Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 9[12] November 2020 :195-208

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.876
Universal Impact Factor 0.9804
NAAS Rating 4.95

REVIEW ARTICLE **OPEN ACCESS**

An overview of the Fluorescence in situ hybridization technique: (Detection of Genetic Mutation)

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ABSTRACT
Fluorescence in situ hybridization technique brings a new era in molecular biology which is based on the complementary DNA or DNA/RNA strands. Probes are made up of complementary sequence and coupled with fluorophore to hybridize with complementary sequence of targeted cells. The results can be visualized by fluorescence light microscopy, conventional light microscopy and autoradiography. It is a combined approach of molecular and cytological to study chromosome structure and function as well as to detect the specific genes sequences. The unique ability of FISH technique is to provide high degree of visualization between DNA analysis and chromosomal investigations while keeping information at the single-cell level. Due to accuracy and versatility of FISH, it is widely used in cancer research to detect the various types of mutations. It has been used in diagnostic and research field. The general use of FISH is to detect one specific region of chromosome by using very small chemical that glows brightly when it detects the specific region on a chromosome. Along these years there are many types of FISH have been discovered which are used for different purposes. In this article we briefly introduced MFISH(MulticolourFISH), QFISH(QuantitiveFISH) and RNA FISH. MFISH is the only type of FISH in which different colours are used to identify more than one regions and genes at the same time. QFISH is mostly used for telomeres to examine length and detect mutations. RNA is detected by RNA FISH. This article explains principle of FISH, common mechanism of FISH, types of FISH with their functions and used probes also applications of FISH in diagnostic and research.
Key Words: *Genetic mutations, Karyotyping, FluorescenceIn Situ Hybridization,Probes, MFISH, RNA FISH, Q FISH*

Received 10.09.2020 Revised 19.10.2020 Accepted 11.11.2020

INTRODUCTION

Chromosomes are organized during interphase into domains and consist DNA, histones and non-histone proteins. In most cells, only during the process of cell division chromosomes are visible by microscopy. G0/G1 chromosomes contain one DNA molecule which gives two sister chromatids by replication that are both replicas of the original DNA molecule in the S phase of the mitotic cell cycle. 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 DNA replication process whichis remarkably accurate. If it 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. These DNA double-strand breaks (which may result in gene malfunction) are considered to be critical primary lesions in the formation of chromosomal aberrations and can occur in both somatic and germ-line cells[1,2].

During the cell cycle, DNA double-strand breaks are readily mediated to alter the super helical 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 replication, transcription, segregation, the maintenance of genome stability andchromosome condensation. However, these enzymes can promotechromosomal aberrations by illegitimate recombination[3]. Through misincorporation 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. Intragenetic 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[4].

Clonal proliferations that arise from an accumulation of mutations and other heritable changes that confer selective growth advantages in susceptible cells lead to cancers. To identify the mutated genes that are causally implicated in oncogenesis is the main aim of cancer. So far, abnormalities in about 350 genes (more than 1% of our genome) have been implicated in human cancers, but the true number is unknown. This illustrates striking features in the types of sequence alteration and protein domains that are encoded in the cancer classes in which oncogenic mutations have been identified. Cancers are caused by various types of mutations shown in fig.1[5].

