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Challenges and solutions in FISH for formalin-fixed paraffin-embedded tissue: A scoping review

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Abstract

Fluorescence in situ hybridization (FISH) has revolutionized molecular cytogenetic analysis since the 1980s, enabling precise localization of DNA sequences in cells and tissues. Despite its relevance, applying FISH to formalin-fixed paraffin-embedded (FFPE) tissue samples encounters significant technical challenges. This review addresses the main issues encountered in this context, such as inadequate fixation, contamination, block and slide age, inadequate pretreatment, and FISH technique. Proposed solutions include optimized pretreatment protocols, monitoring of block-age, careful selection of probes, and thorough analysis of results. Implementing good laboratory practices and quality control strategies are essential to ensure reliable results. Additionally, the use of emerging technologies such as artificial intelligence and digital pathology offers new perspectives for improving the efficiency and accuracy of FISH in FFPE samples. This review highlights the importance of a careful and personalized approach to overcome the technical challenges associated with FISH in FFPE samples, strengthening its role in research and clinical diagnosis.

Research Highlights

- Few FISH studies on FFPE: The scarcity of studies specifically addressing FISH applications in FFPE tissues highlights a critical gap in the literature.
- Troubleshooting FISH in FFPE tissues: Identifying and addressing common challenges in FISH techniques when applied to FFPE samples, such as signal quality and hybridization efficiency.
- Critical aspects of FISH technique: Discuss the main technical considerations crucial for successful FISH in FFPE tissues, including sample preparation, probe selection, and protocol optimization.

KEYWORDS

FISH, fluorescence in situ hybridization, formalin-fixed paraffin-embedded, technical issues

1 | INTRODUCTION

Developed in the 1980s, fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique that marked the beginning of a new era for studying the structure and function of chromosomes (Chrzanowska et al., 2020; Levsky & Singer, 2003).

The DNA FISH technology is used to study chromosomal and genomic alterations in cell suspensions and tissue block preparations.

In oncology, most biological samples are available in formalin-fixed paraffin-embedded tissues (FFPE). The applications cover a wide range of molecular cytogenetic investigations, including the detection of aneuploidies, structural alterations, and the detection of gene deletions and amplifications (Chiecchio, 2020; Price, 1993; Tansatit, 2017).

Two main techniques have been employed for FISH analysis of these FFPE specimen samples: one requires isolation of intact nuclei from thick sections of tumor blocks and hybridization assays conducted on cell suspensions; the other utilizes thin sections from whole paraffin-embedded blocks (Varella-Garcia, 2006).

This methodology allows for the detection of specific targets not only in metaphases but also in interphase nuclei, providing rapid and sensitive detection of chromosomal alterations. FISH in interphase nuclei avoids the need for preparations with dividing cells, overcomes the need for selective cell growth, and allows genomic screening of different tissues that are not amenable to investigation by classical cytogenetics. Additionally, the method is ideal for single-cell analysis and can greatly contribute to understanding the genetic heterogeneity of biological samples (Bishop, 2010; Hackel & Varella-Garcia, 1997).

Indeed, hybridization is a method that capitalizes on the natural tendency of a single DNA strand to reassociate with its complementary strand to form a double helix. Thus, a specific DNA fragment can be located within a heterogeneous mixture as long as a complementary sequence to the fragment is available. This complementary sequence is called a probe, as it is used to "probe" the gene or specific DNA sequence. The probe must be previously labeled in some way to allow for its subsequent identification. Since the hybridization of the probe is specific to the fragment it complements, this method enables the localization of the fragment. Probes are, therefore, segments of nucleic acids (usually DNA), cloned or synthesized, used in hybridization reactions to locate a sequence of interest (Chiecchio, 2020; Farah, 1997).

Currently, there are a myriad of probes available for purchase on the market, developed by various companies, each tailored for a specific type of analysis. However, they consist of three main types: chromosome painting probes (marking either the entire length of the chromosome or only partially), probes with repetitive sequences, such as centromeric and telomeric probes, and locus-specific probes (used in the identification of specific genes, chromosomal regions with alterations in copy number, and structural rearrangements) (Nussbaum et al., 2016).

