Coherent Quenching

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Coherent Quenching of a Fluorophore for the Design of a Highly Sensitive In-Stem Molecular Beacon**

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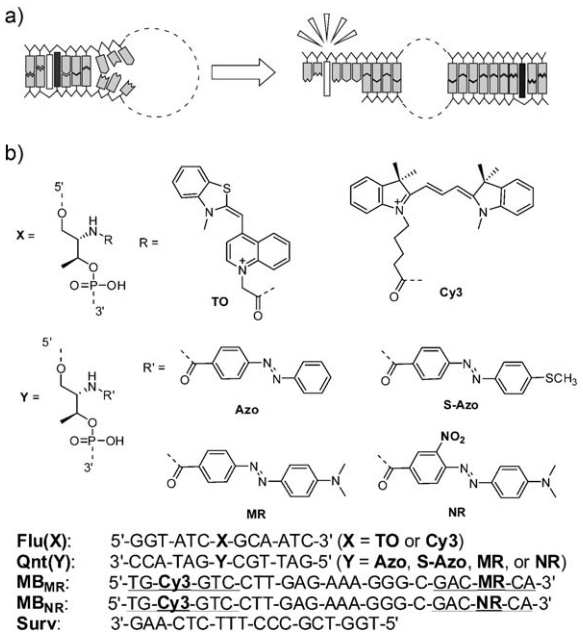
Fluorescence-labeled oligonucleotides provide powerful tools for highly sensitive, sequence-specific detection of target DNA/RNA.[1] A variety of fluorescent probes have been designed for purposes such as genotyping of individuals, identification of pathogens, and real-time monitoring of mRNA within cells.[2] One practical problem with the use of these fluorescent probes is background emission intensity in the absence of the target, because background emission, as well as scattered light of excitation due to a small Stokes shift, critically affects probe sensitivity.[3] Suppression of background emission is particularly important for the design of a highly sensitive molecular beacon (MB); that is, a hairpin oligonucleotide dual-labeled at both 5′- and 3′-termini with a fluorophore and a quencher.[4] Therefore, efforts have been made to efficiently quench a fluorophore in a closed MB.[5]

There are two types of energy transfer that will efficiently quench fluorescence: Förster (dynamic) quenching and contact (static) quenching.[6] The former requires a fluorophore–quencher pair that has sufficient spectral overlap between the emission spectrum of the fluorophore and the absorption spectrum of the quencher, and allows relatively long range (10–100 Å) quenching.[7] The latter is based on the formation of a ground state complex of a fluorophore and a dark quencher by their direct contact,[8] which is favorable for an MB. In this case, a change in their absorption spectra often occurs due to excitonic interaction (coherency). However, Marras also pointed out that any non-fluorescent quencher can be an efficient acceptor for contact quenching.[3] We previously established a novel method for the preparation of a heterodimer or cluster by hybridization of two oligonucleotides, each of which had dyes tethered to D-threoninols (termed threoninol nucleotides).[9] The use of D-threoninol as a scaffold facilitated dimerization or clustering of the dyes in the duplex. We have utilized this base surrogate (threoninol nucleotide) to develop a new in-stem molecular beacon (ISMB) in which both fluorophore (perylene) and quencher (anthraquinone) on D-threoninols are incorporated into the stem region as a pseudo base pair (Scheme 1 a).[10] Direct and strong contact of perylene with the quencher in the stem efficiently removed background emission in the closed ISMB and allowed discrimination of fully matched and one-base deletion mutant pairs. In this case, the absorption spectrum of perylene showed only a slight change with any quencher that we tested, showing that coherency (excitonic interaction) between the perylene and the quenchers was poor. Nevertheless, quenching was very strong, indicating that strong contact by pseudo base-pairing dominated the efficient quenching. However, as Johansson et al. proposed previously,[6,11] stronger coherency (that is, excitonic interaction or ground-state complexation) should enhance the quenching and further increase the sensitivity of the probe.



Scheme 1. a) An in-stem molecular beacon (ISMB), and b) the chemical structure of the dyes and sequences of the modified DNAs. In the MB sequences, the bases in the stem part are underlined.

