Recent Advances in Fluorescence in situ Hybridization

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FISH /gene mapping/ fluorescent labeling

Fluorescence in situ hybridization (FISH) procedures that directly couple molecular and cytological information allow precise visualization of DNA sequences on metaphase chromosomes and interphase nuclei. These techniques can be used to identify chromosomes, detect chromosomal aberrations, and analyze linear and spatial genome organization. FISH procedures are also used to clinical fields for diagnosis of disease-related chromosome changes and tumor biology.

INTRODUCTION

In situ hybridization techniques have been used widely to visualize specific DNA or RNA sequences in preparations of chromosomes, single cells or tissue sections. Initially, the nucleic acid probes were labeled isotopically and detected posthybridization by autoradiography. This procedure has been used most frequently for gene mapping, but radioactive in situ hybridization has several limitations of the scatter of radioactive disintegrations, the long time for autoradiographic exposures, and the health hazards for laboratory personnel. To overcome these limitations of isotopic hybridization, fluorescence in situ hybridization (FISH) has been rapidly developed and become increasingly popular in biological and clinical fields\(^\text{1,2}\). The development of FISH procedures can be attributed to several following factors\(^\text{3-14}\).

1) Probes can be modified with reporter molecules both enzymatically and chemically. The detection of their probes after hybridization can be performed in different ways, by specific ligand interactions by fluorescence or immunoenzymatic assays. Fluorochrome-tagged probes can be visualized by fluorescence microscopy. Chemical modified probes are stable and are not subject to special disposal requirements.

2) Different reporter/detection systems provide simultaneous analysis of several probes in different colors.

3) Hybridization signals can be more precisely localized than isotopic signals that were found in an emulsion overlay, allowing much higher spatial resolution.

4) FISH procedures are considerably faster than autoradiographic ones and in some cases, can provide even greater sensitivity. A variety of amplification procedures can be applied to enhance sensitivity.
(5) Suppression hybridization techniques, which inhibit hybridization signals from the highly repetitive sequence elements present ubiquitously in the genomes, facilitates the direct use of cloned DNA as a probe, which was isolated from cosmid, phage and plasmid vectors or yeast artificial chromosomes (YACs).

(7) Digital imaging microscope can provide further improvement in signal detection and the application of image processing technology provides additional enhancement of signal-to-noise ratios to allow detection of small signals.

In this chapter, a brief overview of the recent technical development and some applications of FISH will be described.

**Brief technical protocol**

Air-dried human metaphase chromosome spreads were produced by standard techniques from phytohemagglutinin stimulated normal peripheral blood cultures. Where needed, 5-bromodeoxyuridine (BrdU) is added to the culture 7 hours before harvesting. After air-dried, slides are stored at -80°C. To denature chromosomal DNA, slides were treated with 70% formamide in salt solution at 70°C for 2–3 min, fixed in cold ethanol, then dehydrated through ethanol and air-dried. Various methods can be available to label probes with reporter molecules nonisotopically (Table 1). For the typical labeling tags, biotin-dUTP and digoxigenin-dUTP are most frequently used. Probes are nick-translated with biotin-16 dUTP or digoxige-

| Labeling tag | Detection tag |
|--------------|---------------|
| Biotin-dNTPs | 2nd antibody  |
| biotin-11-dUTP | goat anti-avidin (biotylated) |
| biotin-16-dUTP | rabbit anti-rabbit-FITC |
|               | goat anti-rabbit-TRITC |
|               | rabbit anti-goat-TRITC |
|               | donkey anti-sheep-FITC |
| Digoxigenin   |               |
| dig-11-dUTP   | sheep anti-digoxigenin |
|               | sheep anti-digoxigenin-FITC |
|               | sheep anti-digoxigenin-TRITC |
| N-acetyl-AAF  | sheep anti-mouse-AMCA |
| (DNP)         | goat anti-rabbit-FITC |

| AMCA = amino methyl coumarine acetic acid; FITC = fluorescein isothiocyanate; TRITC = rhodamine isothiocyanate; AAF = N-acetoxy-N-acetyl-2-aminofluorene |
nin-11 dUTP by standard methodology. After nick translation, sonicated salmon sperm and tRNA are added to probes. Probes are ethanol-precipitated to remove free nucleotides and salt. The pellet is suspended in hybridization buffer added competitor DNA (if required), and denatured at 75°C for 10 min.

Hybridization was performed at 37°C for overnight. Although overnight hybridization is convenient, a few hours of hybridization are sufficient to generate a large signal, at least in the case of highly repetitive sequences. After hybridization, slides were washed in solution containing formamide and salt, and then treated with immunofluorescent reagents to obtain fluorescent signals. As summarized in Table 1, biotin-substituted probes can be labeled with antibiotin antibodies or avidin conjugated fluorochromes. For DNP-, digoxigenin- and AAF-probes each specific antibody, such as rabbit-antiDNP, rabbit-anti-digoxigenin or mouse anti-AAF IgG, is used. After incubation with the primary antibody, slides were treated with secondary antibodies of antiglobulins conjugated with fluorescein isothiocyanate (FITC), rhodamine isothiocyanate (TRITC) or Texas Red. FITC and TRITC are the most frequently used. Other fluorochromes with different spectral properties are becoming more widely available. For enhancement of hybrid signals, a variety of amplification procedures, such as antibody-stacking or sequential antibody sandwich techniques, can be used. Depending on the color of the fluorescent label, a counterstaining may be desired using either 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) or propidium iodide (PI) containing antifade agent and viewed with fluorescence microscope.