DNA probes for centromeres, telomeres, unique sequences, and whole chromosomes can be used to address questions that influence diseases such as cancer and human developmental disorders, aiding in the diagnosis and selection of treatment strategies (Alamri et al., 2017; Yu et al., 2021). In an era of precision medicine, achieving an accurate diagnosis and obtaining a molecular classification of the tumor is essential. In this clinical context, FISH can support, confirm, or exclude a suspected diagnosis, refine the classification of tumor subtypes, indicate disease prognosis, and predict response to therapy. Cancer genomics is considered a subspecialty in pathology, and molecular tests need to be strongly integrated into routine diagnostic practice (Chiecchio, 2020; Horn et al., 2014; O'Connor et al., 2020).

Even though it is possible to work with various types of cells and tissues, the principles of FISH essentially consist of the following steps: fixation of the target DNA on the surface of a microscope slide; pretreatment of the slide to receive the probe; denaturation of the DNA; hybridization of the target sequences and probe; post-hybridization slide washing; addition of the counterstain DAPI (4′,6-diamidino-2phenylindole).

DAPI is the most used dye in the FISH technique (FISH) due to its high affinity for DNA, allowing brilliant staining and high resolution of cell nuclei. However, there are other fluorescent dyes available, such as Propidium Iodide (PI), and Hoechst (HC), which are also used, offering alternatives depending on the specific needs of the experiment and the characteristics of the samples (Jež et al., 2013; Speicher et al., 1996).

Among different types of samples, FFPE tissue is the most challenging to undergo FISH, as many tissue processing variables can influence the quality of results. Differences in pre-analytical and analytical steps can affect the quality and efficiency of hybridization; therefore, a certain level of protocol customization is necessary to obtain optimal results in FISH assays performed on FFPE tissue sections. The variation in enzymatic digestion, denaturation, and hybridization times is a critical aspect in tissue analysis. Different tissues may require varied times for accurate results. Additionally, specific times and temperatures depend on the probe used and must be clearly defined. Internal standardization is essential to optimize and achieve better results (Table 1). Some important factors to consider for successful hybridization include sample fixation time, tissue processing, enzymatic pretreatment, hybridization conditions, and post-hybridization washing conditions (Chiecchio, 2020; Petersen et al., 2004).

Few studies have detailed methodologies for the application of FISH in FFPE. This technique is well-established in pathology laboratories, representing a valuable complementary approach (Faruqi et al., 2012). Therefore, this article proposes troubleshooting solutions for FISH in FFPE tissues by reviewing the main issues that can interfere with the technique.

2 | METHODS

This study consists of a scoping review conducted based on the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement. To conduct this review, we mapped scientific evidence on the use of the FISH technique. Based on the established conditions, the following question was developed: "What are the main problems in performing the FISH technique on FFPE samples?" The review included 15 journal articles on FISH in human paraffin-embedded material, published between 1992 and 2023 (Celep et al., 2003; Chen et al., 2020; Chiang et al., 2012; ElAje et al., 2023; Grushko et al., 2022; Horn et al., 2014; Karlsson & Karlsson, 2011; Küçükodaci et al., 2012; Lim & Lim, 2017; Lutz

TABLE 1 Fluorescence in situ hybridization protocol in selected articles.

Author	Pre-treatment	Denaturation	Hybridization	Post-hybridization	Producer company
ElAje et al. (2023)	NM	NM	NM	NM	PharmDX
Celep et al. (2003)	PI	NM	40°C overnight	PI	Cambio
Chen et al. (2020)	PI	75°C por 10 min	37°C overnight	PI	ZytoVision
Chiang et al. (2012)	PI	80°C por 5 min	37°C overnight	PI	In-house
Grushko et al. (2022)	PI	72°C por 5 min	37°C overnight	PI	Vysis
Horn et al., (2022)	PI	80°C por 10 min	37°C overnight	PI	Vysis
Küçükodaci et al. (2012)	PI	72°C por 5 min	37°C overnight	PI	Vysis
Karlsson and Karlsson (2011)	PI	73°C por 6 min	37°C overnight	NM	Vysis
Lim and Lim (2017)	PI	80°C por 4 min	37°C overnight	PI	NM
Lutz et al. (1992)	PI	90°C por 5 min	37°C overnight	PI	In-house
Pradhan et al. (2015)	PI	75°C por 5 min	42°C overnight	PI	Abnova CO.
Selvarajan et al. (2003)	PI	75°C por 5 min	38°C overnight	PI	Vysis
Tan et al. (2003)	PI	72°C por 5 min	37°C overnight	PI	Vysis
Tantiwetrueangdet et al. (2007)	PI	80°C por 5 min	37°C overnight	PI	Vysis
Ying et al. (2018)	PI	83°C por 5 min	42°C overnight	PI	ZytoVision

Abbreviations: IS, internal standardization; NM, not mentioned.

et al., 1992; Pradhan et al., 2015; Selvarajan et al., 2003; Tan et al., 2003; Tantiwetrueangdet et al., 2007; Ying et al., 2018).

