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Double Displacement: an Improved Bioorthogonal Reaction Strategy for Templated Nucleic Acid Detection

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Abstract

Quenched autoligation probes have been employed previously in a target-templated nonenzymatic ligation strategy for detecting nucleic acids in cells by fluorescence. A common source of background signal in such probes is undesired reaction with water and other cellular nucleophiles. Here we describe a new class of self-ligating probes, double displacement (DD) probes, that rely on two displacement reactions to fully unquench a nearby fluorophore. Three potential double displacement architectures, all possessing two fluorescence quencher/leaving groups (dabsylate groups), were synthesized and evaluated for templated reaction with nucleophile (phosphorothioate) probes both *in vitro* and in intact bacterial cells. All three DD probe designs provided substantially better initial quenching than a single-Dabsyl control. In isothermal templated reactions *in vitro*, double displacement probes yielded considerably lower background signal than previous single-displacement probes; investigation into the mechanism revealed that one dabsylate acts as a sacrificial leaving group, reacting nonspecifically with water, but yielding little signal because another quencher group remains. Templated reaction with the specific nucleophile probe is required to activate a signal. The double displacement probes provided a ca. 80-fold turn-on signal and yielded a 2-4-fold improvement in signal/background over single-Dabsyl probes. The best-performing probe architecture was demonstrated in a two-color, FRET-based two-allele discrimination system *in vitro*, and was shown to be capable of discriminating between two closely related species of bacteria differing by a single nucleotide at a ribosomal RNA target site.

Introduction

Nucleic acid templated chemistry is a versatile approach to a growing number of applications in chemistry, biology and medicine. These reactions have been used to ligate short oligonucleotides (1–3), catalyze prodrug release (4) or cytotoxic drug generation (5), synthesize small molecules (6–9), and evolve modified nucleic acids (10). Templated reactions have also been used to detect nucleic acid sequences both *in vitro* and in cells (11). Unlike more conventional methods for nucleic acid detection such as polymerase chain reaction (PCR) (12) and fluorescence *in situ* hybridization (FISH) (13,14), templated reactive probes can in some cases be employed for cellular RNA/DNA identification without lysing or fixing the cells, and do not require washing steps. This makes probing operationally easier to perform and the results straightforward to interpret. It also allows for the analysis of heterogeneous samples of cells and for sorting cells based on their genetics

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Supporting Information Available

Experimental details, additional data, and characterization of synthetic intermediates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

(15). In addition to templated reactive probes, other classes of fluorogenic nucleic acid probes, including molecular beacons and nanoparticle-based systems, are also under investigation for such applications (16–20).

Several fluorogenic reaction strategies have been investigated for templated nucleic acid detection, including fluorophore uncaging (21–27), quencher release (28), fluorophore synthesis (29,30), fluorophore release (31), and quencher transfer (32,33). Our laboratory has described quenched autoligation (QUAL) probes that rely on an S_N2 reaction to displace a fluorescence quencher, unquenching a nearby fluorophore (34–36). While these probes have been used to discriminate between different strains of bacteria based on small differences in ribosomal RNA sequences and to detect high-copy-number mRNAs in mammalian cells, the reported designs can be limited by a significant background signal; a low background would ultimately be needed to detect rarer RNAs. The background signal from the probes arose largely from non-templated displacement of the quencher by cellular nucleophiles such as water and thiols. Thus we have pursued new strategies that increase specific signals and decrease such background signals. In this light, we describe here double displacement (DD) probes, which require two displacement events to fully unquench a fluorophore.

Double displacement probes were designed to have two fluorescence quenchers as leaving groups on the terminus of a DNA probe. The nonspecific displacement of only one of these quenchers (by water, for example) should not produce a large increase in fluorescence, since another quencher remains. However, the binding of a DD probe adjacent to a nucleophile probe on a target sequence markedly increases the rate at which the quenchers are displaced, leading to signal generation. Herein we describe the synthesis and evaluation of three architectures for double displacement. These new probes are shown to provide enhanced initial quenching and also yield considerably lower background signal during *in vitro* and cellular nucleic acid detection. Two-color DD probes are further shown to discriminate between bacterial cell types in a heterogeneous mixture based on a single-nucleotide polymorphism.

