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Fluorescence in-situ Hybridization: Technology to Detect Genetic Mutation

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ABSTRACT

Introduction: Karyotyping is the traditional method to detect the genetic mutation. Perhaps it has some limitations like tedious, low resolutions of results and strenuous. The fluorescence in situ hybridization technique brings a new era in molecular biology which is based on the complementary Deoxyribonucleic acid or Deoxyribonucleic acid/Ribonucleic acid strands. It is a combined approach of molecular and cytological to study chromosome structure and function as well as to detect the specific genes sequences.

Objectives: To explore more knowledge about applications and different types of FISH. Due to the accuracy and versatility of FISH, it is widely used in cancer research to detect the various types of mutations, diagnostic and research fields. In this article, we briefly introduced MFISH (Multicolour FISH), QFISH (Quantitative FISH) and RNA FISH.

Method: The first step is to make short sequences of single-stranded DNA that should match a portion of the gene that is of interest. Now the tissue should be fixed by different fixers. Before hybridization, probes and sample sequences are denatured. To detect hybridized probes, different techniques are used.

Result: As probes are labelled with different reporters, they can be detected by conventional light microscopy, fluorescence microscopy, etc.

Conclusion: FISH and its types have a wide range of applications in the diagnostic field, genetic research, biomarker research and personalized medicines.

Key Words: Genetic mutations, Fluorescence In Situ Hybridization, Probes, MFISH, RNA FISH, Q FISH

INTRODUCTION

Human cells are estimated to contain approximately 10 double-strand breaks per cell cycle as estimated by the incidence of spontaneous sister chromatid exchanges by the DNA replication process which is remarkably accurate. If it is left unrepaired, such damage results in the loss of chromosomes and/or the induction of cell death. If imprecisely repaired, the damage leads to mutations and chromosomal rearrangements.¹

During the cell cycle, DNA double-strand breaks are readily mediated to alter the superhelical state in DNA by ubiquitous enzymes also known as topoisomerases. For this enzyme temporary cleavage is essential to carry out their primary cellular functions including their roles in DNA rep-

lication, transcription, segregation, the maintenance of genome stability and chromosome condensation. However, these enzymes can promote chromosomal aberrations by illegitimate recombination. Through disincorporation during DNA replication or exposure to exogenous mutagens such as ionizing radiation or endogenous mutagens, mutations can occur in the genomes of all dividing cells. The diversity of mutations (aberration) that can initiate human cancer. Intragenomic mutations highlighted are small nucleotide changes that may be inherited by the cancer progeny from the progenitor cell, resulting in either a loss-of-function of genes or a gain-of-function of genes.

Mutated genes are identified in cancer called oncogenesis. Cancers are caused by various types of mutations shown in fig.1.²

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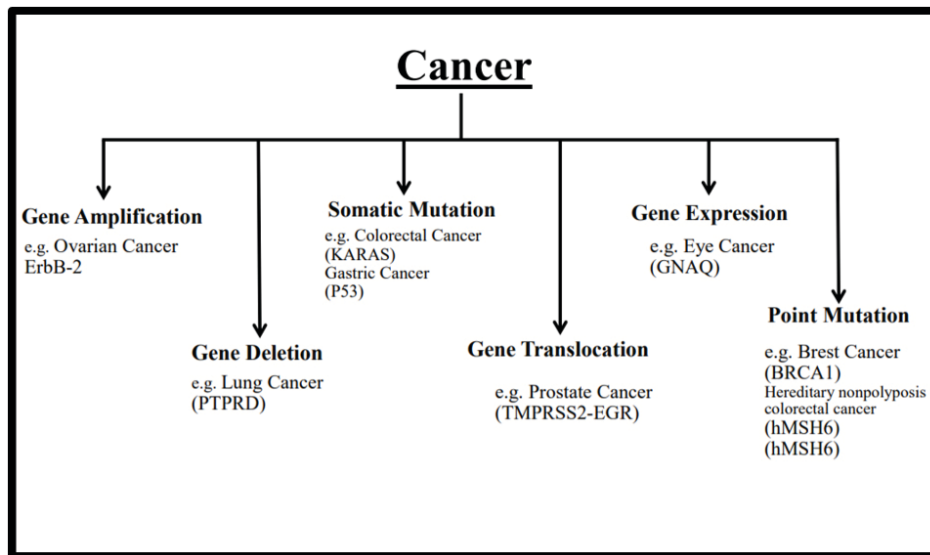