The inclusion criteria were defined as follows: only articles that addressed the FISH technique in FFPE material, with full-text availability, and published between 1992 and 2023, were considered for analysis. The exclusion criteria were articles not published in journals, studies involving FISH in liquid biopsy, nonfluorescent in situ hybridization, frozen tissue, studies not using human samples, and full articles not written in English, Spanish, or Portuguese.

The databases used were the National Library of Medicine (PubMed), Embase, Scopus Preview, Web of Science, and LILACS, with the following strategic keywords: "fluorescence in situ hybridization," "paraffin block," "human tissue", "formalin-fixed paraffin-embedded," "FPPE," and "FISH."

3 | RESULTS AND DISCUSSION

In this review, a total of 577 articles were found, of which 329 were excluded for addressing the technique in liquid biopsy, non-fluorescence material, or nonfluorescent hybridization in the title. After removing 99 duplicate articles, 149 remained. Upon posing the question about the main technical problems encountered in performing the technique, 32 articles were selected for full reading. Nine of them could not be located for reading—three articles full text were not available, five articles did not address FISH but rather pre-analytical factors for basic technique, automation for molecular biology (real-time PCR) and diagnostics, and one article was fully in Hebrew. After the double-check, eleven articles were removed for not presenting data related to technical processes, four weres excluded for not providing the full text, and two for not presenting the text in English, Spanish, or Portuguese. Finally, 15 articles were included in this study (Figure 1).

In this scoping review, we adopted a comprehensive approach to map and synthesize the current knowledge on troubleshooting in FISH on FFPE tissue.

4 | MAIN PROBLEMS THAT INTERFERE WITH FFPE FISH

4.1 | Fixing and processing

Conventional fixation of large surgical tissues is a slow process. Consequently, autolytic damage can occur in tissues if the fixative does not reach the central part of the sample quickly. On the other hand, prolonged formalin fixation can lead to antigen masking (Selvarajan et al., 2003). Inadequate fixation procedures, such as over-fixation, lead to the excessive formation of methylene bridges, and a large proportion of nucleic acids may become trapped in protein–protein cross-links, hindering the binding of the probe to the target DNA sequences (Babic et al., 2010; Yu et al., 2021).

Degraded or cross-linked DNA prevents probe binding, while denatured proteins generate high fluorescence noise, and in both situations, fluorescent signals are either undetected or impossible to score. Additionally, proper fixation ensures the preservation of tissue morphology, which is also a critical parameter for FISH analysis in solid tumors. Fixation time, storage, decalcifying agents, collagen abundance, and extracellular matrix can influence FISH signal intensity and thus complicate pathological diagnosis (Bogdanovska-Todorovska et al., 2018; Varella-Garcia, 2006; Yu et al., 2021). Different histopathology laboratories have various tissue fixation