Experimental Procedures

Double Displacement Oligonucleotide Probe Synthesis

Pac-dA, iPr-dG, and Ac-dC phosphoramidites for UltraMild synthesis (Glen Research) were employed in synthesizing oligonucleotides containing the double displacement linker. The fluorescein label was introduced with fluorescein-dT phosphoramidite (Glen Research). The TAMRA label was introduced using a 3'-TAMRA CPG (Glen Research). Deprotection and cleavage from the CPG support was carried out by incubation in 0.05 M K_2CO_3 in CH_3OH (Glen Research) for 4 hours at room temperature. The oligonucleotides were purified by reverse-phase HPLC (Prosphere C18 300Å 10u 250 mm, eluting with 0.1 M triethylammonium acetate, pH 7.0 and acetonitrile). Probe structure was confirmed by MALDI-TOF mass spectrometry (see Supporting Information).

Preparation of Single-Dabsyl Probes

Single-Dabsyl (butyl linker) probes were prepared and purified as previously described (36).

Templated Double Displacement Reactions

Reactions were performed in 70 mM PIPES buffer (pH 7.0) containing 10 mM $MgCl_2$ and 50 μM dithiothreitol in a Flexstation II 384 microplate reader. Reaction mixtures contained 100 nM each of quencher probe and template and 120 nM of nucleophile DNA. Reactions were initiated by addition of the nucleophile DNA to wells containing the quencher probe

and template DNA. The emission was measured in 2 min intervals at 525 nm with excitation at 494 nm over 8 hours. The control reactions were performed with the template omitted from the reaction mixture.

Monitoring Dabsylate Release by HPLC

Reactions were performed in 70 mM PIPES Buffer (pH 7.0) containing 10 mM MgCl₂ and 50 μ M dithiothreitol at 25°C. At the specified time points, aliquots of the reaction were taken and injected onto an analytical RP-HPLC column (Alltech Platinum C18 100Å 5 μ 250mm), eluting with 0.1 M triethylammonium acetate, pH 7.0 and acetonitrile. The integration of the dabsylate peak was taken and converted to a measure of the number of equivalents of dabsylate based on a standard curve prepared separately with known concentrations.

Testing intracellular RNA Detection

E. coli K12 and *S. enterica* cells were grown to log phase (OD₆₀₀ = 0.4–0.6) in Luria-Bertani (LB) media at 37°C with rapid shaking. Aliquots of media were centrifuged for 5 min. The supernatant was removed and pellets were resuspended in 0.1 mL phosphate-buffered saline solution (pH 7.4). The cells were centrifuged again for five minutes and the supernatant was removed. The pellets were resuspended in 0.1 mL hybridization buffer: 0.9 M sodium chloride and 90 mM sodium citrate (6x SSC) and 0.05% SDS. Aliquots of bacteria suspended in the buffer were treated with quencher probe (200 nM), nucleophile probe (2 μ M), and helper probes (3 μ M each). The reaction mixtures were incubated in the dark at 37°C. Aliquots of incubated bacteria were mixed 1:1 with a 1% agarose solution and spotted on a glass slide without washing or fixation steps. Imaging was performed on a Nikon Eclipse E800 epifluorescence microscope equipped with a Nikon Plan AP 100x/1.40 oil immersion objective and a SPOT RT digital camera.

Results

Design and Synthesis of Double Displacement Probes

Three potential double displacement probe scaffolds were designed to improve the signal/background ratio of the original single displacement probe structure (Figure 1). As with the prior design, the leaving group in this approach (called dabsylate in analogy to tosylate) serves both to activate the substrate for electrophilic attack and to quench a nearby fluorophore.

The first two linkers designed are structural isomers based on a 1,3,5-pentanetriol scaffold. The first (1,5-Pentyl) has the quenchers on the two primary positions, which should make both reactive S_N2 substrates. The second (1,3-Pentyl) has the quenchers at a primary and a secondary position. Since S_N2 reactions are approximately one order of magnitude slower at secondary carbons than at terminal alkyl positions (37), we hypothesized that this scaffold would be less prone to multiple nontemplated reactions. The third new scaffold (Isobutyl) was designed with both quenchers at more hindered positions by using a branched scaffold. Isobutyl electrophiles are expected to react with rates around the same order of magnitude as secondary positions (37), which was expected to make this linker the most challenging S_N2 substrate of the three and therefore allow fewer untemplated reactions and an even lower background signal.

