AR INSIGHTS

Technical Review: *In Situ* Hybridization

ABSTRACT

In situ hybridization is a technique that is used to detect nucleotide sequences in cells, tissue sections, and even whole tissue. This method is based on the complementary binding of a nucleotide probe to a specific target sequence of DNA or RNA. These probes can be labeled with either radio-, fluorescent-, or antigen-labeled bases. Depending on the probe used, autoradiography, fluorescence microscopy, or immunohistochemistry, respectively, are used for visualization. *In situ* hybridization is extensively used in research, as well as clinical applications, especially for diagnostic purposes. This review discusses the basic technique of *in situ* hybridization. The standard *in situ* hybridization process is reviewed, and different types of *in situ* hybridization, their applications, and advantages and disadvantages are discussed. Anat Rec, 297:1349–1353, 2014. © 2014 Wiley Periodicals, Inc.

BACKGROUND

In situ hybridization is a technique that is used for localization and detection of specific DNA and RNA sequences in cells, preserved tissue sections, or entire tissue (whole mount *in situ* hybridization, Fig. 1) by hybridizing the complementary strand of a nucleotide probe to a particular sequence. These hybrids can be visualized by autoradiography for probes labeled radioactively or by development of a histochemical chromogen for probes labeled nonisotopically. The major advantage of *in situ* hybridization is that it enables researchers to determine how the distribution of specific nucleic acids is related to protein products of the target gene and their relation with cellular structures using immunohistochemistry (Coulton and de Belleroche, 1992). Investigation of nucleic acids by *in situ* hybridization was first reported in 1969 (Gall and Pardue, 1969). This approach is currently an important tool in scientific research and in the clinical setting. The spatial information obtained from this method has greatly contributed to researchers, understanding of many diverse areas of research, such as viral infection, gene mapping, cytogenetics, gene expression, and prenatal diagnosis and development.

BASIC PRINCIPLES OF *IN SITU* HYBRIDIZATION

The objective of *in situ* hybridization is to determine the presence or absence of DNA or RNA sequences of interest, as well as to localize these sequences to specific cells or chromosomal sites (Rautenstraub and Liehr, 2002). Particular sequences are identified within cells by taking advantage of a property of nucleic acids (i.e., their ability to specifically anneal to each other to form hybrids). This process can be used for two complementary strands of DNA, and for RNA-to-DNA and RNA-to-RNA combinations. Additionally, hybrids between natural and artificial nucleic acids are possible. After a labeled probe is annealed to matching sequences in fixed cells or tissue, the hybridized probe is visualized. When one of the two strands is labeled, the annealed hybrids can be detected by various methods, including isotopic and nonisotopic (fluorescent and nonfluorescent) approaches. The basic considerations for the process of *in situ* hybridization are discussed below.

Preparation of Tissue

Pretreatment steps can be performed before hybridization to increase hybridization efficiency and reduce nonspecific background staining (Jin and Llyod, 1997). Treatment with proteases (proteinase K is the most common) is an important step to facilitate access of the target nucleic acid. Acetylation of sections with 0.25% acetic anhydride/0.1 M triethanolamine reduces the binding of charged probes to tissues. Optimization of tissue processing, including fixation and storage, is important for detecting intracellular nucleic acids. Fixation for *in situ* hybridization needs to preserve DNA/RNA and tissue morphology. *In situ* hybridization can be performed on cell samples, such as smears and cytospins, and on tissue sections (e.g., frozen and paraffin). Frozen tissue is better than paraffin-embedded tissue for preserving nucleic acids (Egger et al., 1994). Optimally preserved cells should be used for *in situ* hybridization to

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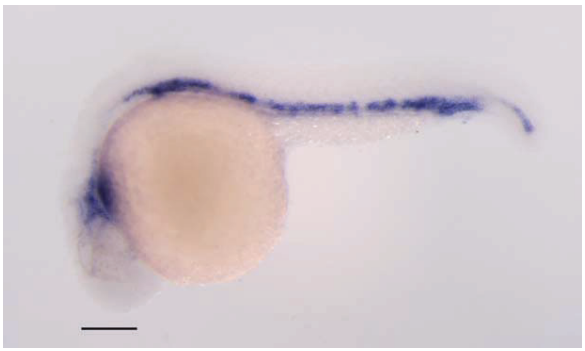

