A stem-less probe using spontaneous pairing between Cy3 and quencher for RNA detection

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ABSTRACT
We herein report a stem-less probe for the detection of RNA that depends on pairing between Cy3 and nitro methyl red. In our design, two Cy3 residues and two nitro methyl red residues were introduced into an oligonucleotide. In the absence of the target, these dyes formed a complex, and emission of Cy3 was efficiently quenched. Hybridization with the target RNA disrupted this interaction and resulted in Cy3 emission. Under optimized conditions, the signal to background ratio was as high as 180. We demonstrated specific detection of target RNA in cells using a wash-free FISH protocol.

1. Introduction
Fluorescent oligonucleotide probes are essential tools in biology, biotechnology, and nano-medicine research. Ideally, a probe should emit a light signal when hybridized to the target. Molecular beacon probes are oligonucleotides with a fluorophore at one end and a quencher at the other. Formation of a stem-loop structure in the absence of a target brings the fluorophore and quencher into close proximity and results in quenching of fluorescence. When the probe is hybridized to target DNA or RNA, a signal is observed. Molecular beacons have been widely used to detect DNA and RNA; however, the stable base pairing of the stem portion of the molecular beacon may result in slow response to target. Linear probes that do not depend on stem-loop structures for quenching have been reported by several groups.

Previously we reported stem-less probes that depend on perylene as a fluorophore. These probes have multiple perylene residues, and emission from perylene is quenched through non-emissive complex formation between perylene residues. This strategy cannot be applied to other fluorophores such as Cy3 due to their low self-quenching efficiencies. Herein we report a new probe design based on spontaneous complex formation between Cy3 and a quencher. We demonstrated specific detection of target RNA in cells using a wash-free FISH protocol.
Upon duplex formation with a complementary target, strong Cy3 emission was observed. Because this probe does not have a stem-loop structure, a high response speed in the presence of a target was expected. Here, we report quenching efficiencies and stabilities of complexes between Cy3 and a quencher in the context of model duplexes. Detection using the stem-less probes was optimized in vitro, and detection of 28S rRNA in cells without washing procedures was demonstrated.

2. Experimental details

2.1. General

All conventional phosphoramidite monomers, controlled-pore glass columns, and reagents for DNA synthesis were purchased from Glen Research (Sterling, VA). Other reagents for the syntheses of phosphoramidite monomers were purchased from Tokyo Chemical Industry (Tokyo, Japan), Wako (Osaka, Japan), or Aldrich (St. Louis, MO). Unmodified oligonucleotides were purchased from Integrated DNA Technologies (Coraville, IA).

All the modified oligodeoxyribonucleotides (ODNs) were synthesized on an automated DNA synthesizer (M-6-MX, Nihon Techno Service Co., Ltd, Tsukuba, Japan) using phosphoramidite monomers bearing Cy3 (Y), 4-dimethylamino-2'-nitroazobenzene (nitro methyl red; R), and anthraquinone (Q) residues. Syntheses of phosphoramidite monomers were reported previously. [17,18] The scheme for synthesis of the phosphoramidite monomer tethering p-nitroazobenzene is shown in Supporting Information. ODNs were purified by reversed phase HPLC and characterized using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS; Autoflex, Bruker Daltonics, Bremen, Germany). MALDI-TOF MS data for the synthesized ODNs (Figure 1): Ra, observed 4109 (calculated for [Ra + H\(^+\)], 4108); Qa, observed 4047 (calculated for [Qa + H\(^+\)], 4046); Yb, observed 4194 (calculated for [Yb + H\(^+\)], 4195); YR2–2, observed 7610 (calculated for [YR2–2 + H\(^+\)], 7614); YQ2–2, observed 7487 (calculated for [YQ2–2 + H\(^+\)], 7490); Y2–2, observed 6683 (calculated for [Y2–2 + H\(^+\)], 6688); YR1, observed 6601 (calculated for [YR1 + H\(^+\)], 6600); YR2–1, observed 7593 (calculated for [YR2–1 + H\(^+\)], 7614); YR2–3, observed 7586 (calculated for [YR2–3 + H\(^+\)], 7614); YR2–4, observed 7590 (calculated for [YR2–4 + H\(^+\)], 7614); YR2–2s, observed 6385 (calculated for [YR2–2s + H\(^+\)], 6403); YR2–2i, observed 8770 (calculated for [YR2–2i + H\(^+\)], 8800); YR2–2_28s, observed 7436 (calculated for [YR2–2_28s + H\(^+\)], 7438).

