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Modern Methods of DNA Probe Synthesis for Fluorescence *in situ* Hybridization (FISH): Technologies and Applications

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ABSTRACT

Fluorescent *in situ* hybridization (FISH) remains an indispensable tool for molecular diagnostics, which makes it possible to detect chromosomal abnormalities underlying many hereditary and oncological diseases with high accuracy. The advancement of medicine towards personalized approaches and the expansion of the spectrum of diagnosed pathologies require constant improvement of methods for synthesizing DNA probes. Despite existing limitations, such as the cost and complexity of synthesis, the future of FISH diagnostics is linked to the development of highly specific, multiplex, and affordable probes that will enable the transition to complex genome and transcriptome analysis. The aim of this article was to reflect the evolution of probe production methods from classical to high-tech ones, including SABER-FISH, CRISPR/Cas9 (CASFISH), and smFISH technologies.

Keywords: fluorescence *in situ* hybridization, molecular cytogenetics, DNA probe synthesis, chromosomal aberrations

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Современные методы синтеза ДНК-зондов для флуоресцентной гибридизации *in situ* (FISH): технологии и применение

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РЕЗЮМЕ

Метод флуоресцентной гибридизации *in situ* (FISH) остается незаменимым инструментом молекулярной диагностики, позволяющим с высокой точностью выявлять хромосомные аномалии, лежащие в основе многих наследственных и онкологических заболеваний. Движение медицины в сторону персонализированных подходов и расширение спектра диагностируемых патологий требуют постоянного совершенствования методов синтеза ДНК-зондов. Несмотря на существующие ограничения, такие как стоимость и сложность синтеза, будущее диагностики с помощью FISH связано с разработкой высокоспецифичных, мультиплексных и доступных зондов, которые позволят перейти к комплексному анализу генома и транскриптома. Данная работа написана с целью отразить эволюцию методов получения зондов от классических к высокотехнологичным, включая SABER-FISH, технологии на основе CRISPR/Cas9 (CASFISH), smFISH.

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Ключевые слова: флуоресцентная гибридизация *in situ*, молекулярная цитогенетика, синтез ДНК-проб, хромосомные аберрации

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INTRODUCTION

Fluorescence *in situ* hybridization (FISH) remains one of the most reliable and sensitive cytogenetic techniques for detecting major chromosomal aberrations, including deletions, amplifications, translocations, and inversions, thereby enabling highly accurate quantification of cells harboring these abnormalities [1]. This powerful cytological method facilitates the detection and precise localization of specific nucleic acid sequences directly on metaphase chromosomes or within interphase nuclei through the use of fluorescently labeled DNA probes. Within clinical diagnostics, FISH has become an indispensable tool for identifying chromosomal abnormalities, such as aneuploidies, translocations, deletions, and amplifications that underpin numerous hereditary disorders and oncological conditions. In basic research, FISH enables the investigation of genome architecture, spatial chromosome organization, gene expression patterns, and chromatin dynamics.

The evolution of the FISH methodology is inextricably linked to advancements in DNA probe technology. This progression has transitioned from the initial use of radioactive labels, which were limited by low resolution and associated health hazards, to contemporary fluorescent detection systems. The adoption of fluorescent probes has provided superior spatial resolution, multiplexing capabilities, enhanced safety, and the potential for quantitative analysis. Consequently, the quality, specificity, and accessibility of DNA probes are critical determinants of the efficacy and broad applicability of the FISH technique.

In light of the rapid advancements in molecular biology and genomics, which demand increasingly sophisticated and specialized probes, the systematization of modern synthesis methods is becoming a task of critical importance. The aim of this review is to conduct a comprehensive analysis of current technologies for synthesizing DNA probes for FISH, to compare their respective efficiencies, and to identify the most promising directions for future development in this rapidly evolving field.

