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Review

Recent Advances in High-sensitivity *In Situ* Hybridization and Costs and Benefits to Consider When Employing These Methods

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In situ hybridization (ISH), which visualizes nucleic acids in tissues and cells, is a powerful tool in histology and pathology. Over 50 years since its invention, multiple attempts have been made to increase the sensitivity and simplicity of these methods. Therefore, several highly sensitive *in situ* hybridization methods have been developed that offer researchers a wide range of options. When selecting these *in situ* hybridization variants, their signal-amplification principles and characteristics must be understood. In addition, from a practical point of view, a method with good monetary and time-cost performance must be chosen. This review introduces recent high-sensitivity *in situ* hybridization variants and presents their principles, characteristics, and costs.

Key words: *in situ* hybridization, RNAscope, SABER FISH, hybridization chain reaction, ClampFish

I. Introduction

In situ hybridization, which detects and visualizes nucleic acids, is a fundamental histological and immunostaining method. Although the reliability of immunostaining depends largely on the antibodies used [8], *in situ* hybridization has the advantage that its sensitivity and reliability can be predicted from the target nucleic acid sequence on which the probes are designed [26, 32]. However, the experimental procedures for *in situ* hybridization are more complex than those for immunostaining, making its practical application difficult. In addition, conventional *in situ* hybridization may not be sufficiently sensitive to detect low-expression genes or short transcripts. Therefore, *in situ* hybridization has been continuously improved to simplify the procedure and increase sensitivity, and several variants have been developed [10, 12, 22, 37]. Using these variants, it is becoming easier for researchers to detect low-expression transcripts that are difficult to visualize with conventional sensitivity. This review provides an overview of the principles and characteristics of high-sensitivity *in situ* hybridization methods currently in use and describes the costs and advantages that should be considered in their application.

II. Comparison of *In Situ* Hybridization and Immunostaining

Immunostaining methods, which mainly target proteins, can determine the subcellular localization of target molecules. However, their limitation is that they depend on the titer and specificity of the antibodies used [8]. Because antibody-based detection is indispensable for pathological diagnosis, antibodies with guaranteed titers and specificity are readily available for human proteins, especially antibodies against pathological markers [33, 35]. However, good antibodies against proteins other than pathological markers and/or non-human proteins are not always avail-

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able. In addition, because the antigen-antibody reaction depends on the conformation of the recognition site, similar conformations can cause false-positive reactions [8, 14].

For *in situ* hybridization, probes can be designed and synthesized based on nucleic acid sequence databases, allowing the targeting of any gene regardless of the animal species, provided that the nucleic acid sequence is known. In addition, the titer and specificity can be predicted from the length of the sequence that can be probed on the target mRNA and from its homology with other genes, respectively [7, 32]. These properties allow *in situ* hybridization to be used not only for transcript localization analysis, but also for verification of specificity of newly developed antibodies in combination with immunostaining [19, 30]. However, compared to immunostaining, *in situ* hybridization is a time-consuming procedure with many steps, which is one of the reasons why its clinical application is limited to a few purposes, such as diagnosing chromosomal aberrations [6, 15].

III. Characteristics of Conventional *In Situ* Hybridization and Signal Enhancement Methods

In situ hybridization employed radiolabeled probes in the early years [9, 16]; however, over the past two decades, digoxigenin (DIG)-labeled RNA probes have been frequently used as "conventional" *in situ* hybridization methods with high detection sensitivity [7, 24, 25]. In conventional *in situ* hybridization, sensitivity can be increased to some extent by increasing the coverage of the target mRNA sequence (Fig. 1). Probes that are too long result in reduced cell penetration [4, 29]; therefore, they are often designed to be 200–1000 base pairs in length for RNA probes, and multiple probes are designed in different regions of the target transcript. Signal amplification using enzymatic reactions such as Tyramide Signal Amplification (TSA) can be combined [2]. Well-designed probes and optimized temperature conditions for hybridization and time for color development allowed for analyzing low-expression genes [17, 18] (Fig. 1C). However, conventional *in situ* hybridization has some disadvantages, one of which is the difficulty in combining it with immunostaining. This is due to decreased antigen reactivity for some proteins, which can be caused via proteinase treatment to increase probe permeability, or by hybridization at temperatures that cause protein denaturation [21]. Therefore, sufficient immunostaining signals may not be obtained unless they target abundant proteins or utilize antibodies at high titers. Double *in situ* hybridization for two gene transcripts is also difficult for some gene combinations. For highly homologous gene pairs, difficulties are faced when designing

