

OLIGONUCLEOTIDE PROBES FOR RNA-TARGETED FLUORESCENCE *IN SITU* HYBRIDIZATION

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1. Abstract

The need for accurate and rapid methodology for detecting cells in environmental and clinical samples has led to the development of *in situ* detection methods, where fixed or intact cells can be imaged directly. In this chapter, we focus on the use of labeled oligonucleotide probes in fluorescence *in situ* hybridization (FISH). We give an overview of FISH probe design, covering issues of affinity and specificity of probes, probe backbone options, cellular targets, and accessibility of target sequences. Decisions that must be made to design optimal probes are evaluated, and available resources to assist in probe design, such as secondary structure, T_m calculation, and site accessibility software, are discussed. We cover different types of FISH probes that have

been reported in the recent literature, including standard fluorescently labeled oligonucleotide probes and newer classes of quenched oligonucleotide probes: molecular beacons and quenched autoligation probes. Advantages and disadvantages of the different probe types are examined and recent literature applications are discussed. The current state of the art in the field as well as limitations and challenges in detection are evaluated.

2. Introduction

Identification and classification of cells in environmental and clinical samples can be critical for determining both prophylactic and curative responses. In dealing with contamination of food or water supplies by microorganisms, bacterial infections in tissues, and cancer-causing mutations in biopsy samples, a rapid diagnosis may be a life or death issue. Standard detection of cells in clinical or environmental samples requires any of a number of methods including culture, antigen detection, serology, nucleic acid amplification, and other biochemical assays [1, 2]. Despite the widespread use of these detection strategies, many can be time-consuming, difficult, and yield inconclusive results. As a result, a great deal of research in clinical and environmental chemistry focuses on developing rapid and specific methods to identify cells and gain information about cell type and characteristics. One of the most promising techniques for doing this is *in situ* detection of nucleic acids.

Oligonucleotide and polynucleotide probes have long been used in detection of nucleic acids in cells; the first report of RNA-targeted *in situ* hybridization came in 1989 by DeLong *et al.* [3], and over the past two decades, RNA-targeted fluorescence *in situ* hybridization (FISH) has become a critical biotechnological tool in environmental sampling and clinical diagnostics. This chapter will focus on short, well-defined oligonucleotide probes, which display much higher sensitivity to small sequence variations in the target than do the much longer enzymatically synthesized polynucleotide probes. The applications of oligonucleotide probes are numerous, and they are becoming commercially available in rapidly increasing numbers.

Despite the growing availability and applications of oligonucleotide probes for *in situ* hybridization, there remain several important factors that limit their use, and these issues are at the forefront of modern research. Among these issues is the difficulty in predicting whether a specific target site will yield useful signal or not; this is important because searching for a target can be costly in time and money, and because detecting genetic differences may only be possible at locally defined sites. A second important issue is sequence specificity; as little as a single nucleotide difference may define

an important genetic variation, and such high specificity is often difficult to achieve by hybridization alone. Third is the problem that many, perhaps even most, potentially useful target RNAs exist in low copy numbers, which means that signal intensity may be low and background signal may interfere. Finally, it is currently difficult to perform FISH in living cells; this would be useful in the study of the dynamics and localization of RNAs, and also might be applied to imaging gene expression in the whole organism. One of the chief aims of this chapter is to define these problems in more detail and to outline current and emerging molecular FISH approaches that are addressing these issues.

This chapter will outline the current FISH standard approach, employing oligonucleotides with fluorescent labels attached, and will then introduce emerging strategies involving the newer classes of quenched probes. The most common strategy for RNA-targeted FISH employs static fluorescent probes to sense sequences, requiring cell fixation and washing steps to remove unbound probes which would otherwise interfere with the desired signal. Quenched probes use a dark quencher, such as dabcy1 or black hole quencher [4], to quench the fluorescence of the fluorophore when the probe is not bound to template. Once the probe hybridizes to its template, the quencher is separated from the fluorophore either by conformational or chemical change in the structure of the probe, thereby establishing fluorescence signal [5]. Nonhybridized quenched probes are therefore nonfluorescent and do not need to be washed out of cells, which raises the possibility of applications in living cells and tissues.

In the following sections, we outline the fundamentals that are critical to understanding and developing oligonucleotide-based FISH techniques, give an overview of the scope and limitations of current and emerging methods, and finally describe applications of FISH probes toward *in situ* diagnostics targeted to cellular RNAs, with an emphasis on probe design and the scope and limitations of various methodologies. We begin with a discussion in [Section 3](#) of the basic principles of RNA-targeted FISH: hybridization affinity and specificity, nucleic acid backbone effects, targets and site accessibility. In [Section 4.1](#), we discuss the most widely used FISH technique, employing oligonucleotide probes, and then we discuss quenched probes—molecular beacons and quenched autoligation probes—in [Sections 4.2 and 4.3](#). Finally, we discuss some of the numerous applications of RNA-targeted FISH probes in molecular diagnostics for bacterial and human cells in [Section 5](#).

3. Principles of Fluorescence *In Situ* Hybridization

Probe design and determination of optimal experimental conditions for hybridization to cellular RNAs are often not trivial, and both are critical to employing FISH successfully. Determining the utility of a given probe set is

TABLE 1
ONLINE TOOLS FOR PROBE DESIGN

	URL	References
Secondary structure and site accessibility prediction		
Dynalign	http://rna.urmc.rochester.edu/	[86]
mFOLD	http://www.bioinfo.rpi.edu/applications/mfold/	[79, 80]
Pfold	http://www.daimi.au.dk/~compbio/rnafold/	[85]
sFOLD	http://www.bioinfo.rpi.edu/applications/sfold/index.pl	[81, 218]
Sequence alignment		
ClusalW	http://www.ebi.ac.uk/clustalw/#	[77]
T_m prediction		
DINAMelt	http://www.bioinfo.rpi.edu/applications/hybrid/hybrid2.php	[17, 18]
HYTHER	http://ozone2.chem.wayne.edu/	[19, 20]
Probe construction		
ARB	http://www.arb-home.de/	[78]
PRIMROSE	http://www.cardiff.ac.uk/biosi/research/biosoft/	[103]
probeBase	http://www.microbial-ecology.net/probebase/	[70]

largely empirical [6], and low fluorescent responses can be caused by a number of factors. Low affinity of the probe for its target can be caused by poor probe design, inappropriate hybridization conditions, and inaccessibility of target sites due to either secondary or tertiary RNA structure or associated proteins. Low signal can also result from low RNA content of the cells and from difficulty permeating cell walls. Some useful probe design tools available online are summarized in Table 1.

3.1. HYBRIDIZATION AFFINITY AND SPECIFICITY

Recent advances in the biophysics and biochemistry of nucleic acids over the past decades have paved the way for the development of hybridization-based approaches in detecting cells. The intricately related issues of affinity and specificity are critical to the design of nucleic acid probes for detection applications [7]. Affinity is the propensity of the probe to bind to its target under a given set of hybridization conditions, defined as the overall free energy change of the probe binding to its target site ($\Delta G^\circ_{\text{overall}}$) [8, 9]. Specificity is the propensity of the probe to bind only to its target and not to other sites with similar sequences. Typically, affinity and specificity are inversely related, meaning that as affinity for the selected target increases, so does the likelihood of binding to similar nontarget sequences [10].

