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Applications of Fluorescent *in situ* hybridization (FISH)

Adna Smajkan[1, 2]
[1]Genetics and bioengineering, International University of Sarajevo
[2]Faculty for chemistry and pharmacy, Ludwig Maximilian University of Munich

*Corresponding author: smajkanadna@gmail.com

Abstract
Fluorescent *in situ* hybridization (FISH) is a molecular technique used for the detection of specific DNA sequences within the chromosome. It relies on the complementary binding between the fluorescently labeled probe and the target sequence. This paper describes how this method was first developed, and the basic principle and the procedure behind it. Furthermore, it covers the basic applications of FISH, including its use in microbiological diagnostics, diagnosis of solid tumors, diagnosis of hematological malignancies, evaluation of sperm and diagnosis of DiGeorge syndrome, along with its applications in plants.

Keywords: Fluorescent in situ hybridization (FISH), microbiological diagnostics, solid tumors diagnosis, hematological malignancies diagnosis, human sperm diagnosis

1. Introduction

1.1. Background information about FISH

Complementary base pairing is possible between two DNA, two RNA, or between DNA and RNA molecules. The process of hybrid molecule formation itself is known as hybridization [1]. Firstly, *in situ* hybridization was described in 1969 by Gall and Pardue, as a technique used for the determination of specific nucleic acid sequences inside cells and tissues [2] [3]. The method relies on the use of nucleotide probes labeled with radio-, fluorescent-, or antigen-labeled bases, which are complementary to the specific target DNA or RNA sequences. The visualization method is determined by the probe being used. Thus, autoradiography, fluorescence microscopy, or immunohistochemistry may be applied correspondingly [2]. Besides being used for the detection of bacterial and mycobacterial pathogens, ISH has been utilized for the indication of viruses such as human papillomaviruses, human herpesviruses (HHV), hepatitis B and C virus, human immunodeficiency virus, adenovirus, as well as the human parvovirus B19 [4] [5]. Developed from in situ hybridization (ISH), the utilization of the fluorescent in situ hybridization (FISH) technique was firstly mentioned in 1980. It relies on the complementary binding of a 3'fluorescence-labeled RNA probe with a specific DNA sequence [6] [7]. According to S. Savic and L. Bubendorf, FISH is described as a technique used for the detection of genomic abnormalities in diagnostic, prognostic, and predictive marker testing [8]. One of the major issues that occurred in the early development of FISH was a high level of background fluorescence which was related to the non-specific binding of the probes. The problem was later on solved by utilizing the pre-treatment with unlabeled probes and by reducing their size [9] [10]. At the early stages of development, FISH was used for the detection of single target sequences in animal cells [7], and later advances allowed the detection of two different DNA sequences at the same time, by utilizing non-radioactive probes in the human and mouse somatic cells [11]. Further modifications lead to the detection of three different DNA sequences simultaneously by the use of blue aminomethyl coumarin acetic acid, in combination with green (FITC) and red (TRITC) fluorescing in human peripheral blood lymphocytes. The

probes were labeled with either amino acetyl fluorene, mercury, or biotin [12].

1.2. FISH principle

The fluorescent in situ hybridization technique follows a few basic steps: fixation, sample preparation, hybridization, washing, and visualization using fluorescence microscopy [13]. To maintain the cellular form and structure, and ensure the optimal retention of target sequences, the fixation of the samples is performed. Several fixatives have been utilized so far, including paraformaldehyde, glutaraldehyde, ethanol, ethanol-formalin, Bouin's solution, and Carnoy's and Zenker's with paraformaldehyde [5] [13]. Additionally, methanol can be used as a fixative in microbiological diagnostics for the production of clear images [14]. In contrast to short-fragment probes which are used in FISH, the non-fluorescent in situ hybridization utilizes longer probes that require unmasking steps [5]. Furthermore, substances such as xylene or formaldehyde can be used for the permeabilization of the cell membrane so that the probe can penetrate the cell. However, since they are very weak, other agents including saponin, Triton X-100, Tween-20, NP40, protease K, lysozyme and streptolysin may be used as well, providing a higher degree of the cell membrane and cell wall permeability. Although permeabilization steps are not required for the Gram-negative bacteria and yeasts, it is needed for Gram-positive pathogens, spore-forming bacteria, and mycobacteria [13]. Lysozyme or enzymatic mixtures may be utilized for the opening of the peptidoglycan layer in Gram-positive bacteria [14]. Permeabilization protocols are based mainly on lysostaphin, the combination of lysostaphin and lysozyme, or a combination of agarose, methanol, and lysozyme without lysostaphin [15] [16]. Hybridization of the probe to the target sequence is mainly influenced by the probe length and GC content. Other important factors include hybridization temperature, salt concentration, and concentration of formamide denaturing agent [5]. However, since the utilization of formaldehyde may be toxic, other hybridization buffers have also been utilized which are based on sodium chloride and urea [17] [18] [19]. Different fluorophores are used for the labeling of nucleotide probes. Among them, the first ones that have been utilized include fluorescein (FITC), rhodamine (TRITC), and aminomethyl coumarin acetate [20]. The utilization of Cy3 and Cy5 cyanine dyes has been shown to have significant benefits over first-generation probes. Probes labeled with Cy3 showed a notable increase in fluorescence intensity, whereas the Cy5-labeled probes are beneficial for multicolor detection [20].

