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The FISH Factor - Resolving Diagnostic Deadlocks in Salivary Gland Pathology

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Abstract

Fluorescence in situ hybridization (FISH) has evolved from a niche cytogenetic technique into a central pillar of modern molecular pathology, enabling the spatially resolved detection of genomic alterations within preserved cellular architecture. Its unique ability to localize chromosomal rearrangements, copy number variations, and gene amplifications at single-cell resolution distinguishes it from bulk genomic techniques and renders it indispensable in diagnostically challenging neoplasms. Salivary gland tumors, characterized by marked morphological heterogeneity and overlapping histopathological features, exemplify the clinical need for such targeted molecular tools. The identification of recurrent gene fusions, including CRTC1–MAML2, MYB–NFIB, ETV6–NTRK3, and EWSR1-associated rearrangements, has fundamentally redefined tumor classification and established FISH as a critical adjunct in routine practice. This review provides an in-depth and critical synthesis of the molecular principles underlying FISH, probe design strategies, technical workflow, interpretative pitfalls, and clinical applications, with particular emphasis on salivary gland neoplasia. The evolving role of FISH in precision oncology, its integration with next-generation sequencing, and future directions involving digital pathology and artificial intelligence are also explored, underscoring its continued relevance in the era of genomic medicine.

Keywords: Fluorescence in situ hybridization; FISH; salivary gland tumors; gene fusion; molecular diagnostics; cytogenetics; precision oncology

Introduction

The incorporation of molecular diagnostics into routine histopathological practice has profoundly transformed the conceptual landscape of disease classification, marking a transition from purely morphology-driven systems to a more precise, genotype-oriented paradigm. Although conventional histopathology remains the cornerstone of diagnosis, it is increasingly evident that morphological evaluation alone is insufficient to encompass the underlying biological heterogeneity of neoplastic processes. This limitation is particularly pronounced in salivary gland tumors, which are characterized by extensive architectural variability and significant cytological overlap across entities, frequently resulting in diagnostic uncertainty and considerable interobserver variability. Within this evolving framework, fluorescence in situ hybridization (FISH) has emerged as a highly valuable adjunct, offering objective and reproducible insights into tumor-specific genetic alterations that complement and refine morphological interpretation [1,2].

Beyond its diagnostic contribution, the true significance of FISH lies in its unique capacity to integrate molecular data with preserved tissue architecture, thereby effectively bridging the divide between traditional morphology and contemporary molecular biology. In contrast to sequencing-based methodologies that rely on bulk nucleic acid extraction and consequently obscure spatial heterogeneity, FISH enables direct visualization of genomic aberrations at the single-cell level within intact histological sections. This attribute is particularly critical in the context of intratumoral heterogeneity, where distinct subclonal populations may harbor divergent genetic profiles with implications for tumor progression, prognosis, and therapeutic response. In salivary gland neoplasia, the identification of recurrent chromosomal translocations has provided a robust molecular foundation for tumor classification, and FISH has consequently become an indispensable tool for the detection and validation of these defining genetic events in routine diagnostic practice [3–5].

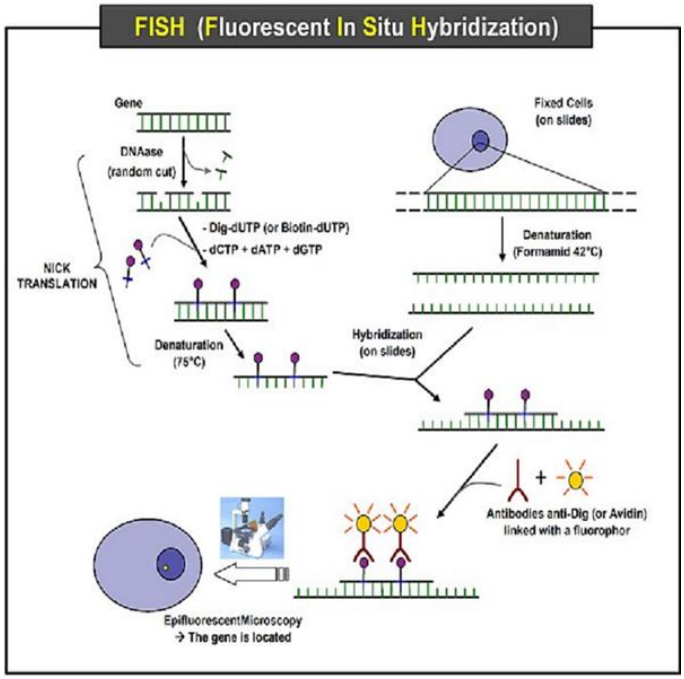
Evolution of Cytogenetics to Molecular Cytogenetics