The traditional approach for determining probe affinity has been the use of probe melting temperature (T_m), the temperature at which half of the

population of oligonucleotides is hybridized, an indicator of the stability of the probe-target duplex [11]. A number of factors affect T_m , including length, GC content, and nearest neighbors, particularly at the site of a mismatch [12–15]. However, because enthalpic and entropic contributions may be very different for unrelated sequences, T_m is not always a good predictor for probe affinity.

More recently, Yilmaz and Noguera demonstrated a free energy-based approach to designing high-affinity FISH probes [9]. The proposed reaction scheme for FISH, shown in Fig. 1, consists of three equilibria: reaction 1 represents binding of the unfolded probe to the unfolded complementary site, reaction 2 represents unfolding or folding of the probe, and reaction 3 represents unfolding or folding of the target site. Probes are typically designed with minimal self-complementarity, so the contribution from reaction 2 is often small, but target RNAs typically have a great deal of higher order structure, so the contribution of reaction 3 may be very significant, depending on the target site (Section 3.4). Using these considerations, the authors were able to demonstrate a relationship between probe affinity ($\Delta G^\circ_{\text{overall}}$) and hybridization efficiency, as measured by probe brightness [9]. Thus, by predicting $\Delta G^\circ_{\text{overall}}$ for a FISH reaction, it may be possible to predict whether a given probe will have sufficient affinity to generate signal. A value of -13.0 kcal/mol is suggested for maximizing affinity while maintaining reasonable specificity. Using this value as a threshold, Yilmaz *et al.* demonstrated that signal-generating probes can be designed even for RNA regions previously believed to be inaccessible [16] (Section 3.4).

While free energy-based approaches show promise for generating high-affinity probes, preparation of extremely high-specificity probes remains problematic. In these applications, T_m remains a useful tool. When comparing closely related systems (same probe length and hybridization conditions, similar sequence), T_m can be a useful predictor in the design of high-sensitivity probes. For cases where only one or two mismatches are present between probe and target, the probe may have significant affinity for the

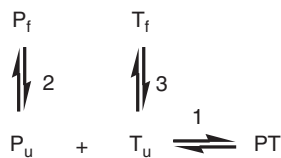


FIG. 1. Proposed reaction scheme for FISH [8, 9]. P, T, and PT denote the DNA probe, the target, and the probe/target hybrid, respectively. The subscripts f and u indicate the folded and unfolded conformations.

mismatched target (and thus low specificity), so the stability of the resulting duplex is significant in determining whether unwanted background signal from binding will be generated.

Several programs for predicting both free energies and T_m 's are available: the DINAMelt Server predicts full melting profiles and thermodynamic parameters for DNA/DNA and RNA/RNA hybridization [17, 18], and HYTHER provides thermodynamic data but not a full melting profile for DNA/DNA, DNA/RNA, and RNA/RNA hybridization [19, 20]. There are a few significant limitations to calculation of T_m by these methods that are worth noting. First, target secondary structure is not considered; only the stability of the probe-target duplex is calculated. Second, although there are a number of studies that determine the effects of mismatches on DNA/DNA duplexes [20–24] and RNA/RNA duplexes [25, 26], there is only one such study for DNA/RNA duplexes [12], and it is not fully exhaustive in the combinations of mismatches examined.

Affinity may be increased by increasing the probe length, or in some cases by altering the hybridization conditions (e.g., raising the concentration of Mg^{2+} or lowering the hybridization temperature). However, increasing specificity while maintaining high affinity tends to be more difficult. A single mismatch has a stronger impact on shorter probes than on longer ones. This may beg the question: why not simply use very short probes when very high specificity is required? The answer is twofold. First, the genetic code is made up of only four letters, so the likelihood for undesired target sequence redundancy is greater for shorter probes. Second, affinity is lowered with decreasing probe lengths, making very short probes incompatible with FISH protocols.

3.2. BASE AND BACKBONE MODIFICATIONS

There are a number of methods for improving affinity of nucleic acid interactions, the most widely studied of which are using backbone- or base-modified probes. Modified bases include C-5 propynyl pyrimidines [27–29], 5-Me-C [30–32], 2-amino-A [30, 32], and 7-propynyl-8-aza-7-deazapurines [33] (Fig. 2). C-5 propynyl pyrimidines, for example, have been shown to increase mismatch penalties by as much as 4.7–10.2 kcal/mol for a single mismatch, resulting in a T_m difference of up to 14°C [34]. Although modified bases can improve melting temperatures of matched sequences by 0.5–3°C per individual substitution, they can be prohibitively expensive, especially when multiple substitutions are desired, and may not be available for incorporation into commercially purchased probes.

Common modified backbones for enhancing affinity include 2'-*O*-methyl (2'-OMe) RNA, 2'-fluoro (2'-F) RNA, locked nucleic acid (LNA), and

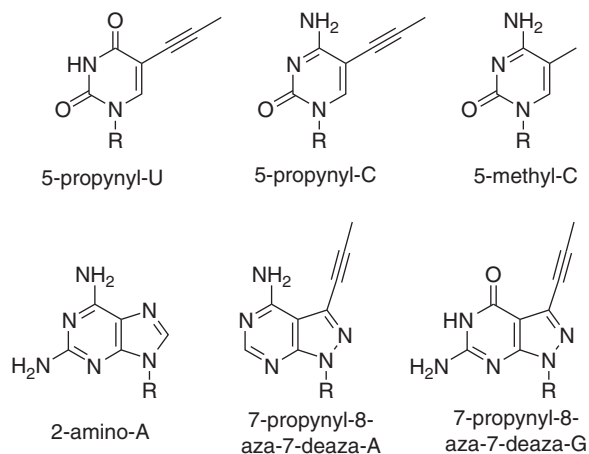
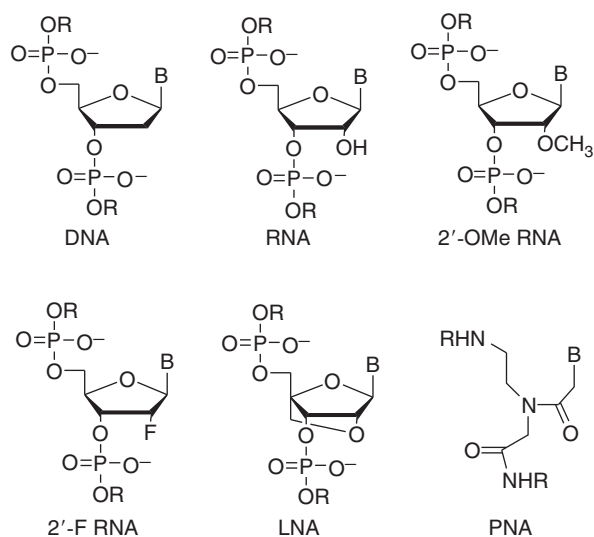


FIG. 2. DNA base modifications for improving mismatch selectivity.