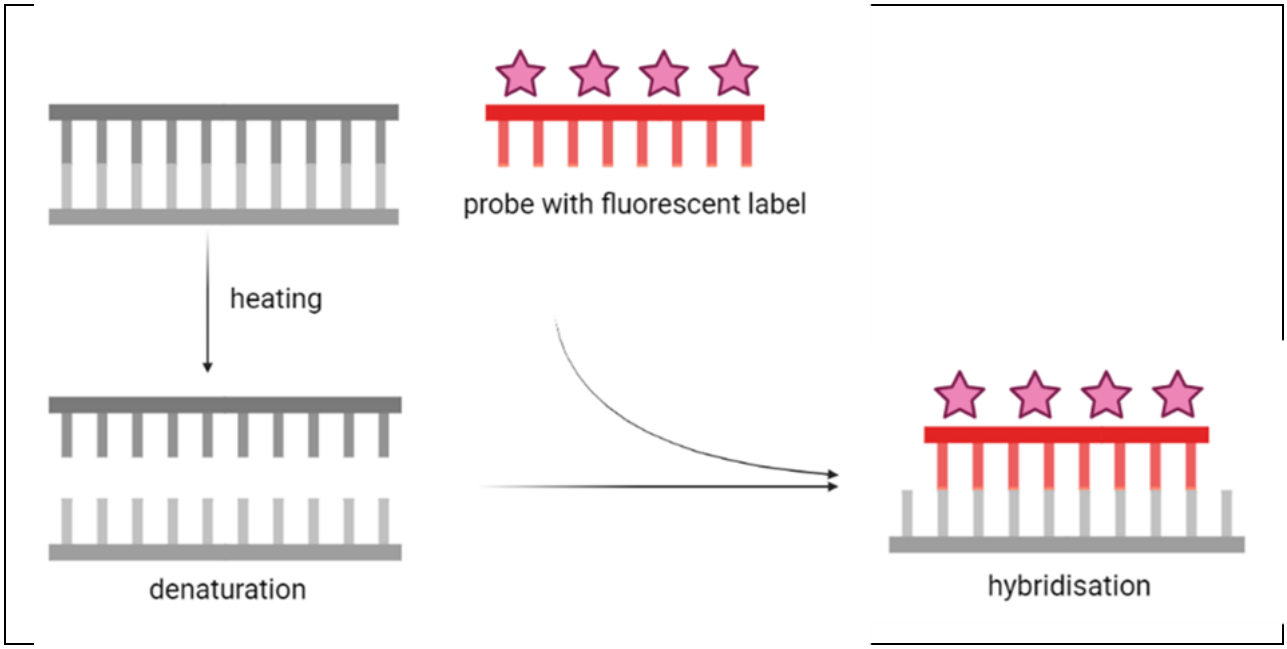

Figure 1. Fluorescent in situ hybridization (FISH) technology. When two DNA strands are heated, they denature or split, which allows fluorescently-labeled probes to attach to complementary sequences in patient's DNA

2. The main applications of FISH

This section discusses some of the main fields where FISH is employed.

2.1. Applications of FISH in the microbiological diagnostics

Our mouth houses a very diverse microbial community, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Fusobacterium*, and others [21] [22]. FISH has been utilized for the detection of diseases in the oral cavity such as dental caries or periodontitis [23] [24]. Furthermore, FISH is utilized for the detection of different pathogens invading the upper and lower respiratory tract, as well as the bloodstream-associated infections, urinary tract infections, genital tract infections, and gastroenteric infections [13]. Mycobacterium tuberculosis has also been successfully detected utilizing this technique. Other applications include the detection of pathogens inside the tissues, as well as the identification of the biofilm species [13] [25] [26]. Anaerobic bacteria pathogens may also be detected, along with the protozoan parasites, viruses, yeasts, and highly pathogenic bacteria [13].

