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Quenched Auto-Ligating DNAs: Multicolor Identification of Nucleic Acids at Single Nucleotide Resolution

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Abstract: We describe the synthesis and study of multicolor quenched autoligating (QUAL) probes for identification and discrimination of closely related RNA and DNA sequences in solution and in bacteria. In these probes, a dabsyl quencher doubles as an activator in the oligonucleotide-joining reaction. The oligonucleotides remain dark until they bind at adjacent sites, and "light up" on nucleophilic displacement of the dabsyl probe by the phosphorothioate probe. Four fluorescent dye conjugates were prepared and tested with probes and targets that differ by one nucleotide. Experiments on polymer beads show clear color-based discrimination of DNAs added in solution. Two-color quenched probe pairs were then tested in the discrimination of 16S rRNA sequences in *Escherichia coli*. Single nucleotide resolution was achieved in the cells with green/red QUAL probes, allowing identification of a one-base sequencing error in the16S rRNA database. Finally, QUAL probes were successfully applied in live bacterial cells. The method requires only incubation followed by fluorescence imaging, and requires no enzymes, added reagents, cross-linking, fixing, or washes. Because probes must bind side-by-side to generate signal, there is little or no interference from unintended protein binding, which can occur with other probe types. The results suggest that QUAL probes may be of general use in the detection and identification of sequences in solution, on microarrays, and in microorganisms.

Introduction

Recent studies have shown that fluorescent-labeled oligonucleotides can be useful in identification of microorganisms, by in situ hybridization to ribosomal RNAs.[1,2] This approach is becoming broadly adopted by microbiologists because of the existence of a growing database of rRNA sequences.[3,4] However, such standard probes exhibit low selectivity between closely related sequences, and this is problematic because ribosomal sequences are highly conserved.[1,2] Second, standard probes require considerable preparation of the cells and manipulation of conditions to remove nonspecific signals.[5] With standard in situ hybridization, for example, cells are permeabilized and cross-linked by formaldehyde; then hybridization is carried out, followed by several washes to remove unbound probes. The fact that bound and unbound probes generate the same signal prevents the use of such probes in live cells. Here, we report on properties of a new class of quenched probes, QUAL probes, that generate signal only on their intended target.[6,7] They therefore require no post-washing, and we find (see below) that they can be applied even without prior cellular preparation. The use of multiple fluorophore-labeled probes results in a clear color-based signal that reports on single nucleotide differences in solution or in bacterial cells.

Quenched autoligation (QUAL) probe pairs consist of an unlabeled nucleophile DNA probe containing a 3′ terminal phosphorothioate group (Figure 1), and an electrophile probe containing a dimethylamino-azobenzenesulfonyl (dabsyl) group on the 5′ hydroxy terminus, along with a fluorophore label. Side-by-side binding on a target sequence of DNA or RNA results in attack of phosphorothioate on the 5′ carbon of the dabsyl-activated electrophile probe. Loss of the dabsyl group results in loss of quenching of the fluorophore, thus switching on its emission. Preliminary experiments showed that QUAL probes were effective in a single color format on beads, using the combination of fluorescein with dabsyl quencher.[6,7]

However, single nucleotide differences are most easily distinguished by use of multiple colors, generating a positive qualitative difference when the target is altered.[8,9] Thus new experiments were undertaken to test (a) whether quenching by dabsyl would be efficient with other dyes in this context; (b) whether competing multicolored probes could correctly identify sequences added in solution; and (c) whether single nucleotide