FIG. 3. Common oligonucleotide backbone modifications used in fluorescence *in situ* hybridization probes.

peptide nucleic acid (PNA) (Fig. 3). The thermodynamic stability of different backbone-containing nucleic acids hybridized to RNA template goes roughly in the order DNA < RNA < 2'-OMe RNA < 2'-F RNA < LNA/PNA [35].

Of these, all have been used for *in situ* hybridization studies except 2'-F RNA, possibly because it is extremely expensive. 2'-F RNA is known to increase T_m 's approximately 1.8°C per substitution, with a slight cooperative effect (i.e., the ΔT_m per substitution increases the more 2'-F RNA bases that are incorporated into the probe) [36], but does not have any other properties that make it superior to other options for FISH. The natural RNA backbone is also not used much for *in situ* hybridization experiments because of its instability and the requirement that RNase-free reagents and techniques must be employed when handling it to prevent degradation. With the development of phosphoramidite chemistry, all of these backbones (except PNA) can be readily incorporated into oligonucleotides in combination [37]. PNA can be synthesized using Boc or Fmoc chemistry for solid-phase peptide synthesis [38].

2'-OMe RNA is an attractive backbone modification because it hybridizes more strongly than DNA and, unlike natural RNA, is extremely stable to RNases and DNases [39]. Oligonucleotide probes containing 2'-OMe RNA are particularly useful in live cell applications because of this stability. The enzymatic resistance is explained by the decreased nucleophilicity and increased steric bulk of the methoxy group as opposed to the hydroxyl group in natural RNA, and even greater stability has been observed with larger alkyl groups at the 2' position [40, 41]. Furthermore, 2'-OMe RNA oligonucleotides have been reported to exhibit faster hybridization kinetics than DNA probes, and due to their increased T_m 's, can in some cases bind to RNA targets that contain significant secondary structure [42].

LNA is a class of conformationally restricted oligonucleotide analogs in which a ribonucleoside is linked with a methylene group between the 2'-O and the 4'-C [43, 44]. It resembles natural nucleic acids with respect to base-pairing and duplex formation, but LNA duplexes with complementary nucleic acids are very thermally stable compared to the respective DNA or RNA duplex [45]. Single or multiple, but separated, LNA modifications appear to have a greater impact on probe affinity than contiguous stretches of LNA bases, so most probes incorporate one or a few LNA nucleotides into a DNA strand for hybridization [46]. The T_m of hybridization to RNA may be increased by as much as 8.3°C for a single LNA nucleotide incorporation [47], and as such, shorter probes can be used, thereby increasing sensitivity to SNPs [48]. The difficulty of using LNA probes lies in the fact that LNA–LNA pairing is extremely strong, and poorly designed probes may self-anneal and be useless [49]. Fortunately, an LNA design tool is available on the web (see www.exiqon.com) to assist in LNA probe design. Like 2'-OMe RNA, LNA has been shown to be resistant to cleavage by nucleases [50–52]. Despite these advantages, LNA has found more adoption in the fields of antisense and RNA interference (RNAi) than for FISH applications [53].

This probably stems from the expense of LNA-spiked oligonucleotide probes, the lack of many facilities that will make them, and the problems often experienced with binding that is too tight, preventing washing away of unbound probes.

In contrast, PNA has been widely adopted in the field of RNA-targeted diagnostics. The PNA backbone is based on 2-aminoethyl glycine units replacing the normal phosphodiester backbone [54]. As such, it is uncharged, yet PNA still recognizes complementary nucleic acid sequences by Watson–Crick base-pairing in an antiparallel geometry [54–56]. Since there is no electrostatic repulsion to destabilize hybridization between PNA and DNA or RNA, the rate of PNA hybridization is increased by up to 48,000-fold (under low ionic strength conditions) relative to that for analogous DNA oligonucleotides [57]. Furthermore, the T_m is substantially raised and is less dependent on the presence of mono- or divalent cations [58]. Specificity is reported to be higher than analogous DNA sequences; a single mismatch in a 16-mer PNA–DNA duplex can reduce the T_m up to 15°C [54]. The increased T_m requires that shorter probes are used relative to DNA oligonucleotides, since the T_m of long PNA probes would be too high and prevent stringent hybridization and washing.

3.3. TARGETS

Ribosomal RNA (rRNA) is currently the most common target for *in situ* hybridization. Ribosomes are made up of two subunits; in prokaryotes, the two subunits are referred to as 50S, which contains 23S and 5S rRNA, and 30S, which contains 16S rRNA. In eukaryotes, the respective subunits are 60S, containing 28S, 5.8S, and 5S rRNA, and 40S, containing 18S rRNA. In *Escherichia coli*, roughly 30% of the cellular mass is accounted for by ribosomes and rRNA accounts for about 80% of the total cellular RNA [59]. In all, a typical *E. coli* cell has between 10,000 and 60,000 ribosomes, depending on growth conditions [59]. The number of ribosomes in eukaryotic cells may range dramatically depending on the cell's function, but is typically in a similar range [60]. The natural abundance of ribosomal RNA makes it a particularly attractive target for *in situ* hybridization assays. There is little need for signal amplification to increase signal-to-background, as observable fluorescence is generated simply by binding fluorescence probes to available target.

Messenger RNAs (mRNAs) are, at present, much less commonly used as a target because they are typically at least an order of magnitude less abundant than rRNA, and in some cases may be present in just a few copies per cell [61]. Sensing such low abundance target is impossible without some sort of amplification strategy, and there are currently no reliable methods

for *in situ* sensing of very low abundance mRNAs without an intermediate amplification step. However, more abundant mRNAs, such as β -actin [62] and dps [63], have been sensed by some FISH techniques.

The widespread availability of RNA sequences for a large number of organisms in databases, such as GenBank [64], EMBL [65, 66], the European ribosomal RNA database [67], the Ribosomal Database Project (RDP) [68, 69], probeBase [70], 5S subunit databases [71], small-subunit rRNA databases [72, 73], and large-subunit rRNA databases [74–76], has significantly enhanced the possibilities for comparative sequence analyses (Table 2). Programs for sequence alignment, phylogenetic analysis, and structure prediction, such as ClustalW [77], ARB [78], and mFOLD [79, 80], respectively, are critical to analysis of the tremendous volume of available sequence data.

3.4. TARGET SITE ACCESSIBILITY

In order for any FISH assay to be successful, the probe must find and hybridize to its target. Unfortunately, this is not always a simple matter, as RNA has complex secondary and tertiary structure elements that can prevent FISH probes from binding to their intended site. Despite the availability of many RNA secondary structure-prediction algorithms [80–87], long-chain RNAs may exist as a population of structures [81], and it is substantially more difficult to predictively determine tertiary folds and the effects of any protein interactions on structure. Even in cases where tertiary structure and protein interactions are minimal, the expected accessibility may not always match the empirical result. Three-dimensional structures for some RNAs have

TABLE 2
ONLINE RNA SEQUENCE DATABASES

Database	URL	References
EMBL-Nucleotide Sequence Database	http://www.ebi.ac.uk/embl/	[65, 66]
European Ribosomal RNA Database	http://www.psb.ugent.be/rRNA/	[67]
GenBank	http://www.ncbi.nlm.nih.gov/Genbank/	[64]
probeBase	http://www.microbial-ecology.net/probebase/	[70]
Ribosomal Database Project II	http://rdp.cme.msu.edu/index.jsp	[68, 69]
5S Ribosomal RNA Database	http://biobases.ibch.poznan.pl/5SData/	[71]

been determined by X-ray crystallography [88–91] or NMR [92–94], and this information can be extremely useful in determining the possible accessibility of a target site.

