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Quenched probes for highly specific detection of cellular RNAs

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Nucleic acid-based RNA detection is a promising field in molecular biotechnology that is leading to the rapid and accurate identification of microorganisms, diagnosis of infections and imaging of gene expression. The specificity of short synthetic DNA probes raises the hope of distinguishing small differences in sequence, ultimately achieving single nucleotide resolution. Recent work using quenched fluorescently labeled oligonucleotide probes as sensors for RNA in bacterial and human cells has overcome several difficult hurdles on the way to these goals, including delivery of probes to live cells, accessing RNA sites containing a high degree of secondary structure, and eliminating many sources of background. Two new classes of quenched oligonucleotide probes, molecular beacons and quenched autoligation probes, have shown the most promise for *in situ* RNA detection. High-specificity detection, at the single-nucleotide resolution level, is now possible in solution with these classes of probes. However, for applications in intact cells, signal and background issues still need to be addressed before the full potential of these methods is achieved.

Introduction

Methods for distinguishing RNA sequences in cells with single nucleotide specificity are of great interest because of their myriad applications in the fields of biochemistry, molecular biology, bioinformatics and pathology. Ribosomal RNA (rRNA) has long been a target for *in situ* studies because of its abundance and its accessibility to oligonucleotide probes. More recently, there has also been substantial research on detecting messenger RNA (mRNA) in cells for obtaining diagnostic or gene expression data.

There is a long history of development of lengthy polymeric DNA or RNA probes for *in situ* hybridization to RNA, and the field continues to move rapidly. However, long probes made enzymatically (hundreds or thousands of nucleotides in length) are not able to distinguish the smallest differences in rRNA or mRNA sequence. Because of the importance of small sequence differences in distinguishing between strains of bacteria, detecting drug resistance and evaluating the early stages of cancer, there is increasing interest in the development of small oligonucleotide-based probes for *in situ* imaging. RNA sequences generally can be identified by using fluorescently labeled oligonucleotide probes (typically 15–30 nucleotides in length), which hybridize to a complementary sequence of interest. Such probes are directly applicable to genus-, or in some cases, species-specific identification of organisms in wastewater, food and water supplies, bioreactors and clinical diagnostics [1]. However, probing for single nucleotide polymorphisms (SNPs) is often difficult. Two or more mismatches are typically required for highly accurate detection using an *in situ* hybridization probe of 15–20 nucleotides; thus, identification of SNPs in bacterial or human cells is usually impossible with traditional methods [2].

However, the need for such specificity is clear. Antibiotic resistance in bacteria is often caused by single base mutations in the rRNA sequence [3]. Closely related bacteria, such as *Escherichia coli* and *Salmonella* species, often have $>97\%$ similarity in their rRNA sequences, with the few variations typically found as SNPs rather than variable domains [4]. Single nucleotide mutations in human cells, such as the well-characterized *H*-ras point mutation, can lead to activation of oncogenes [5]. Unfortunately, the most reliable strategy for highly specific RNA identification, reverse-transcriptase complimentary DNA (cDNA) synthesis, followed by polymerase chain reaction (PCR) amplification, and often sequencing, is labor intensive, time consuming and requires expensive reagents and equipment [6]. This approach is often not practical for applications that require high-throughput or rapid results, such as clinical diagnostics or genomic analyses, and so a great deal of recent research has focused on developing highly specific methods to probe RNA sequences in cells directly.

