Abstract
Fluorescence in situ hybridization (FISH) is the most convincing technique for locating the specific DNA sequences, diagnosis of genetic diseases, gene mapping, and identification of novel oncogenes or genetic aberrations contributing to various types of cancers. FISH involves annealing of DNA or RNA probes attached to a fluorescent reporter molecule with specific target sequence of sample DNA, which can be followed under fluorescence microscopy. The technique has lately been expanded to enable screening of the whole genome simultaneously through multicolor whole chromosome probe techniques such as multiplex FISH or spectral karyotyping or through an array-based method using comparative genomic hybridization. FISH has completely revolutionized the field of cytogenetics and has now been recognized as a reliable diagnostic and discovery tool in the fight against genetic diseases.

Keywords
FISH • Fluorescence microscopy • Chromosomal aberrations • Diversification of FISH • Principle of FISH • FISH probes

16.1 Introduction
The classical cytogenetics used trypsin-Giemsa or fluorescent banding pattern for identification and characterization of different chromosomal abnormalities such as polycentric chromosomes, ring chromosomes, or chromatid interchanges.
Though chromosome banding techniques based on Giemsa staining revolutionized cytogenetic analysis, they did not become popular because of limited resolution involving only >3 Mb of DNA. Certain chromosomal aberrations such as reciprocal translocations and inversions were not easily recognizable with Giemsa stain. Besides that these techniques are very time consuming, and interpretation of karyotype is very cumbersome and uncertain.

In situ hybridization techniques initially developed by Joseph Gall and Mary Lou Pardue in 1960s (Pardue and Gall 1969) and John et al. (1969) have proved to be powerful tools for determining the chromosomal location of hybridized nucleic acid. Soon after that fluorescent labels quickly replaced radioactive labels in hybridization probes because of their greater safety, stability, and ease of detection.

Early in situ studies used radioactive RNA or DNA probes that were labeled with $^3$H or $^{131}$I, and the sites of hybridization were detected by autoradiography. These techniques have been successfully applied to both animals and plants. RNA probes can be designed for any gene or any sequence within a gene for visualization of mRNA, long noncoding RNA and miRNA in tissues and cells. These probes, often derived from the fragments of DNA that were isolated, purified, and amplified for use in Human Genome Project, consist of about 20 oligonucleotide pairs and cover a space of 40–50 bp of target RNA. In 1982, a new method was described to localize DNA sequences hybridized in situ to chromosome. This method utilized a biotin-labeled analogue of thymidine (TTP) which could be incorporated enzymatically into DNA probes by nick translation. The sites of hybridization were detected either cytochemically by using avidin conjugated to horseradish peroxidase, or fluorometrically by using fluorescein-labeled antibodies. Compared to autoradiography this technique decreased the time required for detection, improved resolution, and gave less non-specific background and chemically stable hybridization probes.

Besides that non-isotopic techniques have been developed using DNA probes labeled with amino acetyl fluorene (AAF), mercuration, and sulfonation, which are detected after hybridization by affinity reagents. Recently a very effective system has been described that uses digoxigenin-labeled nucleotides detected by antibodies carrying fluorescent or enzymatic tag. The non-isotopic labeling techniques have also been successfully applied for detection of highly repeated DNA sequences in plant chromosomes. The non-isotopic detection of low- or single-copy genes, however, has not been successful.

Chromosome painting – competitive hybridization using entire chromosome – specific libraries for chromosomes as probes and human genomic DNA as the competitor was one of the first applications of FISH (Fig. 16.1). It provided intense and specific fluorescent staining of human chromosome in metaphase spread and interphase nuclei. A translocation t(9;22)(q34;p11) was first identified in human neoplasia leading to Philadelphia chromosome.

Fluorescence in situ hybridization (FISH) began with the discovery that nucleic acids could be chemically modified to incorporate a hapten such as biotin or digoxigenin, which in turn could be detected with a fluorescently labeled reporter molecule such as avidin or anti-digoxigenin. Since then probe preparation and labeling
techniques have been modified and simplified. Now nucleotides can be labeled with fluors directly and incorporated into FISH probes, eliminating the often laborious detection steps.

### 16.2 Advancement in Fish Techniques

Fluorescence in situ hybridization (FISH) can detect specific sites of specific DNA sequences in metaphase or interphase cells. This technique, initially developed for mammalian chromosome, was first applied to plant chromosomes by Schwarzacher et al. (1989) and Yamamoto and Mukai (1989). FISH has been used to detect 18S.26SrRNA and repeated DNA sequences in plant chromosomes such on *Aegilops*, * Hordeum*, *Oryza*, *Arabidopsis*, *Brassica*, soybean, and barely chromosome.

GISH (genomic in situ hybridization) is a technique in which genomic DNA is used as a probe. In this technique, genomic DNA from one species is used as the labeled probe, while unlabeled DNA from the other species under test is used as the
competitor at a much higher concentration (Fig. 16.2). The technique is very useful for cytological identification of foreign chromatin in interspecific hybrids at the molecular level. In plant molecular cytogenetics, GISH has also been used to detect parental genomes in natural allopolyploid species such as *Millium montianum*, *Triticum aestivum*, *Aegilops triuncialis*, and *Nicotiana tabacum*, and also alien segments in translocations.