As discussed in Section 3.1, probe affinity depends not only on sequence but also on target structure. A straightforward method for designing probes to accessible target sites might involve determining several energy-minimized secondary structures using mFOLD [80] or other program, then considering regions of potential accessibility based simply on predicted secondary structure elements, for example, avoiding hairpins and long regions of base pairs, while targeting loops or bulges [95]. However, such an approach may require several time-consuming trials.

Fortunately, a great deal of empirical data on the accessibility of rRNA to oligonucleotide probes is available, largely from the work of Amann and coworkers [95–101]. Using flow cytometric analysis of fixed *E. coli* using overlapping adjacent probe sets spanning the entire 16S [95, 101] and 23S rRNA [98] subunits, they generated rRNA accessibility maps. This work was expanded to include small subunit rRNA (16S or 18S) of members of the domains *Bacteria*, *Archaea*, and *Eucarya*, enabling the generation of an *in situ* accessibility consensus map [99, 101]. Considering the high sequence conservation of rRNA, these profiles are also useful for rational probe design for other organisms. Furthermore, a significant improvement in target site accessibility can sometimes be obtained by use of “helper probes,” unlabeled oligonucleotides, typically around 18 nucleotides long, which bind adjacently to the labeled probe [97]. Helper probes are intended to disrupt secondary structure around the target site, and were shown to lead to 4- to 25-fold increase in signal when applied to *E. coli* 16S RNA target sites [97].

Much of these data have been integrated into the ARB software package PROBE Design Tool (PDT) [102]. Using this program, the user may select a group or species of interest, indicate probe parameters (length, GC content, and so on), and search for site candidates based on predicted probe quality. Although probes designed using PDT still must be tested empirically, by identifying and excluding inaccessible regions, the number of trials can be reduced. Other software packages for the design of RNA-targeted oligonucleotide probes are also available [103–105].

Because of the large numbers of different mRNAs that may be of interest for FISH applications, accessibility is typically determined by testing probe sets that were rationally designed using secondary structure information. Several labor-intensive biochemical methods for determining site accessibility in mRNA have been reported, but they tend to be more useful for antisense, knockdown, or RNA interference applications, where it is necessary to know beforehand whether probe will bind target [106–111]. In FISH applications, accessibility is to a large extent inherently determined over the

course of the hybridization experiment, so it is unnecessarily laborious to use such methods in most cases. Indeed, FISH is one method that may be used to empirically determine accessibility of mRNA sites for such other mRNA-targeting applications, assuming the mRNA is abundant enough to generate signal.

A study by Yilmaz *et al.* uses an affinity-based approach to generate probes that generate signal across the entire *E. coli* 16S rRNA, including sites that were shown to be inaccessible in the Amann study [16]. Using calculations to design probes with values for $\Delta G^{\circ}_{\text{overall}}$ around -13 kcal/mol, the authors demonstrated that there are no truly inaccessible regions so long as probes with great enough affinity can be designed. This method offers significant new possibilities for rational probe design, but due to several limitations, the need for empirical determination of site accessibility is not yet trumped in many cases. First, different methods for estimating ΔG° , (Fig. 1, unfolding of target site) give different results, so calculation errors can result in errors in the determination of probe affinity. Second, these calculations do not take into account tertiary structure or the influence of associated proteins. Third, the assumption of chemical equilibrium behind the calculations of $\Delta G^{\circ}_{\text{overall}}$ may be invalid after normal hybridization times for some target sites when the kinetics of hybridization are slow [9]. As a result, hybridization times had to be increased to as much as 96 hours in order to generate signal with some target sites, an unacceptably long time for many diagnostic applications.

4. Types of Probes

4.1. OLIGONUCLEOTIDE PROBES

FISH probes several hundreds or thousands of nucleotides long complementary to nearly the entire 16S or 23S rRNA and containing multiple fluorophore labels have long been used to detect microorganisms in environmental samples because such lengthy probes offer reliable hybridization and intense signal. These probes are prepared enzymatically, typically by PCR [112] or *in vitro* transcription [113, 114], at which time multiple fluorophores are incorporated. Although polynucleotide probes allow the visualization of a significantly higher percentage of prokaryotes in a sample compared to singly labeled oligonucleotide probes [115], polynucleotide probes are only able to discriminate between distantly related groups, such as *Bacteria*, *Crenarchaeota*, and *Euryarchaeota* [113, 116], because their sensitivity to small sequence differences is very low. Furthermore, long polynucleotide probes must be produced in the laboratory using cost- and labor-intensive protocols. Typical problems encountered include nonspecific binding of

probe [116], high autofluorescence vs specific fluorescence [117], poor signal-to-background [118], low cellular detection compared to total cell count [119], and enzymatic degradation *in situ* [120].

More commonly, RNA-targeted FISH probes employ oligonucleotides 15–30 nucleotides long, with a DNA, PNA, or modified nucleic acid backbone (Section 3.2), prepared synthetically (Fig. 4A). Fluorescence is typically observed directly, using a fluorophore attached to the 5'-terminus, though in some cases 3'- or internally labeled probes are used. Common fluorophores used in RNA-targeted FISH diagnostics include fluorescein, tetramethylrhodamine (TAMRA), Texas Red, Cy3, and Cy5. Choice of dye is typically determined by its spectral properties and the availability of equipment for imaging. Labeled oligonucleotides are available from a variety of commercial sources, so it is typically not necessary for investigators to synthesize or purify probes.

In some applications, indirect sensing is used instead of directly coupling the fluorophore to the probe. Indirect sensing strategies typically involve coupling an enzyme to the oligonucleotide probe, hybridizing to targets, then

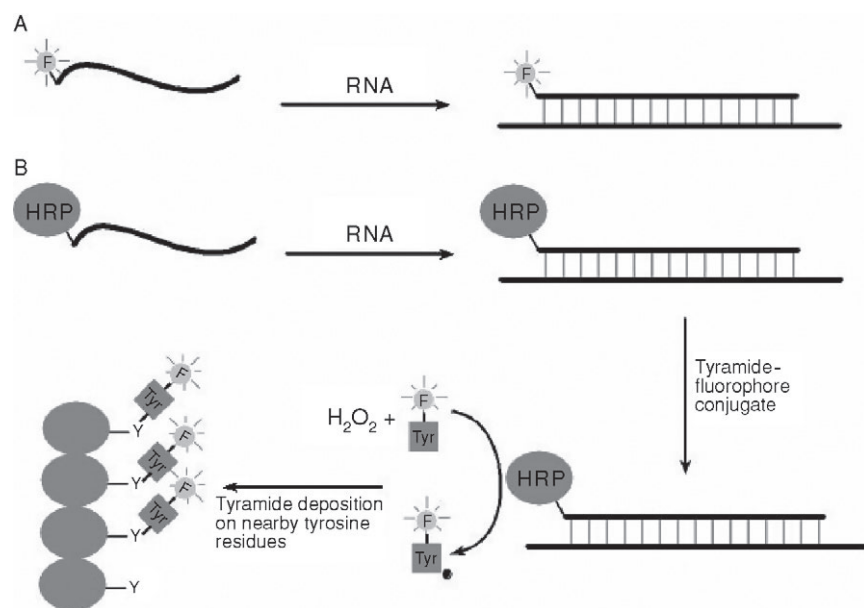


FIG. 4. Standard oligonucleotide FISH probes. (A) Standard probe hybridization. (B) Tyramide signal amplification using oligonucleotide FISH probes conjugated to horseradish peroxidase (HRP).