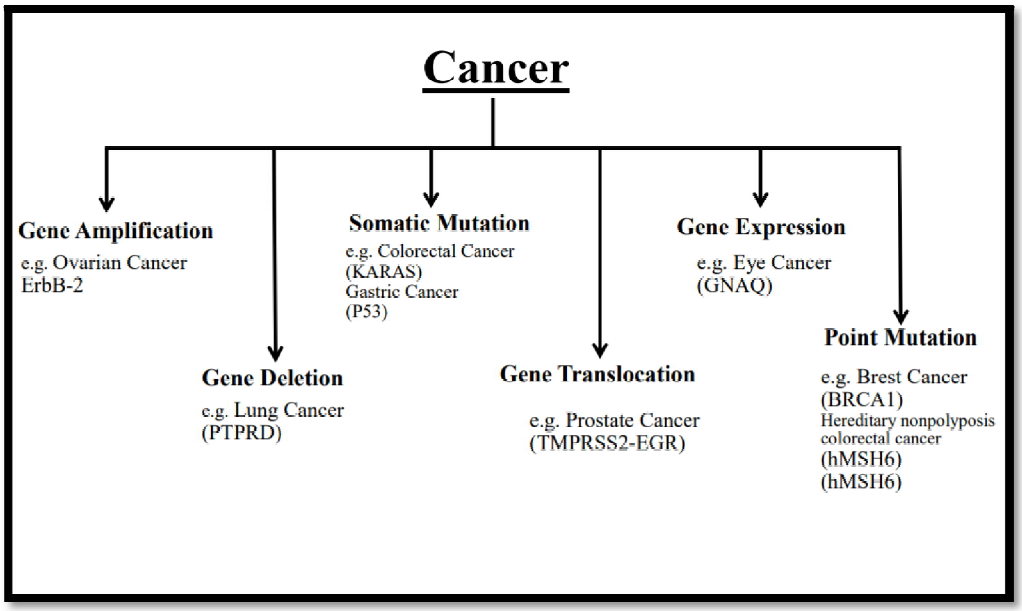


Fig:1 Type of Mutation

karyotyping is considered as the gold standard method which is used to confirm the presence or absence of mutations through counting the number of chromosomes and looking for structural changes(chromosomal analysis)[6]. In spite of practicability of karyotyping as a cytogenetic traditional method for prenatal diagnosis of chromosomal aberrations in laboratories, it also has some considerable limitations such as low resolution of the cell-treated field, time-consuming and laborious cell cultivation, and in detection of chromosome abnormalities which are less than 5mb in length.[7]

Chromosome banding techniques (Giesma staining) revolutionized into cytogenetic analysis and have been pivotal in the understanding of genetic changes in both constitutional and acquired diseases. However, the resolution of banding analysis is such that it can only detect rearrangements that involve 3Mb of DNA. Banding techniques are limited to mitotically active cells with the additional problem of having difficulties in deciphering highly rearranged chromosomes using a monochrome banding pattern. In the late 1980s, the FISH considered as a technique that can readily detect trisomies and translocations in metaphase spreads and interphase nuclei using entire chromosome-specific DNA libraries, was heralded as a further revolution in cytogenetic analysis[8].The high sensitivity and specificity of FISH and the speed with which the assays can be performed have made FISH a powerful technique with numerous applications, and it has considered as a clinical laboratory tool.[9]

The objective of fluorescencein 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.[10] FISH offers the possibility to specifically mark individual chromosomes over their entire length or defined chromosome regions in meta- and interphase preparations. It can be used to detect small deletions and duplications that are not visible. It can also be used to detect how many chromosomes of a certain type are present in each cell and to confirm rearrangements that are suspected after microscope analysis. FISH looks specifically at the one specific area of a chromosome only. 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, 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 labelled the annealed hybrids can be detected by

various methods, including isotopic and nonisotopic (fluorescent and nonfluorescent) approaches. FISH is an effective technique that enables direct visualization of genetic alterations in the cell[11].
'Chromosome painting'competitive hybridizationwas one of the first applications of FISH using entire chromosome-specific libraries for chromosomes asprobes and human genomic DNA as the competitor. It provided intenseand specific fluorescent staining of human chromosomes in metaphase spreads and interphase nuclei, allowing the distinctive identification of chromosomes involved in complex rearrangements. The advent of the Human Genome Project has made available a repertoire of single-locus probes that have provided a significant role to gene mapping strategies and led to the identification of the breakpoints of consistent translocations.[12] In the case of FISH, the target is the nuclear DNA of either interphase cells or of metaphase chromosomes affixed to a microscope slide, although it can also be performed using bone marrow or peripheral blood smears, or fixed and sectioned tissue. Once fixed to a microscope slide, the desired cells are hybridized to a nucleic acid probe.[9] This anneals to its complementary sequence in the specimen DNA and is labelled with a reporter molecule which is either an attached fluorochrome, enabling direct detection of the probe via a coloured signal at the hybridization site.[13]