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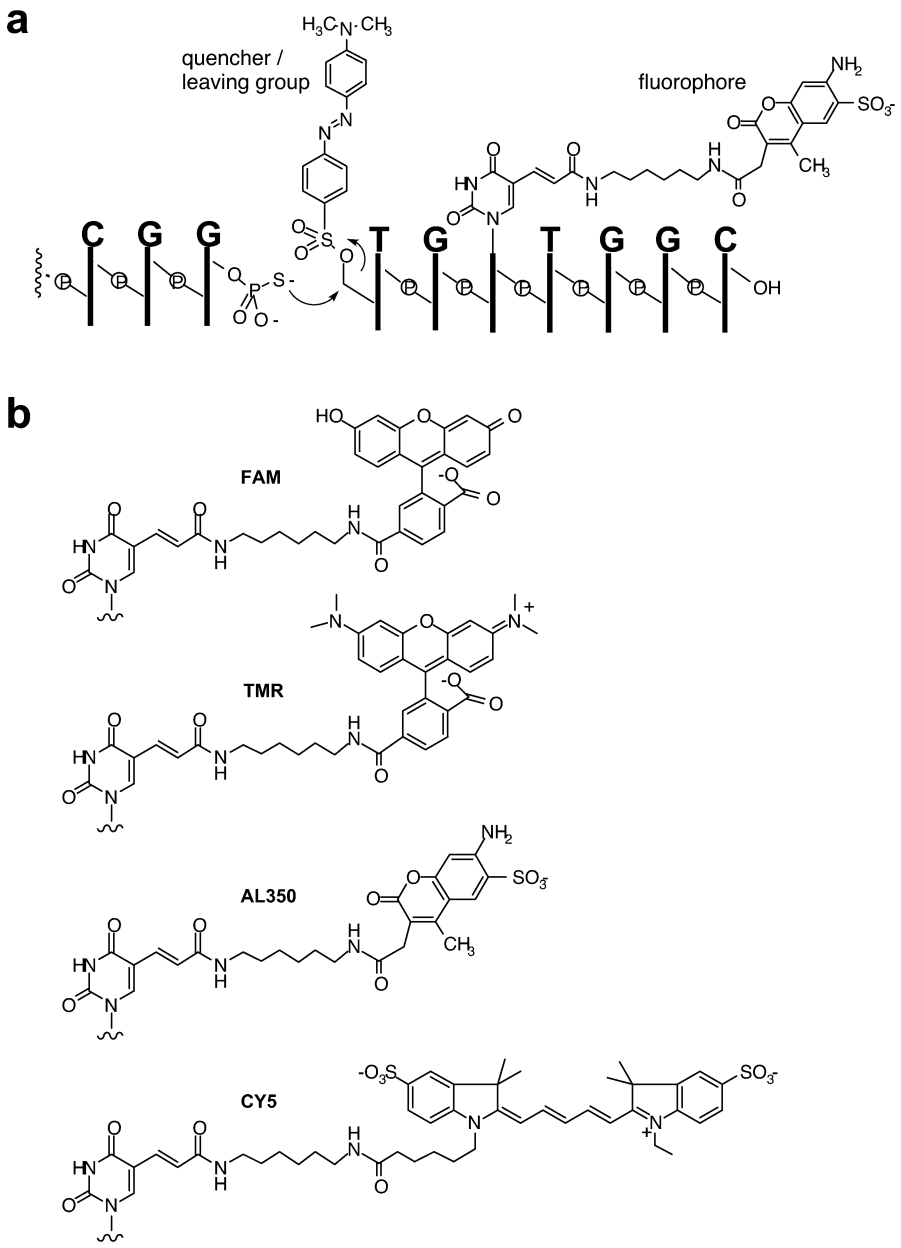

Figure 1. (a) Schematic showing how QUAL probes generate signal by ligation of two probes, displacing a quencher. Ligation is promoted by adjacent binding of the two probes on a complementary DNA or RNA target. (b) Structures of the four dyes and linkers as conjugates of deoxyuridine.

discrimination could be achieved in microorganisms. The data confirm that the QUAL probes can function with at least four colors simultaneously, and that single nucleotide differences in a target are easily distinguished by color. Tests were confirmed both on polymer beads and in bacterial cells.

Results and Discussion

Multicolor Detection on Beads. To examine whether the QUAL probe design could function with multiple colors simultaneously, and with probe sequences differing by only one nucleotide, we prepared a set of four quenched probes as heptamers (Figures 1,2). The conjugated dyes [fluorescein (FAM); Alexa 350 (AL350); tetramethylrhodamine (TMR); cyanine-5 (CY5)] are shown in Figure 1b; they are all attached identically at C-5 of deoxyuridine. The sequences (Figure 2) were derived from the H-*ras* oncogene, and we used the wild-type DNA sequence as well as three possible one-base variants at one site in codon 13. Most of the planned applications of QUAL probes are to be carried out on solid support, including in situ hybridization with cellular RNAs, and reaction on microarrayed DNAs. We therefore carried out the initial tests on solid supports, using PEG-polystyrene beads having the decamer nucleophile probe (Figure 2) conjugated by its 5′ end. This leaves the 3′ terminus (with phosphorothioate group) free to react with an electrophile probe. In this way, the dark beads become brightly fluorescent as reaction occurs, and no washings are needed. The solution around the beads remains dark because unreacted probes remain quenched by the presence of the intact dabsyl group.

We first carried out ligations individually in solution with equimolar amounts of probes and fully complementary target DNAs to test for quenching of the four dyes. The dabsyl group can quench fluorescence by the RET mechanism[8,10] if its spectra overlap sufficiently well with those of the associated fluoro-