Standard fluorescent (non-quenched) oligonucleotide probes in fixed cells

The traditional probes for achieving selective RNA detection *in situ* are standard oligonucleotides carrying one or more fluorescent labels, and with no quenching groups. For example, the simplest and most widely used method for detecting rRNA in cells, fluorescence *in situ* hybridization (FISH), is typically performed with rRNA-targeted oligonucleotide probes, 15–30 bases long, end-labeled with a fluorophore. To remove unbound probes that emit nonspecific signals, careful washing must be performed, hopefully leaving bound probes on their targets [7]. This limits non-quenched probes to applications in fixed (non-intact and dead) cells. Thus, a FISH experiment is comprised of four steps: (i) fixing and

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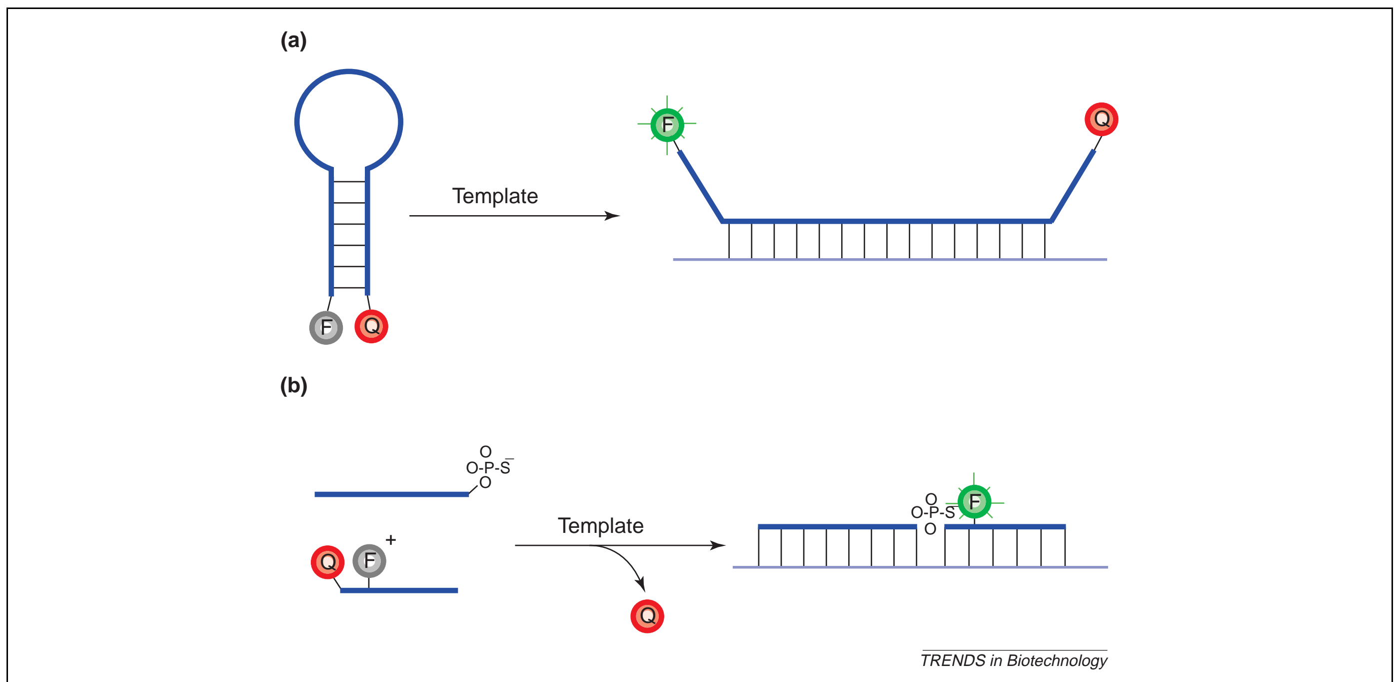

Figure 1. Design strategies for highly specific quenched probes. **(a)** Structure of a molecular beacon in its hairpin state and after binding to template. F refers to a fluorophore and Q is a quencher. The hairpin is disrupted upon binding to template, leading to fluorescence signal. **(b)** Chemistry of QUAL probes. After hybridization to template, the phosphorothioate displaces the quencher, leading to self-ligation of the two probe strands and unquenching of the fluorophore.

