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THE MECHANISM OF FLUORESCENCE IN SITU HYBRIDISATION TECHNIQUE

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ABSTRACT

The Fluorescence in situ Hybridisation (FISH) is a molecular cytogenetic method to detect the presence or absence of specific genes in chromosomes by using radioactive fluorescent DNA dye/ Probes is for binding/hybridizing the exact locations of fixed DNA in which the dye emits bright spots during analysis under fluorescence microscope to see the area of interest. There are different formats of FISH method to investigate the area of interest. The protocol consists of Nine steps such as Preparation of slides, Pretreatment of slides, Probe preparation and additions, Denaturation, Hybridization, Post-hybridization wash, Counter staining, Visualization, Image capture and Analysis.

KEYWORDS: FISH, Probes, Denaturation, Hybridization, Signals.

INTRODUCTION

The conventional karyotyping has been providing a comprehensive observations of the genome. On the other hand, FISH will extrapolate cryptic or molecular level of genetic aberrations like deletions or duplications in interphase cells and metaphase spread respectively by fluorochrome signals binding with nucleic acid. This technique was invented by Joseph G Gall and Mary Lou Pardue in 1969[1] published in a companion papers documenting RNA based probes to label DNA in toad oocyte. Parude treated DNA based probes to label DNA in the same cell. In 1977 Rudkin and Stroller[2] investigated the first fluorescence in situ detection of DNA using indirect immunofluorescence. Bauman et al[3] tested the first direct fluorescence in situ detection of DNA without the need of antibodies in 1980. Therefore the basic principle of this method is hybridization of the DNA probe with the target DNA of either metaphase spread or interphase nucleic acid. The DNA probe is tagged with a fluorescent marker/dye. The denatured single strand fluorescent tagged DNA probe then allow to hybridize with equating target single stranded DNA which can be observed by using fluorescent microscope. The uses of FISH are the identification of chromosomal aberrations, Genetic mapping, Toxicological studies, Analysis of chromosomal structure, aberrations and ploidy determinant, Diagnostic tool along with confirmatory test.

The key component of this technique is the fluorescent probe. They are complementary sequences of target nucleic acids. It have designed the sequence of interest. Probes are tagged with fluorescent dyes like biotin, fluorescein, digoxigenin. Its size is ranges from 20-40 bp to 1000 bp. The hybridization is a zestful reaction in which a denatured target sequence and a complementary single strand DNA and RNA probe from a stable double strand molecule by heat action.[14]

The probes are broadly divided into DNA and RNA probes which further subdivided into different forms according to the regions of the chromosomes.

- *The DNA probe*: For preparation of the DNA probes sequences of DNA synthetic oligonucleotides are used from isolated chromosomes.[5] According to target site DNA probes are divide into chromosomal, satellite, telomere, probes for unique sequence and genome probes.
- *RNA probes*: It can be used for detection of m RNA in tissues by high sensitivity detection procedures because hybrids between m RNA and RNA are highly stable.[6]
- *Locus specific probes*: They bind to particular region of chromosome, which is useful when scientists have isolated small portion/locus of a gene and want to determine on which chromosome the gene is located or how many copies of a gene exists within a particular genome.
- *Centromere repeat probes*: It generated from repetitive sequences found in centromeres in which centromere regions are stained brighter which uses to know the individual has the correct number of chromosomes.

- *Whole chromosome probes*: It can be used to visualize genomic regions, from a single locus to entire chromosomes.[7]
- *Genome probes*: These probes are used for complete detection of chromosomal changes in whole genome by comparative genome hybridization (CGH) method.[9]
- *Telomere probes*: It has specifying the telomeres based on the TTAGGG repeat present on all human telomeres.[8]
- *Satellite probes*: They are specific for certain chromosomes and bind themselves into relatively long repetitive regions where they provide well visible signal. It hybridize with short repeating sequences AATGG located near the centromere in 1,9,15,16 and long arm of Y chromosomes. It could used for detection of trisomies and monosomies.[9]

The probe detection in FISH technique is based on fluorescence observed by means of an epifluorescence microscope. There are two ways of labelling and detection of a probe such as indirect labelling and direct labelling (Fig.1).

In the Indirect way of labelling, the chemically modified nucleotides are incorporated into DNA probes by Rudkin & Stroller etal, Langer 1981 et al, Raap et al.[2,11&12] The basic principle is that biotin or digoxigenin are covalently bound to d UTP that incorporates into the probe sequence instead of thymine. After hybridization with such labelled probe, immunodetection is carried out by means of antibodies conjugated with an appropriate flurochrome.[13] (Fig.1).