The synthesis of all three new reactive linkers was straightforward, with phosphoramidite analogs prepared on the third alcohol group. The synthesis of the 1,3-Pentyl linker did require extra steps to allow for installation of a quencher at the secondary position instead of one of the primary positions, but it was still synthetically accessible. For the initial studies

we prepared identical 18mer DNA probes having the DD reactive linkers at the 5' end, along with a fluorescein moiety at the third position. The syntheses of all three DD linker phosphoramidites and their subsequent oligonucleotide conjugation are described in the Supporting Information and Experimental Procedures, respectively.

Initial Quenching Efficiency

The initial quenching efficiencies of the three double displacement probes were measured and compared to the initial degree of quenching of the single-Dabsyl control probe. All three double displacement scaffolds demonstrated superior quenching of the nearby fluorescein moiety, yielding lower initial fluorescence of the unreacted probe (Supporting Information, Figure S1) by a factor of at least four. Such an effect has been demonstrated with molecular beacons, where adding multiple quenchers improved the quenching efficiency (38). It is unclear why both the 1,3-Pentyl and Isobutyl probes produce more efficient initial quenching than the 1,5-Pentyl probe, as all three have two quenchers approximately equidistant from the fluorophore. Differences in the structure and flexibility of the linkers may account for these small differences in quenching, by allowing different degrees of contact between quencher and fluorophore.

Templated Unquenching of Double Displacement Probes

We tested the reactivity of the probes in the presence of a complementary DNA template. The fluorescence was monitored over eight hours at 37°C with 100 nM probes in a pH 7.0 buffer containing 70 mM PIPES, 10 mM MgCl₂, and 50 μM dithiothreitol (Figure 2). As a control to evaluate background (off-template) signal levels, the template DNA was omitted in separate reactions for each probe type. 15mer DNAs with 3'-phosphorothioate groups were used as the nucleophile probe in this and all subsequent experiments. In initial experiments we also tested 3'-phosphorodithioate nucleophile DNAs; the dithioate moiety is a stronger nucleophile and produces faster displacement reactions (39). However, we found that stability issues at 37°C made them less well-suited for these experiments. Reactions and stability of phosphorodithioate nucleophiles in double displacement reactions are described in the Supporting Information.

In templated reactions with the phosphorothioate nucleophile DNA, results showed that two of the three DD probes yielded a substantial fluorescence turn-on signal. The 1,5-Pentyl and 1,3-Pentyl probes showed very similar fluorescence turn-on kinetics, somewhat slower than the rate of the single-Dabsyl control but reaching the same ultimate fluorescence level (Figure 2). The Isobutyl probe, which has both quenchers linked at hindered positions, gave a significantly slower reaction, reaching only ~30% conversion after eight hours. Importantly, background signals for all three DD probes were substantially lower than that of the single-Dabsyl control. Indeed, the background of the slower-reacting Isobutyl probe did not seem to increase at all over the time of the experiment.

From the kinetic data we plotted the signal/background versus time for each reaction (Figure 3). The 1,3-Pentyl probe showed a higher signal/background at its peak than either the single-Dabsyl or 1,5-Pentyl probe, although the latter had a better ratio over the first hour of reaction. The signal/background ratio for the Isobutyl probe rose more slowly due to its lower reaction rate. However, at long reaction times the value approached that of the best probes. We also evaluated the different probes based on the fold change in fluorescence intensity over time (Figure 4). This metric takes into account the starting fluorescence level of the probes, providing the relative magnitudes of fluorescence turn-on. This is particularly pertinent if the probes will be used for visual evaluations, because the probes with the highest amount change over time will be the easiest to identify by eye. By this measure, the 1,3-Pentyl and 1,5-Pentyl probes produced similar 75- to 85-fold turn-on, both of which

represent a significant improvement over the single-Dabsyl probe (30-fold). These fluorescence enhancements are similar in magnitude to those of the best turn-on values for other templated probes in the literature (23,24,26–28).