Figure 1: Type of Mutation

karyotyping is considered as the gold standard method which is used to confirm the presence or absence of mutations but has some considerable limitations such as low resolution of the cell-treated field, time-consuming and laborious cell cultivation, and in the detection of chromosome abnormalities which are less than 5 mb in length. FISH became a powerful technique that has high sensitivity and specificity as well as with the performance speed it has considered as a clinical laboratory tool.³

The objective of fluorescence In-situ hybridization is to determine the presence or absence of DNA or RNA sequences of interest and to localize these sequences to specific cells or chromosomal sites as well as small deletions and duplications. It offers the possibility to specifically mark individual chromosomes over their entire length or defined chromosome regions in meta- and interphase preparations. 'Chromosome painting competitive hybridization was one of the first applications of FISH using entire chromosome-specific libraries for chromosomes as probes and human genomic DNA as the competitor.'⁴

MECHANISM OF FISH

The main steps of FISH are the binding of fluorescently labelled target-specific nucleic acid probes to their complementary DNA or RNA sequences. Also the visualization of these probes within cells in the tissue of interest. The steps of FISH are as below.⁵

1. PREPARATION OF PROBE AND TARGET SEQUENCE:

Probe and a target Sequence are the basic elements of any FISH technique. The first step is to make short sequences of single-stranded DNA that should match a portion of the gene that is of interest. These are called probes. After cooling of strands, they will anneal with complementary nucleotides making bonds back together with their homologous partners. The higher the number of nucleotides in a probe, it will mostly attach with its homologous target sequences. The choice of the probe is the important thing that must take account in the FISH analysis. There is a wide range of probes available, from whole genomes to small cloned probes (1–10 kb).⁶ (Table:1)

Table 1: Types of probes

TYPES OF PROBE	SIZE	MECHANISM OF PREPARATION
Double-stranded DNA probe	100 – 1000bp	PCR with labelled nucleotide, random primer and nick translation with enzymes like Deoxyribonuclease I and DNA polymerase-I are used.
Single-stranded DNA probes	200-500bp (larger than oligonucleotide probe)	An amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer, reverse transcription-polymerase chain reaction (rPCR) of RNA, and chemical synthesis of oligonucleotides are used.
RNA probes(cRNA probes or riboprobes)	200–600 bases	By using RNA polymerase enzyme a linearized template(DNA) is formed by in vitro transcription method.
Oligonucleotide probes	20-40bp(shorter)	An automated chemical synthesis is used to form oligonucleotide probes.

Now, these probes are usually directly or indirectly labelled.

1. Directlylabelled: Fluorochromes are directly attached to probes.
2. Indirectly Labelled: Here a hapten (such as biotin or digoxigenin) is used for attachment. In indirect labelling, for detection fluorescently labelled antibodies (such as

streptavidin and anti-digoxigenin) are used. Direct labelling is faster than indirect labelling. But in indirect labelling, there is the advantage of signal amplification by using several layers of antibodies and because it is produced by indirect labelling brighter compared with background levels.

Table 2: Properties of Probes

NAME OF PROPERTY	
Construction of probe	It should be highly specific and single-stranded.
Method of labelling	The Klenow fragment of DNA polymerase I is used on heat-denatured DNA(Random primer method) is better than the nicking of DNA with DNAase I and incorporation of nucleotides is done by DNA polymerase I(Nick translation).
Content(%) of G-C base pair	Should be higher.
Strength of RNA versus DNA probes	In decreasing order RNA-RNA, DNA-RNA, DNA-DNA.
Length of probe	Should be shorter.