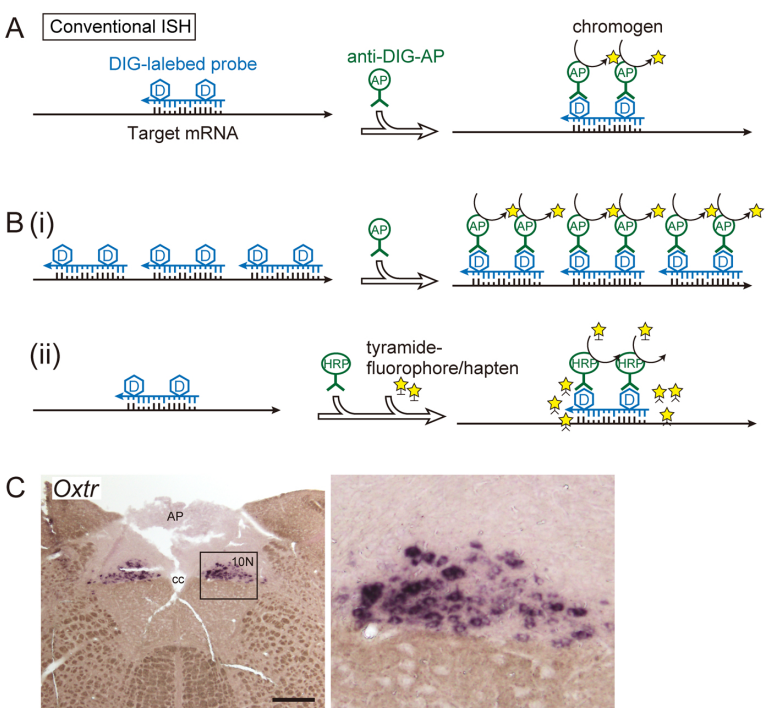

Fig. 1. Schematic of conventional *in situ* hybridization. **A**: DIG-labeled *in situ* hybridization. RNA probes complementary to target sequences were used. A chromogenic reaction using an alkaline phosphatase-labeled antibody is shown as an example of a signal detection method. **B**: Examples of signal amplification methods for conventional *in situ* hybridization. (i) Using multiple probes to increase coverage of the target mRNA sequence. The signal strength is proportional to the total probe length. (ii) Combination with TSA amplification. Tyramide activated by peroxidase is anchored to tyrosine residues of the surrounding protein. **C**: Example of visualization of low-expression genes in the rat brain. *Oxtr*, which encodes the oxytocin receptor, is visualized in the vagus nerve nucleus (10N) of the medulla oblongata by conventional *in situ* hybridization without TSA amplification. The right panel is a magnified image of the framed area in the left micrograph. Bar = 500 μm. AP, area postrema; cc, central canal.

probes with appropriate lengths that ensure sensitivity while maintaining specificity. For gene pairs with widely different GC percentages of mRNA, difficulties are faced when designing probes with similar dissociation temperatures and appropriate probe lengths [20].

IV. Principles of High-sensitivity *In Situ* Hybridization Published Recently

In recent years, many variants of *in situ* hybridization have been developed to achieve higher sensitivity, simplicity, and multiplexed fluorescence. Although these methods differ in the principle of detection, they generally share the following two procedures: the use of a synthetic oligonucleotide with a relatively short strand as a primary probe, and the hybridization of multiple secondary probes against a partial sequence of the primary probe as a linker, resulting in a substantial increase in the signals (Fig. 2).

Some of the newer high-sensitivity *in situ* hybridization methods have been commercialized as kits that include everything from probes to detection reagents. Among these commercialized *in situ* hybridization methods, RNAscope,