Fig. 1. A lateral image of a 24-hr postfertilization zebrafish embryo in which whole mount *in situ* hybridization against *lyve1* mRNA was performed. *lyve1* is expressed in the veins and lymphatic vessels. In this image, the purple staining is present in the main axial veins because the lymphatics have not yet begun to develop. Scale bar = 200 μm. The image was kindly provided by Jonathan Astin, the University of Auckland. See also Okuda et al. (2012) for details of the preparation.

avoid damage to nucleotide sequences. Frozen tissue and formalin-fixed tissue that has been stored for several years may be used for *in situ* hybridization.

Probes

Many different types of probes are used for *in situ* hybridization; these include cDNA, cRNA, and synthetic oligonucleotide probes. When choosing a probe for *in situ* hybridization, the researcher must take into account sensitivity and specificity, production facilities, how easily the probe penetrates the tissue, stability of hybrids, the application, and how reproducible the method is. The optimal size of the probe is 50–300 bases (McNicol and Farquharson, 1997). Probes that can be used include double-stranded DNA probes, single-stranded antisense RNA probes (riboprobes), single-stranded DNA probes generated by polymerase chain reaction (PCR), synthetic oligodeoxynucleotide probes, and oligoriboprobes. The details of these probes, including their advantages and disadvantages, are beyond the scope of this article.

Probe Labeling and Signal Detection

There are two main approaches for labeling a probe: radioisotope labeling and nonisotope labeling. Radioisotope labeling is considered as the most sensitive method of labeling by many researchers, but others believe that nonisotopic methods are just as sensitive. The results of radioisotope labeling are easily quantified or semiquantified using densitometry counting on film or by silver grain counting. Hybridization sites are observed by autoradiography with X-ray film or liquid emulsion. Problems associated with radioisotope labeling include a long exposure time, poor spatial resolution, risk of exposure to radioactivity, and disposal of radioactive waste. For nonisotopic labeling, compounds including biotin, fluorescein, digoxigenin, alkaline phosphatase, or bromodeoxyuridine are used and are visualized by histochemistry or immunohistochemistry (Jin and Llyod, 1997). Problems associated with nonisotopic labeling are that it is generally considered not as sensitive as radioactive labeling, and the hybridization results are difficult to quantify.

Advantages and Disadvantages of *In Situ* Hybridization

A major advantage of *in situ* hybridization is that it enables maximum use of tissue that is difficult to obtain (e.g., embryos and clinical biopsies). Hundreds of different hybridizations can be performed on the same tissue. Libraries of tissues can be formed and stored in the freezer for future use. A disadvantage of applying *in situ* hybridization techniques is the difficulty in identifying targets that have low DNA and RNA copies. However, approaches are continually being developed to improve the sensitivity of *in situ* hybridization by amplifying either target nucleic acid sequences before *in situ* hybridization or by detecting the signal after completion of hybridization (Qian and Llyod, 2003). These approaches are discussed in the following section.

TYPES OF *IN SITU* HYBRIDIZATION

Since the first appearance of *in situ* hybridization, many different methods have been developed. Some of the more common methods are discussed below.

Increasing Sensitivity Before *In Situ* Hybridization

PCR with *in situ* hybridization (*in situ* PCR) is used for amplification of specific DNA or RNA sequences in cells or tissue sections. The copy numbers are increased by *in situ* PCR to detectable levels by standard methods of *in situ* hybridization (Long, 1998; Nuovo, 2001). *In situ* PCR has mainly been used to identify DNA sequences that are not easy to detect using standard *in situ* hybridization. These sequences include human single-copy genes, chromosomal translocations, and rearranged cellular genes. *In situ* PCR is also used for mapping genomic sequences that have a low copy number in metaphase chromosomes. However, *in situ* PCR has multiple problems, including low efficiency of amplification and poor reproducibility.