FIXATION OF TARGET SAMPLE

The fixation methods are formalin-based, paraffin-based or freshly prepared frozen tissue. For adherence of tissue sections, a specially treated glass slide is used to ignore the loss of tissue during the hybridization process. For this purpose, various “adhesives” are available like poly-L-lysine, gelatin chrome alum and aminopropyl-triethoxysilane. For fixation of metaphase chromosome spreads methanol/acetic acid is used. For sections like cryostat 4% formaldehyde (~30 min), Bouin’s fixative, or paraformaldehyde vapour fixation may be used. Most often, tissue specimens are regularly fixed in 10% buffered formalin, procedures overnight in an automatic tissue processor, and embedded in paraffin wax. The optimal fixation time is 8-12 hours. Sections are voided well and then air-dried at room temperature. After de-paraffinization, slides are put down in an alcohol-cleaned staining container of diethylpyrocarbonate water. The staining container is then put down in the heated water bath at 23-37°C. The formaldehyde-based fixatives are used before paraffin embedding to cover up nucleic acid sequences. Digestion enhances probe penetration by expanding cell permeability with minimal tissue degradation.

DENATURATION OF PROBE AND TARGET SEQUENCE

Both the labelled probe and the target DNA are denatured. The annealing of complementary DNA sequences occurring because of the combining sequences of the denatured probe and target DNA. In indirect labelling, one extra step is required for visualization of the non-fluorescent hapten that requires an enzymatic or immunological detection system. Although, the FISH probes are selected according to the diseases or anomalies under the field of interest. Before hybridization, if both the target and the probe are double-stranded,

they must be denatured to make them single-stranded and this can be attained by heat or alkali treatment.⁷

HYBRIDIZATION

In the molecular hybridization process, a single-stranded target sequence is annealed to a complementary single-stranded probe to make a double-stranded hybrid. In the denaturation process,

Single-stranded target and probe sequences are incubated in a hybridization mixture, which supplies an optimal environment for re-annealing of single-stranded sequences. Hybridization is done after denaturation. During cooling, a complementary probe and target sequence forms hydrogen bonding of the two strands of nucleic acids. The probe must make stable hydrogen bonds with the target. At the same time heating the probe and target to high temperatures may increase the stability and sensitivity of detection. For this, care must be taken to fully control this step of the FISH procedure.

DETECTION

For the detection of hybridized probes, enzymatic reactions that generate a coloured precipitate at the site of hybridization is used. The most often used enzymes for this application are alkaline phosphatase (AP) or horseradish peroxidase (HRP). The radioactively labelled probes are detected by autoradiography. This detection procedure is based on the emission of fast electrons or beta-particles from the probe. Autoradiography for radioactive labels is reported to be more sensitive than the immune enzyme systems. After hybridization, fluorophores can be connected with nucleic acid probes by chemical conjugation to the nucleic acid or chemical conjugation of the nucleic acid with a non-fluorescent molecule that can attach fluorescent material. There are four common fluorophore classes-fluoresceins,

rhodamines, cyanines, or coumarins used. For the detection of tumour morphology, conventional light microscopy and fluorescence microscope is also used. For the hapten detection im-

munohistochemically by a fluorophore-tagged antibody against the hapten is used.⁸