Figure 1. Sequences of oligonucleotides used in this study. Chemical structures of dyes and d-threonois linker used to incorporate dyes into oligonucleotides are shown.
2.2. Spectroscopic measurements

Fluorescence spectra were measured on an FP-6500 spectrometer (JASCO, Tokyo, Japan) equipped with a microcell holder. Excitation wavelength was 546 nm. UV-visible absorption spectra were measured on a UV-1800 spectrometer (Shimadzu, Kyoto, Japan) equipped with a programmable temperature controller; 10-mm quartz cells were used. The melting curves were recorded by measuring the change in absorbance at 260 nm versus temperature. The melting temperature ($T_m$) was determined from the maximum in the first derivative of the melting curve. Both the heating and the cooling curves were measured, and the calculated $T_m$ values from these curves agreed to within 2.0 °C. The temperature ramp was 0.5 °C min$^{-1}$.

2.3. Cell culture and fluorescence in situ hybridization (FISH)

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 80 μg ml$^{-1}$ penicillin, and 90 μg ml$^{-1}$ streptomycin on a cover glass placed in the bottom of a well of a 12-well plate. Cells were cultured at 37 °C with 5% CO$_2$ in humidified air. Cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde at room temperature for 30 min. HeLa cells were treated with PBS containing 0.2% Triton-X100, 10 mM glycine, and 0.01% NaN$_3$ for 5 min, and then 1.6 μM probe was applied to cells at room temperature for 1 h. Excess probe was removed by rinsing twice with PBS and the calculated $T_m$ values from these curves agreed to within 2.0 °C. The temperature ramp was 0.5 °C min$^{-1}$.

3. Results and discussion

3.1. Evaluation of quenching efficiency using model duplexes

We first evaluated efficiencies of quenching of Cy3 (Y) in the context of model oligonucleotides by nitro methyl red (R) and anthraquinone (Q), both known to efficiently quench fluorophores.[19,20] Model 13-mer duplexes with Y-R or Y-Q pairs (Ra/Yb or Qa/Yb, Figure 1) were prepared. Emission spectra of Ra/Yb and Qa/Yb duplexes are shown in Figure 2(A). Both R and Q residues strongly quenched the emission of Cy3 compared with a duplex without a quencher (a/Yb). The quenching efficiency of the R residue was slightly higher than that of the Q residue.

Hybridization of Yb with Ra induced remarkable hypochromicity and hypsochromicity of the bands around 550 and 515 nm, respectively, compared with a summation spectrum of single-stranded Ra and Yb (compare red line with blue dotted line in Figure 2(B)). These changes are characteristic of hetero H aggregates. The ratio of absorbance at 550 nm to that at 515 nm, A$_{550}$/A$_{515}$, of the Ra/Yb duplex was 0.73, whereas that of the summation spectrum was 1.42. Thus, complex formation between Cy3 and nitro methyl red can be monitored from changes in absorption bands. In contrast, Qa/Yb exhibited only slight changes in absorption bands, probably due to large difference of absorption bands between Cy3 and anthraquinone (Figure S1).

We estimated the stability of the dye dimers by measuring melting temperatures ($T_m$) of duplexes (Table 1). The Ra/b duplex, a duplex with a single R residue and no Cy3, was 40.2 °C whereas that of a Qa/b duplex was 53.3 °C. This indicated that anthraquinone stabilizes a DNA duplex more than nitro methyl red does. This relationship was reversed when a Cy3 residue was introduced opposite the R or Q residue: Ra/Yb, the duplex with the R-Y pair, had a $T_m$ of 48.2 °C, significantly higher than that of the Qa/Yb duplex, which was 45.7 °C. Thus, interaction between R and Y residues was

Figure 2. (A) Fluorescence spectra of model duplexes a/Yb, Qa/Yb, and Ra/Yb. Conditions: 2.0 μM quencher strands and 1.0 μM Yb in 100 mM NaCl, 10 mM phosphate buffer (pH 7.0), 20 °C. (B) UV-visible absorption spectra of single-strands Ra and Yb, and Ra/Yb duplex. Summation of spectra of Ra and Yb is shown in blue dotted line. Conditions: 2.0 μM each strand, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0), 20 °C.