MACHANISM 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.[14]

1. PREPARATION OF PROBE AND TARGET SEQUENCE:
Probe and a target Sequence are the basic elements of any FISH technique. A probe is a labeled complementary single strand that is related with the DNA/RNA strands of interest. 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.[17] 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 probe, it will mostly attach with its homologous target sequences. [16] The choice of probe is the important thing that must take account in FISH analysis(Types of probes are described in table.1). There is a wide range of probes available, from whole genomes to small cloned probes (1–10 kb).[18]

TYPES OF PROBE	SIZE	MECHANISM OF PREPARATION
Double stranded DNA probe[19]	100 – 1000bp	Methods like PCR with labelled nucleotide, random primer and nick translation with enzymes like Deoxyribonuclease I and DNA polymerase I are used.
Single stranded DNA probes[16]	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 to make ssDNA probe.
RNA probes(cRNA probes or riboprobes)[16]	200–600 bases [20]	By using RNA polymerase enzyme a linearized template(DNA) is formed byin vitro transcription method.
Oligonucleotide probes[21]	20-40bp(shorter)	An automated chemicalsynthesis is used to form oligonucliotide probes.

Table 1 : Types of probes

Now these probes are usually directly or indirectly labelled. 1.Directlylabelled: Fluorochromes are directly attached to probes.2.Indirectlylabelled: Here a hapten (such as biotin or digoxigenin) is used for attachment. In indirect labeling, for detection fluorescently labeled antibody (such as strepavidinand antidigoxigenin) is used [22]. Direct labelling is faster than indirect labeling. But in indirect labelling, there is the advantage of signal amplification by using several layers of antibodies and because of it is produced by indirect labeling brighter compared with background levels.[23]

NAME OF PROPERTY	
Construction of probe	It should be high specific and single-stranded.(oligonucleotide probes are better than others.)
Method of labeling	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 for melting temperature.
Strength of RNA versus DNA probes	In decreasing order RNA-RNA, DNA-RNA, DNA-DNA.
Length of probe	Should be shorter so that it can attach with interested sequence easily.

Table: 2 PROPERTIES OF PROBES[19]

