Biosensors

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Fluorescence Logic-Signal-Based Multiplex Detection of Nucleases with the Assembly of a Cationic Conjugated Polymer and Branched DNA**

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DNA cleavage by nucleases is essential to genetic information processing (e.g., DNA replication, recombination, repair),[1] and is also extensively utilized as a necessary tool in PCR assay, gene mapping, molecular cloning, and medicinal chemistry.[2] The development of sensitive and selective techniques capable of screening nucleases has become a major task in modern molecular biology and drug-discovery processes. The typical assay methods include gel electrophoresis, high-performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA);[3] however, they are time-consuming, laborious, not sensitive, or require the radiolabeling of substrates. To circumvent these limitations, efforts towards the development of fluorescence techniques (e.g., "molecular break light" and "molecular beacon")[4] for nuclease assays have been made. These assays are sensitive and convenient; however, they cannot afford multiplex detection of nucleases simultaneously. In this respect, a molecular ruler for measuring nuclease activity has been developed by using the surface plasmon resonance technique,[5] but it still needs extra treatment of the substrate DNA. There is still a need for a more sensitive and convenient method to simultaneously detect multiplex nucleases in one tube.

Water-soluble conjugated polymers (CPs) are known to have light-harvesting properties and can coordinate the action of a large number of absorbing units with efficient intrachain and interchain energy-transfer mechanisms to amplify fluorescence sensing signals.[6] Another important feature of CPs is that they can form complexes with oppositely charged biomolecules through strong electrostatic interactions and thus avoid covalent labeling, which should significantly reduce the cost. In recent years, CPs have been used as sensitive and convenient optical platforms for the detection of biomacromolecules, such as nucleic acids and proteins.[7–16] Our previous work shows that the conformational change of cationic polythiophene derivatives can be used to monitor the cleavage of single-stranded DNA (ssDNA) by S1 nuclease, but it does not work for restriction nucleases.[17]

Recently, we have developed a fluorescence resonance energy transfer (FRET)-based method to assay both restriction and nonrestriction endonucleases with cationic polyfluorene.[18] However, simultaneous multiplex detection of nucleases will need mixed-multiplex DNA probes in one solution, which results in assay complexity. The Y-shaped DNA (Y-DNA) initialized by Luo's research group consists of three complementary oligonucleotide branches and paves the way for the multiplex detection of DNA.[19] Herein, we present a new design of an electrostatic complex consisting of cationic polyfluorene with negatively charged Y-DNA respectively labeled at the 5′ termini with fluorescein, Tex Red, and Cy5 to generate an energy-transfer cascade. The multistep FRET processes regulate the fluorescence intensities of polyfluorene, fluorescein, Tex Red, and Cy5. Previously, we reported the optical logic systems based on two-step FRET by using a CP/DNA complex, in which a certain logic type is related to specific biorecognition.[20] Herein, different types of logic gate can also be operated in a cationic polyfluorene/Y-DNA assembly by observing the emission intensities of the dyes with multiplex nucleases as inputs upon excitation of cationic polyfluorene. The logic signals are specific to DNA–nuclease interactions, and give rise to a new pathway for the simultaneous detection of multiplex nucleases.

Our multiplex detection of nucleases is illustrated in Figure 1. Water-soluble poly{[(9,9-bis{6-*N*,*N*,*N*-trimethylammonium}hexyl)fluorenylene phenylene] dibromide} (PFP)[7,16] is used as the cationic CP in assay experiments. The Y-DNA labeled at the 5′ termini with fluorescein, Tex Red, and Cy5 includes three nuclease cleavage sites corresponding to Hae III, Pvu II, and EcoRV, respectively. The PFP forms a complex with negatively charged Y-DNA by electrostatic interactions. The fluorescence microscopy image (Figure 2a) shows the morphology of the complex, in which the formation of intense aggregates keeps PFP and dyes in close proximity, thus allowing for efficient intermolecular FRET.[21] Note that no intramolecular FRET was observed among the dyes in Y-DNA in the absence of PFP upon excitation of fluorescein or Tex Red (Figure 2b). PFP acts as the donor for the three dyes, fluorescein acts as the acceptor for PFP and the donor for Tex Red and Cy5, while Tex Red acts as the acceptor for PFP and fluorescein and the donor for Cy5 to satisfy the overlap integral requirement for FRET