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3.2. RNA detection using stem-less probes

We next synthesized stem-less probes containing two Cy3 moieties and two quenchers (illustrated in Scheme 1, sequences given in Figure 1). Nitro methyl red (R) or anthraquinone (Q) residues were incorporated as quenchers since these molecules showed high quenching efficiencies in model duplexes. First, 22-mer stem-less probes YR2–2 and YQ2–2 were investigated. A control probe without quenchers (Y2–2) was also examined. In the absence of the target, background emission of YR2–2 was much lower than that of YQ2–2 (Figure 3(A)); this is correlated with the higher stability of the Y-R pair compared with the Y-Q pair in model duplexes. The A 550/A515 ratio of YR2–2 (0.81) was lower than that of YQ2–2 (1.30), supporting the hypothesis that Y and R residues form a complex in the ground state at 20 °C (Figure 4).

Emission spectra of YR2–2 and YQ2–2 in the presence of target RNA tgt1 are shown in Figure 3(B). In the presence of target, the A 550/A515 ratio of YR2–2 increased to 1.23, demonstrating the dissociation of the Y-R complex. YR2–2 had slightly lower emission in the presence of tgt1 than did YQ2–2, probably due to FRET from Cy3 to nitro methyl red.[21] The signal to background (S/B) ratio (the ratio of emission in the presence much stronger than that between Q and Y residues. This stabilization is likely due to the donor–acceptor interaction between Cy3 and nitro methyl red.

To evaluate how the electron density of the dyes impacts the duplex stability, we also measured T m’s of pairs between Cy3 and two other azo compounds, nitroazobenzene and methyl red. A stronger stabilization of the duplex was observed when the electron-rich azo compounds methyl red and nitro methyl red were introduced into the counter position of Cy3 than when anthraquinone or nitroazobenzene were opposite the Cy3 (Table S1). This strongly supports our hypothesis that a donor–acceptor interaction strongly influences the stability of the dye cluster.

**Table 1.** Spectroscopic and thermal properties of a model duplex that contains a pair between Cy3 and a quencher.

| Duplex | Emission intensitya | S/B ratiob | A350/A495c | Tm/°C | ΔTm/°Cd |
|--------|---------------------|------------|------------|-------|--------|
| Ra/Yb  | 3.9                 | 80         | 0.73       | 48.2  | +8.0   |
| Qa/Yb  | 6.5                 | 48         | 1.64       | 45.7  | −7.6   |

*aEmission intensity at 565 nm in arbitrary units. Conditions were 2.0 μM Ra or Qa, 1.0 μM Yb, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0), 20 °C. bRatio of emission intensity of a/Yb to Ra/Yb or of a/Yb to Qa/Yb. cConditions were 2.0 μM DNA, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0), 20 °C. dDifference in Tm between Ra/Yb and Ra/b or Qa/Yb and Qa/b.

**Figure 3.** Emission spectra (A) without and (B) with RNA target of probes YR2–2, YQ2–2, YR1, and Y2–2. Conditions: 0.2 μM probe, 0.4 μM tgt1, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0), 20 °C.

**Figure 4.** Effects of temperature on UV-visible absorption spectra of YR2–2 (A) without or (B) with target RNA. Conditions: 1.0 μM each strand, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0).
to that in the absence of target) of YR2–2 was 180 at 20 °C, whereas that of YQ2–2 was only 5.7 (Table 2). YR1 that tethers only a single Y-R pair showed much higher background emission in the absence of the tgt1 than did YR2–2 (Figure 3), probably due to insufficient complex formation between Y and R residues (A515/A550 was 1.02). The S/B ratio of YR1 was significantly lower than that of YR2–2 (Table 2). Thus, two R residues and two Y residues are necessary for sufficient signal to background, and the stem-less probe containing the two Y/R pairs detects target RNA with high sensitivity. We then optimized the number of bases between Cy3 moieties. Emission spectra of examined probes in the presence and absence of target are shown in Figure S3, and emission intensities are summarized in Table 2. When a single base was inserted between Y residues (YR2–1), emission intensity in the presence of target decreased relative to that observed with YR2–2 because of self-quenching between two Cy3 moieties. Emission intensity of YR2–4, which has four bases between Cy3 moieties, in the presence of tgt1 was also lower than that of YR2–2. The lower emission of YR2–4 was probably due to the FRET from Cy3 to nitro methyl red, since Cy3 was only nine bases apart from the quencher in the YR2–4/tgt1 duplex. The emission of YR2–3, with three bases between Cy3 residues, in the absence of target was high. Consequently, the highest S/B ratio was attained when two bases were inserted between the Cy3 moieties.