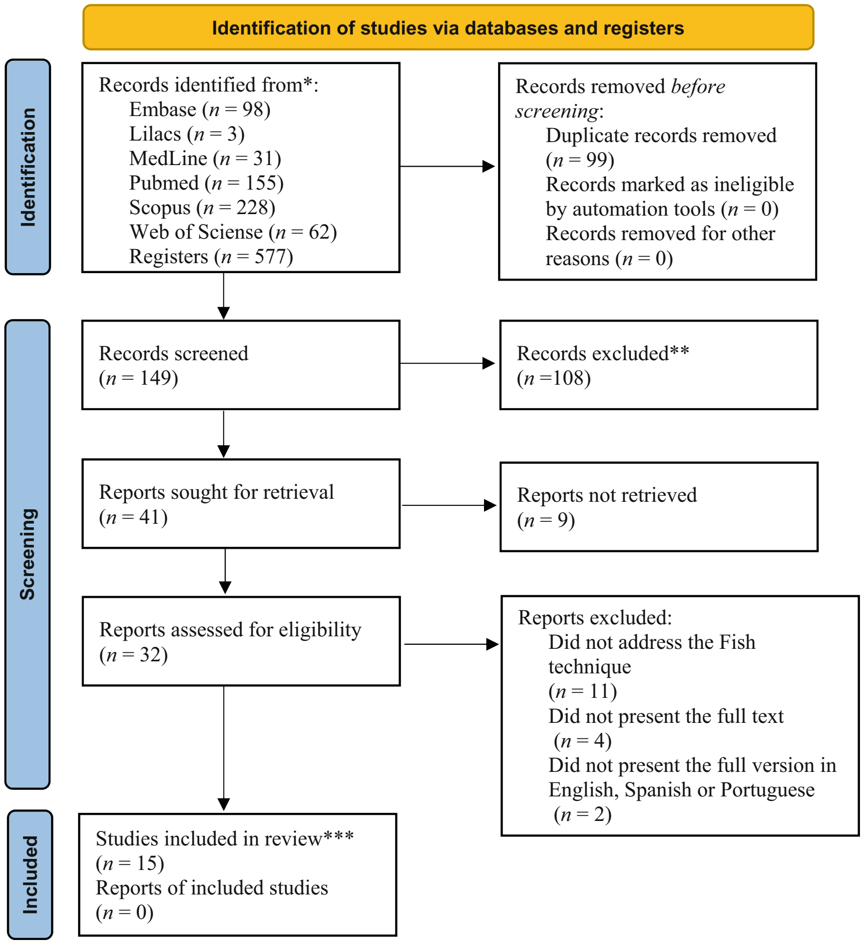

FIGURE 1 PRISMA 2020 flow diagram for new systematic reviews, which included searches of databases and registers only. *Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers). **If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools. ***In this review were included 15 articles each of which was read twice, following the "Double-Check" procedure. From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. BMJ 2021;372:N71 doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/.

methods that must be taken into consideration when evaluating the success of an experiment. For example, it is known that Bouin's fixative can hinder probe hybridization (Bayani & Squire, 2004; Chiecchio, 2020).

The optimal fixation time varies between 12 and 48 h. In a study conducted by KÜÇÜKODACI et al. (2012), involving 30 paraffin blocks in 3 microarrays fixed in 10% formalin, breast tissue samples were analyzed using FISH-HER2, with 100 cells evaluated per sample. Negative effects on FISH quality were more prominent with changes in the fixative pH. It is recommended to maintain the pH of buffered formalin between 7.2 and 7.4.

Chen et al., 2020, discussed the action of formalin on histopathological specimens, highlighting the degradation of DNA and RNA due to cross-linking among various substances. Additionally, it emphasized that inadequate processing may allow the retention of endogenous water in tissue sections. The importance of monitoring pre-analytical factors and implementing quality assessment strategies in sample handling, fixation time, probe selection, controls, and interpretation of results was also emphasized by ElAje et al., 2023.

Selvarajan et al., 2003, reinforced the interference of formalin fixation; therefore, FFPE archive tissues are challenging targets for cytogenetic studies in interphase. It has been suggested that the longer the primary fixation, the more aggressive the pre-treatment and enzymatic digestion steps need to be.

Histological processing varies depending on the type and size of tissues. After fixation, materials undergo dehydration in alcohol, with progressively increasing concentrations starting from 70%. Subsequently, clearing in xylene occurs, although some laboratories opt to replace the latter with isopropyl alcohol due to the toxicity associated with xylene. The risk of xylene to operators and the environment is widely recognized. Currently, the use of less toxic clearing agents, such as isopropanol, is being proposed to mitigate occupational hazards. However, few studies have systematically investigated the effects of xylene compared with isopropanol on tissues. A recent study by Wang et al. (2024) compared isopropanol and xylene in lung tissue processing, finding no significant differences in histological sections, hematoxylin and eosin (H&E) staining, immunofluorescence, and multiplex analysis. Further studies in different tissues are needed to determine if isopropyl alcohol is the best alternative to xylene for the FISH technique. Following this, the infiltration step in paraffin takes place, with temperature being crucial for the success of the FISH technique. According to Carson and Cappellano (2015), the ideal temperature for this process and embedding is 60–65°C.

4.2 | Contamination

Contamination can occur at various stages of the process, from sample reception to interpretation, and may result in incorrect diagnoses. To avoid this problem, rigorous quality control practices are suggested, such as double-checking, proper training, and preventive measures at all stages of the process (Santana & Ferreira, 2017).