CLASSICAL APPROACHES TO CREATING DNA PROBES FOR FISH

DNA probes utilized for fluorescence *in situ* hybridization (FISH) are classified into three principal groups: (1) whole-chromosome painting probes, (2) repetitive sequence probes, and (3) locus-specific probes. Each group fulfills distinct experimental and diagnostic objectives, necessitating divergent methodological approaches for their synthesis.

Whole-chromosome painting probes are used to detect complex interchromosomal rearrangements, which are used in both scientific research and clinical practice [2]. The conventional methodology for their generation involves the microdissection of target metaphase chromosomes or chromosomal regions via laser capture or mechanical scraping with a glass micropipette. The isolated genetic material is subsequently subjected to amplification, followed by labeling with fluorochromes [3]. This technique is notably labor-intensive and requires a high degree of technical expertise. For commercial-scale production, flow cytometric sorting is the predominant method [4]. This approach enables the isolation of all human chromosomes, with the exception of chromosomes 9–12 due to their analogous size and morphological characteristics. Approximately 300 chromosomes are required to generate a single probe. The resultant DNA is fragmented and amplified enzymatically by polymerase chain reaction (PCR), a process that increases template quantity and enhances hybridization specificity by suppressing non-specific binding [5].

For the construction of both whole-chromosome and locus-specific probes, cloning strategies utilizing large-insert genomic libraries, particularly those housed in artificial chromosome vectors, are frequently employed. Among these, bacterial artificial chromosomes (BACs) are accorded significant prominence due to their advantageous properties, which include facile DNA purification,

low incidence of chimeric clones, and high genomic stability [6]. Although the derivation of probes from BAC or plasmid clones necessitates additional procedures involving microbial culture and nucleic acid extraction, this method remains indispensable. It is particularly critical for the development of probes targeting pericentromeric and subtelomeric regions, as the highly repetitive DNA content in these loci presents a substantial limitation for *de novo* enzymatic or chemical oligonucleotide synthesis.

A fundamentally distinct class of FISH probes is represented by oligonucleotide-based probes. Such probes can be engineered to target both repetitive genomic elements and unique, single-copy DNA sequences. For the former, a complete reference genome sequence is not a prerequisite [7]. Signal amplification is achieved intrinsically through the hybridization to multiple identical repeat elements, and the probe length typically ranges from 15 to 30 base pairs (bp), produced via chemical synthesis. While shorter probes exhibit enhanced accessibility to their target sites, their capacity to incorporate fluorescent reporter molecules is proportionally limited [8]. For oligonucleotide probes designed against unique sequences, a complex pool of oligonucleotides must be synthesized. The collective length of this pool must exceed 10–30 kilobase (kb) to ensure the resultant DNA probe generates a signal of sufficient intensity for clear visualization at the specific chromosomal locus during FISH analysis.

Probes for FISH analysis have become an indispensable component of cytogenetic diagnostics for determining the level of genetic mosaicism, identifying marker chromosomes, and elucidating their origin and compositional architecture [9]. Comprehensive, chromosome-specific DNA libraries have been constructed and are continuously refined for the diagnosis of prevalent aneuploidy syndromes, including Patau, Edwards, and Down syndromes (trisomies of autosomes 13, 18, and 21, respectively), as well as Klinefelter syndrome and Turner syndrome (which are associated with numerical abnormalities of the X and Y sex chromosomes).

Locus-specific probes have been developed to identify Robertsonian translocations, Prader–Willi syndrome, and Angelman syndrome among others, and are furthermore employed to detect specific microdeletions and chromosomal rearrangements [10]. Additionally, gene-specific probes are utilized to detect gene amplification events, a common characteristic of neoplastic cells. A prominent example is the assessment of the copy number variation of the human epidermal growth factor receptor 2 (*HER2*) gene, which serves as a critical prognostic biomarker in breast cancer [11] and ovarian cancer [12].