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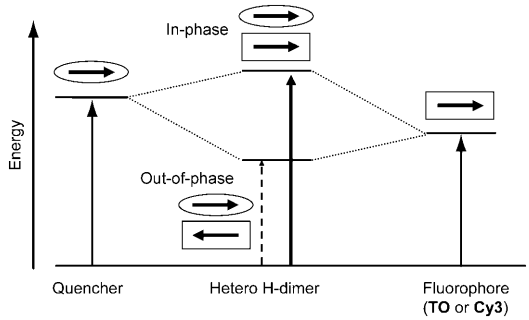
In the present study, which works towards the design of a highly sensitive ISMB, the effect of coherency (excitonic interaction) on the quenching of a fluorophore was systematically examined using azobenzene derivatives as quenchers. We have previously experimentally verified the molecular exciton theory using threoninol nucleotides involving azo dyes that have different absorption maxima, and found that heterodimerization induced hyperchromism of the band of shorter wavelength (in-phase transition),[9b] but hypochromism of the band of longer wavelength (out-of-phase transition; Scheme 2). Herein, we used Thiazole Orange (TO) and



Scheme 2. Energy diagram of the heterodimer of fluorophore and quencher depicted on the basis of the exciton model. Each arrow, outlined by an ellipse or an oblong, designates the transition dipole moment.

pentamethylindocarbocyanine (Cy3) as fluorophores (Scheme 1b; see also the Supporting Information, Scheme S1,S2 for their syntheses), because these are of known practical use in the labeling of biomolecules,[5b,12] and are expected to have stronger coherency with azo dyes than perylene. By systematically varying the absorption maxima of azo dyes with a similar structure as shown in the Supporting Information, Figure S1 (see Scheme 1b for the structures), the quenching efficiency was maximized, resulting in a more sensitive ISMB.

The azobenzene derivatives (Y = Azo, S-Azo, MR, NR) used for Qnt showed a λ_{max} at 334, 394, 480, and 513 nm, respectively, whereas TO and Cy3 in Flu showed a λ_{max} at 516 and 550 nm in single-stranded DNAs at 20 °C (Supporting Information, Figure S1). What we expect for the UV/Vis spectra of Flu/Qnt duplexes based on the molecular exciton theory (Scheme 2) is as follows:[9b] when the quencher that has a higher transition energy is excitonically coupled with a fluorophore of lower transition energy, the excited state splits into two energy levels. The higher energy level corresponds to the in-phase transition in which the transition dipole moments of the quencher and fluorophore have the same direction. In this case, the energy level becomes higher than that of quencher due to the repulsive interaction between the two transition dipoles. The absorption coefficient of the in-phase transition is expected to increase due to the sum of the transitions quencher and fluorophore. In contrast, the energy level of the out-of-phase transition becomes lower than that of fluorophore due to the attractive interaction. However, the absorption coefficient of this transition decreases because the transition moment is partially cancelled due to its antiparallel orientation. The above excitonic interaction (namely coherency) is enhanced when the gap between the transition energies of quencher and fluorophore, this is, $\Delta\lambda_{max}$, decreases. To evaluate coherency, the absorption spectrum of Flu(X)/Qnt(Y) duplexes was compared to that of the individual single strands. The degree of coherency can be estimated from the hypochromicity (decrease in absorbance) of the absorption band of TO (516 nm at 20 °C) or of Cy3 (550 nm), which is at longer wavelengths than that of the quenchers.[9b,13] Figure 1A shows the UV/Vis spectra of Flu(TO)/Qnt(Y) duplexes, Flu(TO) and Qnt(Y) single strands, and a spectrum calculated as the simple sum of the two single strands. When Azo was used as a quencher, the spectral change between the summation spectrum and the Flu(TO)/Qnt(Azo) duplex was small (orange and black lines in Figure 1A(a)); that is, hypochromicity induced by hybridization was small. However, hypochromicity increased as the $\Delta\lambda_{max}$ between the TO and the quencher decreased.[9b] In the case of the TO and NR combination, the UV/Vis spectrum of the Flu(TO)/Qnt(NR) duplex was entirely different from that of the summation spectrum (orange and black lines in Figure 1A(d)):[14] the band of Flu(TO) at 516 nm and that of Qnt(NR) at 513 nm almost disappeared in Flu(TO)/Qnt(NR), and a new strong band and weak shoulder band appeared at 492 and 590 nm, respectively.[15] This large change in the UV/Vis spectrum, namely the hypochromicity of the TO band, was attributed to the strong coherency (excitonic interaction or ground-state complexation) of TO and NR, because the $\Delta\lambda_{max}$ between the two dyes was as small as 3 nm. Similar results were obtained using Cy3: strong hypochromism occurred with MR and NR (Figure 1B(c) and 1B(d), respectively), although this hypochromism was smaller than that of TO due to the larger $\Delta\lambda_{max}$ (37 nm for NR and 70 nm for MR). No spectral change was induced when Flu(Cy3) was combined with Qnt(Azo), because the $\Delta\lambda_{max}$ was as large as 216 nm (note that the Flu(Cy3)/Qnt(Azo) spectrum almost completely overlapped with that of the summation spectrum; Figure 1B(a)). This spectroscopic behavior agreed with our previous results and above expectation based on the model shown in Scheme 2.

