CARD *In Situ* Hybridization: Sights and Signals

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Abstract

During the last decade, several strategies have been developed to improve the detection sensitivity of *in situ* hybridization (ISH) by amplification of either target nucleic acid sequences prior to ISH (e.g., *in situ* PCR), or the detection signals after the hybridization procedures (signal amplification). Here we outline the principles of tyramide signal amplification using the catalyzed reporter deposition (CARD) technique, summarize applications as well as possible limitations of CARD ISH, and discuss some future directions of *in situ* nucleic acid detection using this amplification strategy.

Key Words: *In situ* hybridization; tyromide; signal amplification; mRNA; DNA; catalyzed reporter deposition; interphase cytogenetics.

Introduction

In situ hybridization (ISH) is now an established molecular tool in research and diagnostics and has significantly advanced the study of gene structure and expression at the level of individual cells. Currently, the technique provides an optimal detection sensitivity of approx 1 kb of target DNA in cell preparations using fluorescence approaches in combination with charge-coupled device (CCD) recordings and image analysis [1]. The ultimate mRNA detection limit, however, is more difficult to determine but may reach the level of single mRNA molecules in the most optimal test systems [2]. For detection of nucleic acid sequences in routinely processed tissue sections of paraffin-embedded specimens, these sensitivities may not be reached. As a consequence, ISH detection limits on tissue sections are rather in the range of 40 kb of target DNA and 10–20 copies of mRNA or viral DNA per cell [3–6].

In recent years, several strategies have been developed to improve the sensitivity of ISH. These include the use of increased absolute amounts of hybridized probes (cocktails of oligonucleotides or multiple cRNA probes) [7] and the amplification of either nucleic acid targets (target amplification) [8,9] or of (immuno) cytochemical detection signals (signal amplification) *in situ* [10,11].

In general, target amplification methods combine polymerase chain reaction (PCR) and ISH to visualize specific amplified DNA and RNA sequences within cell and tissue preparations. Theoretically these *in situ* PCR techniques are straightforward, but in practice they are hampered by several obstacles, such as low amplification efficiency (restricted sensitivity), poor reproducibility (restricted specificity), and difficulties in quantification of the results [12–15]. As a consequence, other approaches to increase the sensitivity of ISH have been explored, of which

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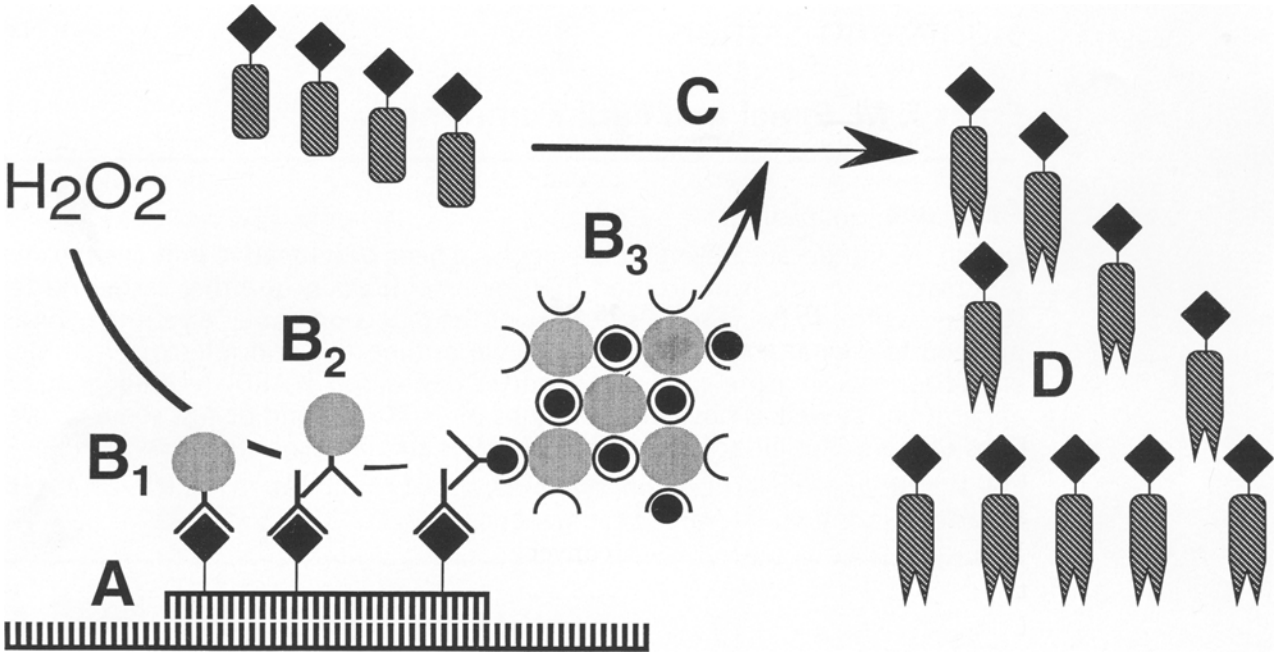

