Biomolecule-to-fluorescent-color encoder: modulation of fluorescence emission via DNA structural changes

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Abstract: A biomolecule-to-fluorescent-color (B/F) encoder for optical readout of biomolecular information is proposed. In the B/F encoder, a set of fluorescence wavelengths and their intensity levels are used for coding of a biomolecular signal. A hybridization chain reaction of hairpin DNAs labeled with fluorescent reporters was performed to generate the fluorescence color codes. The fluorescence is modulated via fluorescence resonance energy transfer, which is controlled by DNA structural changes. The results demonstrate that fluorescent color codes can be configured based on two wavelengths and five intensities using the B/F encoder, and the assigned codes can be retrieved via fluorescence measurements.

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References and links
1. M. Schena, D. Shalon, R. W. Davis, and P. O. Brown, “Quantitative monitoring of gene expression patterns with a complementary DNA microarray,” Science 270, 467–470 (1995).
2. M. Han, X. Gao, J. Z. Su, and S. Nie, “Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules,” Nat. Biotechnol. 19, 631–635 (2001).
3. C. Lin, Y. Liu, and H. Yan, “Self-Assembled Combinatorial Encoding Nanopaticles for Multiplexed Biosensing,” Nano Lett. 7, 507–512 (2007).
4. Y. Li, Y. T. H. Cu, and D. Luo, “Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes,” Nat. Biotechnol. 23, 885–889 (2005).
5. C. Lin, R. Jungmann, A. M. Leifer, C. Li, D. Levner, G. M. Church, W. M. Shih, and P. Yin, “Submicrometre geometrically encoded fluorescent barcodes self-assembled from DNA,” Nat. Chem. 4, 4, 832–839 (2012).
6. Y. N. Teo and E. T. Kool, “DNA-Multichromophore Systems,” Chem. Rev. 112 4221–4245 (2012).
7. K. Wang, Z. Tang, C. J. Yang, Y. Kim, X. Fang, W. Li, Y. Wu, C. D. Medley, Z. Cao, J. Li, P. Colon, H. Lin, and W. Tan, “Molecular engineering of DNA: molecular beacons,” Angew. Chem., Int. Ed. 48, 856–870 (2009).
8. T. Nishimura, Y. Ogura, and J. Tanida, “Fluorescence resonance energy transfer-based molecular logic circuit using a DNA scaffold.” Appl. Phys. Lett 101, 233703 (2012).
9. R. M. Dirks and N. A. Pierce, “Triggered amplification by hybridization chain reaction, Proc. Natl. Acad. Sci. USA 101, 15275–15278 (2004).
10. J. Huang, Y. Wu, Y. Chen, Z. Zhu, X. Yang, C. J. Yang, K. Wang, and W. Tan, “Pyrene-excimer probes based on the hybridization chain reaction for the detection of nucleic acids in complex biological fluids,” Angew. Chem., Int. Ed. 50 401–404 (2011).
11. Y. Jiang, B. Li, X. Chen, and A. D. Ellington, “Coupling two different nucleic acid circuits in an enzyme-free amplifier,” Molecules 17 13211–13220 (2012).
12. W. Tang, D. Wang, Y. Xu, N. Li, and F. Liu, “A self-assembled DNA nanostructure-amplified quartz crystal microbalance with dissipation biosensing platform for nucleic acids,” Chem. commun. 48 6678–6680 (2012).
1. Introduction

Rapid, convenient, and inexpensive biomolecular detection is highly advantageous for clinical point-of-care testing. In this context, fluorescence detection ("readout") is a versatile method for biomolecule sensing. However, the number of fluorescent molecules that can be spectrally resolved often limits the readout performance. Spatial coding technologies such as those used in DNA microarrays can detect numerous molecular species simultaneously [1], but require sets of target molecules on a substrate and expensive readout instrumentation. An attractive alternative method is color coding of biomolecules [2–5], in which combinations of fluorescence wavelengths and intensity levels offer a large number of codes to represent the biomolecular information.

Fluorescence color coding has been achieved by controlling the number of quantum dots or fluorescent molecules that are bound to static nanometer- or micrometer-scale substrates such as micro-beads [2] and DNA [3,4]. Additionally, the substrate has a molecular probe that reports the presence of a preassigned target molecule via an additional color channel. For detection of the biomolecular signals, fluorescent spectroscopy of the small-sized objects that are coded in fluorescent colors is necessary to read out the assigned color codes. For a simple readout scheme, another color-coding method that is not based on the substrates is required.

