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Improved Templated Fluorogenic Probes Enhance the Analysis of Closely Related Pathogenic Bacteria by Microscopy and Flow Cytometry

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Abstract

Templated fluorescence activation has recently emerged as a promising molecular approach to detect and differentiate nucleic acid sequences *in vitro* and in cells. Here we describe the application of a reductive quencher release strategy to the taxonomic analysis of gram-negative bacteria by targeting a single nucleotide difference in their 16S rRNA in a two-color assay. For this purpose, it was necessary to develop a release linker containing a quencher suitable for red and near-infrared fluorophores, and to improve methods for the delivery of probes into cells. A cyanine-dye labeled oligonucleotide probe containing the new quencher-release linker showed unprecedentedly low background signal and high fluorescence turn-on ratios. The combination of a fluorescein-containing and a near-IR emitting probe discriminated *E. coli* from *S. enterica* despite nearly identical ribosomal target sequences. Two-color analysis by microscopy and the first successful discrimination by two-color flow cytometry are described.

Introduction

The development of methods for rapid detection and identification of human pathogenic bacteria is widely regarded as important both in medical diagnostics and in monitoring food safety. The standard analytical approach for most bacteria involves growing cultures followed by microbiological assessment, which is time consuming, labor intensive and requires expert personnel. Consequently, there is significant interest in developing methods to identify microbes directly from clinical samples with a straightforward readout. Of particular appeal with this respect are fluorescence in situ hybridization (FISH) methods,[1,2] which target specific RNA or DNA sequences with labeled hybridization probes followed by microscopic analysis or flow cytometry.[3] Bacterial FISH assays frequently target ribosomal RNAs (rRNAs) because these markers are highly expressed and the target accessibility is well established.[4,5] However, it is challenging to distinguish closely related pathogens with standard-length hybridization probes, because rRNAs are evolutionarily conserved and a difference of only one or two nucleotides in a target sequence has an insignificant influence on the binding of a standard-length probe of 25 nucleotides or more.[6] Furthermore, standard FISH assays involve cell fixation and washing steps, extending the time and care required for analysis.

One possible approach to avoid these limitations of standard FISH probes involves the use of quenched probes that report the presence of a target with a fluorescence turn-on signal.[7]

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Supporting Information Synthesis of quencher release linkers, ^{1}H- and ^{13}C-NMR analysis of synthetic intermediates and products, MALDI-TOF and analytical HPLC analysis of reactive probes, and additional experiments. This material is available free of charge via the Internet at http://pubs.acs.org/BC.

Prominent examples of fluorescence turn-on probes include molecular beacon (MB) probes,[8] forced intercalation (FIT) probes,[9] and templated reactive probes.[10] Because such probes are non-fluorescent before binding their target, they can (at least in principle) be used without washing steps, thus simplifying the genetic analysis considerably. Indeed, MB probes have been employed in the analysis of bacterial RNAs.[11,12] In a different molecular strategy, templated reactive probes allow the identification and analysis of bacteria in a single step requiring as little as 45 min incubation time.[13] In this approach, two modified oligonucleotide probes bind at adjacent sites on a nucleic acid target sequence, bringing two chemically reactive groups together to generate a fluorescence signal.[10] The possibility to use short probes provides this strategy with excellent mismatch selectivity on highly expressed, conserved RNA sequences such as rRNAs.[13–17] This high degree of sequence specificity is a major advantage over standard FISH probes for which single base specificity is possible[18] but is often challenging. Two-color sets of templated quenched autoligation (QUAL) probes enabled discrimination of bacteria by a simple fluorescence color-call.[19] In preliminary experiments, this technique has been applied to distinguish *Escherichia coli* from *Salmonella enterica*[13–17] and *Shigella*[19] pathogens by fluorescence microscopy and single-color flow cytometry.[15] Several laboratories have recently reported advances in templated reactive oligonucleotide designs;[20–25] examples included probes that are based on templated Staudinger reactions.[26–30] Among these, we have described quenched Staudinger reaction triggered α-azidoether release (Q-STAR) probes, which release a quencher after an azide reduction reaction on a linker carrying it.[31–33]

Despite the promise of early experiments, challenges remain for the general application of templated probes in bacteria, including spectral limitations and problems with probe delivery into cells. Previous two-color reactive probe systems for the detection of bacterial rRNAs relied on a FRET-reporter strategy, in which one probe contained fluorescein and the second one fluorescein combined with a TAMRA FRET-acceptor.[17,19,31] This strategy offers direct visual readout under a fluorescence microscope with single-wavelength excitation. However, low emission quantum yield and poor spectral resolution render such FRET-based probes unsuitable for quantitative analytical methods such as flow cytometry, which may benefit from probes with fluorophores that are excited at separate wavelengths. Multi-color QUAL probes have been described and applied to imaging of bacteria,[34] however the dabsyl group intrinsic to the QUAL design quenches red and near-IR emitting dyes inefficiently. Other examples of two-color reactive probes based on two distinct profluorophores have been described but their potential for cellular RNA probing remains untested.[35,36] Delivery of oligonucleotide probes into bacteria is another challenge for the direct detection of genetic markers in microbes. Cell fixation is one possible solution; however, this adds extra time and effort to the experimental protocol, renders bacteria less amenable to separation by flow cytometry, and excludes the possibility of isolating live cells for further analysis. One report described the use of a detergent to aid in delivery of FISH probes into bacteria, offering a simple alternative to fixation.[15] However, it is unknown to what extent various detergents might aid probe uptake into bacteria and how such reagents affect bacterial viability.