2. FIXATION OF TARGET SAMPLE:

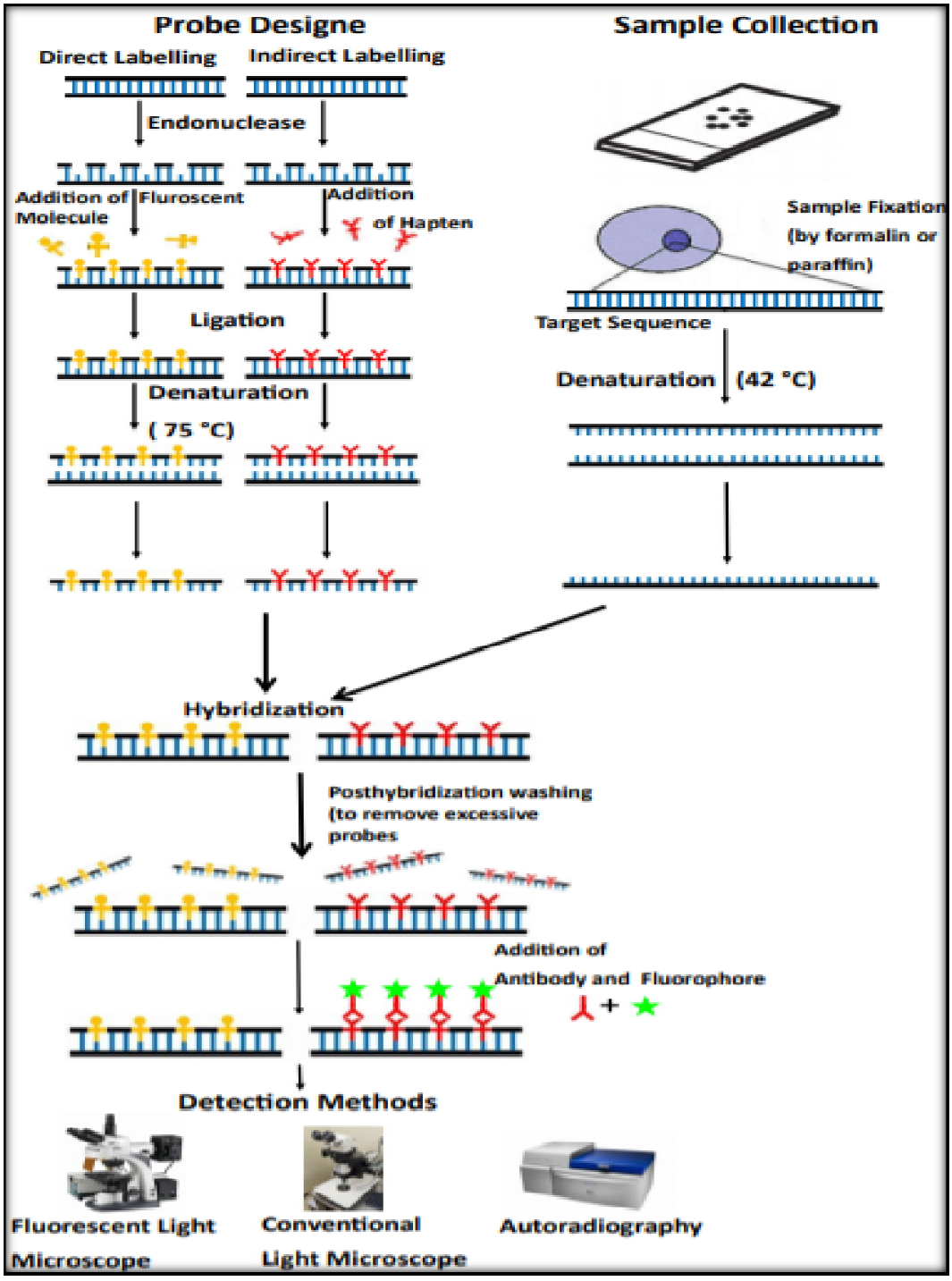
The tissue of interest can either be formalin-fixed, paraffin-embedded sections or fresh-frozen tissue[24]. For adherence of tissue sections a specially treated glass slides is used to ignore loss of tissue during the hybridization process. For this purpose various "adhesives" are available like poly-l-lysine, gelatin chrome alum and aminopropyl-tri-ethoxysilane .For fixation of metaphase chromosome spreads methanol/acetic acide is used. For sections like cryostat 4% formaldehyde (~30 min), Bouin's fixative, or paraformaldehydevapor fixation may be used. This type of fixation also helps to settle the tissue to the slide. Most oftenly, tissue specimens are regularly fixed in 10% buffered formalin, procedured overnight in an automatic tissue processor, and embedded in paraffin wax.The optimal fixation time is 8-12 hours. By using positively charged or hand-coated slides sections are cut at 4-6 µm on an alcohol-cleaned microtome. 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 diethyl pyrocarbonate 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 cover up nucleic acid sequences. Digestion enhances probe penetration by expanding cell permeability with minimal tissue degradation.[16]

2. DENATURATION OF PROBE AND TARGET SEQUENCE:

Both the labeled probe and the target DNA are denatured. The annealing of complementary DNA sequences occuring because of the combining sequences of denatured probe and target DNA. In indirect labeling, one extra step is required for visualization of the non-fluorescent hapten that requires an enzymatic or immunological detection system.[15] Although, the FISH probes are selected according to the diseases, anomalies, 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.[25]

3. HYBRIDIZATION:

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



(Fig: 2 Steps of FISH)

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 done after denaturation. During cooling, a complementary probe and target sequence forms hydrogen bonding of the two strands of nucleic acids. 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.[24]

After hybridization, stringency (post hybridization) washes aim at reducing non-specific binding. Although, it is better to hybridize stringently rather than wash stringently.[26]

4. DETECTION:

Various methods are at hand for visualization of the hybridization. For detection of hybridized probes, enzymatic reactions that generate a colored precipitate at the site of hybridization is used. The most oftenly used enzymes for this application are alkaline phosphatase (AP) or horseradish peroxidase (HRP).[27] The radioactive 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 immunoenzymesystems. 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 fluorophoreclassesfluoresceins, rhodamines, cyanines, or coumarins used. For detection of tumour morphology conventional light microscopy and flurosecnce microscope are also used. For the hapten detection immunohistochemically by a fluorophore-tagged antibody against the hapten is used.[28]

TYPES:

The diversification of real FISH protocol forms different types of FISH. Every type has its unique functions. Here the Table.3 represents the types of FISH, mostly used probes by these types and their functions.