adding a fluorophore moiety that is recognized by and covalently binds to the enzyme [121]. These approaches can offer the significant advantage of brighter signals, but they tend to have low specificity [122, 123]. The most well-studied approach employs horseradish peroxidase (HRP)-labeled oligonucleotide probes [124–127]. HRP reacts with hydrogen peroxide and tyramide to produce a free radical on the tyramide, which covalently binds to a nearby tyrosine residue (Fig. 4B) [128, 129]. A number of fluorophore-conjugated tyramides are available, thus allowing fluorescence detection of enzymatically deposited tyramide [129].

4.1.1. *Standard Oligonucleotide FISH Protocols*

Standard FISH protocols consist of four steps: (1) fixation and permeabilization of the sample, (2) hybridization of fluorescent probe, (3) washing away unbound probe, and (4) detection of labeled cells by microscopy or flow cytometry [116].

The first issue when setting up an RNA-targeted FISH experiment is getting the probes into the cells. Cells typically must be fixed so that high stringency washing steps may be performed to remove unbound probes. However, it is usually desirable to select fixatives that will disrupt cellular morphology as minimally as possible. The most common fixatives fall into two classes: cross-linking reagents, such as aldehydes, and precipitants such as methanol and ethanol [6]. Cross-linking reagents like formalin and paraformaldehyde are quite commonly used for permeabilization of gram-negative bacteria [130] and human cells [131], but may be ineffective in permeabilizing the cell walls of gram-positive bacteria [132]. Several possibilities exist to permeabilize gram-positive bacteria, and often different procedures are required for different species. Treatment of paraformaldehyde-fixed bacteria with cell wall-lytic enzymes, such as lysozyme or proteinase K, has been shown to increase the cellular permeability of *Lactococci*, *Enterococci*, and *Streptococci* [133]. Permeabilization by treatment with ethanol/formalin [134], high concentrations of ethanol or methanol [135], or heat [136], also has been successful in many cases.

Hybridization and washing conditions are highly dependent on the probe affinity and T_m and the cell type being examined, and optimal conditions must be determined empirically. Hybridization is performed at a few degrees lower than the probe T_m , typically in the 40–60°C range, in a buffer containing a relatively high salt concentration. For PNA FISH probes, higher hybridization temperatures can be used when the probes have higher T_m 's [54]. The advantages to using higher hybridization temperatures are better disruption of target structures and better probe specificity. Washing is carried out in similar temperature ranges, often with the addition of higher concentrations of detergents such as SDS, Triton X, or Tween, or

of formamide [116]. More stringent conditions may be required for PNA FISH probes. The washing step is typically difficult to optimize, but the most important in order to minimize false positives from unbound probes.

Insufficiently low signal, on the other hand, can be caused by a number of factors, including low ribosome or mRNA count, poor target accessibility (Section 3.3), or impermeability of cells (see above). Low RNA content potentially can be circumvented by using strategies such as tyramide amplification, but this method requires conditions which tend to cause lysis of fixed cells [122, 137]. When target accessibility appears to be an issue, helper probes can be used or a different target site may need to be selected (see Section 3.3) [97]. In some cases, addition of low concentrations of formamide to the hybridization buffer may improve the result, as formamide lowers the T_m of secondary structures (but also lowers the T_m of the probes) [6]. As a result, hybridizations in formamide-containing buffer must be run at lower temperatures.

After washing, cells may be analyzed by fluorescence microscopy or flow cytometry. Microscopy has the advantage of being rapid and simple, but an untrained eye can lead to incorrect reporting of data, and results are usually qualitative. Flow cytometry provides quantitative data on the fluorescence of individual cell populations, but instruments are quite expensive.

The greatest advantages of standard oligonucleotide probes for RNA-targeted diagnostics are that they are commercially available, relatively inexpensive, and well established in the literature for a plethora of applications. However, standard oligonucleotide probes do have several disadvantages. As discussed in Section 3.1, they are typically unable to distinguish related RNA sequences unless there are multiple nucleotide differences [5, 116, 138]. In addition, careful handling is required to avoid nonspecific signals, especially during washing away of unbound probes [139]. The washing step increases the chances of error and nonspecific signal, and prevents application to live cells.

4.2. MOLECULAR BEACONS

Molecular beacons (MBs) were first developed in 1996 as tools for real-time PCR assays [140, 141]. They have since been developed for multiplex PCR assays [142, 143], solid-phase hybridization assays [144–146], biosensing [147], and FISH applications with both prokaryotic [148] and human cells [149]. Molecular beacons are oligonucleotides, typically with DNA, 2'-OMe RNA, or PNA backbones that have a stem-loop hairpin conformation in their native state, with a fluorophore and quencher at either end such that the probe is quenched while in the hairpin state [140, 150, 151]. The loop region of the probe, or sometimes the loop and part of the stem [152], is

complementary to an RNA target site, and upon binding, the hairpin is disrupted, separating the fluorophore and quencher, enhancing fluorescence signal (Fig. 5A). The quenching efficiencies for several quenchers with many different fluorophores have been examined, allowing for the design of optimal fluorophore–quencher pairs [4].

A careful balance must be reached between the stem length and loop length to design optimal molecular beacons for mismatch discrimination. Solution experiments demonstrated that mismatch discrimination increases as the number of bases in the stem increases [153]. However, if the stem is too long, the kinetics of hybridization to target will be slow [154]. MBs with longer loop lengths tend to have lower hairpin T_m 's, and thus increased kinetics of hybridization and decreased specificity, and MBs with very short stem lengths have lower signal-to-background ratios [154]. Design of MBs is substantially simplified by software, typically offered by companies that sell custom MBs.