2.2. FISH applications in Diagnosis in Tailored Therapies in Solid Tumors

Non-small cell lung cancer is associated with several genomic alterations including point mutations such as EGFR (epidermal growth factor receptor), KRAS (KRAS proto-oncogene, GTPase), BRAF (B-Raf proto-oncogene serine/threonine kinase), MET (MET protooncogene, receptor tyrosine kinase), ALK, RET (ret proto-oncogene), ROS1 (ROS protooncogene 1, receptor tyrosine kinase), and MYC (MYC proto-oncogene, bHLH transcription factor). Various tumorigenesis pathways in NSCLS including rearrangements in ALK, ROS1, and c-MET gene have been detected using Fluorescent *in situ* hybridization [27]. *Ginestet et al*. evaluated the performance of a dual ALK/ROS1 fluorescent in situ hybridization (FISH) probe designed to analyze the two oncogenes simultaneously. ALK and ROS1 rearrangements can be searched concurrently using this probe on a single FISH slide, freeing up tumor tissue for other biomarker tests [28]. The FISH method has also been utilized for the detection of gliomas, which are the most frequent type of brain tumors. Mutations in IDH1/2 (isocitrate dehydrogenase NADP (+)) ½) gene occur in both astrocytic and oligodendroglioma tumors. Additionally, 1p/19q-codeletion is associated with oligodendroglioma, whereas mutations in ATRAX (ATRAX chromatin remodeler) and TP53 (tumor protein p53) genes are associated with astrocytoma. Furthermore, breast cancer is associated with mutations in the ERBB2 (Erb-b2 receptor tyrosine kinase) gene, also known as HER2. This gene is involved in important cellular pathways which regulate cell growth and division, and its locus can be tested using the FISH technique [27]. With FISH, the ERBB2 gene amplification can be quantified at an average level in a tumor, which is a major advantage compared with other methods. Furthermore, there is no need to take large amounts of tumor material, it is very fast and nonradioactive. It has been demonstrated that FISH can distinguish between the increase in ERBB2 copies caused by specific gene duplications from those caused by chromosomal amplification [29]. Another cancer type where FISH can be applied is ovarian cancer, which is associated with high expression of CCNE1gene which can stimulate uncontrolled DNA replication, centrosome amplification, and chromosomal instability (Chrzanowska et al., 2022). Finally, FISH can be utilized for the detection of soft tissue sarcomas (STS) which are a very rare type of cancer. ES is associated with mutations in the EWSR1 (EWS RNA binding protein 1) gene, whereas synovial type (SS) sarcoma is associated with mutations in the SS18 (SS18 subunit of BAF chromatin remodeling complex) gene. Translocations and amplification of gene regions are common characteristics of soft tissue neoplasms that can be evaluated by FISH to assist in diagnosis [27] [30].

2.3. Further applications of FISH in Hematological Malignancies

Fluorescent in situ hybridization has been utilized for the detection of chromosome abnormalities related to chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), and acute myeloid leukemia (AML) [31]. Chromosomes 6, 11, 12, 13, 14 and 17 are most commonly affected by cytogenetic abnormalities in B-CLL [32]. Most common abnormalities related to B cell chronic lymphocytic leukemia include Trisomy 12 and structural abnormalities of band 13q14 to which the retinoblastoma susceptibility gene (RB-1) is mapped [33]. Additionally, a genomic region with a D13S25 locus was found to have a role in the pathogenesis of B-CLL. By utilizing the FISH method, it was found that deleted segment containing the tumor suppressor gene is found in the region between D13S25 and RB-1 [34]. Additionally, in rare cases related to B-cell chronic

lymphoid leukemias, there is the deletion of the short arm of chromosome 17 to which the p53 tumor suppressor gene localizes. Among 100 cases that have been tested utilizing the FISH technique, 17% displayed monoallelic deletion of this gene. Furthermore, patients who had deletion also had considerably shorter treatment-free intervals and short survival times. This confirms the highly sensitive and specific analysis of the FISH technique [31]. Additionally, the Philadelphia chromosome or its counterpart BCR-ABL fusion can be detected by utilizing the FISH technique in both chronic myeloid leukemia and acute lymphoblastic leukemia. BCR-containing YAC clone, firstly described by *Lengauer et al.*, was used for the diagnosis of BCR-ABL in CML and AML patients, successfully detecting the breaks in chromosome 22 [35]. Finally, 50%-60% of patients have chromosome aberrations related to Acute Myeloid Leukemia, including monosomy 7 (-7) and trisomy 8 (+8). These abnormalities can easily be detected by the use of FISH. Although the FISH technique is less sensitive and requires more time than PCR-based diagnostic tests, it can detect chromosome abnormalities that are not possible with PCR [31].