Next, the quenching efficiency of this model Flu/Qnt duplex was evaluated. As shown in Figures 2a,b, all the quenchers that were tested dramatically quenched emission from TO or Cy3 in the Flu/Qnt duplex, demonstrating that close stacking of a fluorophore and a quencher on D-threoninols facilitates quenching. However, the quenching efficiency obviously depended on the type of quencher that was used. The efficiency of TO quenching was in the order: NR $\geq$ MR > S-Azo $\gg$ Azo, which correlated fairly well with the degree of hypochromicity (coherency) as depicted in the Supporting Information, Figure S2a.[13,16] Cy3 was quenched by azo dyes in a similar order: NR $\geq$ MR $\gg$ S-Azo $\geq$ Azo (See the Supporting Information, Figure S2b, for the correlation of coherency and quenching efficiency).[13]

To examine the effect of duplex stability on the quenching efficiency, the melting temperature of the Flu/Qnt duplex was measured (Supporting Information, Table S2). In the case of Flu(TO)/Qnt(Y), the T_m decreased with Y in the order NR >

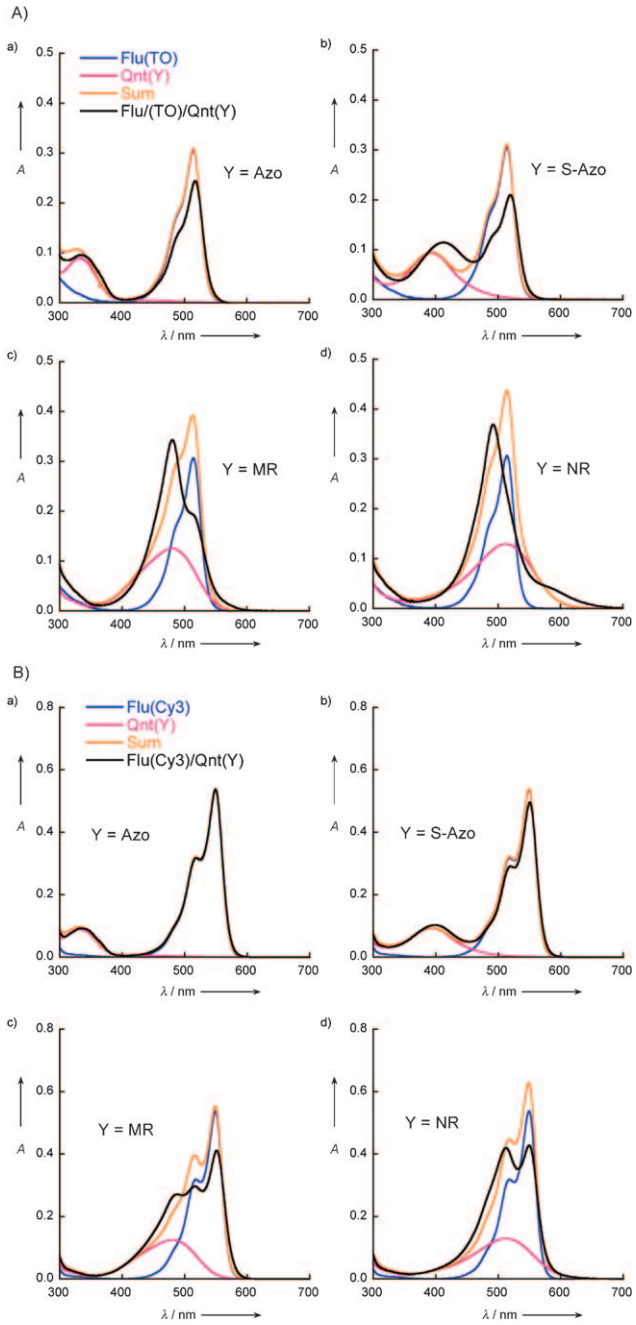


Figure 1. UV/Vis spectra of Flu(X)/Qnt(Y) with A) X = TO, B) X = Cy3. Sum: a simple sum of the spectra. Solution conditions: [Flu(X)] = 4 μM, [Qnt(Y)] = 4 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer); 20 °C.