Now availability of several probe labeling procedures has enabled detection of two or more sequences in the same cell by using fluorochromes of different colors. Reid et al. (1992) were able to visualize seven different DNA probes on human metaphase chromosomes simultaneously by FISH using combinatorial fluorescence and digital imaging microscopy. The multicolor FISH technique has been extensively used in plant molecular cytogenetics. Leitch et al. (1991) demonstrated two highly repeated DNA sequences simultaneously in rye chromosomes. Mukai et al. (1993), using multicolor FISH with total genomic probes and highly repeated sequences, reported simultaneous detection of three genomes of an allohexaploid wheat.

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**Fig. 16.2** The main steps involved in the genomic in situ hybridization are (a) direct or indirect labeling of probe, (b) blocking DNA fragmentation, (c) preparation of slide, (d) denaturation of probe and blocking DNA in a hybridization mixture, (e) addition of the probe and the blocking DNA with the hybridization mixture, (f) chromosome DNA denaturation, (g) hybridization of blocking DNA and probe in the target sequence of the chromosome, (h) detection of the probe in the chromosome of one parent, (i) chromosome DNA molecule of the second parent related to the unlabelled blocking DNA, and (j) visualization of hybridization signals in a fluorescence microscope. Unlabelled chromosomes are visualized with a counterstain (blue) (Taken from [http://www.slideshare.net/kskuldee1995/genomic-in-situ-hybridization](http://www.slideshare.net/kskuldee1995/genomic-in-situ-hybridization))
The combination of biotin, digoxigenin, and fluorescein labeling has allowed us to detect multiple probes and to map sequences relative to each other in single cells. Mukai (1995) detected five DNA probes with different colors on a single chromosome.

### 16.3 Principle Involved in Fish

The basic principle involved is hybridization of nuclear DNA of either interphase cells or of metaphase chromosomes affixed to a microscopic slide, with a nucleic acid probe. The probes are either labeled indirectly with a hapten or directly through incorporation of a fluorophore. The labeled probe and the target DNA are mixed together after denaturation, which allows annealing of complementary DNA sequences. In case the probe had been labeled indirectly, an extra step of enzymatic or immunological detection system will be required for visualization of the non-fluorescent hapten. Finally the signals are evaluated by fluorescence microscopy (Fig. 16.3). The enzymatic detection system involves fluorochrome, which emits colored signals at the hybridization site. The immunological detection system is based on binding of antibodies to specific antigens, which is then demonstrated with a colored histochemical reaction visible by light microscope or fluorochromes with ultraviolet light.

For direct detection, the most frequently used reporter molecules are fluorescein (fluorescein isothiocyanate, FITC), rhodamine, Texas Red, Cy2, Cy3, Cy5, and AMCA. For indirect detection method, the reporter molecules typically used are biotin, digoxigenin, and dinitrophenol.

### 16.4 Preparation of Probes

One of the most important steps in FISH analysis is the choice of probe. A wide range of probes, extending from whole genomes to small cloned probes (1–10 kb), can be used. There are basically three types of probes, each with a different range of applications, whole chromosome painting probes, repetitive sequence probes, and locus specific probes, which are briefly described below.

1. Chromosome painting refers to the hybridization of fluorescently labeled chromosome-specific composite probe pools to cytological preparations. This enables visualization of individual chromosomes in metaphase or interphase cells and the identification of chromosomal aberrations. The whole chromosome painting probes are complex DNA probes derived from a single type of chromosome that has been PCR amplified and labeled to generate a “paint” which homogeneously highlights the entire chromosome. With this probe, the cytologically visible structural and numerical chromosome rearrangement in metaphase becomes obvious. The chromosomal paint is, however, not helpful in the analysis of interphase cells. Whole chromosome painting is now available for every human chromosome, allowing the simultaneous painting of the entire genetic
complement in 24 colors. This has led to the development of two independent FISH techniques – multicolor FISH (M-FISH) and spectral karyotyping (SKY) – which have important diagnostic and research application values.

2. Repetitive sequence probes hybridize to specific chromosomal regions or structures that contain short sequences, which are present in many thousands of copies. For example, pan-telomeric probes target the tandemly repeated (TTAGGG) sequences present in all human chromosomes ends. Centromeric probes target the α- and β-satellite sequences, flanking the centromeres of human chromosomes. Satellite DNA probes hybridize to multiple copies of the repeat sequences present at the centromeres, resulting in two very bright fluorescent signals in both metaphase and interphase diploid cells. These centromere-specific probes

Fig. 16.3  The principles of fluorescence in situ hybridization. (a) The basic elements are a DNA probe and a target sequence. (b) Before hybridization, the DNA probe is labeled indirectly with a hapten (left panel) or directly labeled via the incorporation of a fluorophore (right panel). (c) The labeled probe and the target DNA are denatured to yield single-stranded DNA. (d) They are then combined, which allows the annealing of complementary DNA sequences. (e) If the probe has been labeled indirectly, an extra step is required for visualization of the nonfluorescent hapten that uses an enzymatic or immunological detection system. Finally, the signals are evaluated by fluorescence microscopy (Taken from http://biohorizons.oxfordjournals.org/content/early/2010/02/26/biohorizons.hzq009/F1.expansion.html)
are useful in detection of monosomy, trisomy, and other aneuploidies in leukemias and solid tumors (Fig. 16.4).