Primed *in situ* labeling is a quick, single-step target amplification method. This method incorporates labeled nucleotides mediated by Taq DNA polymerase into

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newly synthesized DNA, using a single primer elongation reaction (Coullin et al., 2002). Primed *in situ* labeling is easily performed, fast, has a reasonable cost, and can be used without cloning technique experience. This method has been improved to identify translocations, as well as staining of human telomeres and multiple chromosomes within the same sample. Primed *in situ* labeling is an alternative option to *in situ* PCR and fluorescence *in situ* hybridization (FISH), and has many potential diagnostic applications. A similar method to primed *in situ* labeling that is used to improve sensitivity before *in situ* hybridization, is called *in situ* transcription. This approach is used for visualization of mRNA by synthesizing labeled complementary cDNA in a cell with a complementary mRNA primer, reverse transcription, and labeled nucleotides.

Amplification of the Signal After *In Situ* Hybridization

One of the signal amplification techniques used by researchers is called catalyzed reporter deposition (CARD; Kerstens et al., 1995). This method involves deposition of activated biotinylated tyramine onto electron-rich moieties (e.g., tyrosine and phenylalanine) at or close to the site of horseradish peroxidase. The CARD technique enables an increase in sensitivity of *in situ* hybridization signals by 500-fold to 1000-fold compared with conventional avidin–biotin complex approaches. The primary advantage of using CARD for *in situ* hybridization is that this technique is performed after probe hybridization and washing. Therefore, the specificity of probe hybridization is not affected. Because of CARD's high sensitivity, there is the potential to amplify background signal. However, CARD is easy, quick, extremely sensitive, and efficient. *In situ* hybridization including signal amplification by CARD is a good option for detecting low copies of nucleic acids and antigens *in situ*.

Another method for amplifying the signal is branched DNA technology. This technique improves detection of nucleic acids by increasing the signal rather than amplifying the target or probe sequence. The branched DNA method uses a series of nonisotopic oligonucleotide probes, sequentially hybridized, for generating chromogenic and fluorescent signals (Urdea, 1994). The branched DNA method can be used to detect either DNA or mRNA, using the same probe set. Advantages of this approach are its specificity and increasing the signal, but not amplifying the target or probe, meaning that it is quantitative.

Fluorescence In Situ Hybridization

FISH is an effective technique that enables direct visualization of genetic alterations in the cell. This technique has many applications and is generally used to examine either imbalances, as a gain or loss of segments of chromosomal materials, or to show specific breakpoints with or without imbalance (Rautenstraub and Liehr, 2002). FISH was initially used for classification of chromosomes (Pinkel et al., 1986) but this technique has since been adopted in a range of applications in the medical and biological fields. The driving force of FISH technology has been increased by geneticists and pathologists interested in human disease because these specialists need to characterize genotype-phenotype correlations. Common uses of FISH in cytogenetic analysis are chromosomal gene mapping, characterizing genetic abnormalities, identifying genetic abnormalities related to genetic disease or neoplasmic disorders, and detecting viral genomes in interphase nuclei or metaphase chromosomes.

Probes for FISH are labeled by fluorescein, biotin, or digoxigenin. Detection of a second signal coupled with fluorochromes is then performed (Jin and Lloyd, 1997). Probes for FISH can mainly be classified into two categories: locus-specific or chromosome paint probes. Locus-specific probes are used for detecting a particular gene or chromosomal area, and are usually applied for evaluating deletion or amplification of DNA sequences. Whole chromosome paint probes are derived from the complete chromosome. These are good for detecting the origin of structurally abnormal chromosomes and for identifying rearrangements involving different (i.e., nonhomologous) chromosomes (Lee et al., 2001). The signals from hybridization (fluorescent spots) are visualized by fluorescent microscopy. FISH tests are highly sensitive.