The foundations of tumor cytogenetics were established with the advent of karyotypic analysis, particularly through chromosome banding techniques that enabled the visualization of largescale chromosomal abnormalities. These early methodologies provided critical insights into the genomic architecture of malignancies and facilitated the identification of recurrent structural alterations associated with specific tumor types. However, their clinical applicability was constrained by several inherent limitations, most notably the requirement for actively dividing cells and a relatively low resolution that precluded detection of submicroscopic genetic changes. The emergence of molecular cytogenetics, and in particular the development of fluorescence in situ hybridization (FISH), marked a transformative advancement by enabling the precise localization of defined DNA sequences within chromosomal structures using fluorescently labeled probes [6,7].

In its initial applications, FISH was primarily performed on metaphase chromosome preparations, thereby retaining dependence on proliferating cells. The subsequent introduction of interphase FISH represented a major conceptual and practical breakthrough, as it allowed for the analysis of non-dividing nuclei within formalin-fixed paraffin-embedded tissue sections. This innovation significantly broadened the clinical utility of the technique, especially in surgical pathology settings where fresh or viable tissue is often unavailable. With

continued technological evolution, refinements in probe design, improvements in hybridization chemistry, and the development of advanced fluorescence imaging systems have collectively enhanced both the analytical sensitivity and specificity of FISH. These advances have facilitated its transition from a specialized research tool to an integral component of routine diagnostic workflows across a wide spectrum of pathological disciplines [8].

Molecular Principles and Hybridization Dynamics



At its fundamental level, fluorescence in situ hybridization is grounded in the principle of complementary base pairing between nucleic acid sequences, whereby fluorescently labeled DNA probes selectively anneal to their corresponding genomic targets following denaturation of double-stranded DNA. This hybridization event generates discrete, locus-specific fluorescent signals that can be visualized within intact nuclei using fluorescence microscopy, thereby enabling direct spatial mapping of genetic material. The fidelity and efficiency of probe hybridization are influenced by multiple physicochemical parameters, including probe length and sequence composition, guanine–cytosine content, hybridization temperature, and the stringency of post-hybridization washing conditions. Achieving optimal hybridization thus necessitates a finely controlled balance between sensitivity and specificity, as overly stringent conditions may compromise signal intensity, whereas reduced stringency can result in nonspecific binding and increased background fluorescence [9].

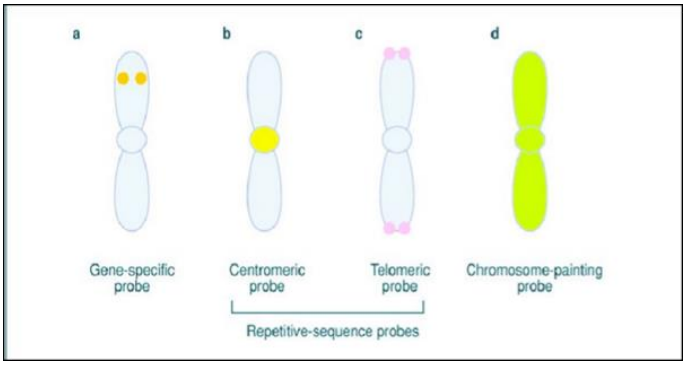
A defining strength of FISH lies in its capacity to detect balanced chromosomal rearrangements, particularly translocations, which do not necessarily alter DNA copy number and are therefore often undetectable by copy number–based techniques such as comparative genomic hybridization. This attribute is especially relevant in neoplasms driven by recurrent gene fusions, where the presence of a specific rearrangement constitutes a diagnostic and sometimes prognostic hallmark. In addition, FISH enables analysis at the single-cell level within preserved tissue architecture, allowing for the identification of intratumoral heterogeneity and the detection of subclonal populations that may have distinct genetic profiles. Such spatially resolved insights into clonal architecture are increasingly recognized as critical determinants of tumor progression, therapeutic response, and disease outcome, further underscoring the unique value of FISH within the broader landscape of molecular diagnostics [10].