Here we describe the preparation and evaluation of quencher release probes suitable for red and near-IR emitting fluorophores and their potential for pathogen analysis in a two-color assay with fluorescein-labeled probes (Figure 1).[31] We found that these probes enable the discrimination of *E. coli* and *S. enterica* with improved signal contrast by fluorescence microscopy and by flow cytometry. Moreover, we investigated the use of various detergents and delivery agents on probe uptake, probe background signals and bacterial viability. Furthermore, we outline and discuss the remaining challenges for development of practical whole-cell microbial detection assays based on templated reactive probes and light-up probes in general.

Experimental Section

Preparation of Reactive Oligonucleotide Probes

Probes were prepared by solid-phase conjugation of the quencher release linker (synthesis described in Supporting Information) to the protected, fluorophore labeled (Quasar 670-dT, Biosearch Technologies) NIR STAR probes and (Fluorescein-dT, Glen Research)) green STAR probes oligonucleotides containing a 5′-terminal amino modifier (5′-amino-modifier 5, Glen Research). NIR STAR probes were synthesized with UltraMILD deprotection phosphoramidites (Glen Research); labeling of these oligonucleotides with **3** provided the NIR STAR probes in lower yield than green STAR probes. The major side product had a mass corresponding to the amine-modified DNA plus a Pac group, possibly resulting from a transfer of a Pac protecting group from nucleobases to the terminal amine under the quencher conjugation conditions. For the introduction of the 2,6-diaminopurine (DAP) DNA monomer, we used 2-Amino-dA-CE phosphoramidite (Glen Research). The trityl protecting group of the terminal amine was removed by repetitive cycles of 2 % trichloroacetic acid in DCM on the DNA synthesizer. Controlled pore glass (CPG) containing the oligonucleotides was incubated with a solution of the quencher release linker (25 mM), PyBOP (25 mM) and DIEA (50 mM) in DMF (250 μL) and gently shaken at room temperature for 5 h, protected from light. The supernatant was removed and the beads washed with DMF (2×) and MeCN (3×) until the solution was colorless. For cleavage from the solid support and deprotection of green STAR probes, the CPG were incubated for 90 min with concentrated aqueous ammonia/methylamine at 55 °C; for NIR STAR probes, beads were incubated in 0.5 M potassium carbonate solution in methanol for 4 h at room temperature. The purity of the probes was assessed by analytical HPLC chromatography (the probes were found to be >95% pure) and the molecular structure was verified by MALDI-TOF mass spectrometry analysis. The triphenylphosphine (TPP)-DNA probe was prepared as previously described.[31] Unmodified template and helper DNAs were obtained from the Stanford Protein and Nucleic Acid Facility.

Kinetic Measurements

Quenched probes (200 nM) and the corresponding template (200 nM) were incubated at 37 °C in Tris-borate buffer (70 mM, pH 7.55) containing $MgCl_2$ (10 mM). TPP-DNA (600 nM) was added and the fluorescence emission (λ_{ex} = 494 nM; λ_{em} = 521 nm for green STAR; λ_{ex} = 644 nm and λ_{em} = 670 nm for NIR STAR) was measured as a function of time on a Fluorolog 3 Jobin Yvon spectrometer equipped with an external temperature controller.

Fluorescence Microscopy

E. coli K12 and *S. enterica* were grown to mid-log phase (OD_{600} = 0.4) in Luria-Bertani (LB) media with rapid shaking at 37 °C. Aliquots of the media (100 – 200 μL) were carefully centrifuged and the supernatant decanted. Pellets were washed with 1× PBS buffer (pH 7.4) and resuspended in 6× saline sodium citrate (SSC) buffer (pH 7.4) containing 0.05% SDS. Green STAR (200 nM), NIR STAR (200 nM), TPP-DNA (2 μM), and helper DNAs (3 μM) were added and the samples incubated at 37 °C for 3 h without shaking and protected from light. Aliquots of incubated bacteria suspensions were mixed with 2% agarose solution and spotted on cover slides without washing or fixation. Imaging was performed on a Nikon Eclipse E800 epifluorescence microscope equipped with a Nikon Plan AP 100×/1.40 oil immersion objective and a SPOT RT digital camera; filter sets were B-2A (excitation: 450–490 nm, dichroic mirror: 500 nm, emission: >500 nm) for green STAR and Cy5 HYQ (excitation: 590–650 nm, dichroic mirror: 660 nm, emission: 663–738 nm) for NIR STAR probes.