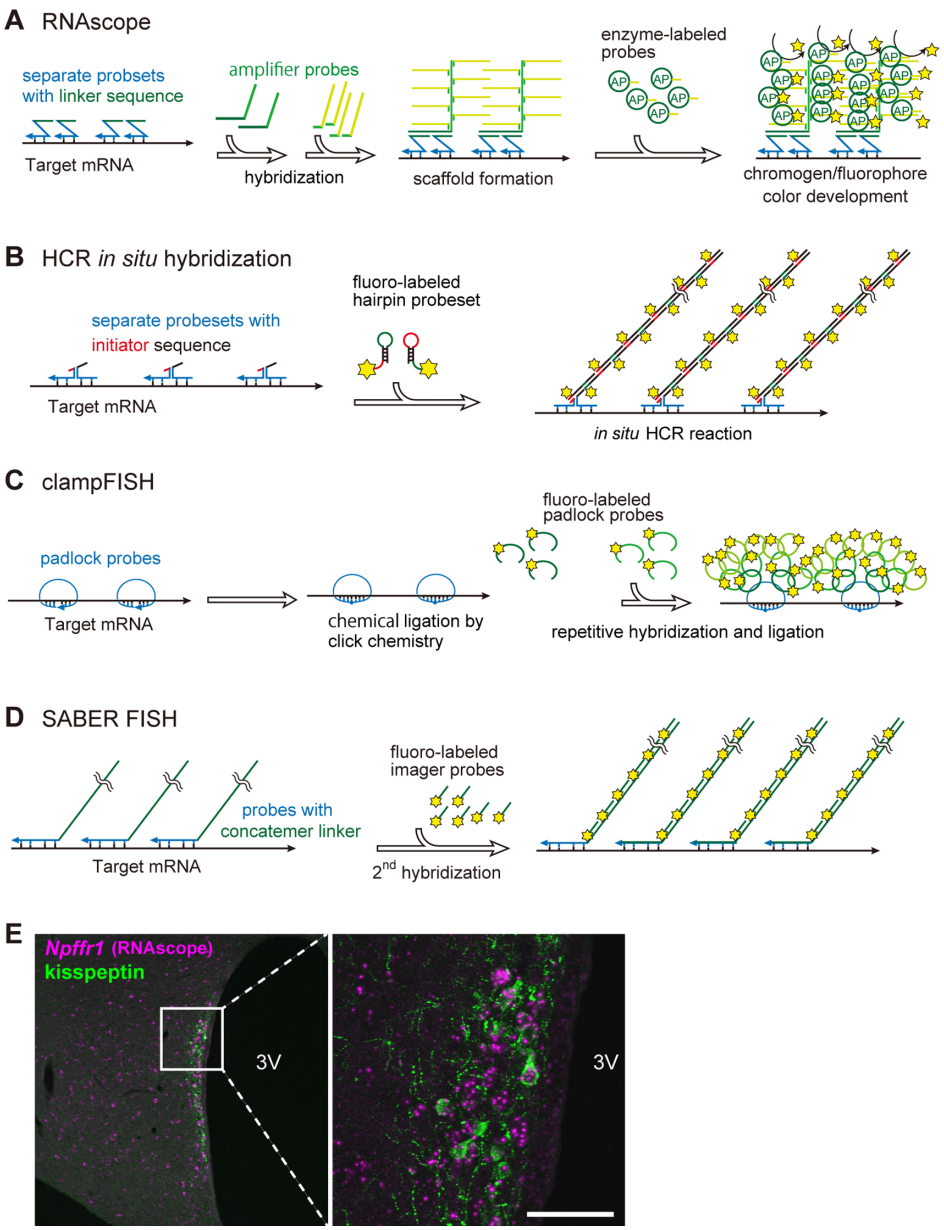

Fig. 2. Schematic of the detection principle of high-sensitivity *in situ* hybridization. The common underlying mechanism of these highly sensitive *in situ* hybridization methods is to use multiple short oligonucleotides as primary probes and then hybridize the probes for amplification, using part of the primary probes as a linker. **A**: RNAscope, **B**: HCR *in situ* hybridization, **C**: clampFISH, **D**: SABER FISH. **E**: Example of high-sensitivity *in situ* hybridization image. Transcripts of the membrane receptor *Npffr1* gene in the rat hypothalamus were visualized by RNAscope (magenta) in combination with immunostaining for kisspeptin (green). Bar = 50 μm. 3V, third ventricle.

Table 1. *Characteristics of each high-sensitivity in situ hybridization method*

Method		DIG-RNA ISH	RNAscope	HCR ISH	clampFISH	SABER FISH
Difficulty of experimental procedures		difficult	easy	moderate	moderate	moderate
Coloration Method		fluorescent chromogenic	fluorescent chromogenic	fluorescent	fluorescent	fluorescent
Multiplex staining		difficult under some conditions	easy	easy	easy	easy
Probe design and synthesis		done by user (can be outsourced)	provided by manufacturer only	done by user (can be outsourced)	done by user	done by user
Automated staining		applicable	applicable	—	—	—
Monetary cost	total	low	high	moderate	moderate	moderate
	per sample	low	high	decreases with increasing sample size	decreases with increasing sample size	decreases with increasing sample size
Time cost	examination of experimental conditions	necessary	mostly unnecessary	necessary	necessary	necessary
	staining time	2–3 days	1 day	1–3 days	1–3 days	2–3 days
Detection of microRNA		difficult	applicable	applicable	—	—

—: not reported.

which provides reagents and probes in drop bottles to simplify and shorten the experimental process, has been the most frequently used recently and has been accepted as a new standard method [37] (Fig. 2A). Because RNAscope is a commercial product, the details of the signal enhancement, including the linker and amplifier sequences, have not been disclosed.