Control of the fluorescent intensity levels is also required for color-coding; one promising method is fluorescence resonance energy transfer (FRET), which depends on the precise positional control of fluorescent molecules attached to DNA strands [6]. For example, molecular beacons for biomolecular detection typically use a structural change that is caused by binding between a DNA probe and a target molecule [7]. Logical readout of biomolecular signals has been demonstrated based on DNA self-assembly and FRET [8]. Hybridization chain reactions (HCR), which produce long double-stranded DNA polymers in the presence of specific initiator molecules [9–12], can enhance the FRET modulation intensities based on the DNA structural changes [13,14]. In HCR, there are no enzymatic reactions, making enzyme-free amplification possible. Therefore, the control of FRET using well-designed DNA reactions might enable modulation and amplification of the fluorescent signals that correspond to biomolecular detection.

In this paper, we propose a biomolecule-to-fluorescent-color (B/F) encoder that transduces a biomolecular signal into an optical signal that is represented by multiple fluorescent wavelengths and intensities. In the B/F encoder, DNA structural changes based on the HCR mechanism alter the relative positions of the fluorescent molecules, which modulate the emission intensities and wavelengths. The B/F encoder generates fluorescent color codes after target molecule recognition dynamically. The original biomolecular signal can be specified from the generated fluorescent color code without the need for special instruments such as a confocal microscope or a flow cytometer. Additionally, because the DNA structural changes occur autonomously, the fluorescence modulation occurs without the need for temperature-controlled enzymatic reactions. These features will contribute to the development of an inexpensive and
convenient method for biomolecular detection. We experimentally demonstrate the generation of an intended fluorescent color code after detection of an assigned target molecule with the B/F encoder.

2. Biomolecule-to-fluorescent-color encoder

Figure 1(a) shows a schematic illustration of the B/F encoder. A biomolecular signal is detected using the specific recognition ability of DNA [7]. The optical signal changes depending on the positions of the fluorescent molecules that are controlled by the DNA structural changes based on the biomolecular signal. The DNA structural changes can be designed using the specificity and predictability of the Watson–Crick base pairing [15]. The efficiency of FRET varies sensitively with the distance between donor and acceptor [16], which makes it possible to modulate the fluorescence intensities. The original biomolecular signal can be retrieved from the detected fluorescent signal using a fluorescence-decoding table that represents the relationships between the color codes and the target biomolecules. Using the specificity of DNA, multiple B/F encoders can be designed to convert each of the specific target biomolecules into individual codes. This means that, as shown in Fig. 1(b), simultaneous detection of different target molecules is possible when using multiple B/F encoders.

![Diagram](image)

Fig. 1. Schematic illustrations of (a) a biomolecule-to-fluorescent-color (B/F) encoder, and (b) a multiple B/F encoder system.

For implementation of the B/F encoder, we use fluorescence amplification based on the HCR mechanism. HCR (which was proposed by Dirks et al.) uses two hairpin DNA strands, H1 and H2, and an initiator strand [9]. A reaction cascade then produces double-stranded DNA polymers in the presence of the specific initiator molecule. Figure 2(a) shows a schematic illustration of the fluorescence amplification process based on the HCR mechanism. In our case, to enable use of the polymerization process in fluorescent signal amplification, H1 is modified
Fig. 2. Schematic illustrations of (a) amplification and (b) modulation of fluorescence in a B/F encoder. (c) Sequences and modifications of the DNA strands used in this study.

with both fluorescent and quencher molecules. To amplify the fluorescence intensity after the detection of a single type of target molecule T, both H1 and H2 are used. When T is absent, H1 and H2 form a closed structure. Because of the close proximity of the two dyes, fluorescence from the hairpin H1 caused by FRET is suppressed. When T is present, H1 binds to T and the structure is changed into an open structure, and the fluorescent and quencher molecules are separated sufficiently to allow fluorescence. A sequence region that binds and opens the closed H2 is exposed in the open H1 structure. The opened H2 also opens another closed H1 structure. Consequently, the reactions are chained to construct a polymer structure. In this chain reaction, the hairpins are opened sequentially and the fluorescence intensity can be amplified. A variety of target sequences can thus be detected by changing the sequences of H1 and H2.