Flow Cytometry Analysis

Bacteria were grown and treated with reactive probes as described for fluorescence microscopy experiments. Bacteria were diluted 10-fold in 6× SSC and the cell suspension immediately analyzed using an LSR I flow cytometer (BD Biosciences). Side angle light scattering was used as the triggering event. Forward angle light scatter, side angle light scattering were recorded as well as fluorescein (λ_{ex} = 488 nm laser; λ_{em} = 530 – 560 nm) and Q670 (λ_{ex} = 640 nm laser; λ_{em} = 666 – 690 nm) fluorescence emission. Flow cytometry data was analyzed with FlowJo software (Tree Star).

Bactericidal Activity of Surfactants

Bacteria cells were grown to mid-log phase (OD_{600} = 0.4), concentrated 10-fold by mild centrifugation and dispersed in SSC buffer (pH 7.0). Bacteria were incubated with different additives for 4 h at 37 °C without shaking. Aliquots of the bacteria samples were spread over solid LB-agar medium in Petri dishes and the dishes incubated at 37 °C overnight. The degree of bacteria growth was assessed visually.

Surfactant-Mediated Effects on Probe Signals

A mixture of Q-STAR (green STAR SE, 200 nM) and TPP-DNA (2.0 μM) probes was incubated in SSC buffer (1×, pH 7.0) containing different additives (1.0% w/v) at 37 °C for 4 h. The fluorescence intensity was measured with a Flexstation II microplate reader (Molecular Devices) in a 96-well quartz microplate (λ_{ex} = 494 nm, λ_{em} = 525 nm). The fluorescence intensity was normalized by dividing by the fluorescence intensity of a sample containing the probes in SSC buffer only.

Results

Design, Preparation and Evaluation of Quenched Near-IR Probes

We aimed to develop a quenched probe with a red-light emitting fluorophore that could be used in combination with fluorescein-labeled green STAR probes[31] for two-color instrumental analyses. The choice of a new long-wavelength dye required pairing it with a compatible quencher, since the original dabsyl quencher (see **1** in Chart 1) is limited to shorter-wavelength fluorophores (λ_{em} < 550 nm). Thus we designed the new quencher-linker **2** containing a Black Hole Quencher™ 1 (BHQ-1) and the same α-azidoether linker structure as the preceding dabsyl-probe **1**. The synthesis of **2** was identical to that of **1** except for the quencher attachment step, which involved HBTU-mediated amide bond formation (Supporting Information). To test the cleavage kinetics, the linker **2** was incorporated at the 5′-terminus of a labeled Q-STAR probe specific to a target sequence of 16S rRNA of *E. coli* (green STAR EC BHQ-1, Table 1).

Evaluation of the quencher-linker **2** showed that the DNA-templated reaction was rapid but that background quencher release is elevated compared with other probes. The presence of the EC DNA template induced a rapid reaction between green STAR EC BHQ-1 and TPP-DNA, eliciting a strong fluorescence turn-on signal (Figure 2a). The reaction kinetics were similar to those observed previously for probes containing linker **1**.[31] Control experiments (mismatch template SE DNA, no template, no TPP-DNA) confirmed the target-specificity of the probes (Figure 2a). Interestingly, the fluorescence-activation was similar for all control experiments, indicating significant phosphine-independent breakdown of probes containing the BHQ-1 release linker **2**. Mass spectrometry analysis revealed cleavage of the α-azidoether group upon incubation of probe alone at room temperature in buffer.

We hypothesized that the carboxamide moiety of linker **2** is responsible for the significant spontaneous decomposition of these probes, possibly by stabilizing the transition state of α-

azidoether hydrolysis[37] via an unidentified cyclic intermediate. Based on this assumption, we synthesized linker **3** (Chart 1) with an additional methylene group to disfavor a possible intramolecular interaction. Concomitantly, we changed the quencher to Black Hole Quencher™ 2 (BHQ-2) because this dye quenches red and near IR fluorophores more efficiently than BHQ-1.[38] The synthesis of BHQ-2 release linker **3** is similar to those of **1** and **2**, affording the product in 6 steps (Supporting Information).

Using the new quencher-linker **3**, we prepared NIR STAR probes containing a Cy5-derivative (Quasar™ 670, Q670) as the fluorophore (Table 1). Probes were complementary to a target site on 16S rRNA containing a single site difference between *E. coli* (EC) and *S. enterica* (SE).[15]

Fluorescence Activation and Sequence-Specificity

Incubation of NIR STAR EC with TPP-DNA and the matched template EC DNA rapidly elicited a strong increase of fluorescence (λ_{ex} = 644 nm, λ_{em} = 670 nm) with a kinetic trace similar to that of green STAR EC BHQ-1 (Figure 2b). The measured fluorescence turn-on ratio (fluorescence intensity after quencher release, divided by fluorescence intensity before addition of TPP-DNA) exceeded 200-fold, establishing that the fluorophore is quenched with >99.5% efficiency. This quenching effect compares favorably with probes containing a dabsyl/fluorescein pair (67-fold fluorescence activation)[31] and is presumably caused by the high quenching efficiency of BHQ-2.[38] Importantly, the generation of background fluorescence for NIR STAR EC was distinctly slower in the absence of TPP-DNA than for green STAR EC BHQ-1 and was below the rate of the reaction in the absence of the template or in the presence of the singly mismatched template SE DNA (Figure 2b). A second NIR STAR probe complementary to the sequence corresponding to *S. enterica* (NIR STAR SE, Table 1) also exhibited sequence-selective fluorescence activation of similar rate and magnitude (Figure S2, Supporting Information) although with significantly lower single-mismatch specificity because of the small energetic difference between the A–T match and the A–G mismatch, one of the most challenging mismatches to discriminate.[39]