3. Locus-specific probes are usually genomic clones, which vary in size depending on the nature of the cloning vector from plasmids (which can carry 1–10 kb) to the larger PAC (P1 bacteriophage-derived artificial chromosome, which can carry 100–300 kb), YAC (yeast artificial chromosome which can carry 150–350 kb), and RAC vectors (which can carry 80 kb to 1 Mb). These probes are particularly useful for detection of translocations, inversions, and deletions in both metaphase and interphase.

16.5 Methodology Involved

In situ hybridization (ISH) involves the following major steps:

16.5.1 Cytological Preparation

Well-spread out and flat preparation ensures best morphology and highest hybridization signals. Most of the ISH studies of plant chromosomes have been made on mitotic root tip preparations. The root tips fixed in ethanol/glacial acetic acid are
stained with 1% acetocarmine and then squashed in 45% acetic acid on the slide. The slides can be stored in −80 °C freezer for at least 1 year. After thawing the chromosomes are dehydrated on the slide before hybridization.

### 16.5.2 Probe Labeling

Several methods for labeling DNA probes for nonradioactive in situ hybridization have become available. The most common approach is to label the probe with reporter molecules (haptens). A variety of haptens are available in the market: biotin, digoxigenin, dinitrophenol, fluorescein, rhodamine, AMCA, and coumarin. These haptens can be incorporated as labeled nucleotides by tagging technique of nick translation, random primer labeling, or PCR according to the routine procedures. Detection of hybridized digoxigenin probes is mediated by anti-digoxigenin antibodies conjugated to enzyme or fluorochrome. The labeled DNA may be separated from unincorporated nucleotides using the spin column or ethanol precipitation methods. The random primed labeling method is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labeled.

### 16.5.3 In Situ Hybridization

#### 16.5.3.1 Nonradioactive In Situ Hybridization

For nonradioactive in situ hybridization, the chromosomal DNA is denatured on the slides in 70% formamide, 2XSSC at 68–70 °C for 2 min. The slides are dehydrated and then air-dried. The hybridization mixture containing DNA probe (20–50 μg/ml) is added to the slide and incubated at 37 °C for 6–12 h. For detection of hybridization sites, the slides are washed in 2XSSC and then PBS. The slide is incubated with 0.6% streptavidin-horseradish complex at 37 °C for 30 min. After washing, 0.05% diaminobenzidine-tetrahydrochloride (DAM) and 0.01% H₂O₂ are placed on the slide and incubated at room temperature in the dark for 5–20 min. Slides are rinsed with PBS and counterstained with 2% Giemsa for one minute and air-dried. Positive hybridization sites should appear dark brown.

#### 16.5.3.2 Genomic In Situ Hybridization (GISH)

The purified isolated genomic DNA is sheared by passing through an 18-gauge hypodermic needle or by ultrasonication. 1 μg DNA is labeled with biotin-16-dUTP through nick translation and then purified by spin column or through ethanol precipitation. The genomic DNA is denatured on the slide by immersion in 70% formamide-2XSSC solution at 68–70 °C for 2 min. The slides are rapidly dehydrated and air-dried. Hybridization mixture containing labeled total genomic probe (1 μg/ml) is added to each slide and incubated in moist plastic chamber at 37 °C for 6–12 h. For detection of hybridization sites, the slides washed in 2XSSC and then PBS are incubated with 0.6% streptavidin-horseradish complex at 37 °C for 30 min.
After washing, 0.05 % diaminobenzidine-tetrahydrochloride (DAM) and 0.01 % hydrogen peroxide are placed on each slide and incubated at room temperature in the dark for 5–20 min. Slides are rinsed with PBS and counterstained with 2 % Giemsa for 1 min and air-dried. Positive hybridization sites should appear dark brown.

### 16.5.3.3 Fluorescence In Situ Hybridization (FISH)

For FISH, the chromosomal DNA is denatured on the slides in 70 % formamide-2XSSC solution at 68–70 °C for 2 min. The slides are dehydrated and then air-dried. The hybridization mixture containing DNA probe (20–50 μg/ml) is added to the slide and covered with cover slip and incubated in moist plastic chamber at 37 °C for 6–12 h. Slides are washed, dried and then immersed in blocking buffer (1X PBS, 0.1 % Triton-100) for 2 min, and rinsed in PBS for 5 min at room temperature. The semidried slide is treated with 100 μl of 1:100 rabbit antibiotin antibodies and incubated in humidity chamber at 37 °C for 5 min. Slides are washed with PBS and immersed in 100 μl of diluted antibody (FITC-conjugated goat anti-rabbit antibody, 1:100 in dilution buffer) and incubated in the humidity chamber at 37 °C for 30–60 min. After washing, 60 μl of an antifade solution (p-phenylenediamine 10 mg/ml, 90 % glycerol, and propidium iodide 1 μg/ml as a counterstain) is added on each slide. The slide is observed with fluorescence microscopy using B2 or B-2A filter cassette.