A distinctive category of probes is constituted by PNA (peptide nucleic acid) probes. A PNA molecule is structurally analogous to DNA, with the fundamental distinction that its sugar-phosphate backbone is replaced by an uncharged N-(2-aminoethyl)-glycine pseudopeptide polymer. This neutral charge confers upon the PNA probe a significantly higher binding affinity for complementary DNA or RNA sequences compared to traditional DNA-DNA or RNA-RNA duplexes [13]. PNA was originally developed by the research group of Peter Nielsen in 1991 [14]. The atypical structure of PNA probes is not a substrate for nucleases or proteases, thereby substantially extending their half-life in both *in vivo* and *in vitro* applications [15], while still permitting direct fluorescent labeling. The implementation of PNA FISH has enabled the precise detection of signals at chromatid ends and the accurate measurement of telomere length in metaphase chromosomes [16, 17].

Methods for Synthesizing DNA Probes

As previously indicated, enzymatic and chemical methodologies for the *de novo* synthesis of oligonucleotide probes have achieved widespread adoption. Enzymatic strategies for DNA probe synthesis encompass techniques such as degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) and long-range PCR.

The PCR necessitates the presence of a target sequence, which serves as the template for probe synthesis. In DOP-PCR, templates most frequently consist of BAC clones or microdissected chromosomal material, as these provide the researcher with an isolated genomic region of interest. The fundamental principle of DOP-PCR involves the utilization of partially degenerate primers. These primers incorporate a core random hexamer sequence flanked on both the 3’ and 5’ ends by defined nucleotide sequences.

The protocol mandates two consecutive PCR amplifications. The second amplification stage employs primers complementary to the fixed sequences introduced during the initial cycle and is conducted at an elevated annealing temperature to enhance specificity [18]. The employment of high-fidelity proofreading polymerases, such as *Pwo*, in conjunction with extended annealing and elongation durations, optimizes assay performance. This results

in superior fidelity, the generation of longer amplicons, and increased genomic coverage [19, 20].

The synthesis of FISH DNA probes via long-range PCR entails a multi-stage process: identification of the target genomic locus; subsequent design and selection of specific oligonucleotide primers for the designated sites; generation of long DNA amplicons (up to 15 kb in length) via long-range PCR; creation of a DNA fragment library through enzymatic or non-enzymatic fragmentation methods; amplification of this library using the pre-designed target-specific primers; and ultimately, the incorporation of a fluorophore into the amplified genomic library. This synthesis paradigm is predominantly employed for the generation of locus-specific DNA probes.

Chemical synthesis of oligonucleotides for *in situ* hybridization applications is categorized into solid-phase and microarray-based synthesis. Contemporary oligonucleotide production is dominated by automated instrumentation utilizing the solid-phase phosphoramidite method, a technology pioneered by Marvin Caruthers in the 1980s [21, 22]. Oligonucleotide synthesis proceeds via a cyclic four-step reaction. In this process, nucleotide phosphoramidites are sequentially added to a nascent oligonucleotide chain that is covalently attached to an insoluble solid support. The cycle initiates with a deprotection step, removing an acid-labile dimethoxytrityl (DMT) group to reveal a reactive 5'-hydroxyl moiety. Subsequently, an activated phosphoramidite monomer is coupled to this hydroxyl group, forming an unstable phosphite triester linkage. Any unreacted 5'-hydroxyl groups are then rendered inert through a capping step with acetic anhydride, a critical measure to prevent the formation of deletion sequences. The cycle concludes with an oxidation step, converting the phosphite triester into a more stable phosphate triester. This iterative cycle is repeated until the desired oligonucleotide length is attained. Upon completion of the synthesis, the full-length oligonucleotide is cleaved from the solid support and subjected to deprotection [23]. Final purification of the crude oligomers is achieved through techniques such as high-performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE).

A logical technological progression from conventional solid-phase synthesis is microarray oligonucleotide synthesis. In the early 1990s, the American company Affymetrix developed one of the first synthesizers for oligonucleotide microarrays utilizing photolabile protecting groups, an innovation that ultimately catalyzed the entire DNA microarray industry [24]. This method is essentially a modification of the standard phosphoramidite chemistry, wherein the acid-labile DMT group is substituted for a photolabile protecting group, allowing for light-directed spatial control of synthesis [25]. The synthesizer from Agilent Technologies (USA) is a leader in terms of both the multiplexing capacity (simultaneous synthesis of up to 1 million unique oligonucleotides on a single substrate) and the achievable oligonucleotide length, which can extend to 200 nucleotides [24].