At the direct way of labelling, the probe is labelled with a d UTP bound flurochrome. The hybridized probe should be observed under microscope immediately after the hybridization reaction[14] (Fig.1).

Types of FISH methods

There are more than fifteen methodologies of FISH. Among them, some are the following

- **QD FISH:** Quantum dot FISH detects target genes in cells. It uses short DNA oligonucleotide attached to quantum dots of nanoparticles is enough which is more brighter and photo sensible for detecting tumor of cells.
- **ACM-FISH:** It is a multiclour FISH technique that detects chromosomal anomalies in sperms.
- **Immuno-FISH:** It is a technique that combines immunofluorescence with FISH for simultaneously visualize both DNA and protein in a sample. It can be used for detecting breast cancer.
- **RNA -FISH:** It detect, visualise, localize and quantify specific RNA sequences, study viral infections, provides transcriptional activity of genes.
- **Raman FISH:** It combines with Raman spectroscopy and FISH to study the structure and functions of single cells and microorganism.
- **Comet FISH:** This method is a technique that detects DNA damage and repair in specific regions of genome caused by UV rays, chemicals, carcinogens, oxidative stress by induction of free radicals.
- **Reverse FISH:** In this method the probe is material of interest which is usually deviate for using to identify and isolate specific DNA sequences in complex genomes.

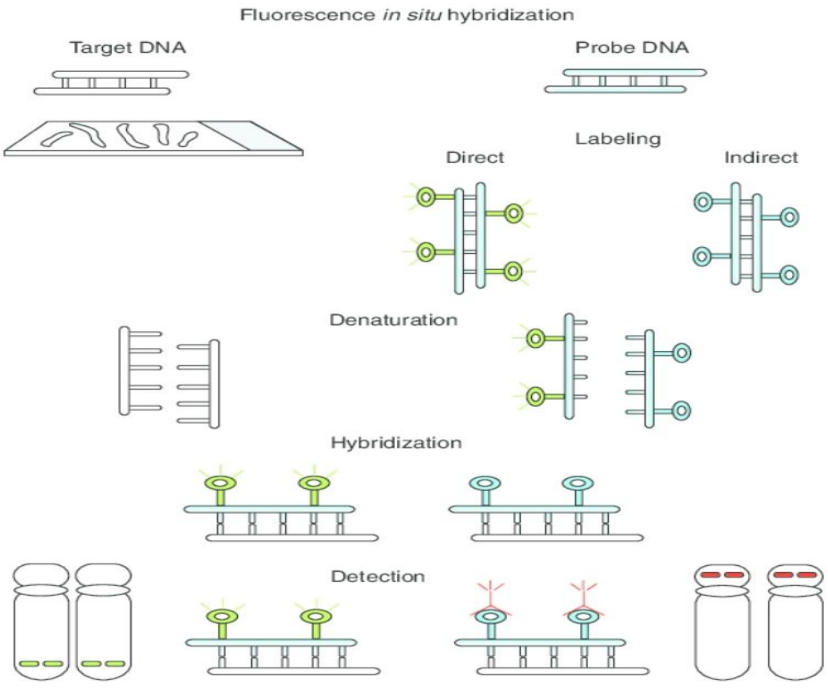

Figure 1: Exhibiting the schematic delineation of fluorescence in situ hybridization procedure.

- **DSB FISH:** It is a specific FISH test to detect double strand breaks (DSB) and its repair in cases of Dunchene muscular dystrophy, cystic fibrosis, Alzheimer's diseases. It also combines with proximity ligation assay (PLA) todetermine protein proximity to DSB transcriptional RNA.
- **Multiplex-FISH:** It develops to stain human chromosomes, 22 autosomes and X&Y sex chromosomes with uniquely multiple colours to facilitate karyotyping for analysing metaphase spreads.
- **Q-FISH:** Quantitative FISH has been measuring the length of telomere repeats sequences in individual chromosomes from single metaphase spread.
- **FLOW-FISH:** It combines with cytometry with FISH used to quantify the copy number of RNA or specific repetitive elements in genomic DNA of whole cell populations.
- **CAT-FISH:** This method identify neuronal populations associated with different behaviours.
- **COD-FISH:** Chromosome orientation and direction FISH to determine the strand modifications to allows single strand probes to hybridize one chromatid of a metaphase, inversion.
- **Cryo-FISH:** This method uses ultra thin cryosections to study the spatial organizations of chromosomes in the cell nucleus and validate results obtained by chromosomes conformation capture on chip technology.
- **LNA-FISH:** It uses locked nucleic acid probes to detect RNA in cells. It offers fast, sensitive and specific detection of chromosomal DNA.