Probe Design and Conceptual Framework

The diagnostic performance of fluorescence in situ hybridization is fundamentally contingent upon the strategic design and selection of probes, which must be precisely aligned with the specific genomic alteration under investigation. Among the most widely utilized probe configurations are break-apart probes, which are particularly advantageous for the detection of gene rearrangements in situations where the fusion partner is either unknown or highly variable. These probes are

designed to hybridize to sequences flanking a target gene locus, such that disruption of the locus through chromosomal rearrangement results in spatial separation of the fluorescent signals, thereby providing a robust indicator of gene breakage. In contrast, dual-fusion probes are engineered to detect defined reciprocal translocations by simultaneously targeting two distinct genes; the resultant co-localization of fluorescent signals reflects the physical juxtaposition of these loci. Although dual-fusion probes offer superior specificity for canonical translocations, their applicability is inherently restricted to wellcharacterized fusion events, limiting their utility in tumors with heterogeneous or novel rearrangements [11].

In addition to rearrangement-focused probes, locus-specific probes play a crucial role in the assessment of gene copy number alterations, including amplification and deletion. These probes are frequently employed in the evaluation of clinically significant oncogenes, such as HER2, where gene amplification carries both diagnostic and therapeutic implications. Centromeric probes, which target highly repetitive alpha-satellite DNA sequences within centromeric regions, are commonly used for chromosome enumeration and the detection of aneuploidy, often serving as internal controls in copy number analyses. The selection of an appropriate probe strategy is therefore not merely a technical consideration but a biologically informed decision that must take into account the molecular landscape of the tumor, the clinical question being addressed, and the diagnostic limitations of alternative methodologies. Such a tailored approach is essential to maximize the interpretive accuracy and clinical utility of FISH in routine practice. [11]



Technical Workflow and Pre-Analytical Considerations

The successful implementation of fluorescence in situ hybridization in diagnostic practice is critically dependent on rigorous optimization of technical parameters across all stages of the workflow, beginning with the pre-analytical phase. Among these variables, tissue fixation plays a pivotal role in preserving nucleic acid integrity and ensuring accessibility of target

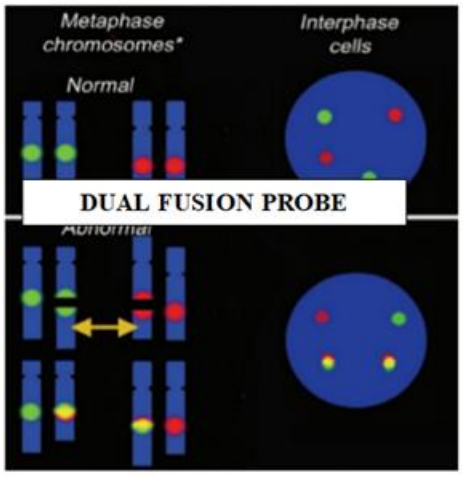
DNA sequences. Formalin fixation remains the standard approach; however, excessive fixation can induce extensive cross-linking between nucleic acids and proteins, thereby hindering probe penetration and reducing hybridization efficiency. In addition, factors such as tissue processing protocols and section thickness exert significant influence on signal quality. Sections that are excessively thin are prone to nuclear truncation, leading to partial loss of target sequences and potential false-negative interpretations, whereas thicker sections may introduce interpretative challenges due to nuclear overlap and increased background fluorescence [12].

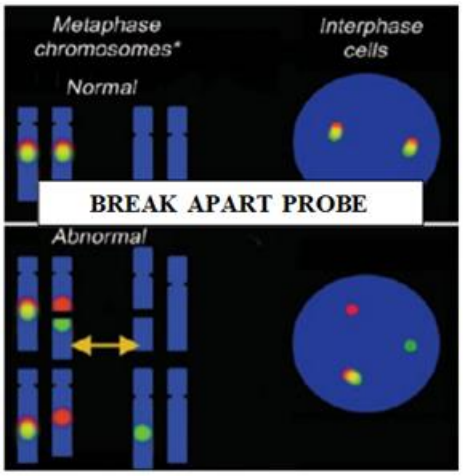
The analytical phase encompasses DNA denaturation, probe hybridization, and posthybridization washing, each of which requires precise calibration to achieve optimal signal specificity and intensity. Hybridization conditions, including temperature, duration, and stringency, must be carefully tailored to the physicochemical properties of the probe and the genomic characteristics of the target sequence. Even minor deviations in these parameters can

significantly impact assay performance, underscoring the need for standardized protocols and quality control measures. Following hybridization, accurate interpretation necessitates the systematic evaluation of an adequate number of nuclei, typically guided by established laboratory thresholds. These cutoff values, derived from validation studies and control populations, are essential for distinguishing true positive signals from background variation or technical artifacts. Importantly, such thresholds are not universally fixed and may vary depending on probe design, tissue type, and analytical context, highlighting the need for assay specific validation to ensure diagnostic reliability. [11]