MBs offer several advantages over standard oligonucleotide probes. Because MBs are quenched, no washing steps are required to remove unbound probe, and they may therefore be applicable to living cells assuming

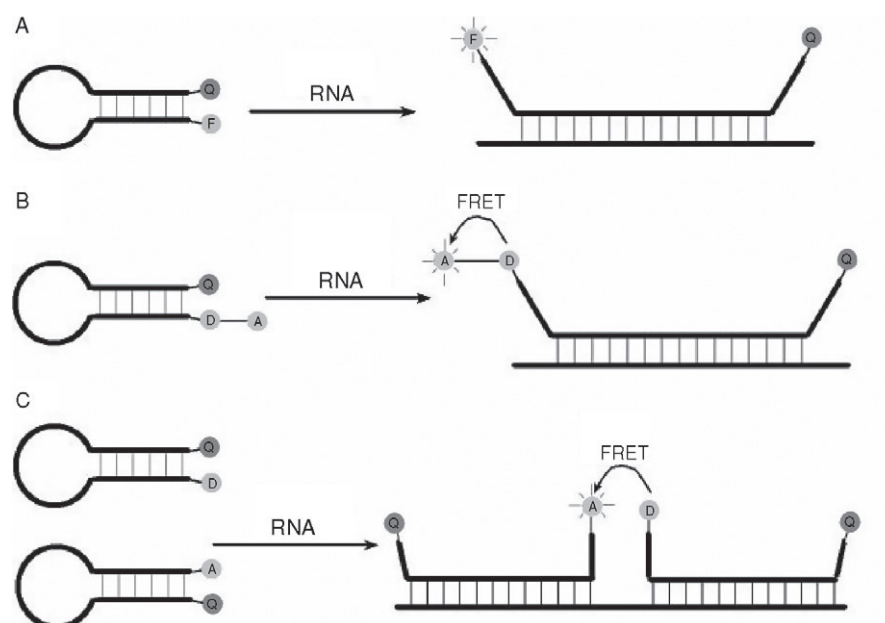


FIG. 5. Molecular beacons. (A) Standard MB hybridization. (B) Wavelength-shifting MB. (C) Dual FRET MB.

the cells can be permeabilized. Second, MBs have higher mismatch sensitivity than standard oligonucleotide FISH probes as a result of their conformational restraints [153]. The main sources of nonspecific signal in MBs are: (1) incomplete quenching, (2) hairpin–hairpin binding between two beacons, (3) nuclease degradation that separates the quencher and the fluorophore, and (4) nonspecific interactions with proteins and other small molecules within the cell that disrupt the hairpin structure [5]. The last may be the biggest problem in cellular diagnostics, since molecular beacons are known to interact with certain nucleic acid-binding proteins, disrupting the MB secondary structure and giving nonspecific signal [155].

Several new approaches to MBs recently have been developed to improve signal-to-background. Most notably, fluorescence resonance energy transfer (FRET) approaches have the potential to decrease background signal if the spectral overlap between the donor and acceptor is minimal [156]. This approach was first examined with the so-called “wavelength shifting molecular beacons,” in which an acceptor fluorophore, such as a rhodamine, was tethered to the fluorescein donor via a linker (Fig. 5B) [157]. Wavelength shifting MBs were found to be useful in multiplex PCR assays, but have not been employed for FISH assays, possibly because the overlap between the donors and acceptors studied is too great to give a substantial signal-to-background advantage. A similar approach uses an acceptor fluorophore instead of a dark quencher, thereby changing the maximum emission wavelength between hairpin and bound states [158]. Going one step further, Bao and coworkers developed “dual FRET MBs,” in which two MBs bind side-by-side, with a donor fluorophore on one beacon thereby being brought into proximity with an acceptor fluorophore on the other beacon (Fig. 5C) [159, 160]. Since both donor and acceptor fluorophores are quenched in their native state, and both need to hybridize adjacently in order for FRET to occur, background from nonspecific hairpin opening is substantially reduced.

4.3. QUENCHED AUTOLIGATION PROBES

Quenched autoligation (QUAL) probes are a relatively new class of *in situ* hybridization probes that were developed for detection of sequences with high specificity [161–163]. Whereas molecular beacons rely on a conformational change to initiate fluorescence signal, QUAL probes utilize a chemical reaction [163]. QUAL probes consist of two oligonucleotide strands, the “dabsyl” probe and the phosphorothioate (thioate) probe (Fig. 4C). The dabsyl probe is short, typically 7–10 nucleotides, while the thioate probe is longer, around 15–20 nucleotides. The dabsyl probe is modified such that it has a dabsyl group at its 5'-terminus, attached through an electrophilic

sulfonate ester linkage, and a fluorescein or other fluorophore internally attached to a uridine base. Dabsyl is a dark quencher that efficiently quenches fluorescein, so the background fluorescence of the dabsyl probe is very low [4]. The thioate probe is modified such that it has a phosphorothioate group at its 3'-terminus. The two probes are designed such that they bind adjacently on their target, bringing the nucleophilic 3'-phosphorothioate close to the electrophilic 5'-dabsyl group. The phosphorothioate reacts to displace the dabsyl group, thereby ligating the two strands and unquenching the fluorophore (Fig. 6A and B). It should be noted that both the 3'-terminal phosphorothioate in the nucleophilic probe and the bridging phosphorothioate linkage formed in the ligation product are quite stable to nucleases and hydrolysis [164]. The 5'-dabsyl group, however, is subject to slow hydrolysis in buffer, particularly in basic conditions or at high temperatures [165].

QUAL probes take advantage of the ability of very short oligonucleotides to sense single nucleotide mismatches while avoiding issues of redundancy and lack of affinity. The highest specificity is achieved when the mismatch is placed in the center of the short probe; substantially less discrimination is observed when the mismatch is at the end of the short probe or in the long probe [161]. Thus, specificity of QUAL probes is determined mostly by

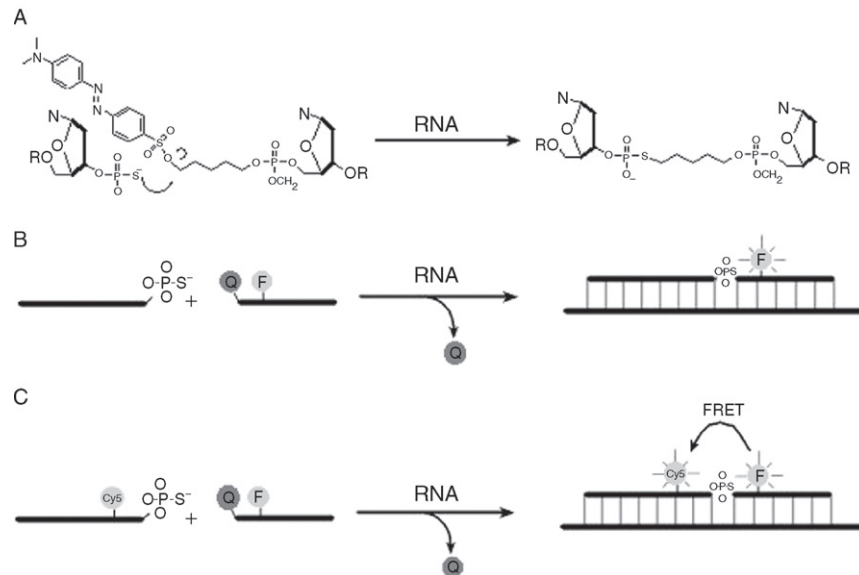


FIG. 6. QUAL Probes. (A) Ligation chemistry. (B) QUAL probe hybridization and reaction. (C) FRET-QUAL probes.

binding affinity as opposed to geometry of the reaction site, as mismatches in the center of short probe lead to the greatest T_m difference for binding to a matched vs mismatched target [161, 162]. Naturally, probing for sites with multiple mismatches will increase the specificity of QUAL probes.