permeabilizing the sample, (ii) hybridization, (iii) washing to remove unbound probe, and (iv) detection of stained cells by microscopy or flow cytometry [8]. Several large databases of rRNA sequences are available on the internet [9,10], and the accessibility of the entire 16S and 23S rRNAs in *E. coli* to hybridization probes has been mapped [11,12]; this makes FISH probes easy to design and use in the study of a wide variety of microorganisms. Furthermore, the use of 'helper' probes – unlabeled synthetic DNAs that bind adjacently to the labeled FISH probe and help disrupt secondary structure – can greatly improve signal for sites that are not highly accessible [13]. Species-specific FISH probes have been used for clinical identification of a variety of microbes, such as *Streptococcus* species, *Enterococcus faecalis*, *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa* [14]. Fluorescent oligonucleotide probes are also being applied in the detection of rRNAs in human cells [15].

Standard FISH probes clearly have numerous well-developed applications. However, there are two substantial limitations of these probes: first, their low sequence specificity due to their large length, which makes them unable to detect subtle mutations and SNPs, and second, their incompatibility with live or intact cells. Thus, several laboratories have turned to quenched probes to address these problems.

Quenched oligonucleotide probes

Over the past decade, many of the improvements in FISH technology have focused on developing ways to (i) reduce background signal and (ii) eliminate the need for washing steps so that live cells can be used. These goals have been achieved by using quenched oligonucleotide probes. Similar to normal FISH probes, quenched oligonucleotide probes are short DNA probes but they are modified with a fluorescence quencher (such as dabcyl) in addition to the fluorophore. Owing to structural or conformational restraints of the probe, the fluorophore and quencher are in close enough proximity for the fluorophore to be quenched by contact and/or resonance energy transfer (RET) quenching. Thus, in an unbound state the probe is dark. Upon binding the target, however, a conformational or chemical change in the probe results in separation of the fluorophore and quencher, leading to a fluorescent signal. Given that unbound probes fluoresce much more weakly, they need not be washed out of cells; therefore, there is no need for fixing the cells, as long as the probes can be introduced by other means. This is a significant benefit because it not only saves the time required to fix the cells but it also enables the possibility of real-time monitoring of RNA expression. Quenched oligonucleotide probes tend to fall into two categories: (i) probes that become unquenched by a conformational change, the best described of which are molecular beacons (Figure 1a), and (ii) probes that are unquenched by a chemical reaction, most notably quenched auto-ligation probes (Figure 1b).

Molecular beacons

Molecular beacons were first developed for monitoring nucleic-acid amplification assays [16] but their potential as *in situ* probes was quickly realized. A molecular beacon is an oligonucleotide that undergoes a conformational change upon hybridizing to a complementary target, resulting in a fluorescent signal. In its native state, the probe is a hairpin with the target sequence in the loop and a sequence that is non-complementary to the target in the stem. A fluorophore is attached to one end of the oligonucleotide, and a quencher is attached to the other terminus. In the hairpin conformation, the two ends of the oligonucleotide are in close proximity, leading to contact

and/or RET quenching of the fluorophore. Upon binding to a complementary target, the stem–loop structure is opened, separating the fluorophore and quencher and resulting in a large increase in fluorescence signal (Figure 1a) [17]. Interestingly, the presence of the stem secondary structure has been reported to increase the sensitivity of the probes to single nucleotide differences in solution-based applications [18,19]. Recently, stemless molecular beacons have also been reported, mostly based on peptide nucleic acid (PNA) chemistry, in which fluorophore and quencher are presumably kept in close proximity by hydrophobic interactions before target binding [20,21].

There are numerous examples in the literature in which molecular beacons are applied *in situ*. In one example of high-specificity detection in bacteria, Xi and coworkers treated *E. coli* and *Methanosarcina acetivorans* cells with species-specific molecular beacons in fixed cells [22]. A fluorescein-labeled molecular beacon was specific for *E. coli* rRNA whereas a tetramethylrhodamine (TAMRA)-labeled beacon was specific for *M. acetivorans*. The authors could differentiate the two bacteria species by color, generating either a green or an orange signal only for the correct microorganism (Figure 2) [22].