Fig. 1. Principles of CARD signal amplification for ISH. (A) Hybridization *in situ* with a hapten-labeled probe. (B) Application of a one-step (anti-hapten antibody conjugated to horseradish peroxidase; B_1), two-step (anti-hapten antibody and a horseradish peroxidase labeled secondary antibody; B_2), or three-step (avidin-biotin horseradish peroxidase complex; B_3) probe detection system. (C) Production of haptenized tyramide radicals by horseradish peroxidase catalyzed CARD signal amplification. (D) Deposition of tyramide radicals to tyrosine moieties of proteins *in situ* in the vicinity of hybridization. Direct visualization of fluorochrome-labeled tyramides and indirect visualization of hapten-labeled tyramides with anti-hapten antibody or (strept)avidin conjugates labeled with fluorochromes or enzymes.

the CARD signal amplification method appears to be the most promising.

Principles of CARD Signal Amplification

The method of CARD signal amplification has been developed by Bobrow et al. [16] for use in immuno-blotting and ELISA assays and is based on the deposition of a large number of haptenized tyramide molecules promoted by peroxidase activity (Fig. 1). Tyramine is a phenolic compound and horseradish peroxidase (HRP) can catalyze the dimerization of such compounds when they are present at high concentrations probably by the generation of free radical intermediates. If applied in lower concentrations, such as used in the signal amplification reaction, the probability of tyramine dimerization is reduced, whereas the binding of the highly reactive intermediates to electron-rich moieties of proteins, such as tyrosine, at or near the site of the peroxidase binding site is favored. In this way, many hapten-labeled tyramine molecules (tyramides) can be deposited at the hybridization site *in situ*. Visualization of deposited tyramides can be performed either directly after the CARD reaction with fluorescence microscopy, if fluorochrome-labeled tyramides are used, or indirectly with either fluorescence or brightfield microscopy, if biotin, digoxigenin, di- or trinitrophenyl are used as haptens, which can act as further binding sites for anti-hapten antibod-

ies or (strept)avidin conjugates (in the case of biotinylated tyramides) [3]. Also fluorescein and rhodamin can be used as haptens, since specific antibodies against these fluorochromes are commercially available from several companies.

Applications and Limitations of CARD Signal Amplification

CARD signal amplification with biotinylated tyramides has been adapted for immunohistochemistry by Adams [17], allowing an increase in sensitivity of up to 1000-fold when compared with conventional avidin biotinylated enzyme complex (ABC) procedures [17–21]. In these studies, the amplification factor was assessed by determining the maximal dilution of the primary antibody leading still to identical staining results as compared with standard reactions. In most cases, however, the increase in sensitivity seems to be rather in the range of 50–100-fold, and sometimes even less. As a consequence of this variability the optimal dilution for every primary antibody needs to be determined. CARD signal amplification has also been applied to visualize antigens or incorporated BrdU in fluorescence microscopy [22,23] or electron microscopy [24,25], and has further been used for double staining with two unconjugated primary antisera raised in the same species [26].