Studies from the American College of Pathologists have shown that tissue contaminants are detected in approximately 0.6% of samples. Molecular approaches, such as Polymorphic deletion probe (PDP) FISH, have been useful in resolving specimen identification issues in problematic cases of contamination and unidentified tissue identification. PDP FISH can distinguish between tissue or cell genotypes from 2 individuals, regardless of sex, and has various applications, such as detecting cellular chimerism in organ transplantation (Chiang et al., 2012).

Lamothe et al. (2023) addresses concern regarding the unwanted presence of harmful substances in samples from surgical pathology laboratories, emphasizing the difficulty in quantifying their prevalence due to a lack of precise measures. The authors suggest categorizing certain contaminants as debris and emphasize the need for standardized criteria for pollution studies. The importance of complementary techniques, such as DNA analysis, in the precise identification of pollutants is highlighted, including the use of XY sex chromosome analysis. In addition, the study also reveals patterns in pathologists' decision-making in investigating contaminants, suggesting the utility of a differentiated approach for different types of pollutants, and proposes an algorithm for classifying and investigating potential tissue pollutants, aiming to improve standardization and risk detection in surgical pathology laboratories. The impact of these contaminants on ancillary molecular studies has not yet been fully understood.

4.3 | Block and slide age

Chen et al., 2020, considered 100 samples of blocks ranging from 1 to 10 years old. It was observed that the intensity of the FISH HER2 signal decreased with the age of the blocks, especially blocks over 5 years old. Grushko et al. (2022), compared old and recent slides of endometrial cancer, emphasizing the importance of avoiding the use of old slides to prevent false positive results. Storing the slide at 4°C was considered superior, and exposure to oxygen, light, humidity, and high temperature are interfering factors among more than 60 preanalytical and analytical variants (Chen et al., 2020; Karlsson & Karlsson, 2011).

Tissues cut into slides for an extended period at room temperature (over 4–6 weeks) may fail when tested by FISH; as such, the tissue may be better stored intact in paraffin blocks and sections cut only as needed. The acceptable storage time for paraffin blocks will depend on environmental factors (Chiecchio, 2020).

Selvarajan et al., 2003, in a study to assess HER/neu gene amplification in archived breast cancer tissue, also reported that they were unable to achieve hybridization with clear signals that could be reproducibly and reliably evaluated for all paraffin-embedded tissues that were stored for more than 12 months. There was also a tendency for autofluorescence in the embedded archive paraffin.

4.4 | Pre-treatment

Correct evaluation of the target area is important before starting the procedure. Most sections will contain tumors as well as areas of normal tissue. Additionally, the tumor itself may be partially necrotic and/or heavily infiltrated by non-tumor cells, such as host immune cells. Thus, it is important to identify the area of interest on a hematoxylin and eosin (H&E) stained slide and mark this area on the unstained slide for FISH analysis. Marking of the area of interest can be done using a pencil/pen with a diamond tip so that the area can still be visualized after post-wash steps (Chiecchio, 2020).

The purpose of pre-treatment is to reverse the formalin-induced cross-linking and make the DNA accessible to the probe during the hybridization phase; this is arguably the most challenging aspect of the technique to standardize (Bogdanovska-Todorovska et al., 2018; Chrzanowska et al., 2020; O'Connor et al., 2020).

For optimal results, it is crucial to optimize pre-treatment methods such as protease or pepsin digestion according to the specific characteristics of each case. A too short digestion time results in poor hybridization signals, possibly because the probe cannot adequately penetrate the nuclei. On the other hand, excessive digestion before hybridization may generate sufficient chromosomal signals but with distortion in nuclear morphology. Tumors with low differentiation tend to be more sensitive to pre-treatment procedures, while fibrotic and mucinous tumors are more resistant (Chiecchio, 2020; Selvarajan et al., 2003; Tantiwetrueangdet et al., 2007).

Celep et al., 2003, addressed pre-treatment for prostate adenocarcinoma, using 70% formamide followed by washes and specific labeling. Lim & Lim, 2017, recommended the use of pepsin and hybridization at 37°C for 16 h. The importance of balancing the pre-treatment time was emphasized, avoiding excesses that may result in tissue morphology loss or alteration. Inadequate protease digestion was also pointed out as a critical point.

