number target antigens. To the authors knowledge the comparative sensitivity of these systems have not been directly compared and manual methods are used as the rolling circle procedure is not available as an automated method.

2. Review

2.1. Revealing the antigen

When unfixed or frozen preparations have been fixed using non-additive fixatives such as acetone, methanol or ethanol then a target antigen will normally be fully available for interaction with a primary antibody. As discussed elsewhere in this issue [4] employing such preparation methods can lead to compromised cytology or morphology. For this reason additive fixation, principally formalin based, is commonly used. Indeed formalin fixation is almost universally employed for surgical tissues that are subsequently processed into paraffin wax for diagnostic purposes. The consequence of even brief formalin fixation is partial or complete masking of the majority of antigens via the introduction of hydroxymethyl adducts and the subsequent formation of methylene bridges linking proteins to proteins and proteins to nucleic acids.

The difficulties of combining formalin fixation with paraffin embedding for IHC where reported over 50 years ago by Saint-Marie [5]. However, the proposed solution to the problem, replacing formalin with alcohol fixation and adopting a special paraffin

* Corresponding author. Address: Cellular Pathology, University of Westminster, 115 New Cavendish Street, London W1W 6UW, United Kingdom. Fax: +44 (0)20 7911 5087.
E-mail address: a.warford@westminster.ac.uk (A. Warford).

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wax processing schedule, effectively put a conceptual road block in the way of the development of formalin fixed paraffin embedded (FFPE) IHC. While Nakane [6] published the successful demonstration of pituitary hormones in FFPE tissues in 1968, it was the seminal paper of Taylor and Burns [7] published in 1974 that rekindled the possibility of applying IHC to routine surgical tissue preparations. Taylor and Burns [7] used unbuffered formalin fixed paraffin embedded sections to demonstrate kappa and lambda light chain immunoglobulins in normal and malignant lymphoid samples. They combined this with the application of chromogenic substrate systems that allowed for simultaneous examination of antigen demonstration with (diagnostic) morphology. The diagnostic possibilities of IHC were quickly realised and a period of intense activity ensued. This proved to be frustrating as antigen masking by formalin, and exacerbated by the use of buffered formulations, was not appreciated.

The application of protease digestion, now referred to as protease induced epitope retrieval (PIER), as a means of reversing the effects of formalin fixed antigen masking was first described by Huang [8,9] who used trypsin to improve the signal/noise ratio of IHC. Subsequent research [10–12] demonstrated that a variety of proteases could be employed, but that careful control was required to avoid either under digestion of preparations and sub-optimal demonstration of antigens, or over-digestion resulting in damage of tissues and, sometimes, destruction of antigens. Thus, undertaking IHC using routine surgical FFPE samples became a reliable methodology providing PIER was carefully controlled. This still holds true and even more care and attention needs to be taken when PIER is applied to relatively delicate formalin fixed cell or frozen section preparations.

The first use of heat induced epitope retrieval (HIER) was described by Shi et al. [13,14]. Whilst potential mechanisms by which this 'super heating' method unmasks antigens in FFPE preparations have been suggested [15,16] the underlying principles are not fully understood. Initially microwave methods using buffer solutions that included metal ions were described [13]. When using microwaves it is important to consider power output and to apply this consistently. The use of pressure cookers [17] to achieve HIER provides an alternative and their use will usually allow a greater number of slides to be treated at any one time. With respect to buffers there has been simplification with two solutions being sufficient, though not necessarily interchangeable, for HIER. These buffers are 10 mmol/L citrate pH 6 and 10 mmol/L TRIS, 1 mmol/L EDTA at pH 9. In comparison with PIER, HIER has expanded the envelope of antigens that can be demonstrated in FFPE preparations. It often allows the demonstration of antigen in preparations that have been fixed for weeks or months in formalin where, typically, PIER is only successful when fixation has been limited to days before paraffin processing. Furthermore HIER is relatively gentle on FFPE sections and destruction of tissue is not normally observed. An important caveat is that for formalin fixed cell and frozen section preparations HIER is too harsh and PIER is recommended. Lastly, for a few antigens optimal demonstration may only be obtained when HIER and PIER are combined [18].

2.2. Blocking non-immunological reactions

A variety of blocking methods are available for use in IHC methods. They fall into two main areas of application; the suppression of non-specific interaction of antibodies with other proteins and the blocking of endogenous components that otherwise would be demonstrated by the detection/visualisation system. Examples of blocking methods and technical notes are provided in Table 1.

2.3. Detection systems

IHC requires an end point that will allow the visualisation of the primary antibody interaction with an antigen by microscopy. Accordingly, a label needs to be introduced to localise the otherwise colourless antigen/antibody interaction – see Section 2.4. Whilst choice of label is very important an equally important decision has to be made as to the complexity of detection system used to introduce the label. When antigen is abundant simple detection methods can be used, but when it is scarce then more complex procedures are usually required.

IHC began with the application of primary antibodies that were labelled with fluorescent molecules [19]. This simplest of detection methods was largely superseded by the introduction of indirect immunofluorescent methods [20]. These offered the advantage of being more sensitive as the ratio of reaction of the secondary labelled antibody with the primary antibody exceeds 1:1. Furthermore, when applied to detect a panel of primary antibodies, indirect detection confers economic advantages as only the secondary detection antibody is labelled. The multi-layering of detection antibodies beyond their use in the indirect method can be problematic as with the addition of every new species of antibody the risk of non-specific interaction with the preparation increases. Exceptions to this are the peroxidase anti-peroxidase – PAP [21] and alkaline anti-alkaline – APAAP [22] methods that offer triple level detection sensitivity with direct level specificity. These methods have proved suitable for cell and frozen section IHC. However, they can lack sensitivity required for use with antibodies applied to FFPE preparations, particularly when used in combination with monoclonal antibodies.

A step change in detection sensitivity was achieved through the introduction of avidin biotin complex [23] and labelled streptavidin biotin [24] systems. Their use required the blocking of endogenous avidin/biotin activity [25]. The introduction of labelled polymer systems, originally as the direct detection methods [26], was quickly followed by their application in indirect systems [27]. The labelled polymer methods use a dextran based polymer backbone onto which antibodies and labels are attached. The system is as sensitive as avidin/biotin methods, but obviates the need for special blocking. Additionally, more than one antibody type can be attached to the polymer, thus allowing for one detection system to be used with primary antibodies of different species. Polymer based detection methods form the basis of many commercial ready to use detection methods that are optimised for use with automated IHC equipment.

At the beginning the 90's and a decade later two potentially exquisitely sensitive detection systems were first reported. The tyramide signal amplification (TSA) method [2] is based on the catalytic local deposition of a reporter (label) via the action of tryramide with horse radish peroxidase. Originally introduced with biotin as the reporter, and requiring considerable skill to produce amplification without high non-specific cell/tissue interactions, the system is now offered in kit form using alternative reporters such as dinitrophenol or fluorescent dyes. The procedure has also been automated. The rolling circle amplification (RCA) method [3] relies on the interaction of two oligonucleotide labelled antibodies. Once the oligonucleotides are ligated a circularised amplification product is generated using a polymerase. The end result, via the inclusion and demonstration of appropriate labels, is to provide a significant amplification of the initial antibody/antigen reaction that is claimed to provide for single molecule detection [28]. An important adaptation of the RCA method is the proximity ligation assay [29] in which primary antibodies against two distinct antigens are applied. Providing the antigens are close to each other ligation and formation of a circle for amplification will occur. Accordingly the proximity of two distinct antigens can