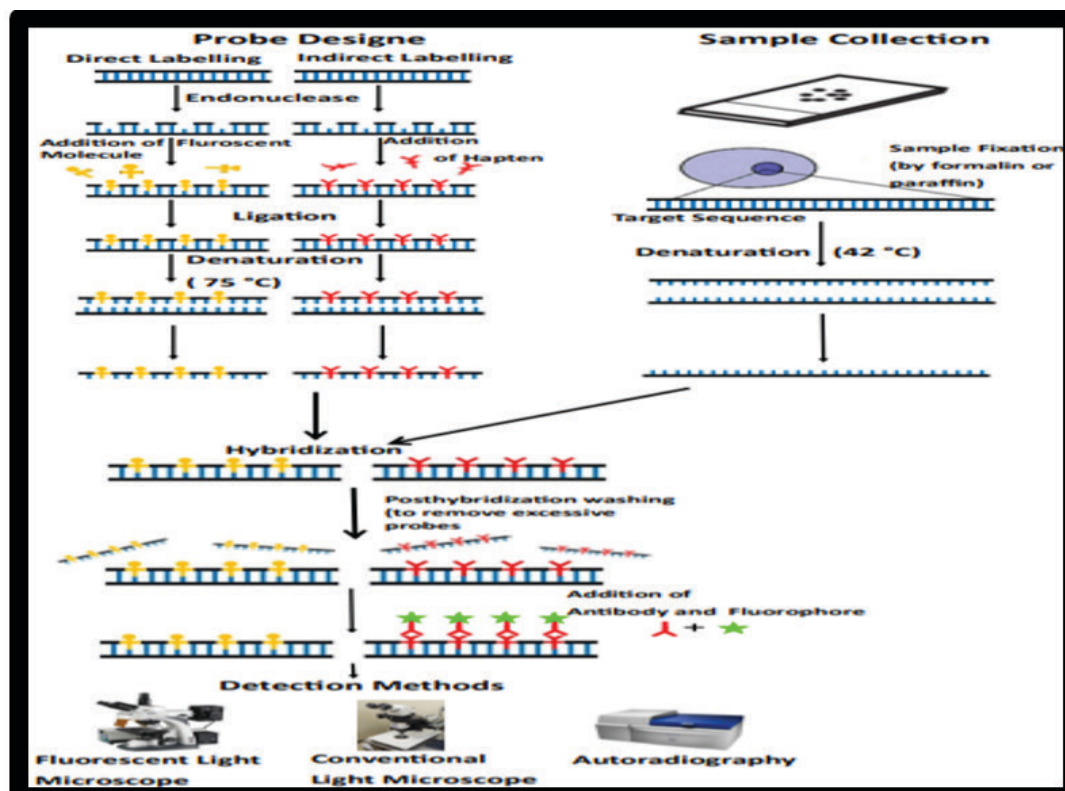


Figure 2: Steps of FISH

TYPES

The diversification of real FISH protocol forms different types of FISH. Here the Table.3 represents the types of

FISH, mostly used probes by these types and their functions.

Table 3: Types of Fish, Probes and Functions⁹

Sr. No	Different Types	Function	Probes
1.	Arm Fish	In this type, a 42-colour M-Fish variant allows the detection of chromosomal abnormalities at the resolution of chromosome arms (P and Q arms).	DNA probes
2.	ACM Fish	Used for the simultaneous detection of numerical and structural chromosomal abnormalities in sperm cells.	DNA probes
3.	CB – Fish	This involves hybridization on binucleated cells in which cytokinesis has been blocked by treatment with cytochalasin -B.	DNA probes
4.	Cat Fish Cellular Compartment analysis of temporal (CAT)	It is an ingenious experimental approach devised to investigate the dynamic interactions of neuronal populations associated with different behaviour of cognitive challenges.	DNA probes
5.	CO – Fish Chromosome Orientation	It was designed to determine repeats within centromere regions of chromosomes.	SSDNA probes
6.	CARD Fish Catalyzed reporter deposition – Fish	It stands for catalyzed reporter deposition Fish, refers to the signal amplification obtained by peroxidase activity through the deposition of a large number of fluorescently labelled tyramine molecules in which the horseradish peroxidase (HRP) – the labelled probe has bound.	polyribonucleotide probes and oligonucleotide probes

TABLE 3: (Continued)