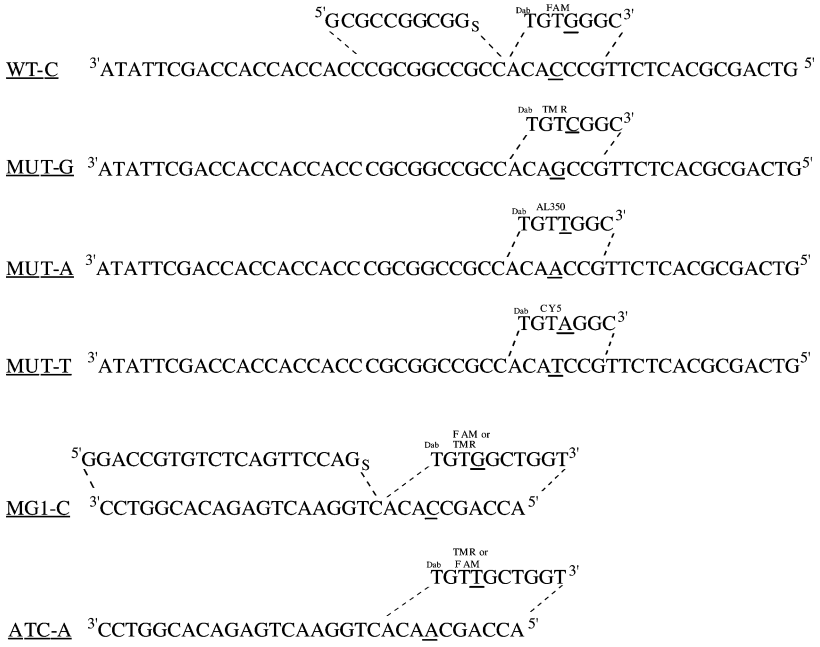

Figure 2. Sequences of targets and probes in this study. The first four targets are derived from the H-ras gene, with the wild-type (WT-C) and three possible sequence variants (MUT-G, MUT-A, MUT-T) at codon 13. The last two targets are DNAs corresponding to the sequence of *E. coli* 16S rRNA (nucleotides 300−330); MG1-C is the sequence of the MG1655 strain, while ATC-A is the sequence previously reported for the ATCC11775 strain. "Dab" refers to the 5′ dabsyl quencher; variable base positions are underlined.

phores. We previously measured >98% quenching of fluorescein by dabsyl in one probe set, for example.[6] However, with a broader range of fluorophores, not all dyes are expected to undergo efficient RET with this quencher because of poor spectral overlap.[8b] Because future uses of QUAL probes may well require sensing of multiple simultaneous targets, we investigated whether this broader set of four dyes could be employed successfully with the dabsyl quencher/leaving group design. Timecourses following the increase in fluorescence over several hours revealed (data not shown) that dabsyl did, in fact, quench each of the four dyes by at least severalfold. This may be explained in part by RET, and in part by charge-transfer quenching, which can be efficient if dye and quencher are in close proximity.[8] Additional studies will be needed to confirm the mechanism of quenching; however, we judged the qualitative degree of quenching to be sufficient to proceed with the four-color probes mixed.

We then tested whether the four probes, differing by only one nucleotide, could successfully identify four variants of the target sequence by fluorescence color. The dark nucleophile-conjugated beads were incubated with an equimolar mixture of the four quenched probes and a single target, in a pH 7.0 buffer (70 mM Na•PIPES, 10 mM $MgCl_2$) at room temperature. Each of the four target DNAs was tested separately; solutions, beads, and probe mixtures were the same in each case. After an 8-h incubation, digital images were obtained under the epifluorescence microscope, taking four images each with four appropriate filter sets matched to the dyes. No washing away of unbound probes was done before imaging, as the unreacted probes in solution remained quenched.

The results are shown in Figure 3, using pseudocolors to reconstruct the filtered data. The images show four strong signals, which correspond to the predicted, correctly matched

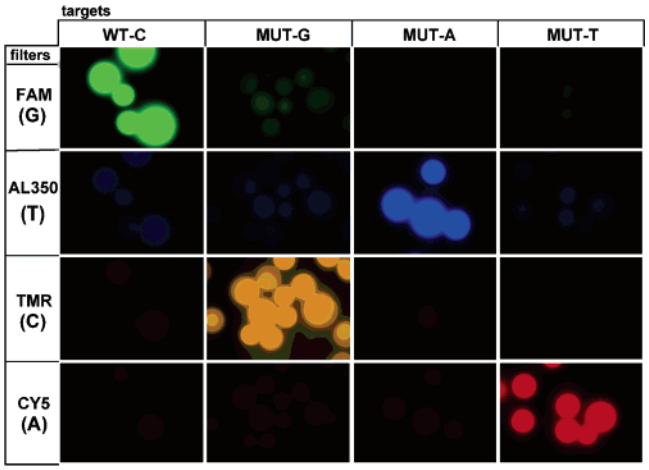

Figure 3. Tests of four-color competing probes having single nucleotide differences for ligation to a nucleophile probe (Ps) on PEG-polystyrene beads. All four probes were mixed with beads carrying the Ps probe. Separate experiments (vertical rows) tested the effect of adding target sequences that vary by one nucleotide. Horizontal rows show the results when filtering for green, blue, orange, or red emission, respectively. See Figure 2 for sequences; conditions are given in the text.

probes and targets. For example, we probed the results with the four targets using the FAM filter set (see first horizontal row of images in Figure 3). There was a strong green signal with the wild-type target (having a C at the variable position), a slight signal (at least 20-fold less bright) for the G mutant, and no visible signal for the other two one-base variants. This is the result expected if the FAM-conjugated probe (containing G at its variable site) out-competes the other three probes and ligates selectively with the fully complementary C target. The faint green signal generated with the MUT-G target presumably resulted either from a small amount of G−G mismatching, or from a slight overlapping of dye emission for green and orange dyes.