To enhance sequence discrimination of SE DNA at this target site, we investigated the use of the 2,6-diaminopurine (DAP) base surrogate, which reportedly enhances single nucleotide discrimination over canonical adenine.[40] We prepared a DAP-containing green STAR probe[1] (green STAR SE DAP, Table 1) and compared its sequence-specificity to a probe with an adenine at this position (green STAR SE, Table 1). The SE DNA-mediated reaction was faster with green STAR SE DAP than with green STAR SE and the reaction mediated by the mismatch-containing EC DNA target was reduced for green STAR SE DAP relative to green STAR EC (Figure 2c). These two favorable effects synergistically yielded a 2-fold enhancement of mismatch selectivity for the DAP probe; after 9.5 min incubation, the sequence-specificity reached a maximum of 19.2 for green STAR SE DAP and only 8.8 for green STAR SE (Figure 2c inset).

Effect of Probe Delivery Protocols on Bacteria Growth

Protocols that enable cellular probe delivery without complex and time-consuming fixation steps would be advantageous to harness the simplicity of fluorogenic probes in bacteria. Previous studies described the introduction of oligonucleotide probes into gram-negative bacteria by incubating the prokaryotes in hyperosomotic buffer (6× saline-sodium citrate buffer, SSC) containing the detergent SDS (sodium dodecyl sulfate).[15,34] Although early studies suggested that bacteria remain viable under these conditions,[15,41] this conclusion remains uncertain.[42] The survival of the bacteria is unnecessary for many diagnostic

[1]Quasar 670 is incompatible with the stringent deprotection condition of commercial 2-amino-dA- CE phosphoramidite.

applications; however, it would be useful for example for culturing colonies from genetically sorted bacteria.

We tested the toxicity of SDS on the bacteria and compared it with the bactericidal effect of a small array of selected surfactants also to be tested for probe delivery. We examined cationic (HDTA), anionic (SDS, STDC), neutral (Triton X-100; Tween 20) and zwitterionic (CHAPS; DPAPS) detergents and selected polymers previously used for cell delivery purposes (polyethylene imine, PEI;[43] Pluronic F-68[44]) (for full names and molecular structures see Table S1, Supporting Information). As anticipated, different additives exhibited widely varying toxicities (Figure 3 and Figure S1, Supporting Information). HDTA was highly toxic at all tested concentrations, in agreement with the bactericidal activity of cationic lipids.[45] The neutral surfactants exhibited low toxicity in the investigated concentration range. Zwitterionic and anionic detergents, including SDS, exhibited a dose-dependent toxicity. SDS significantly inhibited colony formation in 6× SSC buffer; colonies formed only for the sampled with 0.01% ot lrdd SDS but were absent in samples for 1.0% and 0.1% SDS (Figure 3). The data reveal that the previously employed delivery protocol (0.05% SDS in 6× SSC) is toxic to *E. coli*, leaving only a few colonies. The hyperosmotic medium partially accounts for this bactericidal effect; bacteria treated with 1× SSC solutions formed significantly more colonies than those treated with 6× SSC.

Effect of Delivery Agents on Probe Background

For assessment of probe delivery agents, it was important to evaluate whether the reagents engender background signals, for example by sequence-independent aggregation of probes with polymers or lipids. To test this possibility, we incubated Q-STAR (green STAR SE) and TPP-DNA probes for 4 h in the presence of the additives (1% w/v) without template DNA and compared the fluorescence level to a control sample of probes without TPP-DNA. All additives with the exception of Pluronic F-68 generated higher fluorescence levels than the control (Figure 4). For SDS and several other detergents (Triton X-100, Tween 20, CHAPS, DPAPS) the level was approximately 2-fold higher than the control; this level of background fluorescence is acceptable for RNA detection in bacteria. In contrast, background fluorescence for PEI (15.5-fold), HDTA (6.8-fold) and STDC (5.8-fold) substantially exceeded the level of the control sample, ruling out these reagents for use with templated probes. The template-independent background fluorescence may arise from condensation of the polyanionic DNA reactive probes with cationic or zwitterionic surfactants.