Interpretative Complexity and Diagnostic Pitfalls

The interpretation of fluorescence in situ hybridization findings is inherently nuanced and demands a thorough understanding of expected signal patterns in conjunction with an awareness of potential technical and biological artifacts. In normal diploid cells, probe hybridization typically yields paired signals corresponding to homologous chromosomal loci, whereas pathological alterations may manifest as signal separation in the setting of gene rearrangement or as an increased number of signals reflecting gene amplification. However,





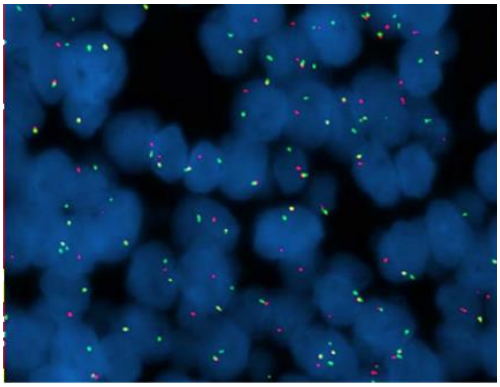
the distinction between true genetic abnormalities and artifactual variations is not always straightforward, and misinterpretation can arise from multiple sources. [10]

One of the most common technical challenges is nuclear truncation, an artifact introduced during sectioning of formalin-fixed paraffin-embedded tissue, which may result in partial loss of nuclear material and consequently reduced signal intensity, leading to false interpretation of deletions. Similarly, in highly cellular or poorly preserved specimens, overlapping or closely apposed nuclei can create the illusion of signal co-localization, thereby mimicking fusion events and generating false-positive results. Variability in hybridization efficiency, influenced by pre-analytical and analytical factors, may further contribute to weak, heterogeneous, or absent signal detection, complicating accurate assessment. These interpretative complexities necessitate a cautious and integrative approach, wherein FISH findings are evaluated in the context of histomorphological features and immunohistochemical profiles. Moreover, strict adherence to quality assurance protocols and the use of validated scoring criteria are essential to ensure reproducibility and diagnostic reliability [10].

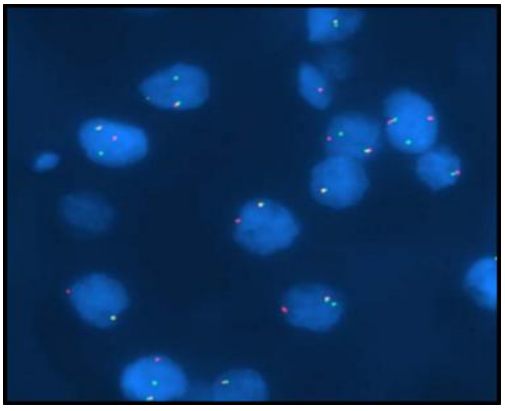
Molecular Pathogenesis and FISH in Salivary Gland Neoplasms

The expanding application of fluorescence in situ hybridization in salivary gland pathology has been fundamentally propelled by the recognition of recurrent, tumor-defining genetic alterations that underpin distinct biological entities. These molecular events not only refine diagnostic accuracy but also provide insights into tumorigenic mechanisms and clinical behavior. Mucoepidermoid carcinoma, one of the most common malignant salivary gland tumors, is typified by a characteristic chromosomal translocation involving the CRTC1 and MAML2 genes, most frequently t(11;19)(q21;p13). This fusion event leads to aberrant activation of the Notch signaling pathway, thereby promoting oncogenic transcriptional programs while paradoxically being associated with a more favorable clinical course compared to fusion-negative counterparts [13,14]. In contrast, adenoid cystic carcinoma is commonly driven by the MYB–NFIB fusion resulting from t(6;9) translocation, which induces persistent activation of MYB-dependent transcriptional networks, contributing to tumor persistence, perineural invasion, and its characteristic indolent yet relentless clinical progression[15].