One frequently reported variation in high-sensitivity *in situ* hybridization was hybridization chain reaction (HCR) *in situ* hybridization [10, 34] (Fig. 2B). This variant utilizes an HCR for signal amplification, in which two fluorescently labeled hairpin DNA strands are hybridized and elongated via a self-folding reaction using a partial sequence of the primary probe as a scaffold [13]. Amplification can be adjusted by the user based on the fact that the degree of amplification is proportional to the time of the chain reaction.

Of the recently developed fluorescent *in situ* hybridization variants, clampFISH and SABER FISH are important [12, 22, 31] (Fig. 2C, D). In clampFISH, primary probes that hybridizes to form a circular structure (padlock probes) are used, then the probes are fixed to the target sequence by ligation using click chemistry [5]. High sensitivity was achieved by repeated hybridization and chemical fixation of a fluorescently labeled probe to the loop portion of the primary probe. In SABER FISH, a primer exchange reaction was used to add a short repeating sequence to the end of the primary probe before hybridization (concatenation) [23], and the short fluorescent probe is hybridized to the repeating sequence. The degree of signal amplification can be adjusted by varying the length of the concatemers; however, longer concatemers are expected to reduce probe penetration into the tissue. In these highly sensitive *in situ* hybridization methods, gene transcripts are visualized as granular signals (Fig. 2E, right panel).

V. Characteristics of High-sensitivity *In Situ* Hybridization Variants

The aforementioned high-sensitivity *in situ* hybridization methods were used to visualize a single transcript molecule as a granular fluorescent signal under ideal conditions. This means that regardless of the method chosen, the signal enhancement is sufficient for practical use. Thus, researchers can select a method based on the cost of implementation. The characteristics of each method, including the monetary and time costs, are summarized in Table 1. For all variants, multicolor fluorescence staining was easier than conventional RNA probe *in situ* hybridization. In addition, the hybridization temperatures of these variants are relatively low, providing high antigen retention and facilitating the combination of these methods with immunostaining (Fig. 2E).

RNAscope has several advantages among *in situ* hybridization variants: it can detect chemical chromogenesis as well as fluorescence and can be applied to automated pathology equipment [1, 37]. The major advantage of RNAscope is its efficiency and ease of operation; the experimental procedure is simple and easy to learn, and the staining itself can be completed in one day. This makes

RNAscope the least time-costly method. The disadvantage of RNAscope is that it has the highest monetary cost per sample, with proportionate increase in costs with increasing number of samples. Therefore, it is more suited for a narrowly focused analysis than for analyzing a large number of samples or targets. For HCR *in situ* hybridization, clampFISH, and SABER FISH, primary probes for detection and fluorescently labeled oligonucleotide probes can be synthesized by outsourcing at moderate monetary costs [12, 22, 34]. The cost per sample decreased with the increasing number of samples, making it close to that of conventional *in situ* hybridization. However, as with conventional *in situ* hybridization, these three methods require time for the experimenter to design the probes and optimize the experimental conditions. RNAscope and HCR *in situ* hybridization have been reported to detect short targets, such as microRNAs [28, 38, 39], whereas clampFISH and SABER FISH have not yet been reported for short targets, although this could be possible. From the aforementioned characteristics of each method, the experimenter should choose a method based on whether multiple fluorescent staining is necessary, the number of samples to be analyzed, number of target transcripts, or length of the target transcripts.

VI. Conclusion

Although conventional *in situ* hybridization is a major histological technique, it is difficult to adopt due to the complexity of the procedure. However, via efforts to increase sensitivity and simplify the procedure described in this review, *in situ* hybridization can be used with relative ease for detecting low amounts of nucleic acids. In addition to the above, methodological improvements are still being actively pursued [3, 11]. Clinical applications in pathology and oncology are gradually increasing, including not only the detection of chromosomal aberrations, but also pathological diagnosis using automated *in situ* hybridization and analysis of intratumor heterogeneity using multiplex fluorescent *in situ* hybridization [27, 36]. *In situ* hybridization has been refined methodologically, and researchers now have a wide range of sophisticated options for *in situ* hybridization variants. Therefore, *in situ* hybridization continues to be a practical and efficient method for research purposes.

VII. Conflicts of Interest

Authors have no conflicts of interest to disclose.

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