2.4. Diagnostics and therapeutic implications of human sperm with FISH

FISH can also be used to evaluate aneuploidies by measuring the frequency of chromosomal abnormalities. Approximately 6% of men suffer from male factor infertility, which affects their ability to reproduce. In such cases, assisted reproductive techniques (ARTs) such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) may be selected for the treatment. Anuploidies, in particular involving the sex chromosomes, may be a risk for the offspring born as a result of this reproductive method [36] [37]. There is a major disadvantage of ICSI in sperm selection. Apart from carefully picking sperm of the highest quality, the genetic integrity of the embryo cannot be guaranteed. Additionally, spermatozoa used in FISH cannot later be used for ART since they become nonviable due to all the steps involved, including formamide denaturing [38]. The chromosomal abnormalities in gametes of infertile individuals are higher than those seen in fertile individuals, contributing to a higher risk of recurrent abortions [39]. Moreover, chromosomal aneuploidy, which may arise from nondisjunction, anaphase lag, or ineffective checkpoint, causes most fetuses to be unviable. In addition to autosomal trisomies , Edwards syndrome, and Down syndrome, aneuploidies of the sex chromosomes, including X monosomy (the only monosomy that can sustain life) and Klinefelter syndrome (XXY-XXXXY), are of the greatest clinical importance [40]. Furthermore, studies have found that with increasing age, chromosomal aberrations are more of a structural nature than a numerical nature [41]. A higher incidence of gonosomal disomy (X-X-8) is observed in aged mice, but no evidence has been found for an increased risk of trisomy. A combination of arrayerative genomic hybridization with pre-implantation genetic diagnosis (PGD) and intracytoplasmic sperm injection (ICSI) is a technique used to detect aneuploid embryos. In order to assess male gametes, FISH is an effective and simple method, but sperm must be examined directly. A sperm FISH cytological assay requires decondenseation of sperm chromatin before the probes can access the spermatozoon DNA [38]. To achieve reliable results, multicolor FISH is used. Two-color FISH is required for autosome analysis, whereas three-color FISH is required for sex chromosome analysis. An advantage of the method is that it relies on sophisticated image analysis software, which is highly sensitive and less operator-dependent. However, disadvantages include the limitations of surveying structural chromosomal aberrations, and detection of more segments of chromosomes (versus the entire chromosome) [42]. It comprises five simple steps: cell fixation, condensation, hybridization, post-hybridization washes, and visualization. A special fluorescence microscope equipped with filter sets for each of the following commonly used colors can visualize the fluorescently labeled DNA probes: FITC, Texas Red, and DAPI/FITC/Texas Red labeled DNA probes [43].

2.5. FISH for diagnosis of DiGeorge syndrome

A complex malformation pattern known as DiGeorge syndrome (DGS) is characterized by congenital heart defects, hypoplastic thymus and parathyroids, and facial dysmorphism in combination. It is known as a 22q11.2 deletion because it involves the removal of a small segment of chromosome 22 [44] [45]. Symptoms of this syndrome usually include delays in learning to walk or talk, hearing and vision problems, mouth and feeding problems, and bone, spine, and muscle problems, among others [46]. In situ fluorescent hybridization (FISH) is an effective tool for easily detecting microdeletions on metaphase chromosomes associated with DGS [47]. It was found that among tested patients, there were chromosome 22 microdeletions with

heterochromatic short arms of different sizes. Cosmid probes hybridize very well, and fluorescent in situ hybridization is now an extremely efficient tool to screen for microdeletion-associated disorders [44].