To generate the fluorescent color codes, the B/F encoder requires modulation of the fluorescence intensities. The fluorescence wavelengths and intensities of the fluorescent color codes are determined by the type and number of fluorescent molecules in the opened H1 structure. Fluorescence modulation is achieved by changing the concentration ratio, \( R_x \), between the modified and non-modified H1 strands. The suffix \( x \) denotes the specific dyes for which the fluorescence intensity is modulated. \( R_x \) is simply defined as:

\[
R_x = \frac{[H1_x]}{[H1_{all}]},
\]

where \([H1_x]\) is the concentration of H1 that has been modified with a fluorescent molecule, \( x \), and \([H1_{all}]\) is the total H1 concentration. \( R_x \) is the probability of opening H1 that has been
modified with $x$ to form polymer structures. Figure 2(b) shows the modulation process when using two fluorescent molecules, carboxytetramethylrhodamine (TMR) and fluorescein (FAM). In accordance with the color code to be generated, $R_{TMR}$ and $R_{FAM}$ are changed. In the presence of the target molecule, the closed H1s are opened, as shown in Fig. 2(b). The fluorescence intensities then increase depending on the values of $R_{TMR}$ and $R_{FAM}$. Thus, for this fluorescent color-coding scheme, only the regulation of the concentration ratio is required. The chained reaction of HCR continues until the supply of H1 or H2 is exhausted [9], indicating the potential for stable control of the fluorescence intensity without having to rely on the target molecule concentration.

The coding capacity of the B/F encoder depends on the available wavelengths and intensity levels. The number of codes, $N$, is a function of the number of fluorescent colors $n$ and the number of intensity levels $m$ [2]:

$$N = m^n - 1. \tag{2}$$

The number of codes that can represent molecular signals in the conventional method is often limited to the number of available fluorescent molecules. In contrast, the B/F encoder offers more effective use of the fluorescence information bandwidth. By changing the sequences of H1 and H2 to adapt them to different target biomolecules [14], multiple B/F encoders can be designed to convert individual and specific biomolecular signals into different fluorescent color codes. A suitable set of color codes is selected from the $N$ potentially available species of color codes for the design of a multiple B/F encoder system.

3. Methods

The sequences and the modifications used in this study are shown in Fig. 2(c). The sequences were the same as those reported by Dirks [9]. The target biomolecule T was a single-stranded DNA molecule (24 nt). In this study, three types of H1 strand, H1$_{TMR}$, H1$_{FAM}$, and a non-modified H1, were prepared for color encoding. H1$_{TMR}$ was labeled with TMR (555 nm peak excitation, 580 nm peak emission) as a fluorescent molecule and used black hole quencher-2 (BHQ-2) as a quencher. H1$_{FAM}$ was modified with FAM (494 nm peak excitation, 517 nm peak emission) as a fluorescent molecule and used black hole quencher-1 (BHQ-1) as a quencher. H2 was not modified to prevent interactions between the fluorescent molecules on the assembled structure.

The hairpin strands were placed in a phosphate buffer containing 0.5 M NaCl. To form the closed hairpin structures, the strands were heated at 95 °C for 5 min and were then rapidly cooled to 4 °C. The target T was then added to the samples and was incubated at 25 °C. The sample volume was 50 μL after the addition of T, so the final concentrations of all H1s and H2 were 500 nM and 600 nM, respectively, and the concentration of T was 250 nM. The concentration ratio was varied in the experiments to generate a variety of color codes.

The fluorescence spectra were measured using a spectrofluorometer (FP-6200, Jasco, Japan) 20 minutes after the addition of T. The FAM and TMR spectra were obtained using 470 nm excitation and 550 nm excitation, respectively. The fluorescent images were obtained using a commercially available digital camera (Lumix, Panasonic Corp., Japan). The samples were excited by an ultraviolet (UV) illuminator (NTM-10, Funakoshi Co., Ltd., Japan) for simultaneous excitation of both fluorescent molecules.

4. Experimental results and discussion

Previously, we have shown that HCR amplifies and modulates the fluorescence intensity of a single type of fluorescent molecule after recognition of the target molecule T by using H1$_{FAM}$, non-modified H1, and H2 [17]. In this paper, to confirm the behavior of the B/F encoder when
using another fluorescence molecule for fluorescent color coding, we also tested the fluorescence modulation with $H_1TMR$, non-modified $H_1$, and $H_2$. Figure 3(a) shows the normalized fluorescence spectra of TMR that were obtained for $RTMR = 0.2, 0.4, 0.6, 0.8, 1$. The spectra are normalized with respect to the averaged value, which was obtained by measuring the samples for $RTMR = 1$ three times. The emission intensities increased with $RTMR$. As shown in Fig. 3(b), a linear relationship exists between $RTMR$ and the intensity at the peak fluorescent wavelength, as well as in the case when using FAM and BHQ-1 [17]. The results here show that HCR-based modulation for the B/F encoder can be applied to multiple wavelength channels simply by modifying $H_1$ with different fluorescent molecules.