For simultaneous visualization of bands and fluorescent signals after hybridization, slides were stained with DAPI that results in Q-bands and fluoresces blue, distinct from the FITC or TRITC. DAPI banding is very simple, reproducible and dose not diminish hybridization signals. R-bands can be obtained from chromosome preparations obtained from BrdU treated cultures. FISH procedures produce an excellent R banding pattern when stained with PI which is generally used in FISH, although the bands vary in size depending on the time that individual cells spent in S phase while exposed to BrdU. From this hybridization simultaneous visualization of bands and fluorescent signals is possible. In two-color FISH, different target sequences can be viewed and photographed simultaneously using a dual-wavelength set, which allows simultaneous detection of FITC and Texas Red signals, avoiding the problems of the image shift that can often occur when filters are changed.

The use of sensitive digital cameras and computerized imaging microscopy provides further improvement in signal detection and additional enhancement of contrast and improvement of the signal-to-noise ratios to allow detection of small signals.

**Probes**

DNA derived from chromosome libraries, chromosome-specific centromere sequences, or single-copy sequences can all be be applied to FISH. DNA from somatic cell hybrids that contained a desired single human chromosome can be used as a probe for chromosome painting. DNA sequences can be obtained from microdissected chromosomal material and be used to paint desired chromosomal segments. As an alternative approach, only human chromosomes in human-mouse hybrid cells can be also painted with total human genomic DNA, as
shown in Fig. 1. This will be useful for detection of chromosomal rearrangements or fragments in hybrid cells. Contiguous cosmid and YAC clones which cover long genomic regions are increasingly available to produce a genome map as part of the human genome research.

**Typical applications**

FISH procedure has been the subject of intensive fundamental research. As a consequence of the increased detection sensitivity, its use in applied research has expanded.

(1) **Mapping of DNA sequences**

The location of DNA sequences can be directly detected by FISH, and it allows the detection of unique sequences of a few kbp in metaphase chromosomes and interphase nuclei using either conventional microscopy or digital imaging microscopy. When genomic probes containing repetitive DNA was used, which will result in non-specific hybridization signals, competition of hybridization to repetitive sequences using total genomic DNA has been applied. This suppression hybridization has been routinely used to map sequences cloned in cosmid, phage and plasmid vectors, and YACs. With cosmid clones, more than 90% of the target sequences is cell populations can be delineated by fluorescence, and on metaphase chromosomes more than 80% showed specific signals on both chromatids of both chromosome...
homologs. When the chromosomal target is smaller than 2 kbp, the percentage of metaphases showing highly specific signals reduces to 20–50%.

Hybridization signals from two probes can be spatially resolved on metaphase chromosomes when the probes are several hundred kilobases apart (Fig. 2), but a minimum of 1–2 Mbp separation is required to order each sequence on the chromosome. The result indicates that the practical limits of metaphase mapping may be closer to a few megabases because the chromosome width is of sufficient dimension to encompass substantial lengths of DNA.

To assess ordering genes or DNA sequences, a potential approach can be available using interphase nuclei or less condensed chromosome state, such as prometaphase or premature chromosome condensation. Multiple-label hybridization techniques with three or more probes will enhance the speed of information acquisition. The order of sequences could be determined by visualizing the interphase configuration of three or more signals simultaneously after multiple in situ hybridization, and measurements of the distances between multiple pairs of probes demonstrate a fairly linear relationship between physical distance and genome order over the range from 30 kbp up to 1 Mbp. The combination of metaphase and interphase nuclear mapping, using multiple probes simultaneously, or overlapping cosmids or YAC clones, might allow relatively rapid means of ordering genomic DNA segments with a resolution presently achieved by gel electrophoretic methods and provide a new bridge to a difficult gap between

Fig. 2. Two-color in situ hybridization of BCL1 and HSTF1 genes. BCL1 (arrows) was labeled with biotin and located proximal, whereas digoxigenin-labeled HSTF1 (arrow heads) was distal. These two genes are mapped at 11q13.3 and seemed to be approximately 1–2 Mb apart. Therefore, each signal often overlapped.
physical and genetic mapping techniques\textsuperscript{1,2,5,10,13}).

(2) Cytogenetic applications:

The speed, convenience, and precision of FISH techniques provide to apply cytogenetic fields for analysis of chromosome aberrations. This feasibility has not been realized during the past decade, because methodological capabilities and availability of desired probes have been developed only recently. Hybridization of DNA probes from chromosome libraries, chromosome-specific centromeres, or single-copy sequences can all recognize readily monosomes or trisomies of special chromosomes. Using multiple labels of several probes, it is possible to screen for several aneuploidies at once. Furthermore, this FISH approach can be done directly in interphase cells, making it unnecessary to cultivate cells, such as amniotic fluid cells. This is so called as interphase cytogenetics\textsuperscript{1,2)}, and using this approach Down syndrome of trisomy 21 is diagnosed\textsuperscript{5).} Translocations and other types of chromosomal rearrangements are also detected by use of chromosome libraries or specific repeats in metaphases as well as interphase cells. There potential applications of FISH procedures will be described in following chapters.

In summary, FISH techniques allow precise visualization of single sequences within individual metaphase and interphase cells, providing important implications for human gene mapping and clinical cytogenetics. The development of FISH techniques provides not only an important tool for determining linear arrangements of genes on individual chromosomes, but also a powerful approach for understanding intranuclear topography of three-dimensional organization and expression within the genome.

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