Sr. No	Different Types	Function	Probes	Reference
1.	Arm Fish	In this type, there is a 42-color M-Fish variant that allows the detection of chromosomal abnormalities at the resolution of chromosome arms (P and Q arms of all 24 human chromosomes, except the P-arm of the Y and acrocentric chromosomes.).	DNA probes	**[28]**
2.	ACM Fish	This is a multicolor fish array for the simultaneous detection of numerical and structural chromosomal abnormalities in sperm cells.	DNA probes	**[29]**
3.	CB – Fish	This involves hybridization on binucleated cells in which cytokinesis has been blocked by treatment with cytochalasin -B.	DNA probes	**[30]**
4.	Cat Fish Cellular Compartment analysis of temporal (CAT)	Cat activity by Fish is an ingenious experimental approach devised to investigate the dynamic interactions of neuronal populations associated with different behaviour of cognitive challenges.	DNA probes	**[31]**
5.	CO – Fish Chromosome Orientation	Chromosome Orientation Fish is the name given to a Fish technique that uses single stranded DNA probes to produce strand specific hybridization. Initially, CO-Fish was designed to determine repeats within centromere regions of chromosomes.	single-stranded DNA probes	**[32]**
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 labeledtyramine molecules in which the horse – radish peroxidase (HRP) – labeled probe has bound.	polyribonucleotide probes and oligonucleotide probes	**[33]**
7.	COBRA FISH	The prefix COBRA stands for combined binary ratio. This particular Fish protocol brings together combinatorial labeling.	DNA probes	**[34]**
8.	Comet Fish	This type of fish is a combination of the comet assay and Fish analysis. The comet assay also called single cell gel test, 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	**[35]**
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	**[36]**
10.	DBD Fish	It detects any sites of DNA damage/breakage in the sample genome.	DNA probes	**[37]**
11.	D- Fish	It is an enhanced version of the fusion signal Fish protocol for the detection of recurring chromosomal	DNA probes	**[38]**

		translocations in hematological malignancies.		
12.	Fusion signal	In peripheral blood and bone marrow 9:22 Philadelphia translocation is detected by this FISH.	DNA probes	**[39]**
13.	Flow-fish	It is used to visualize and measure the length of telomere.	PNA-labeled telomere probes	**[40]**
14.	Fiber Fish	It is used for mapping of genes and chromosomal regions on fibers of chromatin or DNA.	DNA probes	**[41]**
15.	Harlequin Fish	In this type, there is a method for call cycle controlled chromosome analysis in human lymphocytes that allows a precise quantification of induced chromosome damage for human bio dosimetry purposes.	DNA probes	**[42]**
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	**[43]**
17.	Multilocus or ML fish	Used for identifying multiple microdeletionsyndrome in patients.	locus-specific DNA probes	**[44]**
18.	M -Fish (Multiplex – Fish)	The invention of M-Fish (or Multiplex – Fish), a protocol for 24 – colorkaryotyping, based on combinatorial labeling and aimed at facilitation the analysis of complex chromosomal rearrangements and marker chromosomes has signified in molecular cytogenetics, particularly for the study of tumors and prenatal diagnosis.	Chromosome specific DNA probes	**[45]**
19.	PCC Fish	This is a Fish whose application used for bio-dosimetry analysis that relies on the use of chromosome specific painting probes after irradiation.	chromosome-specific painting DNA probes	**[46]**
20.	Q Fish	Used for determining the repeated number of telomere on a specific chromosome.	PNA probes	**[47]**
21.	QD Fish	Used to detect human metaphase chromosomes, human sperm cells, bacterial cells and subcellular mRNA distribution in tissue sections.	oligonucleotide probes	**[48]**
22.	Raman Fish	It is s new technique that combines Fish technology with Raman micro spectroscopy for Eco physiological investigations of complex microbial communities.	16S rRNA probe	**[49]**
23.	RING Fish	Identification of individual genes and detection of halo appearance from fluorescence signals at the bacterial cell at periphery.	16S rRNA-based polynucleotide probes	**[50]**
24.	Reverse Fish	For characterization of chromosomes and chromosome amplifications in cancer.	Chromosome specific DNA probes	**[51]**
25.	RNA Fish	This is a method that allows detection of RNA within cells transcripts can be visualized either in the nucleus or in the cytoplasm.	RNA probes	[52]
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	**[53]**