MATERIALS AND METHODS

The materials using in this technique are depicted in the Table no.1 and the reagents with stock solutions are delineated in the Table no.2. The protocol of FISH technique consists of Nine steps which is explained separately and exhibited as flow chart in figure.2. The principal author had underwent hands on training on Cytogenetic FISH workshop at Regional Cancer Centre abbreviated as RCC Trivandrum in November 2013 which builds the confidence of procedural mastery, creative thinking and problem solving skills of this global demanding technique.[4]

Table 1: Showing the materials required for FISH methodology.

Sl. No.	Equipment's	Company specifications
1	Fluorescent Microscope with appropriate filters & CCD camera	Olympus
2	FISH probes	Vysis Vysis-LS BCR/ABL/PML-RRA/AML-ETO dual color dual fusion translocation probe.
3	Diamidino Phenylin idole (DAPI)	Thermofisher staining AT regions
4	Hybridization chamber	Bioequip
5	Phosphate buffered saline (PBS)IX	Sigma Aldrich
6	ImM MGC12	Sigma Aldich
7	Tween-20	Emulsifying ,Sigma Aldrich
8	20X SSC	Sigma Aldich
9	Centrifuge	Sigma Aldich
10	Pepsin, Absolute ethanol	Sigma Aldich
11	Formamide	Sigma Aldich
12	Fevibond glue	Fevibond 45
13	Quick spin	Labnet
14	Vortex mixer	India mart
15	Micropipetts	Dragon lab
16	Couplin jars	Medikabazaar
17	Ependorf tubes	Medikabazaar
18	Cover slips	Medika bazaar
19	Precleaned slides	Medika bazaar
20	CO_2 Incubator	Sigma
21	Glass marking pencil	Apex Labs

Table 2: Exhibiting the Preparation of Reagent and Working solutions.

Sl. No.	Reagent	Stock solution		Distilled water
		Name of chemical	Requirement	
1	Nucleic acid Hybridization agent	20XSSC (PH- 7)	87.6NaCl 44.1g Trisodium citrate	Make upto 500ml with distilled water
2	Post hybridization wash	2XSSC (PH7±0.2)	20 XSSC solution - 5ml	Tripple distilled water-45 ml
3	Post hybridization wash	2XSSC with 0.1%NP-40 (PH±0.2)	20X SSC-10ml Tween-20-100µl	Tripple distilled water
4	Post hybridization wash	0.4XSSC with 0.3%NP-40(PH7.5) for 50ml		
5	Pre-treating slides before hybridization	Protease solution-50ml	Pepsin0.2g HCl-40µl	Tripple distilled water -50ml
6	Fixative	Neutral buffered formalin	Formaldehyde - 12.5ml 71XPBS 37ml 28MMgCl12 1090MgCl2=500 µl	
7	Washing & Blocking	1XPBS(PH7.4) for 250 ml	NaCl 2g KCl0.05gg NA2HPO4-0.36g KH2PO4-0.06g	Tripple distilled water-45g
8	Denature & Preserve DNA	70% Ethnol	70 ml	30 triple dist water
9	Denature & preserve DNA	80% Ethanol	85 ml ethanol	15 ml triple distilled water
10	Denature & preserve DNA	85% Ethanol	85 ml ethanol	15 ml triple distilled water
11	Denature & preserve DNA	100% Ethanol	100 ml ethanol	-

Protocol of the fish methodology

A. Preparation of slides[17]

- Take fixed (Methnol: acetic acid 3:1) cells cultured lymphocytes in 1.5 ml eppendrof tube and centrifuge at 5000 rpm for 5 minutes.
- Remove the supernatant, add fresh fixative and centrifuge again at 5000 rpm for 5 minutes.
- Remove the supernatant and resuspend the cell pellet in small volume of fresh fixative (According to the pellet size)
- Take 100µl of final cell suspension and put on to the centre of a glass slide (Ice cold), spread the cells by providing heat from back of the hand onto the backside of the slide.
- Check cell density under microscope (circle the target area by a diamond tip marker on the back of the slide)
- Refix the slide 70% acetic acid(flood the slide) for 20-30 seconds
- Dehydrate in 70%, 90% and 100% ethanol series for 3 minutes each at room temperature
- Leave it to dry at room temperature (air dry)

B. Pretreatment of Slides

- Immerse the slides in 2X SSC, PH7.0±0.2 at 73˚C for 2 minutes
- Wash slide in TDW for 2 minutes
- Dip the slides in protease (Pepsin solution) for 15 minutes.
- Wash in PBS to remove pepsin for 2 minutes.
- Dip the slides in neutral buffered formalin for 5 minutes
- Again wash in PBS for 5 minutes
- Dehydrate the slides again in alcohol series 70%, 90%, 100% ethanol series for 3 minutes each at room temperature.
- Dry slides in air.