Determining Double Displacement Reaction Mechanism

In order to gain insight into the mechanism of reaction of the double displacement probes, the displacement of the quencher leaving group was monitored quantitatively by following the increase in dabsylate concentration in solution over time by RP-HPLC. The 1,5-Pentyl linker was chosen as a representative double displacement scaffold for these experiments. The data show that the rates of dabsylate appearance for reactions with and without a nucleophile probe primarily differ in the first few hours of the reaction (Figure 5). At eight hours, the reaction with the nucleophile had lost 1.5 equivalents of dabsylate and the reaction without the nucleophile had lost 0.5 equivalents. From then on, the rates of dabsylate appearance are very similar. By the time the reactions with the nucleophile displace two equivalents of dabsylate, the reaction with no nucleophile present had displaced one equivalent. This supports the intention of the probe design: while background (water) displacement of one of the quenchers does occur, there is a negligible change in signal until a templated nucleophile can react to displace the second one.

Detection of rRNA in Intact Bacterial Cells

In order to determine if the results we obtained *in vitro* translated to cellular conditions, all four probes were used to detect a previously targeted rRNA sequence in *Escherichia coli* cells (39,40). Aliquots of bacteria in log phase growth were treated with 0.9 M sodium chloride and 90 mM sodium citrate (6× SSC) + 0.05% SDS, unlabeled helper DNAs, 3'-phosphorothioate probes and the single-Dabsyl or double displacement probes and incubated at 37°C for three hours. The purpose of the helper oligonucleotides was to disrupt the secondary structure of the rRNA and increase accessibility for the probes (41). The background fluorescence was determined by omitting the 3'-phosphorothioate oligonucleotides from the reaction. No washing or cell preparation steps were taken; the result was simply imaged after three hours. Figure 6 shows representative images of cells for the foreground and background of each probe. Gratifyingly, the 1,3-Pentyl and 1,5-Pentyl probe give the best contrast between the templated and untemplated reactions in the intact bacteria, fully consistent with the results of the *in vitro* experiments.

Two-color Probes for Discrimination Between Bacterial Species

Next we tested whether double displacement linkers could be adapted to a two-color system of probes that allows one to visually distinguish between different types of cells in a heterogeneous mixture. To this end, we synthesized two new probes, both with a 1,3-Pentyl double displacement linker. Both probes are fluorophore-labeled 10mer oligonucleotides that differ only by a single base pair to render them complementary to a known site in *E. coli* and *Salmonella enterica* rRNA that differs by a single base (G for *E. coli*; U for *S. enterica*). The EC (green) probe has only a fluorescein-labeled dT, while the SE (red) probe was designed to have a FRET pair with a fluorescein-labeled dT as the donor and a 3'-TAMRA fluorophore as the acceptor (Figure 7a). Our laboratory has previously used a similar design for QUAL and Q-STAR probes (15, 28, 42).

This set of probes was first tested *in vitro* to validate the two-color system. Both the EC and SE probes were incubated with the 3'-phosphorothioate strand and either the EC or SE template in pH 7.0 buffer containing 70 mM PIPES, 10 mM MgCl₂, 50 μM dithiothreitol at 37°C for one hour. The emission spectra of the individual reactions were measured from 500 to 650 nm with a 494 nm (fluorescein) excitation. When the template containing the complementary sequence for the *E. coli* probe was used, the experiment showed a strong

signal at 520 nm, consistent with fluorescein emission. When the *S. enterica* template was used, the probes showed a clear FRET signal, with emission peaks at both 520 and 580 nm (Figure 7b). The kinetics of these reactions were also measured at 37°C over eight hours with and without the template strands (Supporting Information, Figure S7).