Among all these types, there are some types which have major importance in scientific field. These types are MFISH, QFISH and RNA FISH. There is brief knowledge about these types below.

MFISH(Multicolour FISH)

One of the most appealing aspects of FISH technology is the ability to identify several regions or genes simultaneously, using different colours.[54] In 1996, two groups independently reported a successful 24-colour karyotyping, termed multiplex-FISH (M-FISH) and spectral karyotyping (SKY),respectively.M-FISH (and SKY) allow painting of the entire chromosome complement in a single hybridization through labelling each chromosome with a different combination of fluorophores. Images are collected with a fluorescence microscope that has filter sets for each fluorochrome, and a combinatorial labelling algorithm allows separation and identification of all chromosomes, which are visualized in characteristic pseudo-colours.[55]

Using fluorescence in situ hybridization (FISH) to map molecular markers on chromosomes can afford effective cytological markers for karyotype analysis and compensate for the inadequacies. 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*., *Piceaabies* and *Silenelatifolia.*[56]Ribosomal RNA genes (rDNAs) belong to 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, evolution of karyotype and phylogenetics research. Currently, it is most widely used in the research of molecular markers of chromosomes.[57]

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 tumor diagnostics and research, for example [58], evolutionary cytogenetics[59] and in the study of chemical and radiation-induced aberrations.[60] Impressively, even quite complex karyotypes of individual cells within a nonclonal population can be resolved with a high degree of confidence.[61]

One of the most important considerations in FISH analysis is the choice of probe. Chromosome 'painting' refers to the hybridization of fluorescently labelled chromosome-specific, composite probe pools to cytological preparations, which allows the visualization of individual chromosomes in metaphase or interphase cells and the identification of both numerical and structural chromosomal aberrations in human pathology with high sensitivity and specificity.[62] The whole chromosome painting probes are complex DNA probes derived from a single type of chromosome that has usually been flow-sorted (or microdissected), amplified and labelled by degenerate oligonucleotide polymerase chain reaction to generate a 'paint' which highlights the entire chromosome homogeneously along its length (through this method chromosome arm-specific paints and region-specific paints can also be generated).[63]

QFISH (Quantitative FISH)

Telomeres are nucleoprotein like structures, which cap and protect linear chromosome ends. They comprises of ~1500 TTAGGG repeats, a single strand overhang and complex of six shelter in proteins.[64] Telomeres prevent chromosomes from degradation and fusions by preventing the ends out of 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 numbers of times a cell can divide. For example in normal somatic cells, when few telomeres per cell reach a critical length their capping is compromised and they become dysfunctional. This telomere dysfunction activates replicative cell senescence a feature of getting old. However, in premalignant cells these telomere attrition can cause genomic instability and at the end can promotes tumorgenesis. In fact, 90% of cancer cells survive this crisis due to upregulating telomerase, the enzyme that lengthens telomeres.[65]Still in stem and progenitor cells, telomerase activity is crucial for tissue maintenance and regeneration[66], thus contributes to anti-aging mechanisms.