C. Probe Preparation and Addition

- Centrifuge the probe vials briefly
- Mix well the contents of the probe vials by pipetting up and down several times.
- Aliquot 2µl (per slide) of probe solution to an Eppendorf tube.
- To the same Eppendorf, add 1µl triple distilled water and 7µl hybridization buffer and mix well by pipetting in a PCR tube.
- Spin down the contents of the tube.
- Seal the cover slip with rubber seal on the outer edge.

D. Denaturing step

- Apply probe solution onto denatured slide
- Place coverslip over the probe
- Seal the edges of the coverslip with rubber cement.

- Denature the probe solution in a water bath at 80-90˚C for 10 minutes.

E. Hybridization step

- Take out the slides and keep the slides in moist chamber (hybridization box) at 37˚C overnight.

F. Post Hybridization wash

- Remove the slides from the hybridization chamber.
- Carefully remove the rubber seal and coverslip from the slides by dipping2X SSC.
- Dip the slides in 04X SSC with 0.3% NP -40 (PH 7.5) for 2 minutes at 72˚(±1˚C) without agitation.
- Wash the slides in 2X SSC with 0.1% NP -40 for 2 minutes each at room temperature without agitation.
- Dry in air at room temperature.

G. Counter staining

- Apply 10µl DAPI on the slide and cover with glass cover slip and seal using rubber glue.
- Keep in dark for 30 minutes.
- H. Proceed with Fluorescent microscopy.

H. Visualization

- Observe the slides under a fluorescent microscope to visualise the hybridized probes using appropriate filters.

I. Image Capture and Analysis

Metaphase spreads: Analyse a minimum of 10-15 metaphase spreads and capture a minimum of 3 micrographs/ pictures.

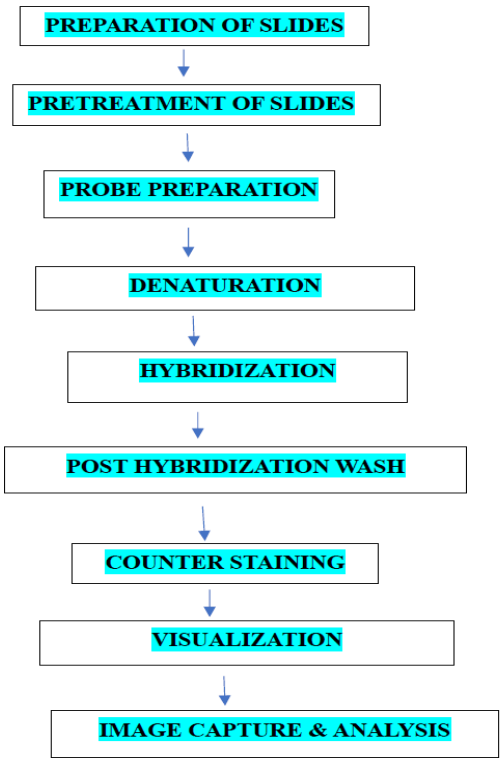

Figure 2: Depicting the flow chart of the protocol of fluorescence in situ hybridization.

New Progression in FISH

They are multicolour technique based on FISH and Comparative genomic hybridization. The multicolour karyotyping procedures are multicolour /multiplex FISH(M-FISH), Spectral karyotyping(SKY), colour changing karyotyping (CCK) as well as multicolour banding(m BAND) techniques were introduced as the modification of FISH. These assays are indispensable for a precise description of complex chromosomal rearrangements by Liehr et a1.[15] They are used in tumor cytogenetics. Comparative genomic hybridization (CGH) invented by Kallioniemi1 et al,[16] using for entire genomic analysis with reference DNA to measure genetic imbalances in solid tumors.

CONCLUSION

This technique is a universally accepted method to diagnose chromosomal aberrations, gene mutations in cancer, gene mapping and RNA -FISH analysis will detect gene expressions during intrauterine life of babies.

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