Effect of Detergents and Polymers on Probe Uptake

We next tested whether milder SDS conditions (1× SSC buffer, low SDS concentrations) or alternative delivery agents promote the uptake of oligonucleotide probes into gram negative bacteria (Figure S3, Supporting Information). For this purpose, we incubated *E. coli* with the reactive probes (green STAR EC and TPP-DNA[*]) and unlabeled helper oligonucleotides (Helper 1 and 2, Table 1; helper oligonucleotides enhance target accessibility by hybridizing adjacent to the probe binding sites and unwinding the local secondary structure[5,15]) in the presence of varying concentrations of the additives (for structures see Table S1, Supporting Information). From the tested surfactants, only SDS provided detectable probe uptake as judged by fluorescence signal associated with cells (HTDC and PEI were not tested because of high toxicity and non-specific fluorescence background, respectively). We then evaluated the effect of SDS and SSC buffer concentration on the bacterial staining (Figure S3, Supporting Information). Samples with 6× SSC buffer showed the characteristic green fluorescence staining of *E. coli* at 0.5% and 0.05% SDS and to a lesser degree at 0.005% SDS. Fluorescence was also observable for the 1× SSC sample with 0.5% SDS but the signal faded considerably with decreasing SDS concentration. Therefore, the nontoxic

conditions deliver oligonucleotide probes with only low efficiency into *E. coli*. For further experiments with Q-STAR probes in bacteria, we used the previously described protocol (6× SSC with 0.05% SDS) with the knowledge that the viability is significantly affected for the majority of the cells.

Two-Color Discrimination of Bacteria by Fluorescence Microscopy

We tested species-specific Q670-containing NIR STAR probes in combination with fluorescein-containing green STAR probes for two-color discrimination of *E. coli* and *S. enterica* (Figure 5). Bacteria were incubated with the Q-STAR and TPP-DNA probes and the helper oligonucleotide probes in hybridization buffer (6× SSC, 0.05% SDS) for 3 h and analyzed by fluorescence microscopy with two filter sets (see Experimental Section). A mixture of NIR STAR SE and green STAR EC probes resulted in a strong and exclusive Q670-fluorescence staining of *S. enterica*, while *E. coli* exhibited strong fluorescein signal without detectable Q670-emission (Figure 5a). The inverse staining pattern was present when we reversed the dye labeling of the probe sequences (green STAR SE DAP and NIR STAR EC). Co-incubation of mixed microbes with NIR STAR and green STAR probes provided images of bacteria stained either red or green, enabling the discrimination of single bacterial cells by a fluorescence color call.

Interestingly, while fluorescein staining for the combined bacteria was selective for the assumed *E. coli* in the mixture, all bacteria exhibited some degree of Q670-emission (Figure 5a, middle row). Possibly under the given conditions, NIR STAR probes after being activated in a sequence specific manner (after release of the BHQ-2 quencher) can exit the host bacteria and reenter another bacterium.

Two-Color Analysis of Bacteria Cultures by Flow Cytometry

Next we evaluated the optimized two-color probes for use in flow cytometry. Previously we described the application of a single monocolor probe using simple fluorescence intensity as the flow cytometry readout.[15] However, a variety of experimental parameters influence the fluorescence intensity besides the presence and the sequence of the target rRNA, for example efficiency of probe uptake or bacteria aggregation. Thus a ratiometric two-color analysis is desirable and is expected to provide more reliable results.

To this end, we employed fluorescein- and Q670-labeled probes (green STAR SE DAP and NIR STAR EC) as described for the microscopy experiments. The bacteria were incubated with the reactive probes and helper oligonucleotides in the delivery buffer (6× SSC, 0.05% SDS) and analyzed by two-color flow cytometry. The signal measured in the fluorescein channel was distinctively higher for *S. enterica* than for *E. coli* (3.7 ± 0.5-fold) for green STAR SE DAP (Figures 6 and 7). Inversely, the NIR STAR EC probe produced a stronger Q670-fluorescence signal in *E. coli* relative to *S. enterica* (6.7 ± 1.6-fold difference). Neither of the probes generated a significant fluorescence signal in the nonspecific detection channel, as expected because of the high spectral resolution of the chosen fluorophore labels. When incubated with a mixture of green STAR SE DAP and NIR STAR EC, bacteria colonies developed distinct fluorescence patterns. *E. coli* exhibited strong Q670-fluorescence, whereas for *S. enterica* fluorescein emission was predominant (Figures 6 and 7). The combination of the probes also provided a better discrimination signal than single probe measurements; taking the ratio of Q670- and fluorescein emission provided a 15.7 ± 3.4-fold difference between the two bacterial strains.

E. coli treated with NIR STAR EC separated into two major subpopulations in flow cytometric analysis, one having approximately 10-fold higher Q670-emission than the other (distinguishable as a shoulder on the histogram in Figure 6, right panel). The fraction of the

low-emission population increased with incubation time and such an effect was absent for green STAR SE DAP. This result agrees with the observation of a small population of doubly stained bacteria in mixtures of *E. coli* and *S. enterica* and points towards incomplete retention of the NIR STAR probes after quencher release.