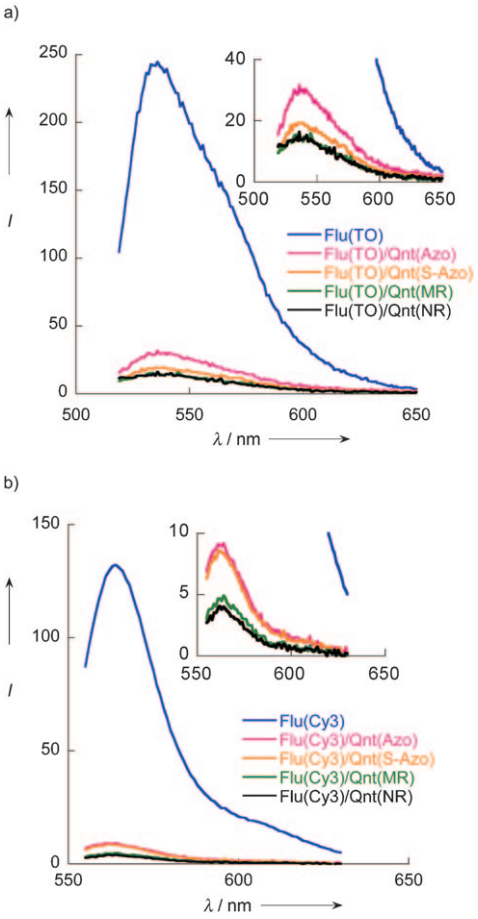


Figure 2. Fluorescence emission spectra of a) Flu(TO) excited at 516 nm and b) Flu(Cy3) excited at 546 nm, and their duplexes with Qnt(Y). Solution conditions: [Flu(X)] = 0.2 μM, [Qnt(Y)] = 0.4 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer); 20 °C.

MR > S-Azo > Azo, which coincided with the order of quenching efficiency. However, combination of these quenchers with Flu(Cy3) completely reversed the order of the T_m decrease with Y to Azo > S-Azo > MR > NR. Therefore, a contribution of the stability of the Flu/Qnt duplex to the quenching efficiency appears to be unlikely. These results strongly suggest that coherency between the fluorophore and the quencher and also direct contact of the fluorophore–quencher pair significantly contribute to effective quenching. Thus D-threoninol may be an ideal scaffold for the pairing of a fluorophore and a quencher because their close contact in the duplex facilitates ground state complexation (that is, coherency or excitonic interaction) to allow rapid electron (or hole) transfer to the quencher. It can now be concluded that to maximize the quenching efficiency $\Delta\lambda_{max}$, the difference in the absorption maxima between the fluorophore and the quencher should be minimized to enhance the coherency.[17]

Based on the above experiments, we chose NR or MR as a quencher of Cy3 to design an ISMB (Scheme 1) targeting the survivin gene.[18] We designed the ISMB as sharing NR or MR strand of the stem region with the target Surv, a synthetic 18 nucleotide-long DNA (see Scheme 1b for the sequence), because sharing of Cy3 or TO strand (that is, intercalation of Cy3 or TO) reduced its fluorescence (data not shown). As the T_m of MB_{NR} was determined to be 63.1 °C,[19] MB_{NR} was available below 60 °C, where the MB was closed in the absence of the target. Figure 3a depicts the fluorescence emission spectra of MB_{NR} involving a Cy3 and NR pair at the stem region in the presence and absence of the target at 20 °C. The ratio of the fluorescence intensities of MB_{NR} with and without the target (I_{open}/I_{close}) at 564 nm was as high as 70 under the conditions employed.[20,21] This high sensitivity was attributed to effective quenching of the Cy3 emission. We also