After verifying that these probes performed correctly *in vitro*, their specificity to the desired cell type was tested. Each probe, along with the 3'-phosphorothioate and the appropriate helper probes were used to treat *E. coli* and *S. enterica* cells in 6× SSC + 0.05% SDS. The intact cells were imaged under an epifluorescence microscope. The results clearly show that the probes were selectively unquenched with the appropriate cell type, as the images of the probes in the incorrect cells showed fluorescence levels that are equivalent to the fluorescence when no nucleophile probe is added (Supporting Information, Figure S8). This study was extended further to show cell-type analysis of a heterogeneous mixture. Both probes were simultaneously added with the same nucleophile and helper strands to a mixture of *E. coli* and *S. enterica* cells. After incubation, the mixture of cells was imaged under a fluorescence microscope, clearly showing two differently colored populations of cells (Figure 8), demonstrating that these probes can be used to distinguish between two cell lines with single nucleotide discrimination.

Discussion

The experiments presented here expand on the previously described quenched autoligation reactions as a viable approach to the detection of nucleic acids. Previous work with this class of reactions showed that non-specific displacement of the quencher was the primary source of background (36,41). Therefore, in designing the double displacement linkers we sought to use scaffolds that would necessitate two reactions to produce a fully unquenched fluorophore, hoping that this would increase the likelihood that a full unquenching of the probe would include a templated reaction. The results with the three new double displacement probes clearly confirmed this, showing strongly enhanced signal/background as a result of the dual reactive groups. The faster-reacting Pentyl probes also showed improved signal/background in a cellular context. An added benefit of the dual-quencher design was the enhancement of the initial quenching of the probes. For two of the new probes this produced a much larger increase in fluorescence intensity over time as compared to the previous probes with a single-leaving quencher/ leaving group.

The third new probe, based on an isobutyl scaffold, reacted more slowly than expected. Although both leaving groups are at primary positions, the linker appears to be too sterically demanding for the S_N2 reactions necessary for unquenching to occur on a useful timescale. This gives these probes very low background levels, but also a very slow turn-on rate. While these properties may make the Isobutyl probes appropriate for other applications (for example, at higher temperature), they do not lend themselves well to cell-based diagnostics at 37 °C.

Our experiments in single-color detection of rRNA in *E. coli* showed similar trends in signal/background as were observed *in vitro*. The 1,3-Pentyl and 1,5-Pentyl probes showed a clearer visual difference between the probes with and without the nucleophile strand than did the Isobutyl or single-Dabsyl probes. The slow-reacting Isobutyl probes yielded cell images that were hardly fluorescent at all, regardless of whether or not the nucleophile was present. In two-color mixed probe experiments, probes were capable of clearly differentiating between these two cell types based on their genetics in a heterogeneous sample. Thus the Pentyl probe designs are potentially very applicable in bacterial discrimination in clinical microbiology laboratories, offering clearer signal and less background than previous designs (42). Although both the 1,3-Pentyl and 1,5-Pentyl probes

performed with similar kinetics and signal/background, from a functional perspective we currently favor the 1,3-Pentyl probes because they appear to give slightly better signal/background in our cellular experiments. However, the 1,5-Pentyl probes do offer a practical advantage, as the synthesis of the phosphoramidite is several steps shorter.