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Similarly, the data for the other three probe colors also show highly selective signals for the predicted cases, where each correctly matched probe is fully complementary to the target DNA (Figure 3, second through fourth rows of images). Thus, we conclude that competing four-color QUAL probes can distinguish single nucleotide differences in a target DNA by clear qualitative color signals. Notably, the reactions were carried out without pretreatments or post-washings, and without enzymes or added reagents beyond the activated probes themselves.

Tests in Fixed Bacterial Cells. We then proceeded to explore the possibility of two-color rRNA hybridizations in fixed *Escherichia coli* preparations. We first tested whether differences in probe sequence would, in fact, result in differences in signal for a given target rRNA. We constructed probes targeted to nucleotides 305−335 in 16S rRNA. Nucleophile probes were 20 nt in length (Figure 2), and the quenched electrophile probes were 10 nt in length. A wild-type quenched probe was constructed with G at the fourth position, making it complementary to the rRNA in the MG1655 strain (and most strains of *E. coli*). A mutant electrophile probe was constructed with T at this position, making it complementary to the reported sequence of the ATCC11775 strain of *E. coli*.[11] We made separate preparations of each probe, carrying TMR or FAM labels.

We confirmed that the two dye-probe conjugates could, in fact, ligate successfully in the presence of complementary targets, and distinguish between these two closely related sequences. Although this had been confirmed in the four-probe mixtures, we considered the possibility that any given target sequence might fold intramolecularly, hindering successful hybridization and ligation. Thus we tested the new probe sequences by mixing them and ligating them to beads in the presence of the two target sequences, using DNA oligonucleotide targets (see sequences in Figure 2) as surrogates for the bacterial RNAs. The results showed (Figure 4a) as before that targets differing by one nucleotide could clearly be distinguished by differently colored signals.

We then proceeded to test the probes in fixed bacterial cells. Standard fixation protocols were carried out, using paraformaldehyde cross-linking.[5] Initial experiments with the two red/green mixed probes showed that the targeted site in 16S RNA generated a signal that was weak as compared to previous experiments at a different ribosomal site.[7] Some sites in ribosomal RNA are known to be poor at hybridization due to competing secondary structures; for example, site 305−335 is predicted to be potentially hindered by three regions of duplex (Figure 4b). To address this we added an unlabeled "helper" probe as described by Fuchs et al.[12] Such helper oligodeoxynucleotides have been reported to assist hybridization of standard fluorescent probes in regions of strong secondary structure. We observed that the 17-mer helper designed to bind adjacent to the dabsyl probe increased signal markedly (Figure 4c).

Single nucleotide selectivity experiments with the *E. coli* cells revealed that either the FAM- or TMR-labeled probes gave

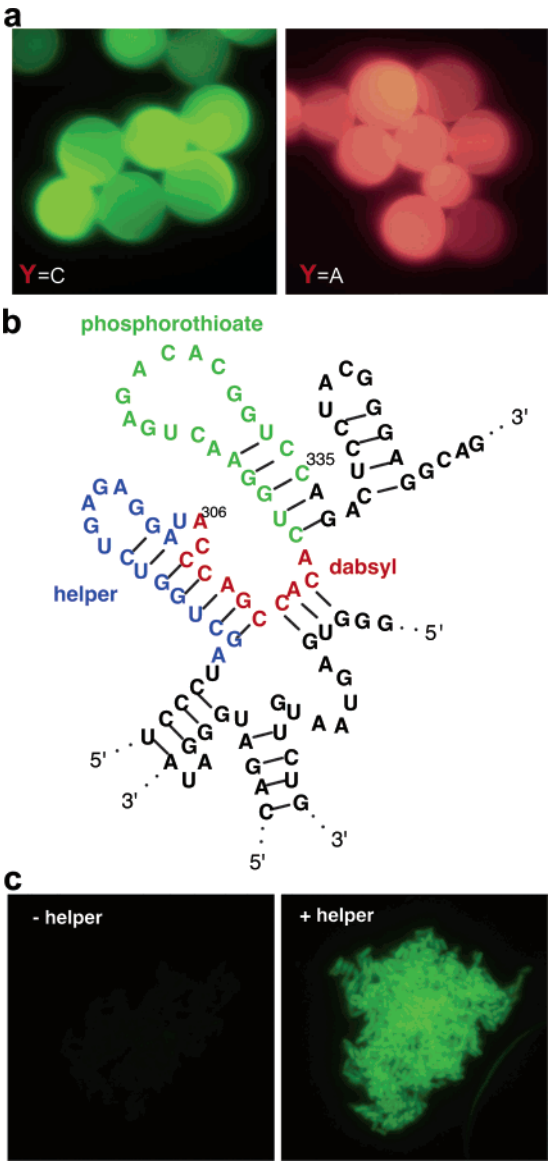

Figure 4. Test of red/green probe pairs. (a) Dabsyl-mediated autoligation on solid support. Dabsyl probes FAM-G and TMR-T were incubated with phosphorothioate probe-immobilized polystyrene beads in the presence of 32mer wildtype16S rRNA sequence ODN (left) and mutant with a single nucleotide difference (complementary to dabsyl probe TAM-T) (right). (b) Predicted secondary structure of 16S rRNA target site. Expected dabsyl probe and phosphorothioate probe binding sites are red and green, respectively. The "helper" oligonucleotide binding site is blue. (c) Effect of helper oligonucleotide in enhancing signal in fixed *E. coli* MG1655 cells.