Enzymatic methodologies for the synthesis of DNA probes are increasingly being used in laboratory practice, a trend partially attributable to the labor-intensive nature of conventional chemical synthesis protocols. Conversely, chemical synthesis affords a superior degree of structural customization: standard nucleotides can be substituted with synthetic analogues, the stoichiometry of incorporated fluorochromes can be precisely controlled, among other modifications. Oligonucleotides generated via chemical synthesis are typically shorter in length (up to 100 nucleotides), a characteristic that facilitates enhanced penetration and accessibility to their target sequences, particularly when hybridization is performed on complex tissue sections rather than individual cell preparations. In contrast, elongated probes synthesized via PCR amplification demonstrate heightened hybridization specificity due to their increased complexity; however, the presence of amplification byproducts can potentially contribute to elevated non-specific background signal. Probes intended for clinical applications – specifically the diagnosis of substantial chromosomal rearrangements associated with various pathologies, which are in high demand – are predominantly manufactured utilizing enzymatic synthesis techniques. The principal consumers of commercially available, chemically synthesized probes are research institutions. These probes command a higher cost but possess a greater multiplexing potential, thereby enabling more sophisticated experimental applications, including the quantitative analysis of individual transcript molecules.

Probe Labeling Methods

Probe labeling methodologies for fluorescence *in situ* hybridization are conventionally categorized into two principal classes: direct labeling, wherein fluorophores are covalently conjugated to the nucleotide chain, and indirect labeling, where a fluorescent signal is produced by a secondary complex that is bound to the probe via an intermediary molecule.

One of the simplest techniques for the direct labeling of DNA/RNA oligonucleotides involves the incorporation of fluorophores during automated chemical synthesis. In this approach, fluorescently tagged phosphoramidite monomers are directly integrated into the growing oligonucleotide chain. This chemical method is highly reproducible as it is independent of the specific activity of labile enzymatic components.

Classical enzymatic strategies for direct labeling exploit the inherent ability of DNA polymerases to extend primer templates. Direct enzymatic labeling is predominantly achieved through techniques such as DOP-PCR, primer extension, and nick translation.

The primer extension method entails the hybridization of a short primer, complementary to a specific genomic locus, to a single DNA strand, followed by its extension by a DNA polymerase. In contrast to PCR-based amplification, this is a linear process with no exponential amplification of the template and, consequently, no concomitant amplification of the fluorescent signal [26].

The nick translation method utilizes a synergistic enzymatic combination: deoxyribonuclease I (DNase I), which introduces single-strand nicks into double-stranded DNA, thereby generating free 3'-hydroxyl groups, and DNA polymerase I, which incorporates nucleotides at these 3' ends. Concurrently, the 5'–3' exonuclease activity of DNA polymerase I degrades the DNA strand ahead of the nick. During this repair synthesis, labeled and unlaneled nucleotides are incorporated, resulting in the generation of double-stranded, uniformly labeled probes [27]. A significant limitation of this technique is that the introduction of nicks can potentially compromise the probe's specificity for its target sequence. Furthermore, the method necessitates a relatively substantial quantity of input DNA (approximately 1 μg).

Direct labeling is more ubiquitously employed due to its procedural simplicity and convenience. Post-hybridization, the protocol merely requires washing steps to remove excess unbound probe.

In indirect labeling schemes, a hapten-modified nucleotide (e.g., deoxyuridine monophosphate conjugated to a reporter molecule) is incorporated into the probe. This reporter molecule subsequently forms a high-affinity complex with a fluorescently labeled ligand. The most prevalent reporter-ligand pairs include biotin–avidin/streptavidin, digoxigenin with anti-digoxigenin antibodies, and estrogen with specific anti-estrogen antibodies [28]. The primary disadvantage of indirect labeling is that it requires additional incubation steps subsequent to the hybridization reaction. However, this is frequently counterbalanced by a significant amplification of the final fluorescent signal, a particular advantage when working with targets of low abundance.