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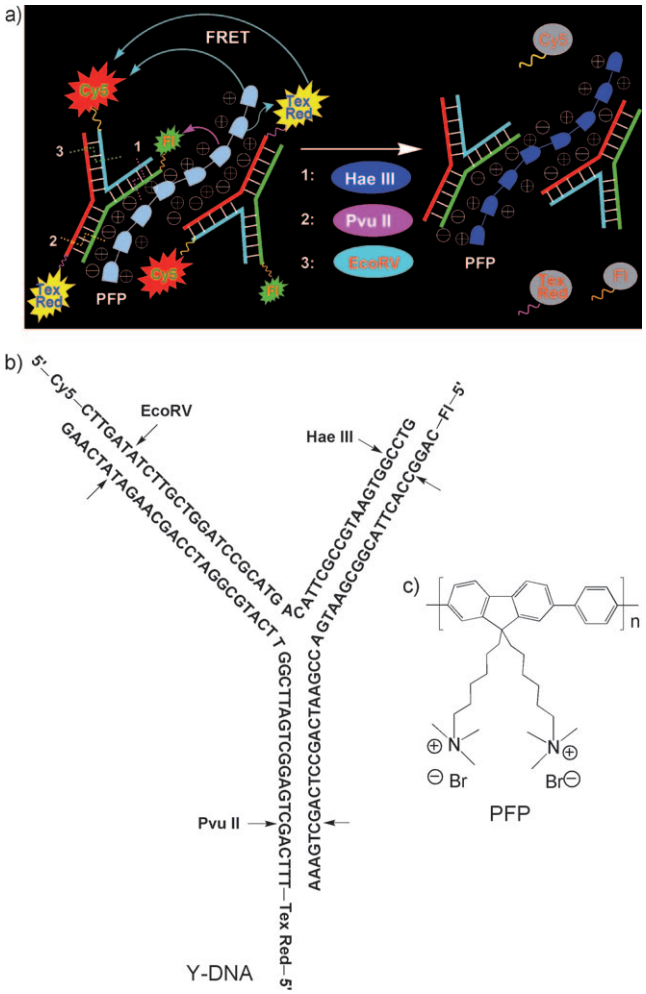

Figure 1. Schematic representation of the multiplex detection of nucleases (a), the sequence of Y-DNA (b), and the chemical structure of PFP (c). The arrows in (a) in cyan and magenta represent the energy transfer. Fl = fluorescein.

(Table 1).[22] Therefore, intermolecular multistep FRET between the chromophores occurs in the PFP/Y-DNA complex upon the excitation of PFP (Figure 2c). Upon addition of Hae III, Pvu II, and EcoRV to the solution of Y-DNA, short fragments carrying fluorescein, Tex Red, and Cy5 are generated. After adding PFP, the relatively weak electrostatic interactions between the DNA fragments and PFP lead the three dyes far away from PFP and Y-DNA and inefficient

Table 1: Data summary of spectral overlap integral (J), energy-transfer efficiency (E), Förster distance (R_0), and donor–acceptor (D/A) distance (R).

D/A pair	J [$M^{-1}cm^{-1}nm^4$]	R_0 [Å][a]	E [%]	R [Å]
PFP→Fl	8.88×10^{14}	41.3	87	30.0
PFP→Tex Red	2.23×10^{13}	22.3	89	15.5
PFP→Cy5	–	–	56	–
Fl→Tex Red	1.53×10^{14}	29.7	6.5	–
Fl→Cy5	5.49×10^{14}	36.8	12	–
Tex Red→Cy5	4.29×10^{15}	58.4	70	56.7

[a] The orientation factor $k^2 = 2/3$ was used to calculate R_0.