Since 1995, CARD has further been implemented in detection procedures of both DNA and RNA ISH on cell preparations and tissue sections. With signal amplification, the ISH sensitivity could be improved in the range of 2- to 100-fold, enabling the detection of up to three different repetitive and single-copy (1–5 kb) DNA sequences in the same cell [22,27–30] as well as low copy viral RNA [31,32] and mRNA ranging from high to low abundancy in cell and tissue preparations [10,33–36].

As an example of the diagnostic potential of CARD ISH, we have implemented CARD signal amplification for our diagnostic nonradioactive oligonucleotide ISH procedure in order to increase the sensitivity of the assay and to shorten its overall turnaround-time [10,33]. This approach allows, e.g., the detection of peptide hormone mRNA in tissue sections from routinely fixed, paraffin-embedded surgical samples within one working day and makes the assay suitable for routine diagnostic purposes. Furthermore, it allows the use of diaminobenzidine (DAB) as a chromogen and, as a consequence, the application of conventional counterstains and the mounting of slides in xylene-based mounting solutions, making the procedure more acceptable to perform in a diagnostic setting.

To date, most of the protocols still use biotinylated tyramides for the amplification step, which can easily be obtained commercially (e.g., NEN Life Science Products, Boston, MA and Dako, Glostrup, Denmark) or synthesized in the laboratory [16,17,27,28,30,37]. However, similar to immunohistochemical procedures, the use of biotin is associated with significant disadvantages, especially when working with tissue sections. Thus, in tissues with high amounts of endogenous biotin, such as liver or kidney, a low signal-to-noise ratio due to high background staining may be encountered. It is therefore desirable to be able to rely on differently labeled tyramides, e.g., with digoxigenin, di- or trinitriphenyl [10,30,33], or fluorochromes [10,22,30,33,34], which is now possible. These tyramide conjugates can also be used in multiple-target ISH approaches [22,29,30] or the combination of immunohistochemistry and ISH with signal amplification.

Although the increase in ISH sensitivity by using CARD signal amplification is obvious from the literature, speculation about the obtained amplification factor is difficult. Moreover, since the tyramide deposition reaction runs very quickly, minor differences in amplification reaction time may lead to variations in the final signal intensities. Nevertheless, an amplification factor in the range of 5- to 10-fold, or possibly higher, together with preservation of distinct localization of ISH signals seems to be a realistic indication for both DNA and mRNA ISH.

Since with CARD signal amplification both specific and nonspecific (background) ISH signals will be amplified, it is essential that nonspecific probe binding and detection have to be avoided in order to successfully apply this procedure [22,30,35,36]. Therefore, we recommend that one should always optimize probe hybridization and cytochemical probe detection when applying CARD signal amplification in order to achieve discretely localized ISH signals of high intensity. In our hands, the number of cytochemical detection layers (e.g., one layer is sufficient for repetitive, centromeric DNA detection but minimal two layers are recommended for DNA targets of 40 kb), the dilution of detection conjugates (usually the first detection layer can be diluted 2–10-fold further than in conventional detection systems), the tyramide concentration in the CARD amplification buffer (usually concentrations in the range of 2–12 μM are used [30]), and the reaction time (usually 5–15 min at room temperature or 37°C) are the most important parameters to consider.

Conclusions

CARD signal amplification using labeled tyramides is an easy-to-perform, fast, highly sensitive, and efficient procedure to increase the detection sensitivity of ISH and immunohistochemistry and appears to become the method of choice for diagnostic laboratories. It will not only promote the detection of viral or mRNA but also facilitate the evaluation of chromosomal aberrations in cytological and histological specimens. Furthermore, it might also help to advance the development of automated ISH spot-counting by computer-assisted image generation and analysis. The now available spectrum of probe labels, detection systems, and tyramide conjugates for CARD signal amplification will further improve the applicability and sensitivity of ISH as well as promote multiple-target nucleic acid detection in situ and procedures combining ISH and immunophenotyping [38].

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