4.5 | FISH technique

Denaturation aims is to render the DNA of both the probe and the sample into single strands; hence, a key advantage of FISH is it's in situ nature, as it preserves cellular morphology and tissue architecture. Co-denaturation of the probe and the target: this step involves the denaturation of the target DNA and the probe into single strands and hybridization. The probe volume needs to be adjusted depending on the size of the section (Chiecchio, 2020; O'Connor et al., 2020).

The post-hybridization wash temperature and/or inadequate washing significantly affect the intensity and stability of the signals. Thus, the temperature should be adjusted with the fixation time and age of the archived tissue samples (Bogdanovska-Todorovska et al., 2018; Petersen et al., 2004).

4.6 | Analysis through fluorescence microscopy

Ideal FISH results are achieved when the tissue exhibits good morphology, bright signal intensity, and very low background "noise." Samples showing evidence of chromatin overdigestion or poor probe penetration are not acceptable, as they may lead to false positive or negative results. These specimens should be retested after technical issues are resolved. Sometimes, a sample may show areas of differential digestion, with tumor cells being either over- or under-digested compared to normal cells (Chiecchio, 2020).

FISH preparations in liquid samples such as blood and bone marrow are characterized by intact and non-overlapping cells; within this context, interpreting FISH in FFPE tissue is more challenging due to signal truncation. Although analysis should be performed on non-overlapping tumor cells, some tissues may show few areas of non-overlapping cells. Gains are usually simpler to assess and less prone to false positive results when consistently visible in different areas. Evaluating copy number losses is more challenging as there may be a large proportion of cells exhibiting one (or none) signal due to signal drop-off and truncation of cell nuclei, which may also depend on the original section thickness, hence the ideal section thickness should be 3–4 μm.

Variation in the signal pattern is also an indication of whether a loss finding is real or a technical artifact; inconsistent signal loss patterns involving different probes in different cells are likely to represent artificial signal truncation. Evaluation of gains and/or losses may vary depending on the type of tumor (Chen et al., 2020; Chiecchio, 2020). Celep et al., 2003, demonstrated the utility of FISH

TABLE 2 Quick troubleshooting guide for fluorescence in situ hybridization in formalin-fixed paraffin-embedded (FFPE) samples (Duffy et al., 2012; Zordan, 2011).

Problem	Possible cause	Solution
Absence of signals or weak signals	Inadequate tissue fixation	Ensure that 10% buffered formalin is used
	Insufficient enzymatic digestion	Ensure that the appropriate digestion temperature was used, and that sufficient time was allowed
	Inadequate denaturation conditions	Ensure that the co-denaturation temperature used was at least 80°C for 10 min.
	Incorrect hybridization conditions	Ensure that hybridization occurs at 37°C for at least 14 h. Repeat with the appropriate temperature and time.
	Probe drying during hybridization	When using a hybridization oven, the use of wet chambers/water baths is mandatory. When using a hybridization oven, the use of a humidity chamber is required. Ensure that the coverslip is perfectly sealed.
	Post-hybridization washing conditions and excess washing	Ensure that recommended times, temperatures, and washing solutions are used. If necessary, decrease the time, or even omit the 2 × SSC/0.1% NP-40.
	The microscope not properly configured	Ensure that an appropriate filter set is in use, a suitable mercury lamp is being used and is not beyond its expected lifespan, and that appropriate fluorescence microscopy oil is being used.
	The signals disappeared	Minimize exposure to strong light sources and check the probe stock.
The region with irregular or absent signals	Insufficient probe usage	Ensure that the probe volume is sufficient to cover the entire area under the coverslip without any presence of air bubbles.
Excessive background	Inadequate tissue fixation	Ensure that 10% buffered formalin was used.
	Incomplete removal of paraffin	Repeat the protocol ensuring complete deparaffinization.
	Inadequate control of post-hybridization washing conditions	Ensure that recommended times, temperatures, and washing solutions are used. If necessary, increase the washing time with 2 × SSC/0.1% NP-40.
Nuclei with a "ghost-like" appearance and poorly defined cell periphery or complete loss of nuclei	Excessive enzymatic digestion	Repeat the protocol with a reduced digestion time.
	High co-denaturation temperature	While co-denaturation temperatures of up to 95°C are occasionally requested, this has an adverse effect on nuclear morphology. Repeat the protocol with a reduced co-denaturation temperature.
Autofluorescence, poor DAPI staining	Insufficient enzymatic digestion	Ensure that the appropriate digestion temperature was used, and that sufficient time was allowed. If necessary, increase the digestion time.
	FFPE samples cut with thickness above 4 μm	Thick sections cause cellular overlap and hinder enzymatic digestion. Ensure that sections are between 2 and 4 μm thick.

in paraffin-embedded material for screening numerical alterations, emphasizing the diagnostic, and prognostic importance of this approach.