Discussion

The present data demonstrate improved methods for identification of two bacterial pathogens with a two-color pair of templated reductive quencher release probes targeting the 16S rRNA. The results show that bacteria can be successfully discriminated with fluorescence microscopy by a simple fluorescence color-call and, for the first time, by two-color flow cytometry. The performed experiments constitute a significant advance over previous assays targeting rRNAs. Standard FISH probes are simple label-containing oligonucleotide hybridization probes, requiring cell fixation and extensive washing steps. However, the necessity for cell fixation makes the protocol more cumbersome, can produce artifacts, and is incompatible with the long-term goal of culturing bacteria obtained from fluorescence-activated cell sorting. Light-up probes provide a powerful tool to overcome these limitations; for example, MB probes have been applied to the analysis of bacteria RNA with FISH and flow cytometry.[11,12] However, those reports used fixed cells and their value for the analysis of intact cells remains to be evaluated. In addition, background fluorescence from nonspecific interactions of MB probes with cellular macromolecules or degradation of the probes complicates analysis.[46] Templated reactive probes in general and the described two-color set of probes in particular offer several possible advantages in bacterial RNA targeting. First, the dual probe design minimizes nonspecific activation of reactive probes, as demonstrated here by the minimal fluorescence background observed in microscopy and flow cytometry experiments. Second, the short length of the probes enables excellent sequence specificity, demonstrated by the ability to discriminate two bacteria by a single nucleotide difference. Finally, templated reactive probes can yield isothermally amplified signals, while MB and FIT are stoichiometric at best.

The current probe set with two distinct fluorophores also offers several advantages over previous templated probe designs that employed either a single color or a FRET pair. Two-color approaches allow for ratiometric analysis of the readout, which in this study provides a significantly better discrimination than a single probe. The two-color ratio further reduces the uncertainty deriving from experimental parameters, such as variations in bacteria size, copy number of rRNAs, and probe delivery efficiency, obviating the necessity of an external standard. During this study, we also noted that under the given experimental conditions, the bacteria tend to aggregate and the *E. coli* more strongly so than *S. enterica*, but again the use of a two-color ratio alleviates problems associated with this effect.

The use of two spectrally separated dyes can be advantageous relative to FRET based probes when using sophisticated equipment such as modern flow cytometers. The current fluorescein and Q670 dyes are bright fluorophores, while FRET-based probes can suffer from low emission quantum yields[47] and suboptimal spectral resolution. Previous attempts to analyze bacteria by flow cytometry were hampered by low fluorescence intensity. Nevertheless, the earlier FRET probes may remain advantageous for certain applications with simpler equipment, such as in cases where there is only a single excitation wavelength and a single filter set available.

A major challenge for *in situ* hybridization-based experiments with live bacteria remains the difficulty of delivering oligonucleotides into cells. The current protocols enable bacterial analysis by microscopy and flow cytometry without fixation; however, our results show that cells are rendered largely nonviable by the studied delivery agents. While many diagnostic

applications require no live bacteria, it may be useful for applications such as sorting and selecting bacteria genetically. Based on the present experiments, it seems possible that conditions for probe delivery and cell viability are orthogonal, likely because the pores induced in the cell wall to allow for probe delivery are also lethal to the cells. In this light, it has been reported that the conjugation of cationic peptides to some oligonucleotide analogues (such as PNAs[48] or morpholino DNAs)[49] enhances bacterial uptake. Whether such an approach is effective with light-up probes in general or with template reactive probes in particular remains to be determined.

Finally, the development of the new quencher-linker **3** expands the utility of templated quencher release (Q-STAR) probes. In combination with the previously established dabsyl release linker, the newly developed BHQ-2 (NIR STAR) probes are highly versatile allowing straightforward design of quenched probes with fluorophores emitting in colors ranging from blue to near-infrared. Furthermore, because of the efficiency of the new quencher, NIR STAR probes reach turn-on values exceeding 200-fold, which is significantly better than previous quencher release probes and rivals the highest turn-on ratios reported to date for any template reactive probes.[21,30] Such multicolor probes may find applications in multiplex detection of nucleic acids in complex samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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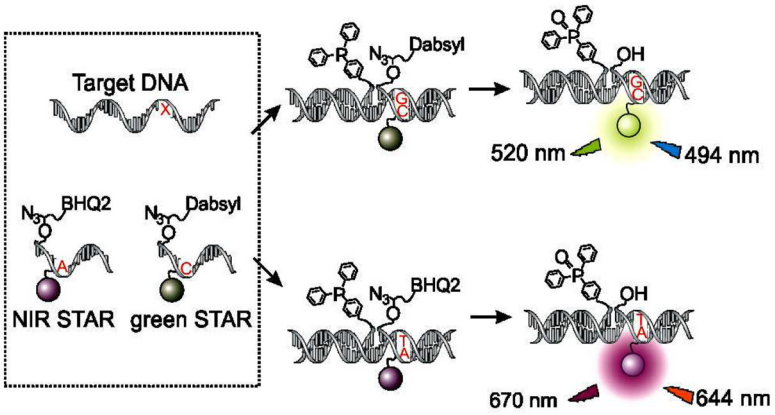

Figure 1.

Schematic illustration of two-color discrimination of DNA targets with single nucleotide specificity. A cyanine dye-labeled quencher release probe (NIR STAR) hybridizes specifically to one target strand, providing a near IR fluorescence signal after templated reaction with a TPP-DNA probe, while a green STAR probe specific for the other target strand provides a green fluorescence signal.