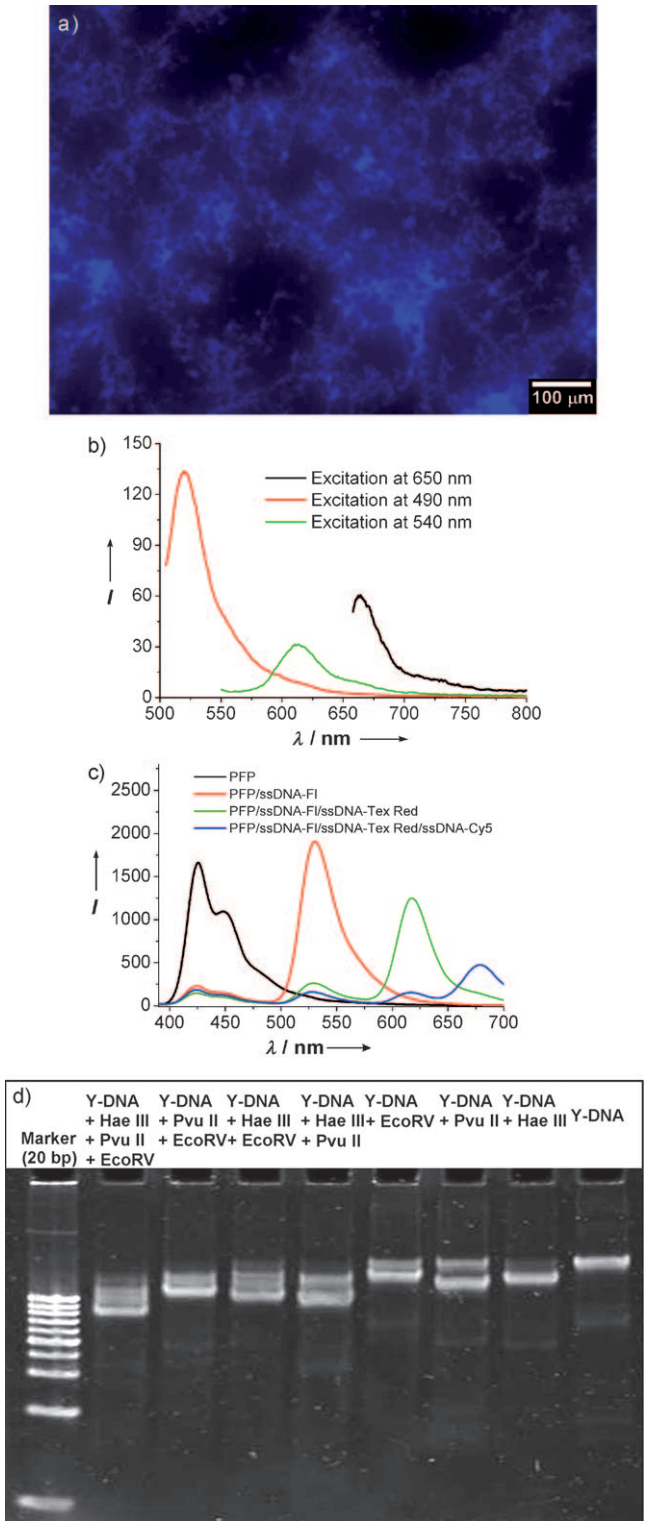

Figure 2. a) Fluorescence microscopy image of PFP/Y-DNA taken at 100 ms exposure with an excitation wavelength of 380/30 nm and emission of 460/50 nm. [PFP] = 1.0×10^{-4} M in repeat units (RUs), [Y-DNA] = 1.0×10^{-6} M. b) Emission spectra of Y-DNA upon excitation of fluorescein, Tex Red, or Cy5 in *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer solution (50 mM, pH 7.4). [Y-DNA] = 1.0×10^{-8} M. c) Emission spectra from solutions containing PFP and dye-labeled DNAs in HEPES buffer solution (50 mM, pH 7.4). [PFP] = 1.0×10^{-6} M in RUs, [DNA] = 1.0×10^{-8} M. The excitation wavelength is 380 nm. d) Electrophoresis analysis of Y-DNA cleavage by restriction nucleases. The gel was stained with SYBR Gold.

FRET between them is present. Thus, the cleavage of DNA by nucleases regulates the multistep FRET process. Different types of logic gate can be operated by observing the emission wavelengths of the dyes with multiplex nucleases as inputs upon excitation of PFP. Thus, the multiplex nucleases can be detected simultaneously in view of the logic signals operated by the PFP/Y-DNA complex in aqueous solution.