When very short probes are used, there is a high probability for sequence redundancy. This issue is made moot in QUAL probes by requiring the two probes that bind adjacently. Thus, even if the short probe represents a sequence that has redundancy in the target RNA, no fluorescence is observed unless the longer probe binds adjacent to it. The lack of affinity of very short probes is dealt with by running the hybridization at relatively low temperatures (37°C or lower) and selecting sequences that have a T_m high enough to hybridize under the desired conditions. Since unbound probes are not fluorescent, no washing steps are required, and ligated fluorescent products are typically 20-mers or longer, which have high affinity for their target.

It should be noted, however, that it is not actually necessary that probes remain bound after ligation occurs in QUAL probes, as ligation permanently switches on fluorescent signal. In fact, if ligated probes dissociate from their target and new probes bind and ligate on the same template, signal amplification may occur [166]. Signal amplification by turnover is highly desirable for the detection of extremely low abundance targets such as mRNAs. A strategy of using “universal linkers” to attach the quencher to the 5'-terminus of the dabsyl probe (Fig. 5A) yielded turnovers of nearly 100-fold on RNA templates in solution [166], and was used to detect mRNAs in human cells [167]. The product of the ligation reaction using the universal linker is destabilized compared to natural DNA, apparently because the alkane linker adds flexibility to the strand. This decreases product inhibition so that the target RNA can become a catalyst for generating multiple signals per target. Furthermore, the linker has been shown to destabilize the product without destabilizing the transition state of the reaction; in fact, the reaction rate is sped up by a factor of 4–5 [166].

In solution and solid-phase assays, QUAL probes were shown to accurately discriminate between all possible mismatches [163, 165]. QUAL probes offer several advantages over other RNA-targeted diagnostics strategies. Like MBs, unbound probes do not need to be washed out of cells, which reduces experimental time and decreases chances for error. QUAL probes may be less prone to nonspecific signals than MBs because turning on fluorescence requires a chemical reaction; thus, binding to proteins should not lead to nonspecific signals.

Disadvantages of QUAL probes include the slow hydrolysis of quencher leading to nonspecific fluorescence, the requirement that multiple probes be used, and the limited number of systems that they have been applied to thus far. The main sources of nonspecific signal in QUAL probes

are: (1) incomplete quenching, (2) nuclease degradation in the 1–3 nucleotides between the fluorophore and quencher, and (3) hydrolysis of the quencher [5]. The last of these appears to be the greatest problem, requiring careful handling and storage of the probe.

Abe and Kool recently described a method to improve signal-to-background by using FRET-QUAL probes (Fig. 6C) [167]. In these specialized QUAL probes, Cy5 was attached internally to the thioate probe. The ligation was monitored by excitation at 488 nm, which gives almost no excitation for Cy5. When the probes ligated, FRET between fluorescein and Cy5 allowed emission to be monitored at 665 nm, beyond the emission wavelength for fluorescein. Thus, background from incomplete quenching or nonspecific hydrolysis of the quencher was minimized, leading to substantially higher sensitivity.

QUAL probes are not yet commercially available, so they have not yet been adopted wide use in diagnostic settings.

5. Applications

5.1. BACTERIAL TARGETS

RNA-targeted FISH may be used for the identification of bacteria in environmental samples, clinical specimens, or cell culture. All three applications are important in diagnostics: environmental detection is critical in identifying contamination, while clinical specimens and cultured cells are important in determining and diagnosing disease.

5.1.1. *Oligonucleotide Probes*

Oligonucleotide FISH probes can be classified as generic, group specific, genus specific, or species specific, depending on the intended targets. Generic probes are designed to target consensus regions across many bacteria groups, while group-specific probes should only hybridize in the presence of related microorganisms. Genus- and species-specific probes are the most highly specific and therefore often the most useful. However, it should be noted that these probes are typically only tested against a few related bacteria, and their general scope of applicability may still be limited.

Early experiments focused on developing generic probes that are complementary to the rRNA in a large number of bacteria species, and generate very strong signal. These probes, such as EUB338 [96], UNIV1392 [168], and ARCH915 [169], can be used in general diagnostic applications or as positive controls to evaluate the brightness or efficiency of new probes. For example, EUB338 is widely used in identification of microorganisms in

plankton [137, 170], biofilms [171–173], sludge [174, 175], sediments [176], soil [177], wastewater [178, 179], and food [180].

A plethora of papers have appeared on development of RNA-targeted FISH probes to identify various organisms or groups in clinical and environmental samples [2, 116, 139, 181, 182], and it would be impossible to cover all examples here. Instead, we will provide some illustrative cases and point out some of the challenges in development of useful diagnostic probes. It is interesting to note that greater than 90% of cases of bacteremia are caused by a very limited number of pathogens including *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Candida* spp. [183, 184], and a significant number of clinical studies focus on developing FISH probes for these microorganisms.

Group-specific oligonucleotide probes have been developed for a wide range of targets, including Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes, Cyanobacteria, Cytophagales, Fibrobacter, Planctomycetes, Verrucomicrobiales, Nitrospira, Acidobacteria, Actinobacteria, domain Bacteria, Crenarchaeota, Euryarchaeota, and Archaea [70, 185]. These probes have good to excellent sensitivities, and target coverage of up to 100% [70, 185]. Some gram-positive groups are difficult to stain using DNA oligonucleotide probes, but PNA probes have shown improvement in some cases [168, 186–188]. It is worth noting that while FISH gives information about the presences of microorganisms in a sample, quantitative data on the abundance of microorganisms in complex samples tends to have low accuracy [120].

Genus-specific identification of *Chlamydiae* [189], *Brachyspira* [190], *Brucella* [191], *Enterobacteriaceae* [192], and *Candida* [193] in culture using specific oligonucleotide FISH probes has been achieved, and in several cases these probes have been applied directly to smears of biopsies. Species-specific oligonucleotide FISH probes have been developed for identification of several species of clinically important bacteria [194]. Particularly useful in the clinic are probes that can be directly applied to clinical samples and do not require culturing the bacteria. This is particularly important in the case of slow-growing organisms such as *Legionella* or *Mycobacteria*. PNA FISH probes are particularly appropriate for use in the clinic because their hydrophobicity makes them more compatible with standard smear preparations [188]. However, cells must still be fixed prior to staining. Species-specific PNA or DNA probes have been applied for identification of *Staphylococcus aureus* [186], *Candida albicans* [195], *Enterococcus faecalis* [196], *Helicobacter pylori* [194], and *Legionella pneumophila* [197], directly from blood smears, giving diagnostic sensitivities of 93–100%.

Rapid diagnosis of bacterial infections can be particularly important in immunocompromised individuals such as individuals with cystic fibrosis.

A recent study by Hogardt *et al.* used RNA-targeted probes and flow cytometry to identify bacteria from 8 groups in sputum samples from 75 cystic fibrosis patients [198]. In this study, they observed 100% accuracy for identifying *P. aeruginosa*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Haemophilus influenzae*, and *Staphylococcus aureus*.