For further amplification of the signal generated via indirect labeling, tyramide signal amplification (TSA) can be employed. TSA is predicated on the catalytic activity of horseradish peroxidase (HRP), which, in the presence of low concentrations of hydrogen peroxide, converts fluorophore-conjugated tyramide substrates into highly reactive radical species. These radicals form covalent bonds with electron-rich tyrosine residues on proteins located in immediate proximity to the enzyme [29]. The TSA protocol consists of sequential steps: fixation of the cell or tissue sample; incubation with a primary biotinylated antibody; incubation with a secondary antibody conjugated to horseradish peroxidase; and finally, incubation with the fluorophore-tyramide substrate. As the tyramide deposition is covalent, multiple sequential rounds of TSA, employing tyramides conjugated to distinct fluorophores, can be performed on a single sample for the multiplexed detection of numerous targets.

Methods for Increasing the Signal-to-Background Ratio

A fundamental prerequisite for successful signal detection in FISH is that the intensity of the specific hybridization signal must significantly exceed the level of background autofluorescence inherent to the sample. Enhancement of the signal-to-noise ratio can be achieved through two principal strategies: suppression of the signal emanating from dispersed repetitive genomic sequences or augmentation of the signal intensity derived from the unique target sequences. The former strategy typically employs methods to competitively inhibit the hybridization of repetitive elements, while the latter focuses on enriching the proportion of unique sequences within the DNA probe preparation [30].

One of the seminal methodologies for suppressing the hybridization of repetitive sequences is chromosomal *in situ* suppression (CISS) hybridization. This technique is based on the pre-hybridization of the labeled DNA probe with a substantial excess of unlabeled C_0t-1 DNA, which is derived from the same species and is enriched for high-copy repetitive elements. This pre-annealing step allows the repetitive sequences within the probe to form duplexes with excess C_0t-1 DNA, thereby preventing them from

hybridizing to the chromosomal target and reducing non-specific background [31]. However, CISS hybridization often proves ineffective for organisms with very large genomes, as the extreme complexity and abundance of repetitive sequences impedes their complete and efficient suppression [32].

A contrasting line of research involves the development of techniques for the physical elimination of repetitive sequences from pre-existing DNA clone libraries prior to probe labeling. One such method employs magnetic separation of macromolecules for this purpose [33]. In this protocol, magnetic nano- or microparticles are utilized to form complexes with specific DNA fragments. In a standard magnetic separation procedure, the material from the original DNA library is first hybridized with biotin-labeled C_0t-1 DNA. The resultant duplexes containing repetitive sequences are then removed from the solution via affinity chromatography using streptavidin-coated magnetic beads. The supernatant now enriched for unique sequences is subsequently amplified by PCR. This process yields DNA samples with a significantly increased proportion of unique sequences, which are then used for labeling [30]. The net effect of this purification is analogous to that of CISS hybridization. Nevertheless, the utilization of C_0t-1 DNA for generating low-repetition DNA probes carries an inherent risk of co-eliminating some adjacent unique sequences through their association with repetitive elements, potentially leading to a loss of genomic coverage in the final probe.

ADVANCED TECHNOLOGIES FOR DNA PROBE SYNTHESIS

Probes synthesized utilizing the classical methodologies previously delineated remain the most extensively employed in both laboratory practice and diagnosis. Despite their widespread adoption, research consortia globally persist in refining existing technologies and pioneering novel approaches for FISH probe generation.