The two-color cellular demonstration of DD probes illustrates one of a number of possible applications of templated chemistry, particularly for single nucleotide discrimination, which is difficult to achieve with classical FISH probes. Continued development of these reactive probes and tests of their application in mammalian cells are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Gryaznov SM, Letsinger RL. Template controlled coupling and recombination of oligonucleotide blocks containing thiophosphoryl groups. *Nucleic Acids Res.* 1993; 21:1403–1408. [PubMed: 8464731]
2. Gryaznov SM, Schultz R, Chaturvedi SK, Letsinger RL. Enhancement of selectivity in recognition of nucleic acids via chemical autoligation. *Nucleic Acids Res.* 1994; 22:2366–2369. [PubMed: 8036165]
3. Herrlein MK, Letsinger RL. Selective chemical autoligation on a double-stranded DNA template. *Nucleic Acids Res.* 1994; 22:5076–5078. [PubMed: 7800502]
4. Jacobsen MF, Clo E, Mokhir A, Gothelf KV. Model systems for activation of nucleic acid encoded prodrugs. *ChemMedChem.* 2007; 2:793–799. [PubMed: 17436260]
5. Arian D, Clo E, Gothelf KV, Mokhir A. A nucleic acid dependent chemical photocatalysis in live human cells. *Chemistry.* 16:288–295. [PubMed: 19894234]
6. Gartner ZJ, Kanan MW, Liu DR. Multistep small-molecule synthesis programmed by DNA templates. *J Am Chem Soc.* 2002; 124:10304–10306. [PubMed: 12197733]
7. Gartner ZJ, Tse BN, Grubina R, Doyon JB, Snyder TM, Liu DR. DNA-templated organic synthesis and selection of a library of macrocycles. *Science.* 2004; 305:1601–1605. [PubMed: 15319493]
8. Liu DR. Translating DNA into synthetic molecules. *PLoS Biol.* 2004; 2:E223. [PubMed: 15252462]
9. Snyder TM, Liu DR. Ordered multistep synthesis in a single solution directed by DNA templates. *Angew Chem Int Ed Engl.* 2005; 44:7379–7382. [PubMed: 16217825]
10. Brudno Y, Birnbaum ME, Kleiner RE, Liu DR. An in vitro translation, selection and amplification system for peptide nucleic acids. *Nat Chem Biol.* 6:148–155. [PubMed: 20081830]
11. Silverman AP, Kool ET. Detecting RNA and DNA with templated chemical reactions. *Chem Rev.* 2006; 106:3775–3789. [PubMed: 16967920]
12. Fey A, Eichler S, Flavier S, Christen R, Hofle MG, Guzman CA. Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism. *Appl Environ Microbiol.* 2004; 70:3618–3623. [PubMed: 15184165]
13. Paillason S, Van De Corput M, Dirks RW, Tanke HJ, Robert-Nicoud M, Ronot X. In situ hybridization in living cells: detection of RNA molecules. *Exp Cell Res.* 1997; 231:226–233. [PubMed: 9056430]
14. Amann R, Fuchs BM, Behrens S. The identification of microorganisms by fluorescence in situ hybridisation. *Curr Opin Biotechnol.* 2001; 12:231–236. [PubMed: 11404099]