![Normalized fluorescence spectra of TMR in HCR for RTMR values of 0.2, 0.4, 0.6, 0.8, and 1.](image)

![RTMR vs. the normalized 580 nm fluorescence intensity.](image)

As a basic function of the B/F encoder, we confirmed whether a single biomolecule can be assigned to multiple different color codes by regulation of $R_x$. In this work, two fluorescent dyes, TMR and FAM, were used at five different intensity levels, $R_x = 0, 0.1, 0.2, 0.3, 0.4$, so that $(5^2 - 1) = 24$ codes were available. The samples for each code are given in Table 1, and Figure 4 shows their fluorescence images. The green and red pixel values of digital images mainly depend on the FAM and TMR emission intensities, respectively. The green and red intensities in Fig. 4 increased gradually with increasing $R_x$.

| Sample# | $R_{FAM}$ | $R_{TMR}$ | Sample# | $R_{FAM}$ | $R_{TMR}$ |
|---------|-----------|-----------|---------|-----------|-----------|
| 1       | 0         | 0         | 14      | 0.3       | 0.2       |
| 2       | 0.1       | 0         | 15      | 0.4       | 0.2       |
| 3       | 0.2       | 0         | 16      | 0         | 0.3       |
| 4       | 0.3       | 0         | 17      | 0.1       | 0.3       |
| 5       | 0.4       | 0         | 18      | 0.2       | 0.3       |
| 6       | 0         | 0.1       | 19      | 0.3       | 0.3       |
| 7       | 0.1       | 0.1       | 20      | 0.4       | 0.3       |
| 8       | 0.2       | 0.1       | 21      | 0         | 0.4       |
| 9       | 0.3       | 0.1       | 22      | 0.1       | 0.4       |
| 10      | 0.4       | 0.1       | 23      | 0.2       | 0.4       |
| 11      | 0         | 0.2       | 24      | 0.3       | 0.4       |
| 12      | 0.1       | 0.2       | 25      | 0.4       | 0.4       |
| 13      | 0.2       | 0.2       |         |           |           |
To evaluate the fluorescence from the B/F encoder in detail, the FAM and TMR spectra of the 25 samples were acquired [Figs. 5(a) and 5(b)]. Fluorescent emission increased when the value of $R_x$ was higher, similar to the behavior for the single dye emission shown in Fig. 3. The emission peak intensities are shown in Fig. 5(c), where they are normalized with respect to the averaged values when $R_{FAM} = 0.4$ for FAM and $R_{TMR} = 0.4$ for TMR. The averaged values were obtained from a total of five measurements. The intensity levels of FAM and TMR were shown to behave linearly with respect to $R_{FAM}$ and $R_{TMR}$, and were independent of each other (i.e., no FRET occurred). This linearity between $R_x$ and the fluorescence intensity would enable the signal from a labeled biomolecule to be decoded. For example, the five intensity levels can be separated; we are therefore able to draw the segmentation lines [the dotted lines in Fig. 5(c)] to specify the dye concentrations. These results show that color-coded molecular information can be obtained by changing $R_x$.

Although the B/F encoder is limited by the number of fluorophores that can realistically be spectrally resolved, one advantage of the encoder is that the number of potentially available codes increases exponentially with the use of intensity as an additional parameter. The number of codes is limited by the capability for discrimination of fluorescence spectra and the scalability of the intensity levels. Because the use of additional fluorescent channels requires good spectral separation, the selection of appropriate molecules is crucial for B/F encoders. Figure 5(d) plots the average intensities of the samples at each concentration ratio. The error bars indicate the standard deviation where the error sources include variations in the procedures, such as the preparation of the DNA concentrations. We were able to distinguish at least five intensity levels. To increase the number of available levels, the intensities at a given $R_x$ should be more uniform. For multiplexed detection, a multiple B/F encoder system similar to that shown in Fig. 1(b) should be designed. In bulk fluorescence measurements, the fluorescence signals from the individual B/F encoders are multiplexed. The assignment of the color codes should be designed to produce multiple biomolecular signals from the multiplexed fluorescent signal, and thus would also meet the specifications needed for specific applications, including point-of-care testing of biomarkers and disease diagnosis. Despite these issues, the experimental results demonstrate the principle of the B/F encoder. The encoders performance is expected to be en-
5. Conclusion

We demonstrated the feasibility of a B/F encoder for encoding of biomolecular signals into fluorescent color codes. The amplification and encoding of the biomolecular signal are carried out at a molecular level by manipulation of the fluorescent molecules through DNA structural changes. The encoded molecular information can be decoded through characterization of the fluorescence spectra and intensities. As proof of our concept, we constructed a B/F encoder enhanced by multiplexing and optimization in our future work.
using two dyes and five intensity levels. The process is performed without the use of enzymatic reactions or DNA microarrays, thus offering an inexpensive and convenient method for biomolecular detection. With further improvements, this technique will contribute to the realization of on-site biomolecular screening for clinical diagnosis and patient care applications.

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