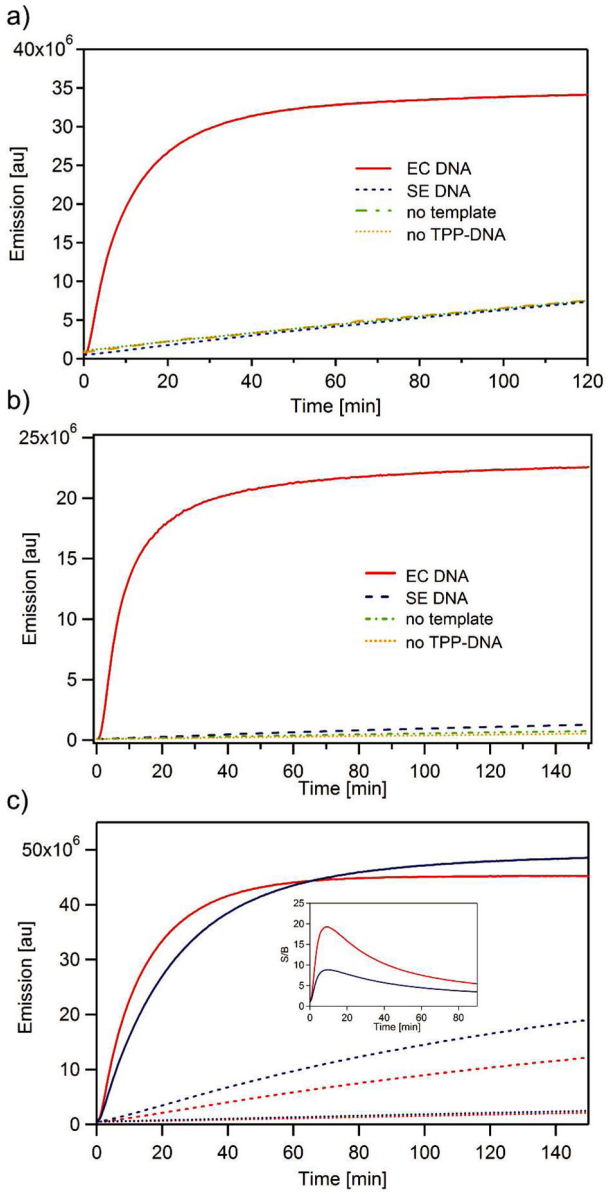

Figure 2.

In vitro DNA-templated fluorescence activation of several green- and NIR-STAR probes by TPP-DNA. a) Green STAR EC BHQ-1 containing linker **2**; b) NIR STAR EC containing linker **3**; c) Comparison of probes with either 2,6-diaminopurine (green STAR SE DAP, red traces) or adenine (green STAR SE, blue) in the presence of the matched template (SE DNA, solid traces), singly mismatched template (EC DNA, dashed traces), and without template (dotted traces). Inset shows mismatch selectivity (ratio of matched/mismatched signal) for the probes as a function of time.

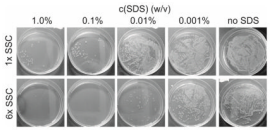

Figure 3.

Effect of delivery protocols (buffer salinity (saline sodium citrate, SSC) and concentration of sodium dodecyl sulfate (SDS)) on the viability of *Escherichia coli* K12. Bacteria were incubated with the indicated buffers for 4 h before spreading on the culture plates; cultures are shown 12 h post-treatment at 37 °C.

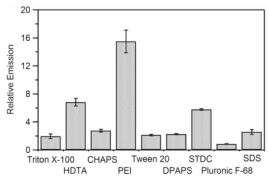

Figure 4.

Template-independent fluorescence activation of green STAR SE probes by TPP-DNA mediated by selected additives in solution (in the absence of bacteria). Data are taken after 4 h at 37 °C with TPP (2.0 μM) and green STAR SE (200 nm). Values are fluorescence signals normalized to controls with probes in 1× SSC buffer alone. Error bars are standard deviations from triplicate experiments. (For full name of additives and molecular structures see Supporting Information).

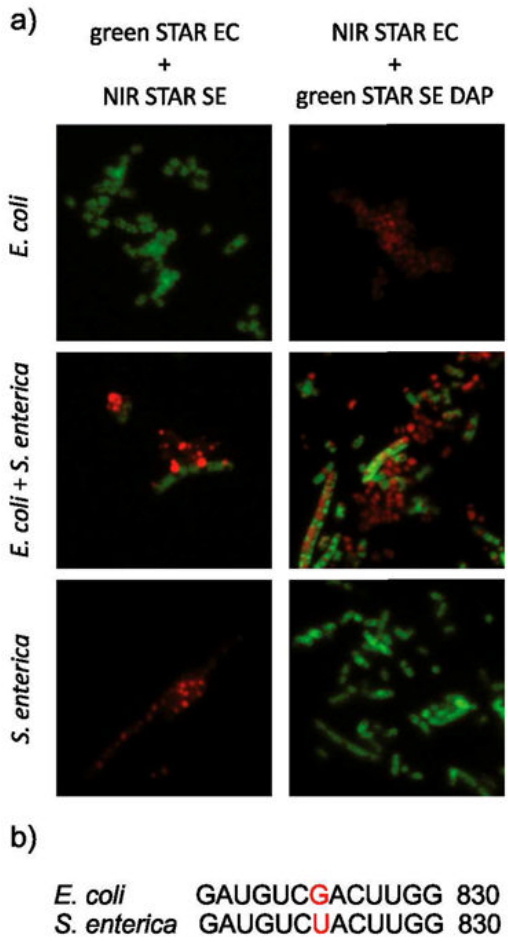

Figure 5.

a) Discrimination of *E. coli* and *S. enterica* by two-color excitation/emission fluorescence microscopy with a combination of green STAR and NIR STAR probes targeted to a single nucleotide difference in 16S rRNA. Signals are shown after 3 h incubation. b) 16S RNA sequences of the two bacteria at the target site (Reference 15).