positive signals if perfectly complementary to the target RNA (Figure 5). By contrast, a single nucleotide mismatch in a probe with the opposite label led to significantly lower signal under the same conditions. Experiments lacking the nucleophile probe also showed significantly lower signal, indicating that fluorescence did not arise from accidental protein binding or nonspecific RNA binding (data not shown). Also yielding little signal were control experiments in which nucleophile and electrophile probe were complementary to nonadjacent sites in 16S RNA. Ongoing experiments suggest that background signals are typically severalfold lower than specific signals, and that they arise either from incompletely quenched probes that are sequestered by the cells, or from background hydrolysis of the dabsyl group from the quenched probes. Further experiments are underway to investigate origins of background as well as

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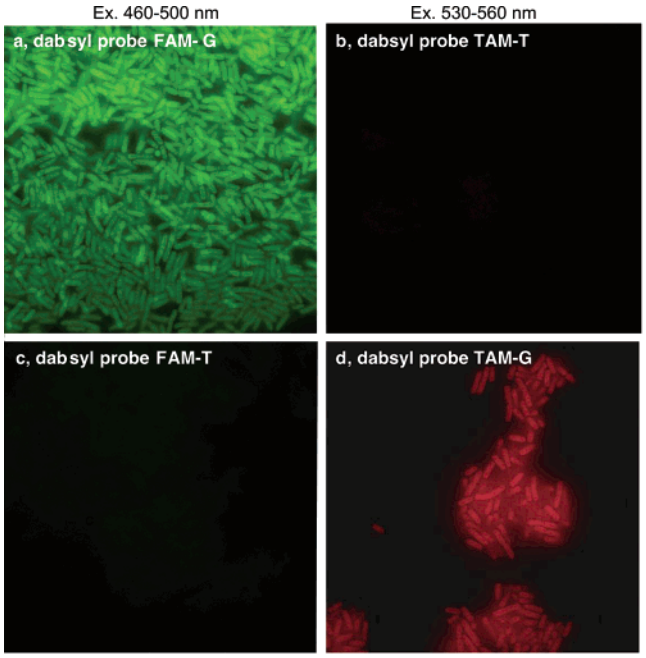

Figure 5. Single nucleotide discrimination by two-color autoligation. Fixed *E. coli* cells (MG1655 strain) were incubated for 18 h at 37 °C with (a) dabsyl probe FAM-G, (b) dabsyl probe FAM-T, (c) dabsyl probe TMR-G, and (d) dabsyl probe TMR-T in the presence of phosphorothioate probe and helper oligonucleotide. FAM and TMR were excited at 460−500 nm (left) and 530−560 nm (right) light, respectively. Probe sequences are in Figure 2.

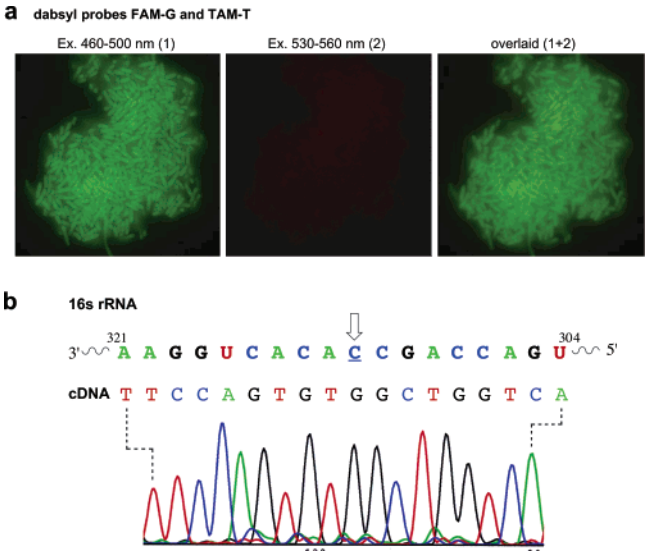

Figure 6. Single nucleotide specificity of QUAL probes leads to identification of a single-base sequence error in the public 16S rRNA database. (a) Fluorescence color-based sequence identification of 16S rRNA in fixed *E. coli* with two-color QUAL probes. Fixed *E. coli* cells (ATCC11775 strain) were incubated for 18 h at 37 °C with dabsyl probes FAM-G and TAM-T in the presence of phosphorothioate probe and helper oligonucleotide. Unquenched fluorescein and TAMRA dyes were excited with 495 and 530 nm light, respectively. (b) Re-sequencing of target 16S rRNA using normal RT-PCR-based method. Sequence data between nucleotide positions 304 and 321 are shown.