Telomeres are very prone to oxidative DNA damage. Abundance of epidemiology studies report relation between oxidative stress conditions and shortened telomeres. Various studies in human tissue, animal models and cell culture revealed the inflammation mediating oxidative and genotoxic stress quicken telomere shortening and dysfunction that can promote premature aging, cancer and age-related disease conditions.[67,68]Hence telomeres may represent a powerful readout of oxidative stress, potential bio-marker of exposures and disease risk. Three common techniques widely used by laboratories to measure telomere length.[89] The telomere restriction fragment (TRF) analyzed by southern blot is basically considered the gold standard. Although this technique only yields average telomere length, generally requires one to three micrograms of genomic DNA. Quantitative PCR is other common method which 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 length and integrity of individual telomeres.[70] 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, hence allowing the recording of multiple telomere parameters in a single experiment.[71]

Q-FISH uses peptide nucleic acid (PNA) probes they are synthetic molecules formed by DNA bases linked to a backbone by peptide bonds. These characteristics allow for an absence of repulsive charges which can occur between two DNA molecules and hence increases their binding efficiency. [70]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-color staining required for chromatid orientated FISH.[71] This method was adopted from previously published protocols prepared by Lansdorp and colleagues [72], which include a description of telomere length analysis utilizing Nikon NIS Elements

Advanced Research software. Q-FISH has been utilized extensively to quantitative information regarding telomere length distribution and associating it with various illnesses. In this context, Q-FISH is specifically relevant because it is able to identify and quantify critically short telomeres. It has been revealed that it is the frequency of these critically short telomeres, other than the average telomere length, which is important in telomere dysfunction.

RNA FISH

RNA molecules have a extensive range of characters in the cell. Coding RNAs (messenger (m)RNAs) works as templates for the protein translation, whereas noncoding RNAs (including microRNAs (miRNAs) and long non coding RNAs (lncRNAs)) manage gene expression programmes on many levels.[73] RNAs control all features of cell metabolism and thus have been shown to be important regulators of physiologic and disease activity.[94]

Current research have shown that the genome is crowded with a huge amount of long RNA molecules which does not encode proteins. These long noncoding RNAs (lncRNAs) have been ccorrelated with a large number of cellular activities as well as differentiation and beginning of disease and progress of disease. RNA fluorescence in situ hybridization (RNA FISH) is a technique that in numerous ways provides a convenient supplement to biochemical evaluation labelling RNA molecules in the cell by labeled nucleic acid probes hybridization with target RNA. [76]

In 1980 the very first application of fluorescence in situ detection was invented, when RNA that was directly labelled on the 3′ end with fluorophore was used as a probe for a particular DNA sequences.[76]RNA-FISH is a method which can be used for the detection of RNA present in cells. Transcripts are present either in the nucleus or in the cytoplasm. The method, also called as expression-FISH, has been used to examine the transcriptional action taken by endogenous genes [77], exogenous genes like those belonging to integrated viral genomes and transgenes.[78] The method allows investigation into allelic-specific expression on cell basis [79] and is anticipated to provide a policy for gene expression profiling studies in single cells. [80] RNA-FISH has also been used in studying different functional aspects of genome organization and nuclear architecture. Also, as a technique it is being inspected as a prenatal diagnosis tool for myotonic dystrophy type.

Single-particle RNA FISH dependent on pools of short, marked DNA oligonucleotides [81] can identify singular atoms of RNA in situ, in this way managing it the 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 unraveling 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. The issue is that a solitary oligonucleotide in the pool may dilemma to a high abundance, profoundly confined off target inside the cell, mainly inside the nucleus. Due to the serious extent of sequence contamination with repeat elements lncRNA is more inclined to these "rogue" oligonucleotides conversely with mRNA. Real lncRNA signal frequently shows up as brilliant masses in the nucleus making it more difficult to limit such signals as nonspecific foundation.

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. Due to this property of FISH makes easy diagnosis and also increases the life span of people.

Table.4 and 5 show applications in diagnosis field and in genetic research field.