### 16.6 Diversification of Fish Techniques

Ever since widespread recognition of FISH as a physical mapping technique to support massive nucleotide sequencing is involved in the Human Genome Project; it has become a more convenient and popular technique in other areas of biological and medical research including clinical genetics, neuroscience, reproductive medicine, cellular genomics, and chromosome biology.

The diversification of the original FISH protocol into a variety of remarkable procedures developed over the years has come about due to the improvement in sensitivity, specificity, and resolution of the technique (Volpi and Bridger 2008). These improved techniques along with the advancements in fluorescence microscopy and digital imaging have helped in better understanding of the chemical and physical properties of nucleic acids and chromatin.

Some of the techniques listed below, which have been inspired by the glossary of Volpi and Bridger (2008), show the versatility of FISH.

#### 16.6.1 Centromere-FISH (ACM-FISH)

ACM-FISH is a multicolor FISH assay for detection of chromosomal abnormalities in sperm cells. The abbreviation ACM refers to the simultaneous hybridization of DNA probes for the alpha (centromere), classical (1q12) satellite and midi (1p36.3)
satellite of chromosome 1 for the specific detection of duplications and deletions of 1pter and 1cen and for the identification of chromosomal breaks within the 1cen-1q12 region in human sperm. The discovery of chromosomal break/damage in the human sperm provided explanation for infertility in oligozoospermic men.

16.6.2 armFISH

armFISH is a 42-color M-FISH variant that allows the detection of chromosomal abnormalities in the p- and q-arms of all 24 human chromosomes, except the p-arm of the Y and acrocentric chromosomes.

16.6.3 Catalyzed Reporter Deposition-FISH (CARD-FISH)

CARD-FISH refers to the fluorescein tyramine signal amplification mediated by horseradish peroxidase (HRP)-labeled oligonucleotide probe (Fig. 16.5). This technique is very useful for detection, identification, and quantification of microorganisms involved in bioleaching processes.

16.6.4 Cellular Compartment Analysis of Temporal (Cat) Activity by Fish (catFISH)

catFISH uses FISH to immediate early rRNA genes and confocal microscopy to identify neuronal population activated at two distinct times. This technique is used to determine the interactions of neuronal populations associated with different behaviors.

Fig. 16.5  Schematic representation of mRNA in situ hybridization detection using tyramide signal amplification (T5A) in the presence of horseradish peroxidase (HRP) and hydrogen peroxide; tyramide radicals are formed (red box) that can covalently react with nearby residues (Taken from http://www.authorstream.com/Presentation/chhabra61-443431-insitu-hybridization/)
16.6.5 Cytochalasin B (CB-FISH)

CB-FISH involves hybridization on binucleated cells in which cytokinesis has been blocked by treatment with cytochalasin B (CB). Figure 16.6 shows increased ratio of mosaic diploid cells in vivo in trisomy 21 cases. Analysis of the chromosomal content of micronuclei can be facilitated by combining the standard CB-FISH protocol with the 24-color SKY technology.

16.6.6 Chromosome Orientation (CO)-FISH

CO-FISH uses single-stranded DNA probes labeled with 5-bromodeoxyuridine during S phase to produce strand-specific hybridization. It allows to determine the relative orientation of two or more DNA sequences along a chromosome. Initially, this technique was designed to determine the orientation of tandem repeats within centromeric regions of chromosomes. This technique has also been useful in assessing chromosomal translocations and inversions.

16.6.7 Combined Binary Ratio (COBRA)-FISH

COBRA-FISH enables recognition of all human chromosome arms on the basis of color and mapping of gene and viral integration site in the context of chromosome arm painting. COBRA-FISH protocol brings together combinatorial labeling which allows different ratios of label to distinguish between probes. This permits the use
of fewer fluorochromes to produce up to 48 color combinations for differential painting of human chromosome arms within a specimen.

### 16.6.8 Chromosome Orientation and Direction (COD)-FISH

This protocol is similar to CO-FISH except for the information about the directional organization of telomeric sequences. It can also stand for concomitant oncoprotein detection-FISH which allows visualization of loci signals for a particular oncogene and also the protein product derived from this gene. Another technique that has been termed COD-FISH is the combined CaCO₃ optical detection-FISH, in which FISH is used to detect calcifying microorganisms in open ocean.

### 16.6.9 Combinatorial Oligonucleotide (COMBO)-FISH

COMBO-FISH is used for specific labeling of genomic sites. It takes advantage of homopurine/homopyrimidine oligonucleotides that form triple helices with intact duplex genomic DNA. This will not require prior denaturation of the target sequence, which is usually a prerequisite for probe binding in the standard FISH protocols. Homopurine or homopyrimidine regions of DNA are usually longer than 14 bp, representing 1–2% of the human genome, with an average of 150–200 of such stretches in a 250-kb segment of the genome. Accordingly, specific probe sets can be constructed to target genomic regions of interest in that size range.

### 16.6.10 Comet-FISH

Comet-FISH is a combination of comet assay and FISH analysis. It is used to detect genome region-specific DNA damage. It involves attachment of DNA onto agarose-coated microscope slide prior to in situ hybridization and allows specific sequences to be delineated in the comet head or tail. This will permit the assessment of sensitivity to DNA damage/breakage in the specific genomic region, which has been shown to be associated with the gene density of a chromosome rather than the chromosome size. This technique has been successfully used to determine the sensitivity of telomeres to damage.