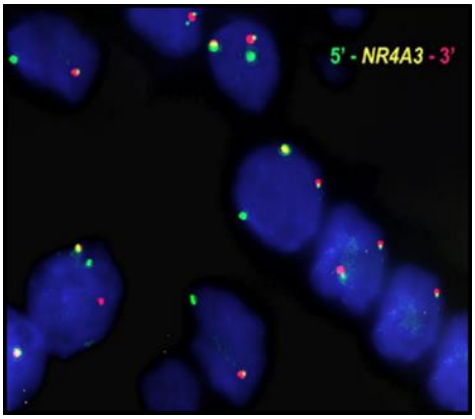
Secretory carcinoma of the salivary gland represents a paradigm of molecularly defined neoplasia, being consistently associated with the ETV6–NTRK3 fusion gene. This chimeric tyrosine kinase confers constitutive signaling activity, rendering the tumor amenable to targeted inhibition by TRK inhibitors, thereby establishing both diagnostic and therapeutic relevance. In routine practice, demonstration of ETV6 rearrangement by FISH has become a defining criterion for confirming this entity. Similarly, hyalinizing clear cell carcinoma is characterized by recurrent EWSR1 gene rearrangements, most commonly involving ATF1, which serve as a crucial molecular discriminator from other clear cell–rich salivary neoplasms and metastatic mimics. Acinic cell carcinoma, although historically defined on morphological grounds, has more recently been shown to harbor activation of the NR4A3 transcription factor, frequently driven by enhancer hijacking mechanisms, further emphasizing the evolving role of molecular diagnostics in refining tumor classification. Collectively, these discoveries underscore the pivotal role of FISH in not only confirming lineage-specific genetic alterations but also in integrating molecular pathology into routine diagnostic algorithms for salivary gland tumors [16–18].



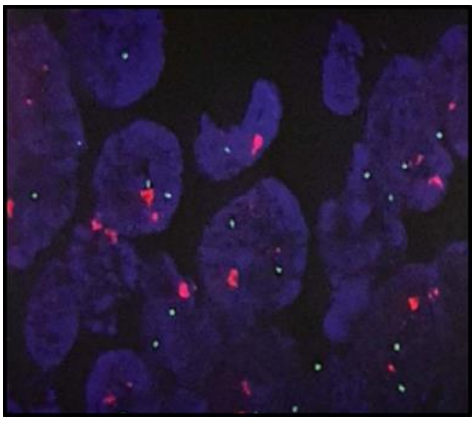
Fluorescence in situ hybridization in Adenoid Cystic Carcinoma reveals rearrangement of the v-myb avian myeloblastosis viral oncogene homolog (myb) locus (separation of the red and green signals).



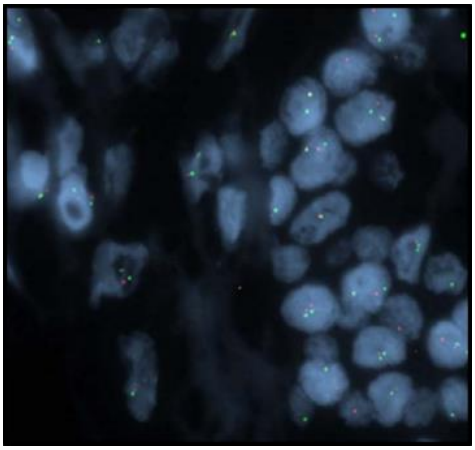
Fluorescence in situ hybridization in Mucoepidermoid Carcinoma reveals rearrangement of the mastermind-like 2 (MAML2) locus (separation of red and green signals).



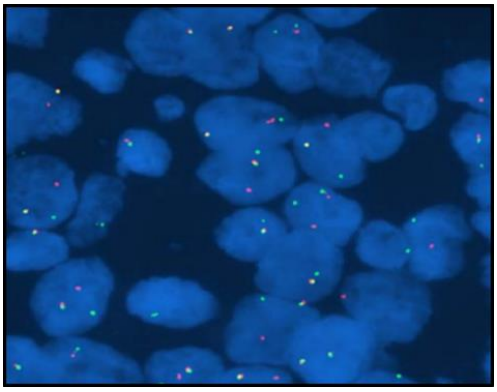
Dual-color break-apart interphase. Split red and green hybridization signals suggest the presence of a genomic NR4A3 rearrangement in Acinic Cell Carcinoma.