Multicolor FISH

An efficient strategy to search for chromosomal abnormalities, even though it is expensive, is the use of multicolor FISH techniques (Nath and Johnson, 1999; Lee et al., 2001). In multicolor FISH, two or more probes are each specifically labeled, combined, and then identified with different fluorescent colors (Fig. 2). Using this method, scientists can evaluate multiple chromosome sites. Types of multicolor FISH include multiplex-FISH, spectral karyotyping, cross-species color banding, and comparative genomic hybridization. In multiplex-FISH, spectral karyotyping of the human genome is performed using 24 different colors. Multiplex-FISH enables detection of many chromosomal changes in the genome in just one hybridization reaction. Additionally, multiplex-FISH is a reliable approach for diagnosis. The two systems of multiplex-FISH and spectral karyotyping have different ways of acquiring and processing images of chromosomes, but they both provide the same information. The multiplex-FISH systems and spectral karyotyping are accurate for detecting abnormalities of chromosomes (Garini et al., 1999). Cross-species color banding is a combination of the sensitivity of the traditional method of G banding with the objectivity and

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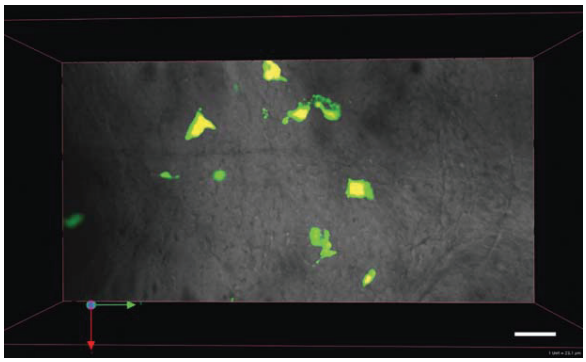

Fig. 2. A dual fluorescent whole mount *in situ* hybridization image. The image is a confocal z-section within the head of a 3-day postfertilization zebrafish larvae. Expression of immunoresponsive gene 1 (*irg1*), a marker of activated immune cells (red fluorescence), and apolipoprotein Eb (*apoeb*), a marker of microglia (green fluorescence), can be seen 1 day after injection of *Salmonella* into the head. Overlapping expression (yellow) shows that these microglia in the head (which are the resident immune cells of the brain) have become activated. The image was kindly provided by Christopher Hall, the University of Auckland. See also Hall et al. (2013) for details of the preparation.

efficiency of a molecular method (chromosome painting; Müller et al., 1998). This approach uses a chromosome painting probe set originating from a species of ape, the gibbon, to examine human chromosomes. Cross-species color banding is inferior to that of multiplex-FISH/spectral karyotyping for identifying translocations between different chromosomes, but it can be useful for identifying certain intrachromosomal rearrangements (Harrison et al., 2001). Comparative genomic hybridization is a good method for investigating the genetic composition of cells from two different sources. This approach is used for investigating variations in DNA copy number (e.g., gains and losses). Comparative genomic hybridization can reveal genetic alterations in all areas of the genome in one experiment by FISH and image analysis by computer (Kallioniemi et al., 1992). However, comparative genomic hybridization is technically difficult and resolution is limited.

Chromogenic *In Situ* Hybridization (CISH)

CISH enables examination of gene amplification, gene deletion, chromosomal translocations, and chromosomal number. This approach uses conventional peroxidase or alkaline phosphatase reactions using bright-field microscopy on tissues fixed by formalin and embedded in paraffin (Tanner et al., 2000). These peroxidase- or alkaline phosphatase-labeled reporter antibodies interact with a hybridized DNA probe, and are then observed with an enzymatic reaction. Tissue morphology and genetic abnormalities can be viewed at the same time with CISH. Advantages of CISH are the fact that signals do not diminish over time, its low cost, the ability to use a light microscope, and permanent staining. CISH is an appropriate alternative method to FISH, and is broadly used by pathologists because it uses bright-field microscopy. A recent study reported a combination approach that exploited the advantages of FISH and CISH (Paz et al., 2013).

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