### 16.6.11 Cryo-FISH

Cryo-FISH makes use of ultrathin cryosections (150 nm thick) of sucrose-embedded cells. The spatial interrelationship of chromosome territories and the genome organization in the cell nucleus has been successfully studied with this technique.
16.6.12 Double Fusion FISH (D-FISH)

In this FISH, a secondary color is observed since the adjacent colors overlap. The secondary color will be present or absent in the cases under study (Fig. 16.7). An example is the detection of BCR/ABL translocations, where the secondary color indicates disease. The opposite situation, where the absence of secondary color is pathological, is illustrated by an assay for translocation where only one of the breakpoints is known. Locus-specific probes are made for one side of the breakpoint and the other intact chromosome. In normal cells secondary color is observed, but only the primary colors are observed when the translocation occurs. This technique is called “break-apart FISH” (Fig. 16.7).

![Fig. 16.7](image_url)
16.6.13 DNA Breakage Detection FISH (DBD-FISH)

DBD-FISH has been used to determine DNA fragmentation levels in sperms. Cells are normally stabilized in agarose beads and incubated with the unwinding buffer to form single-stranded DNA in the sample that can be hybridized with the appropriate probes.

16.6.14 e-FISH

e-FISH is a BLAST-based FISH simulation program, which can predict the outcome of hybridization experiments. This program was developed as a bioinformatics tool for selecting appropriate genomic probes for hybridization experiments.

16.6.15 Fiber-FISH

Fiber-FISH is a technique in which DNA fibers or chromatin fibers are released from cell nuclei by salt or solvent extraction and stretched on a microscope slide prior to hybridization. This technique allows high-resolution mapping of chromatin fibers or DNA such as physical ordering of DNA probes, assessment of gaps and overlaps in contigs, and copy number variants.

16.6.16 Flow-FISH

In this technique, the in situ hybridization is combined with flow cytometry for measurement of the telomeric signals from cells in suspension. The PNA-labeled telomere probes are used to visualize and measure the length of telomere repeats. This technique has been used in aging studies.

16.6.17 Fusion-Signal FISH

This technique was initially used for identification of the 9;22 Philadelphia translocation in peripheral blood and bone marrow cells of CML patients to detect minimal residual disease after bone marrow transplantation. BCR and ABL gene fragments, each flanking one of the two breakpoints, were used as probes for the detection of the BCR/ABL fusion product, hence the name fusion-signal FISH.

16.6.18 Halo-FISH

In halo-FISH the cells are first permeabilized and then extracted with high salt to remove soluble proteins. The chromatin/DNA that is not fixed to an internal
structure within cell nucleus is consequentially released, forming a halo around a residual nucleus. FISH can then be performed on these preparations using any type of probe to delineate specific DNA sequences such as α-satellite, telomeres, scaffold attachment regions (SARs), matrix attachment regions (MARs), gene loci, and whole chromosomes.

16.6.19 Harlequin-FISH

Harlequin-FISH is a method for cell cycle-controlled chromosome analysis in human lymphocytes that allows a precise quantification of induced chromosome damage for human biodosimetry. This technique combines FISH painting with differential replication staining of sister chromatids, either with Giemsa and/or fluorescent dyes, after BrdU treatment of lymphocyte cultures. After a few cell divisions, the chromosomes acquire an asymmetrically striped appearance, to which the term harlequin refers.

16.6.20 Immuno-FISH

Immuno-FISH is a combination of standard FISH and indirect or direct immunofluorescence. With this technique, the antigens can be visualized within the sample. Moreover, both DNA and proteins can be analyzed on the same sample. It is often used to investigate co-localization of genomic regions with proteins in the interphase nuclei such as nucleoli or promyelocytic leukemia (PML) bodies.

16.6.21 Locked Nucleic Acids (LNAs)-FISH

The in situ hybridization efficiency is remarkably improved by using locked-nucleic-acid (LNA)-incorporated oligodeoxynucleotide probes (LNA/DNA probes) without compromising specificity. LNA/DNA oligonucleotide heteroduplexes show a structural shift from a B-like helix toward an A-type helix, which has higher thermal stability. LNA/DNA probes are more useful for the detection of mRNA and genes on the chromosomes.

16.6.22 Multiplex (M)-FISH

One of the most fascinating aspects of FISH technology is the ability to identify several regions or genes simultaneously using different colors. The entire chromosome can be painted in a single hybridization by labeling with a different combination of fluorophores. This technique consists of labeling each probe with a unique combination of five spectrally separable fluorochromes in a 1:1 ratio. Originally these probes were used for simultaneous detection of the 24 human chromosomes.
(22 autosomes and the X and Y chromosomes), but was subsequently used to analyze specific chromosomal subregions, like centromeres and sub-centromeres. M-FISH and SKY differ only in the method of discriminating differentially labeled probes. SKY uses CCD camera and Fourier transform spectrometry.