strategies for suppressing it. Overall, the data show that only completely complementary probes binding side-by-side on an RNA target yielded the brightest signal, and that both dye-quencher pairs were functioning in situ. The use of two colors allows for best discrimination of specific signals from background. Overall, the results demonstrate a clear example of single-nucleotide specificity in in situ hybridization. This is distinct from standard in situ hybridization experiments in two respects: the first is the high sequence specificity, and the second is simplicity, since in the current experiments we carried out no post-hybridization washes to remove unbound and nonspecifically bound probes.

Two-Color Tests for Distinguishing Bacterial Strains. The above experiments tested sequence specificity by varying probe sequence. To test the sequence specificity with varied targets, we obtained the ATCC11775 strain of *E. coli*, which (as mentioned above) the sequence database reported to have a single nucleotide difference in the 16S rRNA (5′-GCAAC at sites 310−314 compared to 5′-GCCAC in the previous MG1655 strain).[13] We then tested two of the above probe pairs, using the FAM-labeled probe complementary to the MG1655 RNA and the TMR-labeled probe complementary to the ATCC11775 strain (see Figure 2 for sequences). Results with the ATCC bacterial strain using the two probes mixed together are shown in Figure 6a. Surprisingly, the two probes consistently yielded a distinctly green signal in multiple experiments (Figure 6a, third panel) even though the red probe was complementary to the reported rRNA sequence. To investigate this independently we isolated total RNA from the cells, carried out RT-PCR amplification using 16RNA-specific primers, and sequenced the resulting DNA. The sequencing results showed clearly that the RNA contains a C at position 312, in contrast to the database listing of an "A" at that position (Figure 6b). (Note that there are seven rRNA operons in *Escherichia coli*, so we cannot rule out the possibility that one or two operons may have a different sequence. However, clearly the majority of operons have the same sequence as the earlier MG1655 strain, which is reasonable considering the high homology of ribosomal RNAs across bacterial strains.) Thus, the QUAL probes again demonstrated single nucleotide specificity, albeit in an unexpected way, allowing the identification of a single nucleotide sequencing error in the database.

Results with Nonfixed Cells. Standard fluorescent oligonucleotide probes require fixation and permeabilization of bacterial cells because they must be carefully washed after hybridization to allow for removal of unbound or nonspecifically bound probes. In the present approach, there is no requirement for this washing; thus we tested the possibility of targeting rRNAs in intact bacterial cells. Complementary FAM-G and control TMR-T probes were incubated with live MG1655 *E. coli* cells at 37 °C in the presence of 0.01% SDS to aid in uptake. This dilute detergent solution is reported not to affect the viability of *E. coli*.[14] No prior fixation and permeabilization steps were performed, and again, no post-washing steps were carried out. Images were acquired directly in the probe solution.

Results are shown in Figure 7. Distinct green signals are seen for the bacteria after 3 h, as expected for the probe complementarity. No signal is seen in the control, where a singly mismatched probe pair was used. An overlay of white-light and fluorescence images revealed a bimodal distribution, in which approximately half of the cells are stained and half are not (data not shown). Notably, literature reports of oligonucleotide/rRNA hybridization with fixation and analysis by flow cytometry also

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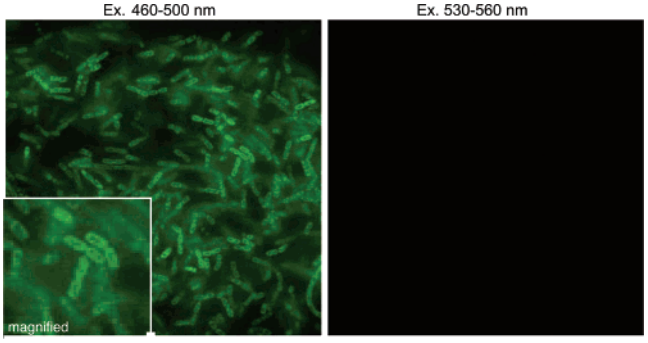

Figure 7. Quenched autoligation probes applied in nonparaformaldehyde-fixed *E. coli* cells (MG1655 strain). Dabsyl probes FAM-G and TMR-T, phosphorothioate probe, and helper oligonucleotide probe were incubated with cells in the presence of SDS (0.01%) surfactant. Image with excitation of FAM is shown on left; no red signal was observed with excitation of TMR (right).

show such a bimodal response, with approximately half of cells yielding signals.[15] It is possible that cells are differently permeable to the probes depending on their age or on clumping with neighbors. Regardless of the origin of this, our experiments establish that intact, nonfixed bacterial cells can be stained at single nucleotide resolution. To our knowledge, this is the first use of in situ hybridization in a nonfixed microorganism. This approach makes rRNA-based bacterial identification much more straightforward than has been previously possible, as the lack of requirement for pre-preparation and post-washes greatly speeds and simplifies the process. Moreover, QUAL probes yield much higher sequence specificity than standard fluorescent probes.