• SABER-FISH

This methodology is predicated on primer exchange reaction (PER) technology, initially described by J.Y.Kishi et al. in 2017 [34]. A subsequent modification termed SABER-FISH (signal amplification by exchange reaction) was advanced by the same research group in 2019 [35]. Within this paradigm, the foundational probe oligonucleotide incorporates a sequence complementary to the target molecule (30–50 nucleotides) and an auxiliary 9-nucleotide sequence at the 3’ end, which functions as a primer for the PER cascade. This primer anneals to a catalytic hairpin structure, which subsequently serves as a template for the enzymatic synthesis of concatemers. Polymerases possessing strand-displacement activity, such as *Bst* LF, mediate this synthesis without hydrolyzing the DNA backbone, elongating the chain. The amplification cycle is terminated by thermal denaturation at 80 °C. Each catalytic cycle appends approximately 10 nucleotides; iterative cycling enables the concatemer to attain lengths of several hundred nucleotides, a parameter dictating the ultimate fluorescence signal intensity. Following hybridization of these concatemerized probes to their chromosomal targets, a secondary hybridization with fluorescently labeled oligonucleotide ‘imagers’ (20–30 nucleotides complementary to the concatemer) is requisite for signal detection. This technology facilitates a 5 to 450-fold signal amplification and enables highly multiplexed analyses on a single sample.

• LNA Probes

Locked nucleic acids (LNAs) represent structural analogs of RNA wherein the ribofuranose ring is constrained in a C3’-endo conformation via a methylene bridge linking the 2’-oxygen and the 4’-carbon atoms. LNA nucleotides obey the Watson–Crick base-pairing rules and are integrated into oligonucleotides via a standard phosphodiester backbone, permitting the chemical synthesis of chimeric LNA-DNA or LNA-RNA oligonucleotides using conventional phosphoramidite chemistry.

The conformational rigidity imparted by the bicyclic structure enhances the thermal stability of duplexes formed with complementary DNA or RNA sequences, significantly increasing binding affinity [36, 37]. Analogous to PNA probes, the atypical structure of LNA oligonucleotides confers considerable resistance to degradation by nucleases.

• smFISH

Single-molecule FISH (smFISH) is a quantitative methodology that enables the formulation of precise mathematical models, thereby facilitating the investigation of biological processes at an exceptionally high resolution. The quantitative visualization of RNA, for instance, permits the exact enumeration of transcript copies within individual cells, subcellular compartments, and distinct ribonucleoprotein complexes [38].

A considerable diversity of commercial, ready-to-use *in situ* hybridization solutions is now available from numerous entities, including Thermo Fisher Scientific (Affymetrix) with its ViewRNA and PrimeFlow platforms, Bio-Techne (Advanced Cell Diagnostics, ACD) with the RNAscope system, and LGC Biosearch Technologies with Stellaris FISH probes. Despite this commercial availability, the development of novel probe labeling strategies, frequently aimed at cost reduction, remains an active area of research.

An exemplar of a comparatively cost-effective and efficacious technology is the $3P^3$ (three-pot probe production assay), delineated by its authors in 2018 [39]. This methodology comprises three principal stages: (1) conjugation of the fluorophore to a modified terminator nucleotide (NH2-ddUTP); (2) enzymatic labeling of oligonucleotide-DNA probes utilizing terminal deoxynucleotidyl transferase (TdT); (3) purification of the final labeled probe. The foundational principle relies on the template-independent addition of nucleotides to the 3'-hydroxyl end of DNA oligonucleotides catalyzed by TdT. Although this enzyme possesses the capacity for indefinite elongation, the strategic incorporation of dideoxynucleotides ensures the incorporation of precisely a single labeled nucleotide, thereby terminating further extension. While TdT activity has been historically exploited for labeling, these authors pioneered the application for labeling complex pools of standard oligonucleotides via PCR, employing a custom-synthesized biotin-conjugated ddUTP. This reaction achieves a high yield, exceeding 90%, of probes bearing a single label.