16.6.23 Multilocus or ML-FISH

The ML-FISH refers to the simultaneous use of multiple probes in multicolor FISH. This FISH assay was initially designed to screen for multiple microdeletion syndromes in patients with unexplained developmental delay and/or mental retardation.

16.6.24 Premature Chromosome Condensation (PCC)-FISH

PCC-FISH is used for determination of chromosome damage after irradiation. It relies on the use of chromosome-specific painting probes. This technique refers to the effect obtained by virus-mediated cell fusion or phosphatase inhibitors to prematurely condensed chromosomes of cells in G1 and G2 phases. PCC-FISH was initially devised as an assay to estimate/predict the in situ radiation sensitivity of individual human tumors. It has subsequently been used to estimate the effect of whole-body high- or low-dose exposure to human peripheral lymphocytes.

16.6.25 Peptide Nucleic Acid (PNA)-FISH

PNAs are synthetic analogues of DNA in which the deoxyribose phosphate backbone is replaced with a noncharged peptide backbone. As a result of this unique structural property, there is no electrostatic repulsion when PNA oligomers hybridize to complementary DNA or RNA sequences. The PNA-DNA and PNA-RNA duplexes become more stable than the natural homo- or heteroduplexes. FISH with PNA probes was first used to measure individual telomere lengths on metaphase chromosomes.

16.6.26 Quantitative-FISH (Q-FISH)

This method has been used mainly for measuring the number of telomere repeats on a particular chromosome, using PNA-conjugated probes (Fig. 16.8). Typically, metaphases are imaged and then analyzed using software TFL-TELO. Q-FISH has become an important tool in studying the role of telomeres in aging and cancer.
16.6.27 Quantum Dot (QD)-FISH

Quantum dots are nanometer-sized inorganic fluorophores, characterized by photo-stability and narrow emission spectra. These have been successfully used for FISH analysis on human metaphase chromosomes, human sperm cells, and bacterial cells. QD-FISH has also been used to detect subcellular mRNA distribution in tissue sections.

16.6.28 Rainbow-FISH

Rainbow-FISH allows simultaneous detection and quantification of up to seven different microbial groups in a microscopic field. This protocol uses specific 16S rRNA-targeted oligonucleotide probes for discrimination of different phylogenetic groups of microbes. As a result, by the combined application of seven DNA probes, each labeled with up to three fluorochromes, seven kinds of microbial strains can be distinguished simultaneously.

16.6.29 Raman-FISH

It is a technique in which FISH is combined with Raman microspectroscopy for ecophysiological investigation of complex microbial communities. The shift in the

**Fig. 16.8** The length of telomere repeats at individual chromosome ends is highly variable. Telomere repeats in a normal human lymphocyte are visualized using quantitative fluorescence in situ hybridization (Q-FISH) using peptide nucleic acid probes. Telomeres are shown in yellow, whereas the DNA of chromosomes, counterstained with DAPI, is shown in blue (Taken from http://physrev.physiology.org/content/88/2/557)
resonance spectra in Raman microscopy, after anabolic incorporation of $^{13}$C isotope, compared with $^{12}$C, into microbial cells is the basis of this procedure. This metabolic labeling with stable isotope is combined with in situ hybridization with specific 16S rRNA probe for identification of microbial species. This allows structural and functional interrelated analyses of microbial communities at a single-cell resolution.

### 16.6.30 Replicative Detargeting FISH (ReD-FISH)

The replication timing of specific sequences can be determined by ReD-FISH. If BrdU is incorporated in the sequence of interest, the newly formed DNA strand will be detargeted, and each oligonucleotide probe will only be able to hybridize to one of the parental strands, and only one chromatid will display a signal. However, if the sequence of interest has not replicated and has not incorporated BrdU, then a FISH analysis will reveal the standard double signal on both chromatids of the metaphase chromosome. ReD-FISH provides qualitative and quantitative information about replication timing, including the relationship between defects in replication timing and defects in chromosome condensation, sister chromatid cohesion, and genome stability.

### 16.6.31 Reverse-FISH

Reverse-FISH is the process whereby the FISH probe comprises DNA from the material of interest. Reverse-FISH has been useful for characterizing marker chromosomes and chromosome amplifications in cancer.

### 16.6.32 Recognition of Individual Genes (RING)-FISH

RING-FISH utilizes high concentrations of polynucleotide probes in order to increase the visualization and sensitivity of any part of the genetic material in a bacterial cell, regardless of copy number. It was designated as ring-FISH because of the characteristic halolike, ring-shaped hybridization signal in the cell periphery obtained with this method.

### 16.6.33 RNA-FISH

RNA-FISH allows simultaneous detection, localization, and quantification of individual mRNA molecules either in the nucleus or cytoplasm at the cellular level in fixed samples. This RNA FISH technology provides a method to achieve allelic-specific expression on a single-cell basis. It has the potential for investigating gene expression profiling in single cells.
16.6.34  Cross Species Color Banding (Rx)-FISH

RxFISH, also known as chromosome bar coding, is based on sequence homologies between human and the apes, such as gibbon (98 %). This technique produces, by cross species hybridization using differentially labeled gibbon chromosome probes, a specific banding pattern on human metaphase chromosomes. If the probes are labeled with a number of fluorochromes, usually three, this allows a colorful and reproducible banding to be observed and analyzed. The color bands make it easier to see intrachromosomal rearrangements, compared to G-banding. However, in combination with G-banding, RxFISH can provide detailed information about the chromosomal breakpoints.