15. Abe H, Kool ET. Flow cytometric detection of specific RNAs in native human cells with quenched autoligating FRET probes. *Proc Natl Acad Sci U S A*. 2006; 103:263–268. [PubMed: 16384914]
16. Nitin N, Santangelo PJ, Kim G, Nie S, Bao G. Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells. *Nucleic Acids Res*. 2004; 32:e58. [PubMed: 15084673]
17. Santangelo PJ, Nitin N, Bao G. Direct visualization of mRNA colocalization with mitochondria in living cells using molecular beacons. *J Biomed Opt*. 2005; 10:44025. [PubMed: 16178658]
18. Santangelo PJ, Nix B, Tsourkas A, Bao G. Dual FRET molecular beacons for mRNA detection in living cells. *Nucleic Acids Res*. 2004; 32:e57. [PubMed: 15084672]
19. Nitin N, Bao G. NLS peptide conjugated molecular beacons for visualizing nuclear RNA in living cells. *Bioconjug Chem*. 2008; 19:2205–2211. [PubMed: 18939859]
20. Seferos DS, Giljohann DA, Hill HD, Prigodich AE, Mirkin CA. Nano-flares: probes for transfection and mRNA detection in living cells. *J Am Chem Soc*. 2007; 129:15477–15479. [PubMed: 18034495]
21. Cai J, Li X, Yue X, Taylor JS. Nucleic acid-triggered fluorescent probe activation by the Staudinger reaction. *J Am Chem Soc*. 2004; 126:16324–16325. [PubMed: 15600325]
22. Pianowski ZL, Winssinger N. Fluorescence-based detection of single nucleotide permutation in DNA via catalytically templated reaction. *Chem Commun (Camb)*. 2007:3820–3822. [PubMed: 18217658]
23. Abe H, Wang J, Furukawa K, Oki K, Uda M, Tsuneda S, Ito Y. A reduction-triggered fluorescence probe for sensing nucleic acids. *Bioconjug Chem*. 2008; 19:1219–1226. [PubMed: 18476727]
24. Franzini RM, Kool ET. 7-Azidomethoxy-coumarins as profluorophores for templated nucleic acid detection. *Chembiochem*. 2008; 9:2981–2988. [PubMed: 19035374]
25. Furukawa K, Abe H, Hibino K, Sako Y, Tsuneda S, Ito Y. Reduction-Triggered Fluorescent Amplification Probe for the Detection of Endogenous RNAs in Living Human Cells. *Bioconjug Chem*. 2009
26. Furukawa K, Abe H, Wang J, Uda M, Koshino H, Tsuneda S, Ito Y. Reduction-triggered red fluorescent probes for dual-color detection of oligonucleotide sequences. *Org Biomol Chem*. 2009; 7:671–677. [PubMed: 19194582]
27. Pianowski Z, Gorska K, Oswald L, Merten CA, Winssinger N. Imaging of mRNA in Live Cells Using Nucleic Acid-Templated Reduction of Azidorhodamine Probes. *J Am Chem Soc*. 2009
28. Franzini RM, Kool ET. Efficient nucleic acid detection by templated reductive quencher release. *J Am Chem Soc*. 2009; 131:16021–16023. [PubMed: 19886694]
29. Huang Y, Coull JM. Diamine catalyzed hemicyanine dye formation from nonfluorescent precursors through DNA programmed chemistry. *J Am Chem Soc*. 2008; 130:3238–3239. [PubMed: 18302371]
30. Jentzsch E, Mokhir A. A fluorogenic, nucleic acid directed "click" reaction. *Inorg Chem*. 2009; 48:9593–9595. [PubMed: 19769395]
31. Cai J, Li X, Taylor JS. Improved nucleic acid triggered probe activation through the use of a 5-thiomethyluracil peptide nucleic acid building block. *Org Lett*. 2005; 7:751–754. [PubMed: 15727432]
32. Grossmann TN, Roglin L, Seitz O. Target-catalyzed transfer reactions for the amplified detection of RNA. *Angew Chem Int Ed Engl*. 2008; 47:7119–7122. [PubMed: 18677727]
33. Grossmann TN, Strohbach A, Seitz O. Achieving turnover in DNA-templated reactions. *Chembiochem*. 2008; 9:2185–2192. [PubMed: 18752239]
34. Sando S, Kool ET. Quencher as leaving group: efficient detection of DNA-joining reactions. *J Am Chem Soc*. 2002; 124:2096–2097. [PubMed: 11878946]
35. Sando S, Abe H, Kool ET. Quenched auto-ligating DNAs: multicolor identification of nucleic acids at single nucleotide resolution. *J Am Chem Soc*. 2004; 126:1081–1087. [PubMed: 14746476]
36. Abe H, Kool ET. Destabilizing universal linkers for signal amplification in self-ligating probes for RNA. *J Am Chem Soc*. 2004; 126:13980–13986. [PubMed: 15506759]
37. March, SA. *March's Advanced Organic Chemistry*. John Wiley & Sons, Inc.; 2007. p. 479

38. Yang CJ, Lin H, Tan W. Molecular assembly of superquenchers in signaling molecular interactions. *J Am Chem Soc.* 2005; 127:12772–12773. [PubMed: 16159250]
39. Miller GP, Silverman AP, Kool ET. New, stronger nucleophiles for nucleic acid-templated chemistry: Synthesis and application in fluorescence detection of cellular RNA. *Bioorg Med Chem.* 2008; 16:56–64. [PubMed: 17502150]
40. Silverman AP, Kool ET. Quenched autoligation probes allow discrimination of live bacterial species by single nucleotide differences in rRNA. *Nucleic Acids Res.* 2005; 33:4978–4986. [PubMed: 16284198]
41. Fuchs BM, Glockner FO, Wulf J, Amann R. Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. *Appl Environ Microbiol.* 2000; 66:3603–3607. [PubMed: 10919826]
42. Silverman AP, Baron EJ, Kool ET. RNA-templated chemistry in cells: discrimination of *Escherichia*, *Shigella* and *Salmonella* bacterial strains with a new two-color FRET strategy. *Chembiochem.* 2006; 7:1890–1894. [PubMed: 17031884]

Figure 1. Structures of the reactive parts of double displacement and control (single displacement) probes.