Molecular beacons have also been used for detecting RNA in human cells [23,24]. Live cell applications can be particularly difficult because DNA molecular beacons might be cleaved by nucleases. Several research groups have tried to enhance the *in situ* stability of molecular beacons using 2′-*O*-methyl RNA beacons. These studies found that 2′-*O*-methyl RNA beacons do have enhanced stability compared with DNA beacons but their target specificity is slightly reduced [25,26].

A recent scheme for human cell application has shown success in lowering background signal: dual fluorescence resonance energy transfer (FRET) molecular beacons, which are designed so that two beacons bind side-by-side, bringing together a donor dye and an acceptor dye (Figure 3). By detecting signal with a FRET filter, background caused by non-specific binding and degradation is substantially lowered. For example, Bao and coworkers use dual FRET molecular beacons to detect and monitor expression levels of K-ras and survivin mRNAs in live HDF and MIAPaCa-2 cells [23]. Another useful modification – wavelength shifting molecular beacons, which are molecular beacons with a FRET acceptor tethered to fluorescein – enables multiple colors to be visualized with a single wavelength excitation, which might be useful for the detection of samples containing several different targets [27]. These have not yet been applied to cellular RNAs.

Molecular beacons offer several clear advantages over standard FISH probes. Most significantly, because molecular beacons are quenched before they bind their target, no washing steps are required and the overall background signal is therefore substantially lower. Furthermore, if the probes are delivered to live cells through a non-lethal permeabilization method or through microinjection, the cells need not be fixed before the hybridization experiment. Successful methods for permeabilizing live cells for molecular beacon applications have included using sodium dodecylsulfate (SDS) or other detergents [22], use of a peptide–beacon conjugate [28] or the use of uncharged peptide nucleic acid (PNA) molecular beacons [20,21,29].

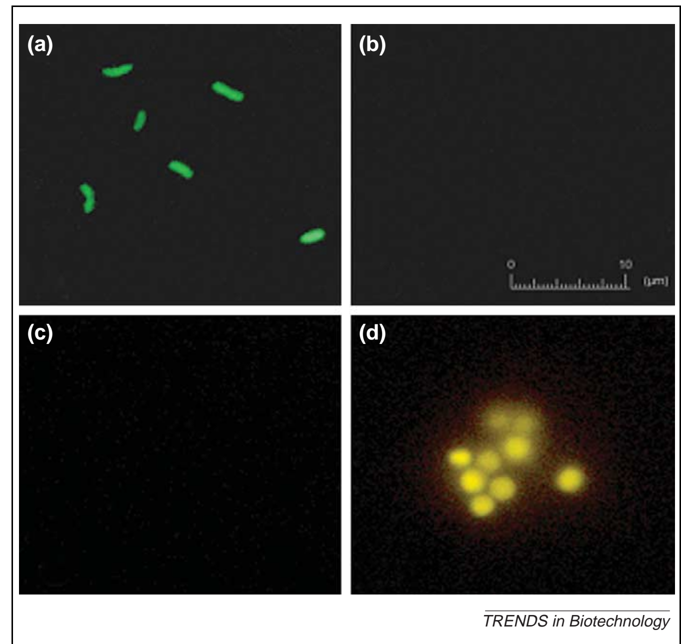

Figure 2. Epifluorescence micrographs showing the use of DNA and PNA molecular beacons to detect bacterial and archaeal cells. Pure culture of *E. coli* (a and b) and *M. acetivorans* (c and d) were fixed and incubated with DNA MB Bact0338 (compliment to *E. coli* 16S RNA; panels a and c) and DNA MB Arch0915 (compliment to *M. acetivorans* 16S RNA; panels b and d). Reproduced with permission from [22] copyright 2003 American Society for Microbiology.