16.6.35  Split-Signal FISH

It is a dual-color FISH assay for detection of frequently occurring chromosome translocations affecting specific genes in hematopoietic malignancies. The assay involves differential labeling of two probes on the flanking regions of the translocation breakpoint. The signals normally co-localize and appear fused, but they split in the translocative event. This technique has been used for the detection of Burkitt translocation in B cell lymphomas and mantle cell lymphomas.

16.6.36  Stellaris RNA FISH (Single-Molecule RNA FISH)

It is a method of detection and quantification of mRNA and other long RNA molecules in a thin layer of tissue samples. The binding of up to 48 fluorescent-labeled oligos to a single molecule of mRNA provides sufficient fluorescence to detect and localize each target mRNA. Figure 16.9 shows RNA FISH for Cre mRNA in genetically identical cells, in which expression is epigenetically controlled.

16.6.37  T-FISH

The three versions of T-FISH – tyramide-FISH, tissue-FISH, and telomere-FISH – are discussed in the order of their arrival in the field.

Tyramide-FISH: Tyramide is a compound that binds to peroxidase and greatly increases the sensitivity in FISH experiments, with the use of only one or two layers of reagents for visualization. The first layer uses a peroxidase-conjugated antihapten antibody or a compound such as streptavidin to bind to the labeled probe (Fig. 16.5). Fluorochromes or haptens, such as biotin, are conjugated to tyramine derivatives. This leads to massive buildup of fluorochromes that make the visualization and detection ultrasensitive. The technology has been used to map gene loci and look for specific transcripts in cell.
Tissue-FISH: Tissue samples collected from patients or experimental animals are frozen, fixed, or embedded in paraffin wax and used for FISH analysis.

Telomere-FISH: It is FISH using telomeric probes.

16.6.38 3-D FISH

3-D FISH has been developed to analyze spatial positioning and relative organization of chromosomes and sub-chromosomal regions within the cell nuclei. Paraformaldehyde is usually used as cross-linking fixation reagents to preserve nuclear architecture and chromatin organization. Due to cross-linking of proteins, an efficient permeabilization step would be required to allow the probes to penetrate the sample.

16.6.39 Zoo-FISH

Zoo-FISH, also known as cross species chromosome painting, involves hybridizing libraries of DNA sequences of one species to the chromosomes of another species, to identify regions of synteny. The first Zoo-FISH study used human and mouse whole chromosome painting probes on primates, rodents, even-toed ungulates, and whales.
One of the most significant developments in FISH technology in relation to genome-wide screening was the introduction of comparative genome hybridization (CGH) in 1992. In CGH, the genomic DNA from the specimen and the control DNA extracted from an individual with a normal karyotype (46,XX or 46,XY) are differentially labeled with green and red fluorochromes, respectively, mixed in equal amounts and co-hybridized to reference human metaphase chromosomes (Fig. 16.10). The relative difference in DNA content between the normal and specimen DNA is represented by a difference in the green/red fluorescence ratios. For example, if the chromosomal material is present in identical copy numbers in both the reference and the specimen genome, the observed fluorescence is a blend of an equal contribution.

**Fig. 16.10** Comparative genomic hybridization. Genomic DNA is isolated from both the tumor sample and the normal reference sample, labeled with different fluorochromes and mixed in the presence of excess Cot-1 DNA to prevent binding of repetitive sequences. In conventional chromosomal CGH, these are hybridized to normal metaphase chromosomes, and the ratio of fluorescence intensities along each chromosome is analyzed. Increased DNA copy number (amplification) in the tumor sample will be detected by increased red fluorescence, whereas decreased copy number in the tumor sample will allow more binding of the normal DNA and increased green fluorescence. On the right, a similar hybridization to a cDNA array permits measurement of copy number at a higher resolution. The red and green spots on the fluorescence image represent increased and decreased copy number changes, respectively (Taken from http://biohorizons.oxfordjournals.org/content/early/2010/02/26/biohorizons.hzq009/F7.expansion.html)
of red and green fluorescence. If chromosomes are lost or chromosomal subregions are deleted in the specimen genome, the resulting color is shifted to red. A gain in the certain chromosome in the specimen, such as amplification of oncogenes, is reflected by a more intense green staining of the respective chromosome in the reference metaphase preparation. The ratios of the test to reference fluorescence along the chromosomes are quantified using digital image analysis.

In array CGH, metaphase chromosomes are replaced as the target by large number of mapped clones that are spotted onto a standard glass slide greatly increasing the resolution of screening for genome copy number gains and losses. In array CGH, the test and the normal reference genomes, which are used as probes, are differentially labeled and co-hybridized to a microarray before being imaged. The fluorescence intensities are calculated for each mapped clone, with the resulting intensity ratio reflecting the DNA copy number difference (Fig. 16.11). Despite some limitations, array CGH has become one of the most widely used cytogenetic techniques in both basic research and molecular diagnosis. This technique has enabled us to understand that tumors of the same type have similar patterns of DNA gains and losses and that the frequency of changes increases with tumor progression.