To study intermolecular FRET processes between PFP and the three dyes, the emission spectra of the assemblies of PFP with ssDNAs labeled with different dyes were measured in HEPES buffer (50 mM, pH 7.4) as shown in Figure S1 in the Supporting Information. The results for the spectral overlap integral (J), energy-transfer efficiency (E), Förster distance (R_0), and donor–acceptor distance (R) are summarized in Table 1. The emission maximum of PFP itself appeared at around 425 nm. For the PFP/ssDNA-Fl complex, the efficient FRET from PFP to fluorescein led to a significant quenching of PFP emission at 425 nm and the appearance of the fluorescein emission at 529 nm upon exciting PFP at 380 nm (Figure S1a). A FRET efficiency of 87% is estimated by quenching of the PFP emission,[22] and the separation distance between donor and acceptor is 30.0 Å obtained from $R_0 =$ 41.3 Å. For the PFP/ssDNA-Tex Red complex, FRET occurred with an efficiency of 89% and the Tex Red emission at 618 nm was observed. As there is no overlap integral between PFP and Cy5, the efficient energy transfer between them (efficiency: 56%) may originate from their orbital interactions through the Dexter mechanism.[6c] The PFP/Tex Red pair shows the shortest separation distance (15.5 Å) among all donor–acceptor pairs. Note that although there are good overlap integrals and efficient Förster distances for fluorescein/Tex Red and fluorescein/Cy5 pairs, inefficient energy transfer between them in the presence of PFP was observed.

In the presence of PFP, direct excitation of fluorescein showed that its fluorescence quantum yield was considerably reduced from 75 to 30%, which indicated that the self-quenching of fluorescein was prior to energy transfer to Tex Red or Cy5.[23] Moreover, direct excitation of Tex Red in the presence of PFP showed a much less reduced quantum yield. Although the Tex Red/Cy5 pair has the longest Förster distance in the assembly, the large overlap integral leads to efficient FRET between them with an efficiency of 70%. Thus, for PFP/ssDNA-Fl/ssDNA-Tex Red and PFP/ssDNA-Fl/ssDNA-Cy5 complexes, irradiation at 380 nm exclusively excited PFP, and FRET occurred favorably from PFP to Tex Red (Figure 2c) or Cy5 (Figure S1b). For PFP/ssDNA-Tex Red/ssDNA-Cy5, two-step FRET occurred: from PFP to Tex Red followed by FRET from Tex Red to Cy5, as shown in Figure S1b. For PFP/ssDNA-Fl/ssDNA-Tex Red/ssDNA-Cy5 complexes, multistep FRET between the favorable donor–acceptor pairs occurred (Figure 2c), as outlined in Figure 1.

Direct evidence that shows the cleavage of Y-DNA by nucleases is provided by electrophoresis analysis with a 15% nondenaturing polyacrylamide gel. As shown in Figure 2d, the Y-DNA itself moves as a single band, whereas it yields another, faster-moving band upon treatment with any one of Hae III, Pvu II, and EcoRV nucleases. Upon simultaneous treatment with two or even three nucleases, the Y-DNA similarly yields the corresponding faster-moving bands. These results indicate that the Y-DNA can be cleaved by specific nucleases, which provides a solid foundation for following fluorescence experiments.

Single-nuclease detection was performed firstly with the PFP/Y-DNA complex. Upon adding Hae III, a DNA fragment carrying fluorescein was generated and the emission intensity of fluorescein at 529 nm was sharply decreased (Figure 3a). By probing the emission intensity change of

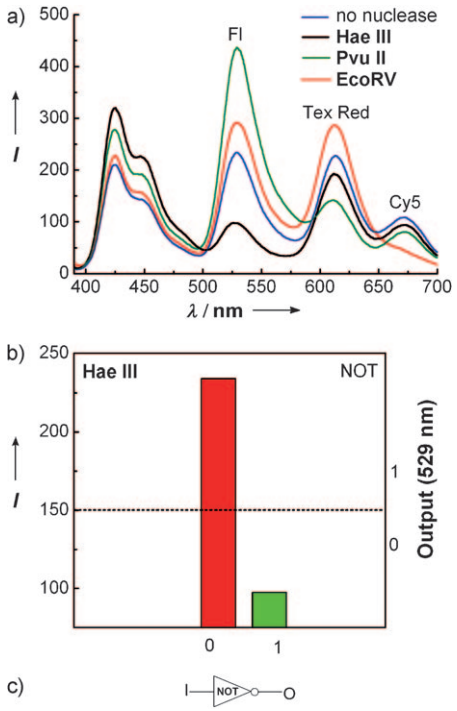

Figure 3. a) Emission spectra of PFP/Y-DNA before and after the addition of Hae III (10 U), Pvu II (20 U), or EcoRV (20 U) in HEPES buffer solution (50 mM, pH 7.4). b) Emission intensity of the output of the NOT logic gate at 529 nm from the two input combinations. c) NOT logic scheme and truth tables of the gates. [PFP] = 1.0×10^{-6} M in RUs, [DNA] = 1.0×10^{-8} M. The excitation wavelength is 380 nm.