A poignant example of the challenges of employing FISH in clinical diagnostics is the development of probes for identifying *Mycobacterium tuberculosis*. *Mycobacterium tuberculosis* remains one of the most important clinical targets because 8 million new cases of tuberculosis (TB) occur annually, producing nearly 3 million deaths, mostly in developing nations [199]. Diagnosis of *Mycobacterium tuberculosis* is extremely time-consuming, requiring initial examination of sputum stained for acid-fast bacilli, followed by differentiation of *Mycobacterium tuberculosis* from nontuberculosis mycobacteria using biochemical tests on cultures, which often takes 4–6 weeks [200]. Several academic laboratories and companies are working to develop FISH probes specific for *Mycobacterium tuberculosis* 16S rRNA [200–203]. The first difficulty in applying FISH to *Mycobacteria* is their extremely robust mycolic acid-containing cell wall, which is extremely difficult to permeabilize to oligonucleotide probes without destroying cellular morphology. As a result, most attempts at *Mycobacterium tuberculosis*-specific probes have employed PNA. While such probes offer reasonable results, sensitivities may be as low as 57% because of contamination by non-*Mycobacterium tuberculosis* *Mycobacteria* species which are not identified by the probe set [204]. Furthermore, the non-*Mycobacterium tuberculosis* probes have been shown to nonspecifically identify species of *Actinomyces* and *Rickettsia* [201]. Thus, these probes have not been adopted for widespread use because they are not sufficiently accurate for identifying *Mycobacterium tuberculosis* or non-*Mycobacterium tuberculosis* *Mycobacteria* infections. Nevertheless, initial probe sets with reasonable sensitivity prove that FISH is a potential method for more rapid identification of *Mycobacteria* infections.

5.1.2. Quenched Probes

MBs have been used primarily indirectly in real-time PCR monitoring of rRNA or mRNA sequences, but several attempts have been made for using MBs for *in situ* detection of ribosomal rRNA in bacteria. Most FISH applications of MBs use PNA beacons. Xi *et al.* were able to discriminate cultured and fixed *E. coli* from *Methanosarcina acetivorans* very accurately [148]. Another study used MBs to detect *Lactobacillus reuteri* in stomach biopsies [205]. Despite their potential for use with intact organisms, there are presently no reports of applications of MBs with live nonfixed bacteria.

QUAL probes targeted to their 16S rRNA of *E. coli* have been shown to generate signals within 3 hours of incubation [206]. No signal was observed when the thioate probe was not included, nor when probes that bound nonadjacently were used. Similarly, live *E. coli* could also be detected with QUAL probes, as the cells can be permeabilized with 0.05% sodium dodecyl sulfate (SDS) [165].

QUAL probes have been used to discriminate three different species of closely related gram-negative bacteria, *E. coli*, *Salmonella enterica*, and *P. putida*, based on very small differences in their 16S RNA sequences [207]. The *E. coli* and *Salmonella enterica* probes differed by a single mismatch, and the *P. putida* probes differed by a few mismatches, yet bacteria treated with probes only fluoresced when treated with fully matched sequences. Thus, QUAL probes have been shown to be useful for rapid and specific detection of closely related bacteria species based on very small rRNA sequence differences [207]. It remains to be seen whether QUAL probes will work as well in discriminating clinically important strains.

5.2. HUMAN CELL TARGETS

RNA-targeted FISH in human cells is typically aimed at identification of mRNAs. Although mRNA expression may not directly correlate with expression of the corresponding protein, mRNA expression levels are quite useful for gaining an understanding of cellular activities [208, 209]. In particular, tumor cells are known to express or overexpress mRNAs for certain oncogenes [210], and detection of such mRNAs could be extremely useful for rapid diagnosis of cancers. Furthermore, a reliable method to quantitatively or even qualitatively monitor mRNA expression levels would be extremely useful for understanding cellular behavior in response to stimuli, thereby potentially helping determine treatment options. Although no such method exists now for native cells, FISH holds a great deal of promise. Standard oligonucleotide probes cannot be used in live cells, so any mRNA detected is the expression level at the time of fixation, though it is likely that the fixation event triggers cellular responses that alter mRNA expression levels. MBs and QUAL probes, on the other hand, give a great deal of promise for real-time detection of mRNA expression levels. However, only a handful of papers on this topic have appeared in the literature, and results are largely preliminary.

Part of the difficulty in sensing mRNAs lies in the fact that in applying FISH probes to live cells, not only the abundance of target but also its stability must be considered. Ribosomes are quite stable and degradation is typically not an issue on the timescale of the FISH experiment, but mRNA is potentially more problematic since its half-life may be as short as 3 min [61].

In addition, introducing probes into human cells can be quite difficult, requiring microinjection [62, 211, 212], attachment of a cell-permeable peptide [213, 214], fixation [215], or use of reversible permeabilization such as by streptolysin O (SLO), which can severely disrupt cellular morphology [159, 216].

The first examples of MBs being used to detect abundant mRNAs in human cells have appeared in the literature over the past few years. In one of the earliest examples, human trabecular cells were treated with an MB complementary to basic fibroblast growth factor (bFGF) mRNA fluoresced, while cells treated with a noncomplementary MB gave less signal [217]. This initial study suggested that mRNA levels could be qualitatively examined using MB probes. More recently, MBs have been applied to detect c-fos mRNA in transfected Cos7 cells [211], β -actin mRNA in K562 human leukemia cells [212], PTK2 cells [62], and fibroblasts [213], GAPDH and survivin mRNA in HDF cells [214], cyclin D1 and survivin in human breast cancer cells [215], and K-ras and survivin in HDF and MIAPaCa-2 cells [159]. The latter study employed dual FRET MBs (see Section 4.2), which helped reduce nonspecific background signals [159]. mRNA-targeted studies using MBs have been largely preliminary, but suggest that MBs, and especially dual FRET MBs, merit more study for real-time monitoring of cellular mRNA expression levels.

QUAL probes have been reported to be applicable for sensing abundant mRNAs in intact human HL-60 cells [167]. This study employed FRET-QUAL probes to minimize background in sensing GAPDH, β -actin, histone H3, and JUND mRNAs. The RNAs were detected both by fluorescence microscopy and by flow cytometry. Not surprisingly, there was a high dependence of probe target site accessibility on signal-to-noise, suggesting that better methods for determining mRNA accessibility would still be useful.

6. Conclusions

RNA-targeted FISH has become an extremely significant diagnostic method over the past two decades, and recent advances in protocols and probe design have had a tremendous impact on maximizing the utility of the approach. The empirical determination of RNA accessibility and availability of probe design tools have made FISH probe design substantially more facile, and the many approaches available for improving the affinity, specificity, and *in situ* stability of probes give researchers a great deal of flexibility in developing new probes. The development of quenched probes has substantially added new possibilities for live cell and very high-specificity detection.

Nevertheless, there are still many challenges to overcome. In the area of microbiology, preliminary results have shown promise for highly specific detection of closely related microorganisms, but it remains to be seen if these probes will be applicable to a wide range of systems or be useful in a clinical setting. Specific detection of gram-positive bacteria, particularly those with very robust cell walls, remains problematic. There are no examples in the literature demonstrating specific detection of low abundance mRNAs in bacteria, an advance that is critical for detecting many virulence factors such as the Shiga-like toxin in *E. coli* O157:H7. Early results in human cells using quenched probes have demonstrated the possibility of detecting abundant mRNA in living systems; it remains to be seen whether expression levels can be quantitated or if very low-abundance transcripts can be observed, and whether cancerous cells can be differentiated from normal cells based on mRNA expression.

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