Next, we optimized the length of the probe by varying the number of nucleotides; emission spectra of the probes ranging in length from 18 to 26 nucleotides are shown in Figure S4. When the chain length was 26 residues (YR2–21), background emission was higher than that of the 22-mer YR2–2. Because of relatively high A550/A455 ratio (0.95), Y-R complexation appeared to be suppressed in the context of the longer chain (Table 2). In contrast, a shorter probe, the 18-mer YR2–2s showed less emission than YR2–2 in the presence of tgt1. This low emission was mainly caused by the low Tm of the YR2–2s/tgt1 duplex (28.8 °C). From these results, we concluded that a 22-mer probe with two Cy3 moieties separated by two bases is the most efficient probe for RNA detection.

In order to compare the self-quenching probe to a conventional molecular beacon, we compared response speed of YR2–2 with that of a shared-stem molecular beacon with R and Y on the termini (MBcon). The emission intensity of YR2–2 reached a plateau within 500 s after the addition of target RNA, whereas the molecular beacon signal did not plateau for over 5000 s (Figure 5). We also evaluated the affinity of YR2–2 for mismatched targets and found that the emission intensity in the presence of an RNA target with a single mismatch was only one tenth that for the fully matched target (Table S2). In addition, almost no emission was observed in the presence of an RNA with two bases mismatched to the probe. Thus, our self-quenching probe has better response speed than a conventional molecular beacon and high specificity.

### 3.3. RNA detection in cells

Finally, we evaluated the ability of our probe to detect RNA in cells. We designed a probe targeting a region of 28S rRNA (YR2–2_28S). The precursor of mature 28S rRNA is processed in the nucleolus and incorporation into the 60S ribosomal subunit follows.[22] Hence, if YR2–2_28S specifically detects 28S rRNA, bright

### Table 2. Detection abilities and duplex stabilities of probes synthesized in this study.

| Sequence | Fluorescence intensity b/ |
|----------|--------------------------|
|          | w/o tgt1 | with tgt1 | S/B ratio | w/o tgt1 | with tgt1 | Tm/°C |
| YR2–2   | 2.73     | 491      | 180       | 0.81     | 1.23     | 51.9   |
| YQ2–2   | 103      | 589      | 5.7       | 1.30     | 1.41     | 61.1   |
| Y2–2    | 486      | 646      | 1.3       | 1.33     | 1.41     | 61.2   |
| YR1     | 32.6     | 467      | 14        | 1.02     | 1.45     | 61.7   |
| YR2–1   | 4.61     | 260      | 56        | 0.80     | 0.97     | 55.9   |
| YR2–3   | 9.54     | 487      | 51        | 0.86     | 1.32     | 56.6   |
| YR2–4   | 4.20     | 343      | 82        | 0.85     | 1.39     | 52.2   |
| YR2–2s  | 3.96     | 97.6     | 25        | 0.82     | 1.05     | 28.8   |
| YR2–2l  | 24.9     | 358      | 14        | 0.95     | 1.29     | 57.0   |

a Emission intensity at 565 nm in arbitrary units. Conditions were 0.2 μM probe, 0.4 μM tgt1, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0), 20 °C.

b Conditions were 1.0 μM DNA, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0), 20 °C.
FISH protocol. Moreover, since quenching of Cy3 by nitro methyl red occurred spontaneously without the assistance of base pairing, this strategy will be applicable to the design of peptide-based probes.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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4. Conclusions

We successfully prepared an oligonucleotide probe that relies on spontaneous pairing between Cy3 and nitro methyl red to quench emission in the absence of target. Melting analyses of a model duplex tethering a Cy3-quencher pair showed that this dimer has a high stability. From results in vitro, we concluded that a 22-mer probe, which has two Cy3 and two nitro methyl red residues separated by two natural bases, detected RNA with high efficiency. This optimized probe design enabled sensitive detection of 28S rRNA in HeLa cells using a wash-free FISH protocol. Moreover, since quenching of Cy3 by nitro methyl red occurred spontaneously without the assistance of base pairing, this strategy will be applicable to the design of peptide-based probes.
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