fluorescein, a NOT logic gate can be operated to detect the presence of Hae III as illustrated in Figure 3b. NOT logic gates pass input bits to the reverse output signal.[24] The input (I) is Hae III; gate output (O) is determined by measuring the emission intensity of fluorescein at 529 nm with an excitation at 380 nm. The fluorescence intensity at 529 nm is high (1) in the presence of Hae III, and is low (0) when Y-DNA is cleaved by Hae III. Similar to the case of Y-DNA cleavage by Hae III, the addition of Pvu II reduced the Tex Red emission intensity and recovered that of fluorescein. For EcoRV, the emission intensity of Cy5 was reduced and that of Tex Red was recovered. Thus, the NOT logic gates can also be constructed with Pvu II or EcoRV as input (I) and the emission intensity of Tex Red at 613 nm or Cy5 at 672 nm as output (O) with an excitation at 380 nm (Figure S2). Figure 3c shows the scheme for NOT logic together with the truth tables of the gates. Thus, the single nuclease can be detected as a result of the NOT logic signals operated by the PFP/Y-DNA complex.

Figure 4 demonstrates the ability of the PFP/Y-DNA complex to detect two nucleases simultaneously with excitation at 380 nm. Upon simultaneous addition of Hae III and Pvu II, DNA fragments carrying fluorescein and Tex Red

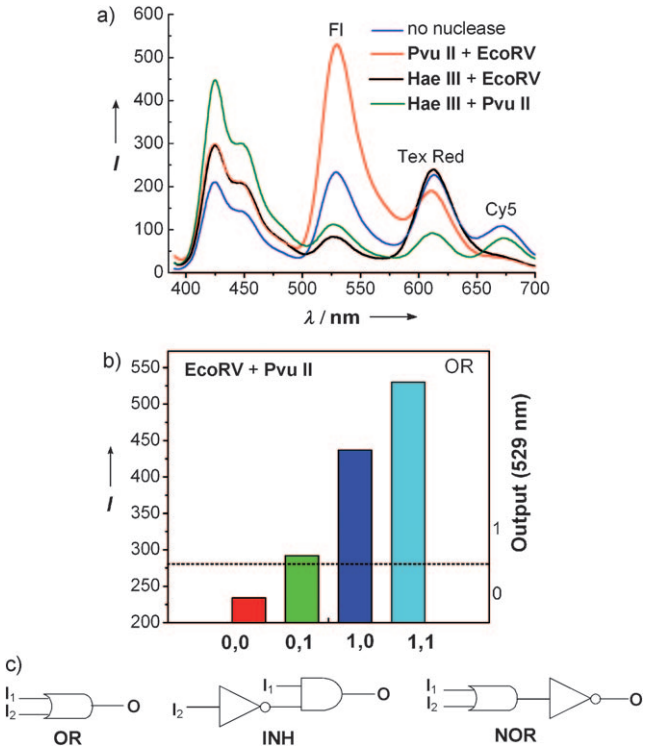

Figure 4. a) Emission spectra of PFP/Y-DNA before and after the addition of nuclease combinations EcoRV + Pvu II, EcoRV + Hae III, and Pvu II + Hae III in HEPES buffer solution (50 mM, pH 7.4). b) Emission intensity of the output of the OR logic gate at 529 nm from the four input combinations. c) Logic schemes and truth tables of the OR, INH, and NOR gates. [PFP] = 1.0×10^{-6} M in RUs, [DNA] = 1.0×10^{-8} M. Amounts of nuclease: 10 U for Hae III, 20 U for Pvu II, 20 U for EcoRV. The excitation wavelength is 380 nm.

were generated. As shown in Figure 4a, the emission intensity of fluorescein at 529 nm and that of Tex Red at 613 nm were sharply decreased and that of PFP was increased. Note that the apparent change of Cy5 emission intensity was not observed, which indicates negligible cross-interferences among the nucleases. For the Hae III/EcoRV and Pvu II/EcoRV combinations, similar results to those for Hae III/Pvu II were observed in which the intensity changes of the dyes reflected the identity of the specific nucleases. By measuring the emission intensity of fluorescein at 529 nm, we can achieve an OR gate with EcoRV (I_1) and Pvu II (I_2) as inputs.[24] The four possible input combinations [(0,0), (0,1), (1,0), (1,1)] for the OR gate are shown in Figure 4b. The INH gate is an AND gate with one of the inputs inverted through a NOT function. As illustrated in Figures 4c and S3a, an INH gate can be created by monitoring the emission intensity of Tex Red at 613 nm with EcoRV (I_1) and Hae III (I_2) as inputs. When the two inputs are Pvu II (I_1) and Hae III (I_2), a NOR gate was realized through observing the emission intensity of Cy5 at 672 nm (Figures 4c and S3b). Thus, the cleavages of DNA by nucleases regulate the multistep FRET processes and the OR, INH, and NOR logic signals can be used to simultaneously detect EcoRV/Pvu II, Hae III/EcoRV, and Hae III/Pvu II pairs, respectively.