2.6. Applications of FISH in plants

FISH was first applied to plants nearly 30 years ago, and it has been widely used in plant cytogenetic research [48] [49]. DNA robust probes have been used in recent FISH experiments to produce highly efficient results. The most common FISH probes for plants have been repetitive DNA sequences and large-insert genomic DNA clones, such as bacterial artificial chromosomes (BAC) Recently, synthetic oligos have been discovered to be useful as FISH probes, which can either be constructed from repetitive DNA elements, repetitive DNA elements, or single-copy DNA sequences. For the first time, synthetic oligo-FISH probes were developed to map plant simple-sequence repeats (SSRs). FISH probes are synthesized from synthetic oligos containing repeated di-, tri-, or tetra-nucleotide motifs, such as (AG)12 or (AGG)5. These synthetic oligos can be end-labeled with digoxigenin-dUTP or biotin-dUTP [50]. It is also possible to conjugate them with fluorochromes during the synthesis [51]. There have been a number of plants species for which SSR-related oligo probes have been used for DNA analysis and chromosome identification. In plants, satellite repeats, or tandem repeats, are popular FISH probes since they can often be used to identify chromosomes using FISH signals derived from them. There is a possibility that oligo probes based on a short sequence motif unique to a satellite repeat can be synthesized for FISH analysis if a short sequence motif is identified [51]. Compared to conventionally prepared probes derived from cloned satellite repeats, these short synthetic probes offer several advantages, including a consistent probe quality and a reduction in probe preparation time and cost. Furthermore, some of these probes do not require denaturation of the chromosomal DNA [52] [53]. It is not necessary to have a fully sequenced reference genome in order to design synthetic oligo probes, as analytical satellite repeats can be identified directly from genomic sequence data. It is also possible to design oligo probes from single-copy DNA sequences, although numerous single-copy oligo probes may be required to visualize a specific chromosomal region. As an alternative, a pool of oligos specific to a particular chromosomal region or to a particular chromosome could be computationally identified and synthesized. Sequence tags can be added at both ends of each oligo during synthesis, which allows the entire pool to be amplified using PCR. In a subsequent step, FISH probes can be generated from this pool via amplifying oligos that have been labeled directly with a fluorescence or indirectly using deoxyuridine-dUTP or digoxigenin-dUTP [54]. The most common application of FISH involves the mapping of DNA probes to chromosomes, which enables the determination of the physical location of the probes. FISH can be applied for the identification and validation of satellite repeats in plant genomes, essays such as the one for chromosome synteny and evolution, and gene duplication and amplification can be applied. Among the applications of FISH are the identification and validation of satellite repeats in plant genomes, essays such as those helping to understand chromosome synteny and evolution, and gene duplication and amplification [50].

3. Conclusion

This essay has discussed the basic principle behind the fluorescence in situ hybridization (FISH) procedure as well as its most important applications. The FISH technique is used routinely for detecting genetic abnormalities. Through the use of a simple FISH procedure, tumor-specific abnormalities can be identified. However, it is limited to designed probes. Using formalin-fixed paraffin-embedded tissue samples (FFPE), FISH can be used to assess the genomic status of cells derived from a primary tumor or metastasis. Although numerous more sophisticated techniques are available, such as Real-Time PCR and new generation sequencing, FISH continues to be the most widely used method in genetic laboratories. It is increasingly being applied to the cytogenetic analysis of human cancers, as well as in biological research. It is possible to delineate specific numerical and structural aberrations in interphase cells using FISH compared to conventional cytogenetic analysis. The development of genomic DNA probes for the study of chromosome aberrations associated with chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), and acute myeloid leukemia (AML) has been investigated in some studies. Based on the findings, researchers will have an easier time monitoring residual disease following chemotherapy with disease-specific probe sets when DNA probes become more complex, digital microscopy becomes more advanced, and automated image analysis becomes more sophisticated. The FISH assay is also useful for estimating the frequency of chromosomal abnormalities. An analysis of FISH can also be used to screen for mutations that cause the

DiGeorge syndrome, which is characterized by congenital heart defects, hypoplastic or absent parathyroid glands, and physical abnormalities. Moreover, FISH has been used extensively in plant biology to study issues related to the structure, mutation, and evolution of whole genomes, as well as individual chromosomes. Recent advances in FISH have been made due to the development of synthetic oligonucleotide probes that are being applied to a wide range of plants species.

4. Abbreviations and acronyms

FISH - Fluorescent *in situ* hybridization, DNA - Deoxyribonucleic acid, RNA – ribonucleic acid, ISH – *in situ* hybridization, HHV – human herpesvirus, FITC - Fluorescein isothiocyanate, TRITC – Tetramethylrhodamine, CCL - chronic lymphocytic leukemia, CML - chronic myeloid leukemia, AML - acute myeloid leukemia, ART – assisted reproductive technique, IVF – *in vitro* fertilization, ICSI – intracytoplasmic sperm injection, DGS – DiGerorge syndrom, dUTP - deoxyuridine triphosphate

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