Recent studies have begun to test other classes of quenched probes for in situ hybridization, including DNA molecular beacons,[8a,16−18] PNA beacons,[19] and acridine-labeled DNAs.[20] Like the current probes, those probes also give increased fluorescent signal on binding a target. Beacons offer rapid response since the signal arises from simple binding as opposed to a chemical reaction. However, such probes can suffer from nonspecific signals, which are often serious with beacon-type probes because of unintended protein binding.[21,22] A large fraction of proteins in a cell have affinity for nucleic acids, and such binding to beacons can often yield a positive signal. In the present approach, protein binding is extremely unlikely to yield a signal because the quencher must be displaced chemically. In addition, we find that millimolar levels of thiols do not yield a significant false positive signal with QUAL probes.

Future experiments will be directed at determining the sensitivities of QUAL probes to less abundant RNAs, and whether other RNAs, such as mRNAs, can be targeted by this approach. The current results strongly suggest that these probes merit testing in the rapid identification of pathogenic strains of bacteria by rRNA sequence detection. Work is underway to test this possibility.

Experimental Section

QUAL Probes. 3′-phosphorothioate, FAM-, and TAMRA-labeled dabsyl probes were synthesized as previously described.[6,7] Pac-protected dA, ^{i}Pr−Pac-protected dG, and acetyl-protected dC phosphoroamidites for ULTRA MILD SYNTHESIS (Glen Research) were employed in oligonucleotides containing a dabsyl group. The FAM and TMR labels were introduced with fluorescein-dT and TAMRA-dT phosphoroamidites (Glen Research), respectively. Deprotection and cleavage from the CPG support was carried out by incubation in 0.05 M potassium carbonate in methanol for 12 h at room temperature, and the resulting oligonucleotides were purified by reverse-phase HPLC (ZORBAX ODS 9.4 × 250 mm, 5 μm, Agilent Technologies, eluting with 0.1 M triethylammonium acetate pH7.0/acetonitrile). For labeling with Alexa350 and Cy5, Amino-Modifier dT (Glen Research) was incorporated into oligonucleotides. The oligonucleotides were treated with concentrated ammonia for 3 h at room temperature, dehydrated and purified by reverse-phase HPLC. To incorporate AL350 and Cy5, according to the procedure from Molecular Probes, 100 nmol of the probes in 430 μL of 0.1M sodium tetraborate (pH 8.5) were incubated for 4 h at room temperature with 3 μmol of dye in 70 μL DMSO. Activated dye precursors used were Alexa Fluor 350 (AL350) *N*-hydroxysuccinimidyl ester (Molecular Probes) and Cy5 Mono *N*-hydroxysuccinimidyl (NHS) ester (Amersham Bioscience). Unreacted dye was removed by ethanol precipitation. Finally, probes with AL350 or Cy5 were purified by reverse-phase HPLC. Probe structures were confirmed by MALDI-TOF mass spectrometry. ^{Dab}TG^{FAM}TGGGC: calculated mass, $C_{122}H_{129}N_{37}O_{56}P_7S$ 3258.4; found 3180.1. ^{Dab}TG^{TMR}TCGGC: calculated mass, $C_{125}H_{139}N_{37}O_{54}P_7S$ 3272.5; found 3296.5. ^{Dab}TG^{AL350}TTGGC: calculated mass, $C_{113}H_{129}N_{35}O_{57}P_7S_2$ 3170.4; found 3199.7. ^{Dab}TG^{Cy5}TAGGC: calculated mass, $C_{134}H_{158}N_{39}O_{56}P_7S_3$ 3523.9; found mass 3544. 6.

Bacterial Strains. *E. coli* strains MG1655 and ATCC11775 were purchased from ATCC. Complete sequences of seven rrs operons (rrs A, B, C, D, E, G, and H) of *E. coli* MG1655 strain (GenBank: U00096) were obtained from the web at http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/chrom?gi = 115&db = G. A mean sequence of seven rrs operons of ATCC11775 strain was taken from EMBL databank: ×80725.

Autoligation in Paraformaldehyde-Fixed *E. coli* Cells. *E. coli* cells (MG1655 or ATCC11775) were fixed with paraformaldehyde prior to the reaction.[7] 100 μL aliquots of the fixed *E. coli* stock suspension were taken into 1.5 mL vials, and the fixed cells were collected by centrifugation at 10 000 rpm for 5 min. The cells were washed once with 100 μL PBS, and were resuspended in 100 μL Hybridization buffer (20 mM Tris-HCl pH 7.2, 0.9 M NaCl, and 0.1% SDS). To the suspension were added 2 μL of 20 μM dabsyl-probe, 6 μL of 20 μM phosphorothioate probe, and 1 μL of 500 μM helper oligonucleotide. The mixture was incubated at 37 °C for 18 h. After incubation, the suspension was directly spotted on glass slide without any washing steps and was covered with micro cover slide. Fluorescence images were obtained through a fluorescence microscope (Nikon Eclipse E800 equipped with 100× objective Plan Fluor apo) with super high-pressure mercury lamp (Nikon model HB-10103AF), using a SPOT RT digital camera and SPOT Advanced imaging software. Typical microscope settings were as follows: fluorescein, ex. 460−500 nm with no ND filters; TMR, ex. 530−560 nm with ND filters 4 and 8. Typical digital camera settings were as follows: fluorescein, exposure time Green 4 s, binning 2 _ 2, gain 1; TAMRA, exposure time Red 5 s, binning 2 _ 2, gain 2.