Quenched auto-ligation (QUAL) probes

Similar to molecular beacons, QUAL probes were designed to be highly sequence specific for RNAs and show a simple on or off signal in the presence or absence of the sequence of interest. Unlike molecular beacons, the fluorescence

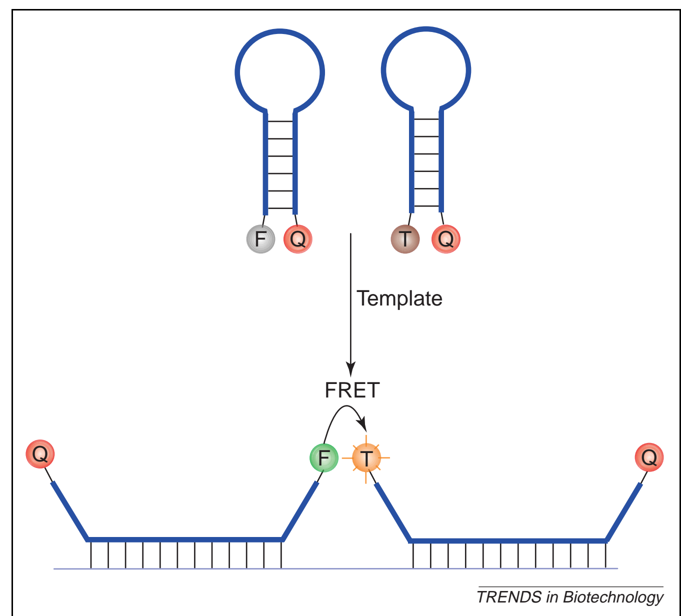

Figure 3. Dual-FRET molecular beacons bind to adjacent sites on a target, leading to unquenching of the fluorophores and FRET. In the example shown, fluoroscein (F) acts as the donor and TAMRA (T) is the acceptor.

signal of QUAL probes is the result of a chemical reaction, not a conformational change. QUAL probes consist of a probe pair: one probe contains an internal fluorophore and a 5′-terminal quencher (typically dabsyl) attached by an electrophilic sulfonic acid linkage; the other probe has a nucleophilic phosphorothioate at its 3′ terminus. The probes are designed so that they bind adjacently on their target RNA, putting the 3′ phosphorothioate in position to displace the 5′ quencher on the other probe, thereby ligating the two probes and generating a fluorescent signal (Figure 1b) [30,31].

Short oligonucleotide probes of 7–10 nucleotides often have excellent discriminating ability for single nucleotide differences when the site of interest is in the middle of the strand, and ligation reactions might enhance such specificity even further. Sando and coworkers demonstrated that by placing the mismatch in the center of a 7-mer probe and ligating it to a 13-mer, more than 100-fold specificity for this single nucleotide difference could be obtained in solution experiments [31]. They also showed a high degree of single nucleotide specificity using multiple colors (Figure 4), by using phosphorothioate probes attached to beads and four different quenched 7-mers, each containing a different base in the central position and a different fluorophore [32].

QUAL probes were also successful at detecting bacterial RNAs with single nucleotide resolution [32,33]. When paraformaldehyde-fixed *E. coli* were treated with a fully complementary 5′-dabsyl-containing probe labeled with fluorescein or TAMRA, along with phosphorothioate and helper oligonucleotides, a green or red signal was observed. However, when mismatched labeled probes were used, a much lower signal was observed (Figure 5) [32]. For distinguishing a single nucleotide difference, establishing this two-color approach enables a simple call of sequence by qualitative color differences.

QUAL probes might be less susceptible to non-specific signals in cells than standard FISH probes or molecular beacons because a chemical reaction is required to turn on the fluorescent signal. Therefore, the binding of probes to cellular proteins or degradation by nucleases should not result in a signal. Furthermore, once the probes ligate, hybridization is no longer necessary for signaling, creating the possibility of generating more than one ligation product per RNA template. This property of QUAL probes has enabled their use in intact bacteria [33]. Such probes have not yet been tested in human cells.