A more intricate protocol for smFISH probe generation was detailed by a separate research consortium in 2022 [40]. The initial phase involves the *in silico* design of a substantial pool of primary oligonucleotides complementary to discrete regions of the target RNA molecule. These oligonucleotides subsequently undergo two rounds of PCR amplification followed by *in vitro* transcription. The resultant oligonucleotide-RNA products are then labeled via hybridization with short LNA oligonucleotides that are directly conjugated to fluorophores. A significant advantage of this platform is its inherent capacity for multiplexed, simultaneous detection of numerous distinct RNA targets.

• Quantum Dot Methodology

Conventional organic fluorophores, while widely employed, exhibit photophysical limitations, such as photobleaching and blinking, that can constrain their utility in FISH applications. Inorganic nanocrystals, or quantum dots (QDs), represent a next-generation alternative, offering exceptional photostability and enabling more reliable transcript quantification due to their superior brightness (high signal intensity). A primary challenge in deploying QDs as FISH labels pertains to their substantially larger hydrodynamic diameter (~25–35 nm) compared to organic dyes (~1 nm) [41]. Contemporary research efforts are consequently directed toward the synthesis of more compact QD constructs [42]. The second challenge is the propensity for non-specific adsorption of QDs, inherent to their solid-state colloidal nature, onto cellular structures. This phenomenon can be effectively overcome by the inclusion of blocking agents such as bovine serum albumin (BSA) and polyanions, within the hybridization buffer.

• CASFISH

In 2015, W. Deng and colleagues [43] pioneered the adaptation of the CRISPR/Cas9 system for FISH, designating the technology CASFISH. In their approach, a HaloTag ligand covalently conjugated to a fluorophore was attached to a recombinant catalytically inactive Cas9 protein (dCas9). Concurrently, the guide RNA (sgRNA) designed to target a specific satellite DNA sequence was itself fluorescently labeled. Initial hybridization experiments on mouse embryonic fibroblasts required merely 30 minutes, successfully yielding detectable signals at pericentromeric regions via fluorescence microscopy. Subsequent investigations have demonstrated the exceptional thermodynamic stability of the resultant dCas9/sgRNA/target DNA ternary complex; fluorescence intensity remains unaltered even in the presence of a hundredfold molar excess of competing non-target DNA. These properties – speed and high specificity – endow the CASFISH platform with significant potential for performing highly multiplexed analyses, allowing for sequential or simultaneous hybridizations with multiple distinct probes on a single sample.

CONCLUSION

The analysis of modern methods for synthesizing DNA probes for FISH demonstrates a variety of technologies with unique advantages. Classical enzymatic approaches, such as nick translation and PCR incorporating labeled nucleotides, maintain their relevance for the generation of long-range probes essential for the comprehensive mapping of extensive genomic loci. Both chemical and enzymatic labeling strategies provide significant flexibility

for the structural modification of oligonucleotides. The most transformative advancement has emerged from the field of oligonucleotide synthesis, enabling the engineering of sophisticated constructs with set properties, enhanced specificity, and superior multiplexing capabilities. These are critical for the analysis of complex genomic rearrangements and for applications in spatial transcriptomics.

Despite these advancements, considerable limitations persist. The synthesis of long, highly specific probes remains a labor-intensive and costly endeavor. Although oligonucleotide-based methods are becoming increasingly accessible, they necessitate sophisticated bioinformatic design and extensive empirical optimization to mitigate non-specific background hybridization, a challenge particularly acute in regions rich in repetitive sequences.

Future prospects for the field are anticipated to evolve along several pivotal trajectories: 1) the enhanced automation and standardization of probe synthesis and purification processes to reduce costs and improve inter-laboratory reproducibility; 2) the development of novel fluorophores with improved brightness and photostability, coupled with advanced detection systems; 3) the deeper integration of computational biology and bioinformatic tools to optimize probe design *in silico* and accurately predict hybridization kinetics and specificity; 4) the creation of innovative hybrid methodologies that synergistically combine the strengths of disparate synthesis technologies. It is a reasonable projection that the continued refinement of DNA probe synthesis methodologies will serve as a primary catalyst for the evolution of FISH technology, thereby substantially expanding its diagnostic and research applications.

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