Application in diagnosis	Symptoms	Use of FISH	Reference
Histiocytoid Sweet Syndrome	Fundamental hematologic myeloid issue or strong tumor malignancies or fiery gut illness or gastrointestinal lot or upper respiratory parcel diseases.	To decide the nearness of BCR/ABL quality combination. FISH led to survey the nearness of a chromosomal irregularity in the cutaneous invade of the underlying biopsy example.	**[80]**
Pseudomosaicism from True Mosaicism Differential Diagnosis	Chromosomal abnormality and clinical miscarriage.	FISH can be utilized as an indispensable apparatus for a pseudomosaicism and mosaicism differential determination in isochromosome 20q location.	**[81]**
Dedifferentiated Liposarcoma (DDLPS)	Pain, Swelling, Weakness, constipation.	MDM2 fluorescence in situ hybridization gave astounding information to recognizing the sicknesses.	**[82]**

Streptococcus Pneumonia	Bacteremia in kids and grown up.	FISH strategy can recognize *S. pneumonia*in blood culture without enzymatic treatment.	**[83]**
Aneuploidies	Severe microcephaly , growth deficiency and short stature, 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.	**[84]**
Chronic Myeloid Leukemia (CML)	Fatigue, anaemia, malaise, or night sweats, bleeding, bruising, pallor, reduced ability to exercise, shortness of breath, or weight loss.	Distinguishing these chromosomal movements, and in this manner, it very well may be utilized as an imperative apparatus in choosing a focused on treatment in various leukemias.	**[85]**
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 test for representation of cytogenetic variations from the norm in MM.	**[86]**
Pulmonary Adenocarcinomas	Raspy voice, Persistent cough, Chest pain, Shortness of breath, Unexplained weight loss, Cough that produces blood.	EML4-ALK quality combination can be recognized through FISH.	**[87]**
Prostate Cancer	Pain in the bones, excessive urination at night, difficulty starting and maintaining a steady stream of urine, dribbling of urine, frequent urination, urge to urinate and leaking, urinary retention, or weak urinary stream.	Four-concealing FISH test was used for the acknowledgment of either TMPRSS2 or ERG modifications.	**[88]**
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.	**[89]**
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.	**[90]**
Cholangiocarcinoma (CC)	Pain areas: in the abdomen Whole body: fever or weaknessAlso common: dark urine, itching, pale faeces, weight loss, or yellow skin and eyes.	FISH technique on brushing smears can detect numerical and structural deformity of four chromosomes in patients having documented extrahepatic CC.	**[88]**
Melanoma	Bigger mole diameter, more color changes, darkening of the skin, or skin mole with irregular border.	There are four tests focusing on 6p25 (RREB1), 6q23 (MYB), 11q13 (CCND1), and centromere 6 (CEP6) are used. The ideal calculations for differentiating positive FISH results dependent on these four tests are likewise settled.	**[87]**

(Table 4: Application of FISH for diagnosis)

Application in research	Description	Reference
New non-random abnormalities identification (by M-FISH or SKY)	The application of the SKY technique as an adjunct to G-banding and FISH studies in the clinical cytogenetics laboratory could potentially help delineate the more complex chromosome aberrations seen in MM and provide new clinical insights.	**[90]**
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.	**[91]**
Identification of regions of amplification or deletion by CGH	One of the main advantages of CGH is its use as a discovery tool. CGH has also contributed significantly to 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 the lymphomas.	**[91]**
The identification of translocation breakpoints	Multicolor FISH analysis identified an array of breakpoints responsible for locus- and region-specific translocations.	**[92]**
The study of 3D chromosome organization in interphase nuclei	Detection of fluorescent probes by fluorescence in situ hybridization in cells with preserved three-dimensional nuclear structures (3D-FISH) is useful for studying the organization of chromatin and localization of genes in interphase nuclei. Fast and reliable measurements of the relative positioning of fluorescent spots specific to subchromosomal regions and genes would improve understanding of cell structure and function.	**[93]**

(Table 5 Application of FISH in research field)

CONCLUSION

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

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CITATION OF THIS ARTICLE

M Prajapati, K Dave, D Pathak ,L Alisagar, K Suthar· An overview of the Fluorescence in situ hybridization technique: (Detection of Genetic Mutation). Bull. Env. Pharmacol. Life Sci., Vol 9[12] November 2020 :195-208