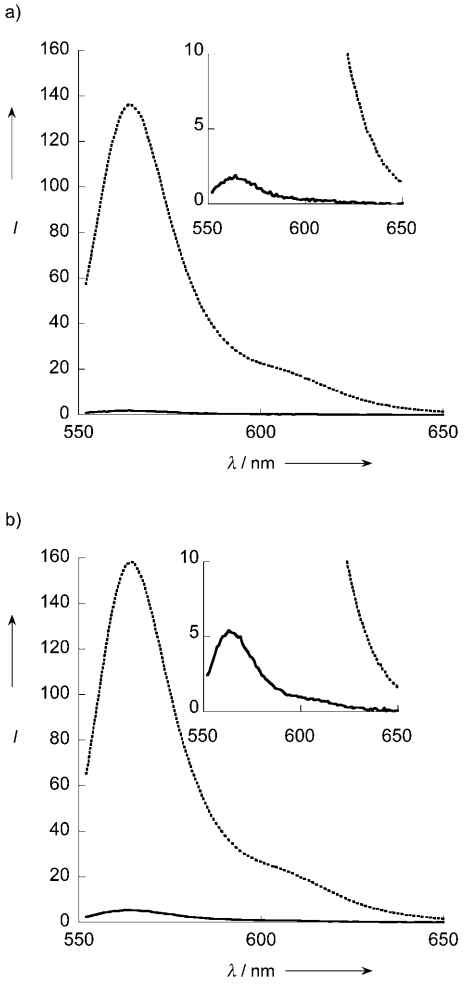


Figure 3. Fluorescence emission spectra of a) MB_{NR} and b) MB_{MR} excited at 546 nm with (••••) and without (——) the presence of Surv. Solution conditions: $[MB_{MR}]=[MB_{NR}]=0.2\ \mu M$, [Surv] = 0.4 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer); 20 °C.

tested an MB_{MR} bearing MR (Dabcyl) that is used as a conventional quencher, and found that I_{open}/I_{close} was as high as 30 (Figure 3b). However, this value was still less than half that obtained with MB_{NR}.[22] Undoubtedly, strong coherency (excitonic interaction) facilitated the quenching that strongly enhanced the sensitivity of the ISMB.[23]

In conclusion, excitonic interaction (coherency) was utilized to design a highly sensitive ISMB. Based on the results of a systematic study, we conclude that minimizing the $\Delta\lambda_{max}$ maximizes quenching efficiency due to maximization of coherency. We are currently spotting this ISMB on a solid surface and testing its potential as a capture probe on DNA chip.