Fish analysis is positive for erbb2 (also called her2) gene amplification, showing numerous red signals (erbb2) versus a normal number of green signals (centromere 17) in Salivary Duct Carcinoma.



Fluorescence in situ hybridization reveals rearrangement of the EWS RNA binding protein 1 (ewsr1) locus (separation of the red and green signals).



Fluorescence in situ hybridization in MASC reveals rearrangement of the ets variant 6 (etv6) locus (separation of red and green signals).

FISH as a Bridge to Precision Oncology

The utility of fluorescence in situ hybridization extends well beyond its established role in diagnosis, encompassing significant prognostic and predictive dimensions that are integral to contemporary oncologic practice. The identification of actionable genomic alterations through FISH has facilitated the transition from empiric treatment strategies to mechanism-based targeted therapies, thereby substantially improving clinical outcomes in selected patient populations. A paradigmatic example is provided by tumors harboring NTRK gene fusions, in which constitutive activation of TRK signaling pathways drives oncogenesis. The detection of such fusions by FISH enables the deployment of highly selective TRK inhibitors, including larotrectinib and entrectinib, both of which have demonstrated remarkable and durable response rates across a broad spectrum of tumor types irrespective of histological origin [19]. In a similar vein, assessment of HER2 gene amplification using FISH remains a clinically validated approach for identifying patients who are likely to benefit from HER2-targeted therapies, thereby serving as a critical determinant of therapeutic stratification. Likewise, the detection of RET gene rearrangements has direct therapeutic implications in the

context of emerging RET inhibitors, further underscoring the expanding relevance of FISH in guiding precision-based interventions. Collectively, these applications illustrate the evolving role of FISH as a pivotal interface between molecular diagnostics and targeted therapeutics, reinforcing its central contribution to the implementation of precision oncology and individualized patient care. [19,20]

Integration with Next-Generation Sequencing and Future Directions

Although next-generation sequencing has revolutionized oncologic genomics by enabling comprehensive and high-throughput characterization of genetic alterations, it inherently lacks the spatial resolution necessary to contextualize these changes within the architectural framework of tissues. Sequencing-based methodologies typically rely on DNA extracted from bulk tumor samples, thereby averaging signals across heterogeneous cell populations and potentially masking the presence of biologically significant subclonal alterations. In contrast, fluorescence in situ hybridization offers the distinct advantage of single-cell analysis within intact histological sections, permitting direct visualization of genetic abnormalities in their spatial context. This capability is particularly valuable in the assessment of intratumoral heterogeneity, where discrete subpopulations of tumor cells may harbor divergent genomic profiles that influence disease progression and therapeutic response. Consequently, FISH and next-generation sequencing are increasingly viewed as complementary rather than competing modalities, with FISH providing rapid, targeted validation of specific genomic events and sequencing platforms delivering a broader, discovery-oriented overview of the tumor genome [20].

Looking forward, the evolution of FISH is likely to be shaped by its integration with digital pathology and advances in artificial intelligence, which hold promise for enhancing both the accuracy and reproducibility of signal interpretation through automated detection and quantification. In parallel, the development of multiplex FISH techniques capable of simultaneously interrogating multiple genomic loci within a single assay is expected to expand its analytical scope and efficiency. The convergence of FISH with other molecular and imaging modalities may further facilitate a more comprehensive, multi-dimensional understanding of tumor biology, thereby reinforcing its role within integrated diagnostic frameworks and ensuring its continued relevance in the era of precision medicine. [1,20]

Conclusion

Fluorescence in situ hybridization continues to occupy a central and enduring role in contemporary diagnostic pathology, particularly in the evaluation of salivary gland neoplasms, where morphological complexity often necessitates molecular corroboration. Its unique capacity to detect clinically relevant genetic alterations within preserved tissue architecture affords critical insights into tumor pathogenesis while simultaneously enhancing diagnostic precision, prognostic stratification, and therapeutic decision-making. In an era increasingly defined by precision oncology, the value of FISH lies not only in its diagnostic robustness but also in its ability to function as a rapid, targeted, and spatially informative modality within integrated diagnostic algorithms. As molecular technologies continue to advance, FISH is unlikely to be supplanted; rather, it will persist as a complementary and indispensable technique that bridges histomorphology with genomic data, thereby reinforcing its pivotal contribution to personalized patient care and the broader evolution of translational pathology.

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