Sr. No	Different Types	Function	Probes
7.	COBRA FISH	The prefix COBRA stands for combined binary ratio, which brings together combinatorial labelling.	DNA probes
8.	Comet Fish	A combination of the comet assay and Fish analysis. The comet assay also called the single-cell gel test, is used to evaluate the amount of DNA breakage within single cells by running the DNA out of the nuclei into an agarose gel.	DNA probes
9.	COD Fish	This fish is an abbreviation that has been used to describe three different hybridization techniques the most common use is for chromosome orientation and direction.	DNA probes
10.	DBD Fish	It detects any sites of DNA damage/breakage in the sample genome.	DNA probes
11.	D- Fish	An enhanced version of the fusion signal Fish protocol for the detection of recurring chromosomal translocations in haematological malignancies.	DNA probes
12.	Fusion signal	In peripheral blood and bone marrow, 9:22 Philadelphia translocation is detected by this FISH.	DNA probes
13.	Flow-fish	It is used to visualize and measure the length of the telomere.	PNA-labelled telomere probes
14.	Fibre Fish	It is used for the mapping of genes and chromosomal regions on fibres of chromatin or DNA.	DNA probes
15.	Harlequin Fish	In this type, there is a method for cell cycle controlled chromosome analysis in human lymphocytes that allows precise quantification of induced chromosome damage for human biodosimetry purposes.	DNA probes
16.	ImmunoFish	It is a combination of two techniques one being standard Fish either on flattened chromosome preparation (2- D Fish) or on three-dimensionally preserved nuclei (3 - D fish) and the other indirect or direct Immuno - Fluorescence.	dual-fusion probes
17.	Multilocus or ML fish	Used for identifying multiple microdeletion syndrome in patients.	locus-specific DNA probes
18.	M -Fish (Multiplex - Fish)	The invention of M-Fish (or Multiplex - Fish), a protocol for 24 - colour karyotyping, based on combinatorial labelling and aimed at facilitating the analysis of complex chromosomal rearrangements and marker chromosomes has signified in molecular cytogenetics, particularly for the study of tumours and prenatal diagnosis.	Chromosome specific DNA probes
19.	PCC Fish	This is used for bio- dosimetry analysis that relies on the use of chromosome-specific painting probes after irradiation.	chromosome-specific painting DNA probes
20.	Q Fish	Used for determining the repeated number of the telomere on a specific chromosome.	PNA probes
21.	QD Fish	Used to detect human metaphase chromosomes, human sperm cells, bacterial cells and subcellular mRNA distribution in tissue sections.	oligonucleotide probes
22.	Raman Fish	It is a new technique that combines Fish technology with Raman microspectroscopy for Ecophysiological investigations of complex microbial communities.	16S rRNA probe
23.	RING Fish	Identification of individual genes and detection of halo appearance from fluorescence signals at the bacterial cell at the periphery.	16S rRNA-based polynucleotide probes
24.	Reverse Fish	For characterization of chromosomes and chromosome amplifications in cancer.	Chromosome specific DNA probes
25.	RNA Fish	This is a method that allows the detection of RNA within cells transcripts can be visualized either in the nucleus or in the cytoplasm.	RNA probes
26.	T - Fish	Tyramide is a compound that binds to peroxidase easily and thus has been used to increase the sensitivity greatly in fish experiments with the use of only one or two layers of reagents for visualization.	DNA probes and oligonucleotide probes

Among all these types, some types have major importance in the scientific field. These types are M FISH, Q FISH and RNA FISH.

MFISH (MULTICOLOUR FISH)

By using different colours, FISH contains the ability to find several regions or genes simultaneously. Multicolour FISH, which can locate many different probes on chromosomes simultaneously, is an important branch of FISH techniques. Using M-FISH to construct FISH karyotype has been applied in many plant species, for example, *Lotus japonicus*, *Pinus sp.*, *Picea abies* and *Silenelatifolia*. Ribosomal RNA genes (rDNAs) belong to a highly conservative repeat sequence family with hundreds of copies and locate on one or more pairs of chromosomes. FISH mapping of rDNA on chromosomes can provide important clues for molecular markers of karyotype analysis, the evolution of karyotype and phylogenetics research.¹⁰

The power of M-FISH (and SKY) lies in its ability to resolve complex karyotypes and identify the origin of marker chromosomes as evidenced by its many applications in tumour diagnostics and research, evolutionary cytogenetics and the study of chemical and radiation-induced aberrations. Impressively, even quite complex karyotypes of individual cells within a nonclonal population can be resolved with a high degree of confidence. One should give more attention before choosing probes in FISH analysis. The pe of the chromosome. This type of probes are flow-sorted (or microdissected), amplified and labelled by an oligonucleotide polymerase chain reaction to generate a 'paint' that highlights the entire chromosome.¹¹

QFISH (QUANTITATIVE FISH)

Telomeres are nucleoprotein like structures, which cap and protect linear chromosome ends. They comprise ~1500 TTAGGG repeats, a single strand overhang and a complex of six shelters in proteins. Telomeres prevent chromosomes from degradation and fusions by preventing the ends from being falsely recognized as DNA double-stranded breaks. However, the intrinsic inability of the replicative machinery to complete replication of linear DNA leads to progressive telomere reduction upon cell division which imposes a limit on the number of times a cell can divide. 90% of cancer cells survive this crisis due to upregulating telomerase, the enzyme that lengthens telomeres. Still, in stem and progenitor cells, telomerase activity is crucial for tissue maintenance and regeneration.¹²

Telomeres may represent a powerful readout of oxidative stress, a potential biomarker of exposures and disease risk.