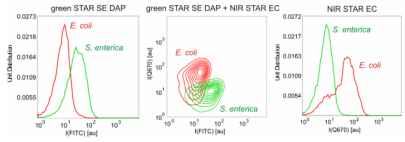

Figure 6.

Flow cytometric analysis of *E. coli* (red) and *S. enterica* (green) populations stained with Q-STAR probes. Histograms of cell fluorescence intensity for single probe experiments with either green STAR SE DAP (left graph) or NIR STAR EC (right graph). The center graph shows a two-color plot of the fluorescence intensities of either bacteria in an experiment containing both probes. (FITC: λ_{ex} = 488 nm laser; λ_{em} = 530 – 560 nm; Q670: λ_{ex} = 640 nm laser; λ_{em} = 666 – 690 nm)

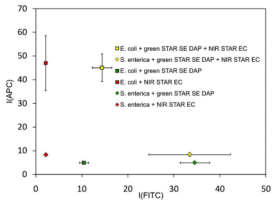

Figure 7.

Quantitative analysis of flow cytometric results of bacteria incubated with Q-STAR probes. Values indicate mean fluorescence intensity; error bars are standard deviations of three independent experiments. I(FITC) = Intensity in FITC channel; I(APC) = Intensity in Cy5 channel.

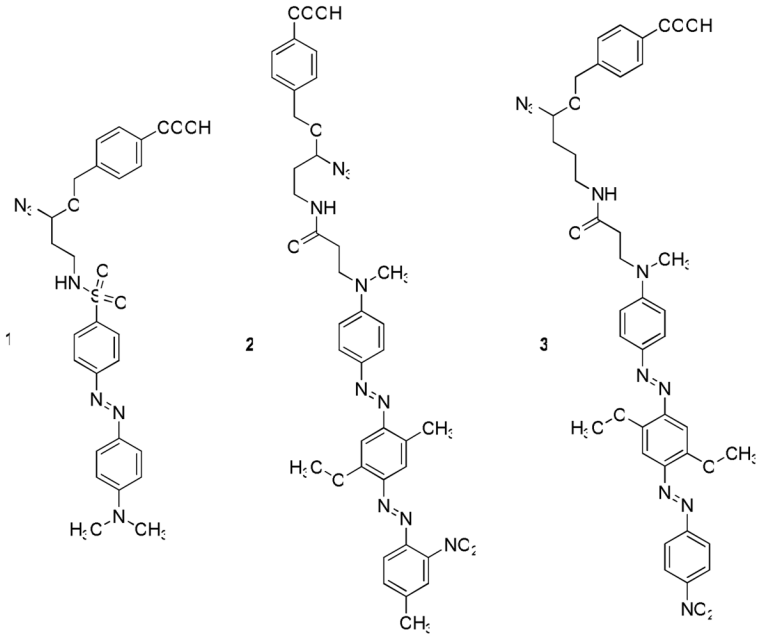

Chart 1.
Structures of releasable quencher linkers designed for Green STAR and NIR-STAR probes.

Table 1

Sequences of reactive probes, templates and helper oligonucleotides used in this study.

Probe[a]	Sequence[b]
green STAR EC	5′-**1**-AGT^{Fl}CGACA-3′
green STAR SE	5′-**1**-AGT^{Fl}AGACA-3′
green STAR SE DAP	5′-**1**-AGT^{Fl}DGACA-3
green STAR EC BHQ-1	5′-**2**-AGT^{Fl}CGACA-3′
NIR STAR EC	5′-**3**-AGT^{Q670}CGACA-3′
NIR STAR SE	5′-**3**-AGT^{Q670}AGACA-3′
TPP-DNA	5′-CAACCTCCA-TPP-3′
TPP-DNA*	5′-AGGGCACAACCTCCA-TPP-3′
EC DNA	5′-GATGTCGACTTGGAGGTTGTG-3′
SE DNA	5′-GATGTCTACTTGGAGGTTGTG-3′
Helper 1	5′-TCGTTTACGGCGTGGACT-3′
Helper 2	5′-GCTCCGGAAGCCACGCCT-3′

[a] TPP-DNA is used for in vitro experiments, TPP-DNA* is used for experiments with bacteria;

[b] Fl: fluorescein, Q670: Quasar 670, TPP: Triphenylphosphine, D: 2,6-diaminopurine; for the chemical structure of 1, 2 and 3 see Chart 1.