A

B

Figure 2. Fluorescence Timecourse of Double Displacement and Single-Dabsyl Ligation Reactions at 37°C

(A) Sequences of DNA probes and targets (^f = fluorescein dT; ^Q = single-Dabsyl or Double Displacement linker; Nuc = phosphorothioate) (B) 100 nM of 1,3-Pentyl (blue), Isobutyl (green), 1,5-Pentyl (yellow) or single-Dabsyl (red) probe and 120 nM phosphorothioate probe were incubated with or without 100 nM template DNA at 37°C in pH 7.0 buffer containing 70 mM PIPES, 10 mM MgCl₂, 50 μM dithiothreitol. The background (no template) reactions for each probe are shown as broken lines. The fluorescence was measured every 120 s with 494 nm excitation and 525 nm emission. Reactions were repeated in triplicate; representative traces are shown.

Figure 3. Change in Signal:Background for Double Displacement and Single Dabsyl Probes Over Time

The signal/background ratios for the 1,3-Pentyl (blue), Isobutyl (green), 1,5-Pentyl (yellow) and single-Dabsyl (red) reactions over an eight hour time period. The reactions were run at 37°C. 100 nM quencher probe and 120 nM phosphorothioate probe were incubated with or without 100 nM template DNA at 25°C in pH 7.0 buffer containing 70 mM PIPES, 10 mM MgCl₂, 50 μM dithiothreitol. The fluorescence was measured every 120 s with 494 nm excitation and 525 nm emission. The data was smoothed using a 7-point running average.

Figure 4. Fold Change in Fluorescence Intensity Over Time

100 nM of 1,3-Pentyl (blue), Isobutyl (green), 1,5-Pentyl (yellow) or single-Dabsyl (red) probe and 120 nM phosphorothioate probe were incubated with 100 nM template DNA at 37°C in pH 7.0 buffer containing 70 mM PIPES, 10 mM MgCl₂, 50 μM dithiothreitol. The initial quenching was calculated before the phosphorothioate DNA was added to initiate the reaction. The fluorescence was measured every 120 s with 494 nm excitation and 525 nm emission. Reactions were repeated in triplicate and the traces shown represent the average fold change in fluorescence at each time point.

Figure 5. Monitoring Dabsylate Release in Double Displacement Reaction

The amount of free dabsylate released in solution, as monitored by HPLC from reactions containing 1 μM DD probe, 1 μM template strand and 1.2 μM phosphorothioate probe (blue) at 25°C. The DD probe and template with no nucleophile probe present is shown in pink.

Figure 6. Comparison of Double Displacement and Single Dabsyl Probes for Detecting Cellular 16S rRNA

The indicated double displacement or single-Dabsyl probe (200 nM), helper DNAs (3 μ M) and 3'-phosphorothioate probes (2 μ M) were incubated with *E. coli* cells in 6 $\times$ SSC buffer with 0.05% SDS at 37°C for three hours. Images were taken with a black/white camera and false-colored green.

A

B

Figure 7. FRET probes for Two-color Detection Scheme

(A) Sequences of DNA probes and targets (T^f = fluorescein dT; DD = 1,3-pentyl linker; Nuc = phosphorothioate); (B) Normalized fluorescence emission spectra of a mixture of EC and SE probes incubated with 3'-phosphorothioate probe and either EC DNA or SE DNA. Conditions: 200 nM EC probe, 200 nM SE probe, 600 nM phosphorothioate DNA and 200 nM EC (green) or SE DNA (red). The reactions were incubated for 1 h at 37°C in pH 7.0 buffer containing 70 mM PIPES, 10 mM MgCl₂, 50 μM dithiothreitol. The excitation wavelength was 494 nm.

Figure 8. Two-color Discrimination of *E. coli* and *S. enterica* Based on a Single Nucleotide Polymorphism in the 16S rRNA

EC and SE double displacement probes (200 nM each), helper probes (3 μ M) and 3'-phosphorothioate probes (2 μ M) in 6 $\times$ SSC + 0.05% SDS buffer were incubated in the indicated cell type or mixture of cells at 37°C for five hours. A B-2A filter was used for imaging. (a) *E. Coli* cells (b) *E. Coli* and *S. enterica* cells (c) *S. enterica* cells