![Fig. 16.11](https://en.wikipedia.org/wiki/Comparative_genomic_hybridization#/media/File:Array-CGH_protocol.svg)
16.7 Applications of Fish

FISH has now become an essential tool for gene mapping and characterization of chromosome aberrations. Since the target DNA remains intact, unlike in molecular genetic analysis, information is obtained directly about the positions of probes in relation to chromosome bands or to other hybridized probes. Using differentially labeled probes, chromosome aberrations on particular chromosomes or chromosomal regions can be easily defined. The diseases that have been diagnosed using FISH include Prader-Willi syndrome, Angelman syndrome, 22q13 deletion syndrome, chronic myelogenous leukemia, acute lymphoblastic leukemia, Cri-du-Chat syndrome, velocardiofacial syndrome, and Down syndrome. The analysis of chromosomes 21, X, and Y can identify oligozoospermic individuals at risk.

In medicine, FISH can be used for diagnosis, evaluation of prognosis, and evaluation of remission of a disease such as cancer. FISH can be used to detect diseased cells more easily than standard cytogenetic methods. High-resolution FISH mapping and ordering of probes relative to one another can be performed on released chromatin fibers and is termed fiber-FISH. Fiber-FISH has a wide range of resolution (I kb–I Mb).

One of the major advantages of FISH over conventional molecular biology is the provision of molecular information in the context of cell morphology. Targeting nuclear RNA and the corresponding genes within cells or within a single cell or from a single allele can provide important information about gene expression, processing, and transport of transcripts in normal and mutant cells. The use of RNA FISH for studying the intracellular localization of RNA has increased over understanding of in situ physical characteristics of DNA transcription and transport of RNA transcripts. Similarly FISH can be used to examine many interesting biological questions about nuclear organization. Three-dimensional nuclear DNA FISH can provide high-resolution information about sub-chromosomal domains, gene position, and the relationship of genes and their transcripts in different cells and during different stages of the cell cycle. Accurate analysis of three-dimensional FISH is highly dependent on excellent quality confocal microscopy and image analysis procedures.

FISH technology also allows genome-wide screening of chromosomal gains and losses, which is comparative in in situ hybridization (CGH). It is based on the comparison of genomic DNA from two different genomes and identifies chromosomal gains and losses of one genome relative to the other. CGH is performed in normal chromosome metaphase spreads, which is a distinct advantage for studying tumor samples. The resolution of identifying chromosomal gains and losses on metaphase chromosomes is several Mbs. However, this technique has been modified to increase the resolution to several Kbs by the technique of matrix or array CGH, in which the targets are cloned DNA fragments immobilized on the glass surface. This allows detection of low copy number gains and losses and may be used diagnostically to identify microdeletions or amplifications affecting only one or two genes.

Cancer cytogenetics has benefitted greatly from FISH technology, and hence the clinical laboratories have benefitted from the technique, since it is rapid and can be
performed on tissues (fresh frozen or formalin-fixed paraffin-embedded), touch preparations, cytospins, or cell cultures. Since it is usually difficult to get chromosome spread from tumor cells, the use of interphase FISH directly on tumor samples (biopsies, section, and archived paraffin-embedded material) enables the determination of chromosomal aberration without the need for interphase chromosomes preparations. Numerical chromosome aberrations, chromosome deletions, and translocations can all be identified in interphase nuclei providing important diagnostic and or prognostic information.

The advent of spectral dyes and imaging has made FISH more colorful and even more powerful. Using multiple probes simultaneously provides important additional information that can now be obtained for a single sample using multicolor FISH techniques. The techniques allow for both a genome-wide screen of aberrations and a gene or chromosomal regain-specific analyses of specific aberrations in chromosomes and can be adopted for use in the analysis of interphase nucleic. Similarly, genome-wide screen for mRNA expression differences or for genomic aberrations can be performed by microarray FISH, which is based on the comparative hybridization of two samples onto arrays that represent either specific sets of genes or the whole genome. The targets used come as oligonucleotides, cDNA, or genomic arrays.

Glossary

A

**Allopolyploid** An individual or strain whose chromosomes are composed of more than two genomes, each of which has been derived more or less complete but possibly modified from one of two or more species.

C

**Chromosome painting** The use of fluorescent-tagged chromosome-specific dispersed repeat DNA sequences to visualize specific chromosomes or chromosome segments by in situ DNA hybridization and fluorescence microscopy.

**Confocal microscopy** It is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light. It enables the reconstruction of three-dimensional structures from the obtained images.
Hapatens are small molecules that elicit an immune response only when attached to a large carrier such as a protein; the carrier may be one that also does not elicit an immune response by itself.

Immunodetection The use of antibodies to identify proteins or other chemicals.

Neoplasia The presence or formation of new, abnormal growth of tissue.

Nick translations Nick translation is a tagging technique in molecular biology in which DNA polymerase I is used to replace some of the nucleotides of a DNA sequence with their labeled analogues, creating a tagged DNA sequence which can be used as a probe in fluorescent in situ hybridization or blotting techniques. It can also be used for radiolabeling.

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