Signal amplification by turnover would be extremely useful for detecting low abundance RNAs, which might have copy numbers two orders of magnitude less than rRNA. A recent modification to the standard QUAL probe strategy led to such a signal amplification without slowing the reaction rate. By attaching the dabsyl quencher/activator to a short 'universal linker' that forms a bulge upon ligation, the dissociation of reacted probes from the target was enhanced and, in addition, the reaction rate was increased by up to fourfold. Using probes with the universal linker, nearly 100 signals per target were observed [34]. Conveniently, the universal linker can be added in automated fashion to any oligonucleotide as the last step in its synthesis.

Limitations of current methodologies

The most fundamental problems of cellular RNA detection methods that must be addressed are those of signal and background. Some improvements and modifications to existing strategies will be required to fully maximize signal (for detection of low-copy-number RNAs) and to minimize background (to avoid false positives).

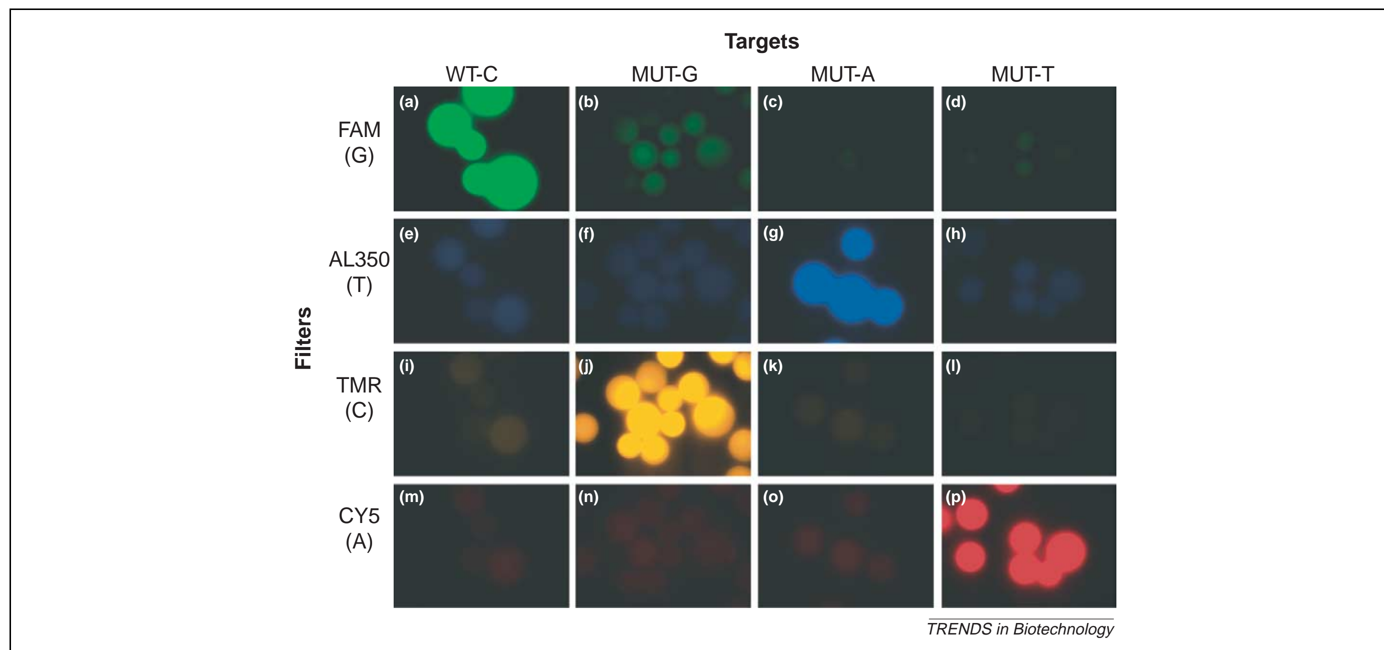

Figure 4. Multi-color detection on polystyrene beads using competing QUAL probes. The base at the target site is indicated in the top horizontal row, and the fluorophore and corresponding base in the QUAL probe is indicated in the vertical column (FAM=fluorescein, AL350=Alexa 350, TMR=tetramethylrhodamine). Bright signals are only observed for matched sequences (a, g, j and p), whereas only minor background is observed for mismatches (b-f,h,l,k,l,m,n and o). Reproduced with permission from [32], copyright 2004 American Chemical Society.