In situ hybridization requires for its final stage a fluorescence microscope with a set of filters whose wavelengths (nm) must match those of the fluorochromes used in the probe labeling and detection system (Guerra, 2012). The fluorescence microscope needs to be of high quality and located in a dust-free, vibration-free area with well-adjusted filters. Fluorescence microscopy requires a bright light source at a specific wavelength that will excite the fluorochromes used (O'Connor et al., 2020).

As recommended in the literature, FISH results should not be analyzed if there is excessive background fluorescence that may mask the signal in more than 10% of cells, if the signals are weak and non-uniform in more than 25% of cells, if autofluorescence is high, or if nuclear morphology is not visible. FISH signals fade over time, so it is advisable to capture representative images of each case whenever possible. Slides can be stored at −20°C for at least 12 months (Bogdanovska-Todorovska et al., 2018).

ElAje et al. (2023) emphasized that although FISH HER2 is considered the gold standard for borderline cases, its execution is technically complex, time-consuming, and costly, mainly due to the high cost of fluorescence microscopes and imaging systems. The shortage of qualified labor is a limiting factor (Chrzanowska et al., 2020), and the availability of technical training courses is insufficient. To overcome these difficulties, virtual simulators and gamification techniques in healthcare are being developed (Do et al., 2023; Rinner et al., 2020), aiming to fill this gap.

In the era of digital pathology, high-resolution machines scan slides, and store information, while various artificial intelligence software aids in cell analysis. While high throughput approaches like next generation sequencing (NGS) are increasingly utilized in tumor FFPE sample analysis, FISH retains its unique ability to offer precise information on the localization and copy numbers of specific nucleic acid stretches at the single-cell level. Despite its limitation of addressing only one question per cell, FISH continues to be indispensable and serves as a gold standard in daily. This article highlights the effective resolution of key challenges in FISH on FFPE samples, providing a quick guide (Table 2).

4.7 | Innovation and FISH

Increasing research has reported the importance of the spatial context of tumor architectures to resolve the mechanisms of tumor initiation, progression, metastasis, and therapeutic response, and several spatial omics technologies have recently been developed and are being applied to various cancer research. Therefore, FISH has been widely used to map precise spatial information of tumor-specific biomarkers in various tissue samples. Spatial genomics is crucial in cancer biology as it allows the detection of underlying genetic aberrations. By hybridizing fluorescence probes with target genes, FISH identifies these aberrations and maps their spatial and chromosomal location within the nucleus. Advances such as DNA seqFISH+ increase the multiplexity of target DNA loci, allowing a more detailed analysis of genomic organization (Lee et al., 2024).

Spatially resolved transcriptomics is transforming pathology, allowing detailed assessment of gene expression in a spatial context and providing unprecedented insight into tissue organization and function. Techniques such as smFISH, combined with state-of-the-art spatial analysis platforms, are making these studies more accessible and efficient (Marx, 2021). In the future, the integration of spatially resolved gene expression data with in silico research promises to revolutionize the understanding of biology and disease, allowing gene expression patterns to be explored in a deeper and more integrated way.

5 | CONCLUSION

A thorough understanding of the technical challenges associated with FISH in FFPE samples is essential to ensure reliable results and precise interpretations. Diligently addressing these issues through the application of optimized protocols and appropriate pre-treatment strategies is crucial to enhancing the use of this technique in molecular studies and diagnostics. This underscores its relevance in the scientific and clinical landscape, highlighting the critical importance of a careful approach to ensure the robustness of the results. We hope that this review contributes to addressing the difficulties encountered in performing FISH on FFPE samples.

AUTHOR CONTRIBUTIONS

Cássia Campanhol Lemes: Conceptualization; investigation; writing – original draft; methodology; data curation; validation. **Andressa Germano da Silva:** Conceptualization; investigation; writing – original draft; methodology; validation; data curation. **Daniel Araki Ribeiro:** Validation. **Andréa Cristina de Moraes Malinverni:** Validation; writing – review and editing; supervision.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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