Three common techniques are widely used by laboratories to measure telomere length. The telomere restriction fragment (TRF) analyzed by the southern blot is considered the gold standard. Although this technique only yields average telomere length, generally requires one to three micrograms of genomic DNA. The quantitative Polymerase chain reaction is another common method that requires very low amounts of DNA but yields a ratio of telomeric DNA content relative to a single-copy gene. Telomere length analyzed by quantitative fluorescence in situ hybridization (qFISH) on metaphase chromosomes provides the ability to examine the length and integrity of individual telomeres. Q-FISH allows for the evaluation of the telomere length heterogeneity within the cell population and distinguishing of critically short telomeres. Moreover, qFISH also offers the ability to assess telomeric aberrations like telomere fragility, losses and fusions, associated with any illness, hence allowing the recording of multiple telomere parameters in a single experiment.¹³

Q-FISH uses peptide nucleic acid (PNA) probes; they are synthetic molecules formed by DNA bases linked to a backbone by peptide bonds. Telomeric PNA probes can be Supportive to the G-rich or to the C-rich telomeric DNA strand which provides the possibility for simultaneous application and two-colour staining required for chromatid orientated FISH.¹⁴ In this context, Q-FISH is specifically relevant because it can identify and quantify critically short telomeres.

RNA FISH

RNA molecules have an extensive range of characters in the cell. RNAs control all features of cell metabolism and thus be important regulators of physiologic and disease activity. Genome is crowded with a huge amount of long noncoding RNAs (lncRNAs) that have been correlated with a large number of cellular activities like differentiation and the beginning of the disease. RNA fluorescence in situ hybridization (RNA FISH) is a technique that provides a convenient supplement to label RNA molecules in the cell by labelled nucleic acid probes hybridization with target RNA for the detection of RNA.

Transcripts are present either in the nucleus or in the cytoplasm. The method also called expression-FISH, has been used to examine the transcriptional action taken by endogenous genes, exogenous genes like those belonging to integrated viral genomes and transgenes. RNA-FISH has also been used in studying different functional aspects of genome organization and nuclear architecture, also as a prenatal diagnosis tool for myotonic dystrophy type.

Single-particle RNA FISH depends on pools of short, marked DNA oligonucleotides that can identify singular atoms of RNA in situ, by managing its affectability to distinguish even low abundance lncRNA. In addition, it

is enabling quantification of the number and area of each target molecule inside the cell. Specialists have utilized single-atom RNA FISH to recognize lncRNA with progress and in two cases, it has demonstrated basic to unravelling the system of activity. In an ongoing methodical investigation of the use of single-particle RNA FISH to lncRNA, researchers found that lncRNAs can introduce special difficulties regarding recognizing nonspecific background from valid signals.¹⁵

APPLICATIONS

There are diverse applications on FISH based which are from different fields of investigation, including clinical genetics, evolutionary biology, neuroscience, cellular genomics, toxicology, microbial ecology, reproductive medicine, comparative genomics, and chromosome biology. FISH consumes less time than other techniques so makes easy diagnosis and also increases the life span of people.