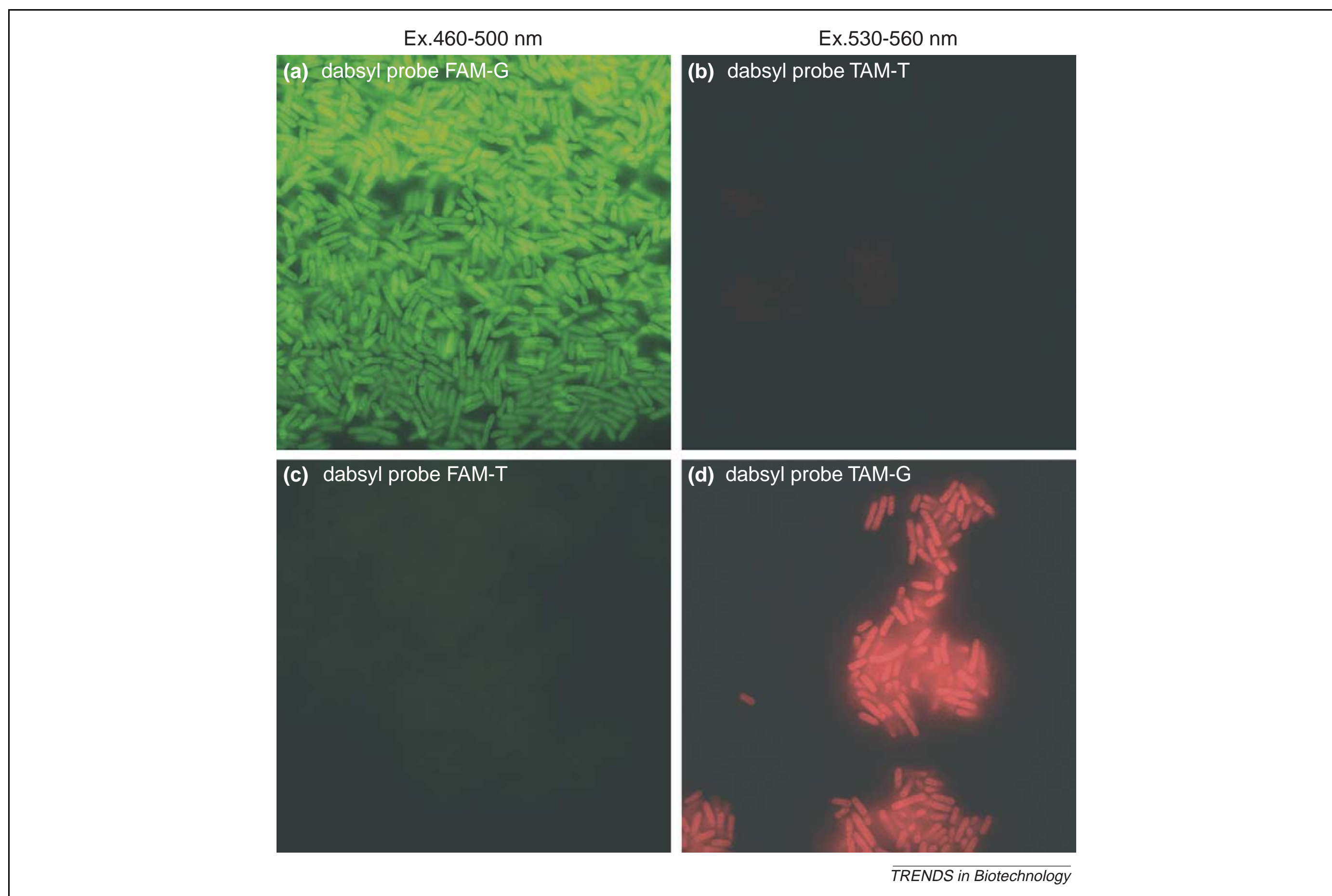

Figure 5. Single nucleotide discrimination of 16S RNA by two-color quenched autoligation. Fixed *E. coli* cells were incubated for 18 h at 37 °C with **(a)** complementary fluorescein-labeled probe FAM-G, **(b)** mismatched TAMRA-labeled probe TAM-T, **(c)** mismatched fluorescein-labeled probe FAM-T, or **(d)** complementary TAMRA-labeled probe TAM-G in the presence of phosphorothioate probe and helper oligonucleotide. Fluorescein and TAMRA were excited at 460–500 nm (left) and 530–560 nm (right) light, respectively. Reproduced with permission from [32] copyright 2004 American Chemical Society.

Positive signal can be limited by the intensity of the fluorescent label on the unquenched probe, by the number of target molecules, and by the numbers of probes introduced into the cell. The typical methods used for introducing probes into live cells are detergents [33], streptolysin O [23] or microinjection [35]. Although all of these methods lead to signal in many contexts, there have been few quantitative and comparative cellular uptake studies. This information is particularly crucial for accurate live cell studies in which the intention is to track mRNA expression, location and degradation over time. Molecular beacons are apparently faster at generating signal in cells, a specific signal being generated in less than one hour [22], whereas QUAL probes required at least 2–3 h before substantial signal was observed [32].

In cases where signal is generated from one probe per target, not enough signal might be generated for many applications when the target is in low abundance, such as for some mRNAs. A detection limit of only ~10 molecules of mRNA was estimated for molecular beacons [36], and several papers have reported using molecular beacons to detect mRNA in living cells [23,35]. However, substantial background is observed in these studies and controls have only included beacons with random sequences, so no data on single-mismatch specificity has been obtained. Nevertheless, this preliminary