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[1] a) E. T. Kool, *Acc. Chem. Res.* **2002**, *35*, 936; b) A. A. Martí, S. Jockusch, N. Stevens, J. Ju, N. J. Turro, *Acc. Chem. Res.* **2007**, *40*, 402.
[2] W. Tan, K. Wang, T. J. Drake, *Curr. Opin. Chem. Biol.* **2004**, *8*, 547.
[3] S. A. E. Marras, *Mol. Biotechnol.* **2008**, *38*, 247–255.
[4] S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303.
[5] a) O. Seitz, *Angew. Chem.* **2000**, *112*, 3389; *Angew. Chem. Int. Ed.* **2000**, *39*, 3249; b) S. Tyagi, S. A. E. Marras, F. R. Kramer, *Nat. Biotechnol.* **2000**, *18*, 1191; c) B. Dubertret, M. Calame, A. J. Libchaber, *Nat. Biotechnol.* **2001**, *19*, 365; d) H. Du, M. D. Disney, B. L. Miller, T. D. Krauss, *J. Am. Chem. Soc.* **2003**, *125*, 4012; e) C. J. Yang, H. Lin, W. Tan, *J. Am. Chem. Soc.* **2005**, *127*, 12772; f) C. J. Yang, M. Pinto, K. Schanze, W. Tan, *Angew. Chem.* **2005**, *117*, 2628; *Angew. Chem. Int. Ed.* **2005**, *44*, 2572; g) L. Wang, C. J. Yang, C. D. Medley, S. A. Benner, W. Tan, *J. Am. Chem. Soc.* **2005**, *127*, 15664; h) C. Crey-Desbiolles, D.-R. Ahn, C. J. Leumann, *Nucleic Acids Res.* **2005**, *33*, e77; i) D. M. Kolpashchikov, *J. Am. Chem. Soc.* **2006**, *128*, 10625; j) Y. Kim, C. J. Yang, W. Tan, *Nucleic Acids Res.* **2007**, *35*, 7279; k) T. N. Grossmann, L. Röglin, O. Seitz, *Angew. Chem.* **2007**, *119*, 5315; *Angew. Chem. Int. Ed.* **2007**, *46*, 5223; l) E. Socher, L. Bethge, A. Knoll, N. Jungnick, A. Herrmann, O. Seitz, *Angew. Chem.* **2008**, *120*, 9697; *Angew. Chem. Int. Ed.* **2008**, *47*, 9555; m) S. Nakayama, L. Yan, H. O. Sintim, *J. Am. Chem. Soc.* **2008**, *130*, 12560; n) E. Socher, D. V. Jarikote, A. Knoll, L. Röglin, J. Burmeister, O. Seitz, *Anal. Biochem.* **2008**, *375*, 318; o) S. Berndl, H.-A. Wagenknecht, *Angew. Chem.* **2009**, *121*, 2454; *Angew. Chem. Int. Ed.* **2009**, *48*, 2418.
[6] M. K. Johansson, R. M. Cook, *Chem. Eur. J.* **2003**, *9*, 2466.
[7] T. Förster, *Ann. Phys.* **1948**, *2*, 55.
[8] J. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd, ed., Plenum, New York, **1999**.
[9] a) H. Kashida, T. Fujii, H. Asanuma, *Org. Biomol. Chem.* **2008**, *6*, 2892; b) T. Fujii, H. Kashida, H. Asanuma, *Chem. Eur. J.* **2009**, *15*, 10092.
[10] H. Kashida, T. Takatsu, T. Fujii, K. Sekiguchi, X. Liang, T. Takase, Y. Yoshida, H. Asanuma, *Angew. Chem.* **2009**, *121*, 7178; *Angew. Chem. Int. Ed.* **2009**, *48*, 7044.
[11] M. K. Johansson, H. Fidder, D. Dick, R. M. Cook, *J. Am. Chem. Soc.* **2002**, *124*, 6950.
[12] a) W. R. Algar, M. Massey, U. J. Krull, *J. Fluoresc.* **2006**, *16*, 555; b) R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis, A. S. Waggoner, *Bioconjugate Chem.* **1993**, *4*, 105; c) G. A. Korbel, G. Lalic, M. D. Shair, *J. Am. Chem. Soc.* **2001**, *123*, 361; d) O. Seitz, F. Bergmann, D. Heindl, *Angew. Chem.* **1999**, *111*, 2340; *Angew. Chem. Int. Ed.* **1999**, *38*, 2203; e) F. Menacher, M. Rubner, S. Berndl, H.-A. Wagenknecht, *J. Org. Chem.* **2008**, *73*, 4263.
[13] Quantitatively evaluated degree of hypochromicity (coherency) and quenching efficiency for the Flu(X)/Qnt(Y) duplex are summarized in Table S1 of the Supporting Information.
[14] Pseudo pair of TO (or Cy3) and Y is stacked in the duplex; see Ref. [9b].
[15] This large spectral change is explained from the molecular exciton theory. In the case of the Flu(TO)/Qnt(NR) or Flu(TO)/Qnt(MR) combinations, strong excitonic interaction due to the small $\Delta\lambda_{max}$ induced large blue shift of TO (in-phase) band and red shift of NR (out-of-phase) band, which resulted in the appearance of new bands. However, as $\Delta\lambda_{max}$ of the Flu(TO)/Qnt(S-Azo) or Flu(TO)/Qnt(Azo) combination was much larger, such large shift did not occur. See: T. Kobayashi, *J-aggregates*, World Scientific, Singapore, **1996**, pp. 1–40.
[16] Excitation spectra of the Flu(TO)/Q(NR) duplex was very similar in shape to the excitation and absorption spectra of Flu(TO) (Supporting Information, Figure S3), demonstrating

that the heterodimer did not emit fluorescence at all; see Ref. [11].
[17] In other words, spectral overlap between the quencher and fluorophore should be maximized.
[18] X.-H. Peng, Z.-H. Cao, J.-T. Xia, G. W. Carlson, M. M. Lewis, W. C. Wood, L. Yang, *Cancer Res.* **2005**, *65*, 1909.
[19] Melting temperatures of MB_{MR} and MB_{NR} in the presence and absence of the target Surv are listed in Table S3 of the Supporting Information.
[20] The response profile of MB_{NR} under these conditions is depicted in Figure S4 of the Supporting Information.
[21] MB_{NR} showed sufficient sequence-specificity; see Figure S5 of the Supporting Information.
[22] MB_{MR} and MB_{NR} were available below 60 °C, at which temperature the molecular beacon was closed in the absence of a target. See Figure S6 of the Supporting Information.
[23] Note that I_{open}/I_{close} of previous ISMB, composed of a single pair of perylene and anthraquinone, was 26 under similar conditions.