Table 4: Application for diagnosis¹⁶

Application in diagnosis	Symptoms	Use of FISH
Histiocytoid Sweet Syndrome	Fundamental hematologic myeloid issue or strong tumour malignancies or fiery gut illness or gastrointestinal lot or upper respiratory parcel diseases.	To decide the nearness of the BCR/ABL quality combination. FISH led to a survey of the nearness of a chromosomal irregularity in the cutaneous invade of the underlying biopsy example.
Pseudomosaicism from True Mosaicism Differential Diagnosis	Chromosomal abnormality and clinical miscarriage.	FISH can be utilized as an indispensable apparatus for a pseudo mosaicism and mosaicism differential determination in isochromosome 20q location.
Dedifferentiated Liposarcoma (DDLPS)	Pain, Swelling, Weakness, constipation.	MDM2 fluorescence in situ hybridization gave astounding information to recognizing the sicknesses.
Streptococcus Pneumonia	Bacteremia in kids and grown-up.	FISH strategy can recognize <i>S. pneumoniae</i> in blood culture without enzymatic treatment.
Aneuploidies	Severe microcephaly, eye abnormalities, developmental delay, mild physical abnormalities, problems with the brain and central nervous system, seizures, and intellectual disability.	Identification of chromosome flags in interphase cores is conceivable.
Chronic Myeloid Leukemia (CML)	Fatigue, anaemia, malaise, or night sweats, bleeding, bruising, reduced ability to exercise, shortness of breath, or weight loss.	Distinguishing these chromosomal movements, very well may be utilized as an imperative apparatus in choosing a focused treatment in various leukaemias.
Multiple Myelomas (MM)	Pain in the back or bones anaemia, fatigue, or loss of appetite, constipation, hypercalcemia, increased risk of infection, kidney damage, or weight loss.	FISH is attention for investigation of interphase cores and small chromosomal deviations, which are perceived as the majority of energetic hereditary tests for representation of cytogenetic variations from the norm in MM.
Pulmonary Adenocarcinomas	Raspy voice, Chest pain, Shortness of breath, Unexplained weight loss, Cough that produces blood.	EML4-ALK quality combination can be recognized through FISH.
Prostate Cancer	Pain in the bones, excessive urination at night, difficulty starting and maintaining a steady stream of urine, frequent urination, urge to urinate and leaking, urinary retention, or weak urinary stream.	A four-concealing FISH test was used for the acknowledgement of either TMPRSS2 or ERG modifications.
Breast Carcinomas	Breast discomfort, lumps, inverted nipple, or nipple discharge, redness, swollen lymph nodes, or thickening or puckering of the skin.	FISH examines are utilized for estimating HER2 overexpression.
Renal Mesenchymal Neoplasm	flank pain, an abdominal mass, and hematuria.	With needle biopsy conclusion can be troublesome; here, immunohistochemistry and in-situ hybridization are used for the exact finding.
Cholangiocarcinoma (CC)	Pain areas: in the abdomen Whole-body: fever or weakness	FISH technique on brushing smears can detect numerical and structural deformity of four chromosomes in patients having documented extrahepatic CC.
Melanoma	Bigger mole diameter, more colour changes, darkening of the skin, or skin mole with an irregular border.	Four tests are focusing on 6p25 (RREB1), 6q23 (MYB), 11q13 (CCND1), and centromere 6 (CEP6) are used.

Table 5: Application in research field ¹⁷

Application in research	Description
New non-random abnormalities identification (by M-FISH or SKY)	SKY technique could potentially help delineate the more complex chromosome aberrations seen in MM and provide new clinical insights.
Gene mapping	In species for which the genome has not been sequenced, FISH and related in situ hybridization methods continue to provide important data for mapping the positions of genes on chromosomes.
Identification of regions of amplification or deletion by CGH	CGH is used as a discovery tool. CGH has also been significant for the analysis of haematological malignancies in the identification of (previously unrecognized) high-level amplifications, particularly in chronic lymphocytic leukaemia, non-Hodgkin lymphoma and as an aid to classification schemes for lymphomas.
The identification of translocation breakpoints	The multicolor FISH analysis identified an array of breakpoints responsible for locus- and region-specific translocations.
The study of 3D chromosome organization in interphase nuclei	Fish in cells with preserved 3D nuclear structures (3D-FISH) is useful for studying the organization of chromatin and localization of genes in interphase nuclei.

CONCLUSION

Fish has been developed to the next level and is a powerful technique for detecting mutation and alternation in gene expression at the microscopic level. Moreover, the application of Fish lies in the diagnostic and research. The use of fish is a game-changing method for the diagnosis of diseases like cancer as where chromosomal abnormality detection became very crucial for further treatment and monitoring of disease. Novel FISH techniques and types like M FISH, Q FISH, and RNA FISH are introduced with their wide range of applications that have been discussed in genetic research. The single-molecule FISH technique in cell-based genetic diagnosis is expected to enhance the capacity of the spectrum of genetic defaults from chromosomal and genetic abnormalities. Also, this technique helps to develop biomarker research and personalized medicine.

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Authors Contribution:

Dave Kruti: Overall review and compilation

Prajapati Mitali: Methods and M-FISH

Pathak Deepika: Q-FISH

Alisagar Luluaa: R-FISH

Suthar Krutish: Application

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