work has raised the possibility of using molecular beacons or QUAL probes for real-time monitoring of mRNA populations and sequences. However, given that the half-lives of most cellular mRNAs are in the order of minutes [37], probes must be able to find their targets and unquench rapidly if meaningful data are to be obtained. An additional concern is that FISH probes, molecular beacons and QUAL probes are all antisense oligonucleotides, so the probes themselves could conceivably affect the stability or expression levels of the mRNA that they are being used to detect [38].

Background and non-specific signal can come from a variety of sources. In the case of molecular beacons, sources of background include: (i) incomplete quenching; (ii) hairpin–hairpin binding; (iii) nuclease degradation that separates quencher from fluorophore; and (iv) non-specific interactions with proteins and other small molecules within the cell that disrupt the hairpin structure. This last effect might be the most substantial *in vivo*; for example, molecular beacons bind to certain cellular proteins, which can give a large background or false positive signal [26,39].

Sources of background signal for QUAL probes include: (i) incomplete quenching; (ii) nuclease degradation in the 1–3 nucleotides between the quencher and fluorophore; and (iii) the slow autohydrolysis of the quencher. This last

source might be the strongest, and can lead to non-specific signal within cells or in solution [32].

Despite these issues, nucleic acid-based detection has made great advances over the past few years, and quenched oligonucleotide probes are beginning to approach the efficiency required for many useful applications. The biggest remaining challenges – maximizing specificity, increasing signal on rare targets and reducing background – still need to be better addressed. Creative design innovations for probe molecules and in protocols for their use and analysis will no doubt be useful in overcoming these last barriers.

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