Autoligation in Nonparaformaldehyde-Fixed *E. coli* Cells (Surfactant Introduction). *E. coli* cells (MG1655 or ATCC11775) were grown at 37°C in LB broth (DIFCO). When an optical density at 600 nm reached 0.5, the suspension was chilled on ice for 5 min, 0.5 mL aliquots were taken into 1.5 mL vials, and cells were harvested by

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centrifugation for 5 min at 10 000 rpm at 4°C. Supernatant was removed and cells were resuspended in 100 μL Hybridization buffer (20 mM Tris-HCl pH 7.2, 0.9 M NaCl, and 0.01% SDS). To the suspension were added 2 μL of 20 μM dabsyl-probe, 6 μL of 20 μM phosphorothioate probe, and 1 μL of 500 μM helper oligonucleotide. The mixture was incubated at room temperature for 3 h. After incubation, the suspension was directly spotted on a glass slide without any washing steps and was covered with a micro cover slide. Typical microscope settings were as follows: fluorescein, ex. 460−500 nm with no ND filters; TAMRA, ex. 530−560 nm with ND filter 8. Typical digital camera settings were as follows: fluorescein, exposure time Green 6 s, binning 2 _ 2, gain 1;TAMRA, exposure time Red 3.5 s, binning 2 _ 2, gain 2.

Re-sequencing of Target 16S rRNA. *E. coli* cells (ATCC 11775) were grown up in LB medium (GIBCO) at 37 °C until the absorbance at 600 nm reached to 0.3. Total RNA was isolated from the *E. coli* using QIAGEN RNeasy Kit. RT-PCR amplification of a 401 nucleotide sequence between nucleotide position 40 and 440 of the 16s rRNA was carried out with forward primer 5′-d(TAC TCC CTT CCT CCC CGC TG)-3′ and reverse primer 5′-d(CGG CAG GCC TAA CAC ATG CAA)-3′ under the following conditions: Total volume 50 μL, reaction solution containing dNTPs (0.4 mM each), QIAGEN OneStep RT-PCR buffer (10 μL), QIAGEN OneStep RT-PCR Enzyme Mix (2 μL), Promega RNasin (20 units), total RNA (400 ng), forward primer (5 pmol), and reverse primer (5 pmol). Samples were amplified with iCycler (BIO RAD). Initial reverse-transcription reaction was carried out at 50 °C for 30 min, followed by PCR activation step at 95 °C for 15 min, 40 cycles of 3-step amplification (denaturation step at 94 °C for 1 min, annealing step 61 °C for 1min, and extension step 72 °C for 1min), then final extension step at 72 °C for 10 min. PCR products were purified using 1% agarose gel electrophoresis and were sequenced using an ABI377 DNA sequencer (Taq cycle).

Autoligation on Solid Support. 3′-Phosphorothioate oligonucleotide-immobilized PS beads were soaked in 40 μL pH 7.2 Tris-HCl (20 mM) buffer containing 0.9 M NaCl and 0.1% SDS. To the suspension were added 0.5 μL of 100 μM 32-mer target DNA, 5 μL each of 20 μM dabsyl-probes. After incubation at 37 °C, the supernatant was removed and the beads were re-suspended in H_2O. A 5.0-μL reaction suspension containing PS beads was directly spotted on glass slides. Microscope filters were as follows: For AL350, UV-2B (Nikon, ex. 330−380); for FAM, B-2A (Nikon, ex. 450−490); for TMR, HYQ TRIC (Nikon, ex. 530−560); for CY5, HYQ Cy5 (Nikon, ex. 590−650). Typical digital camera settings (color) were as follows: fluorescein, exposure time R:G:B = 0:400:200 ms, binning no, gain 1; TMR, exposure time R:G:B = 300:100:100 ms, binning no, gain 1. For gray scale imaging with pseudocolor, images were taken through a clear filter at 8 bpp (monochrome). AL350: exposure time = 10 ms, binning no, gain = 1; FAM: exposure time = 50 ms, binning no, gain = 1; TMR: exposure time = 2 ms, binning no, gain = 1; Cy5: exposure time = 50 ms, binning no, gain = 1. Images of AL350, FAM, TMR, and CY5 were colored with blue, green, orange and red, respectively, using SPOT Advanced Imaging software.

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