Figure 5 demonstrates the ability of the PFP/Y-DNA complex to detect three nucleases simultaneously upon excitation at 380 nm. Simultaneous additions of Hae III,

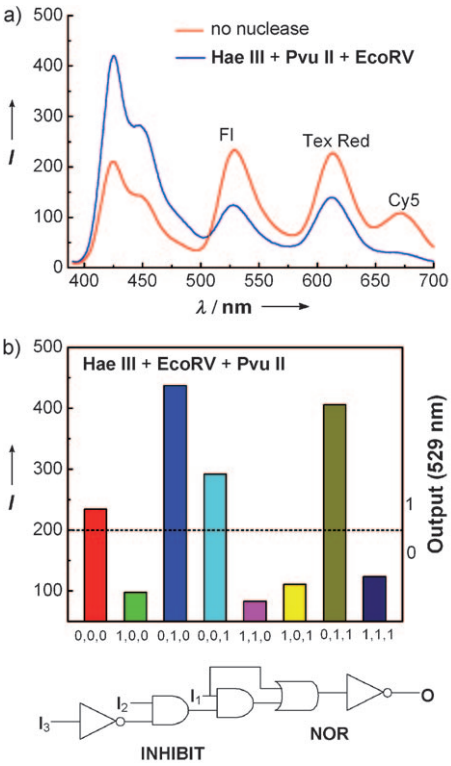

Figure 5. a) Emission spectra of PFP/Y-DNA before and after addition of a three-nuclease combination of Hae III + Pvu II + EcoRV in HEPES buffer solution (50 mM, pH 7.4). b) Emission intensity of the output of the integrated INHIBIT and NOR logic gates at 529 nm from the eight input combinations. c) Logic scheme and truth table of the integrated INHIBIT and NOR gates. [PFP] = 1.0×10^{-6} M in RUs, [DNA] = 1.0×10^{-8} M. Amounts of nuclease: 10 U for Hae III, 20 U for Pvu II, 20 U for EcoRV. The excitation wavelength is 380 nm.

Pvu II, and EcoRV generated DNA fragments carrying fluorescein, Tex Red, and Cy5. As shown in Figure 5a, the emission intensities of fluorescein, Tex Red, and Cy5 were all clearly decreased and that of PFP was increased. Through monitoring the emission intensity of fluorescein at 529 nm, the complicated and integrated INHIBIT and NOR logic gates[24] can be achieved with Hae III (I_1), EcoRV (I_2), and Pvu II (I_3) as the inputs. The eight possible input combinations for the integrated gate are shown in Figure 5b. Figure 5c shows the scheme for the integrated logic gate, together with the truth table. Thus, the multiplex nucleases can be detected simultaneously as a result of the integrated logic signals operated by the PFP/Y-DNA complex upon excitation at 380 nm. Furthermore, the PFP/Y-DNA complex works well when the amounts of the three nucleases are very different, such as Hae III/Pvu II/EcoRV = 5:20:20, 10:10:10, or 1:5:10 U

(Figure S4). The detection limit of the nucleases was estimated to be 0.05 U μL^{-1}, which is better than that of traditional methods, for example, gel electrophoresis, chromatography, and UV-based assays.

In summary, a new electrostatic complex of cationic CP with negatively charged Y-DNA respectively labeled at the 5′ termini with fluorescein, Tex Red, and Cy5 was designed as an energy-transfer cascade.[25] Multistep FRET processes regulate the fluorescence intensities of the CP and three dyes. Different types of logic gate can be operated by observing the emission wavelengths of different dyes with multiplex nucleases as inputs. The logic signals give rise to a new method for the simultaneous detection of multiplex nucleases. Furthermore, the combination of the CP/Y-DNA assembly with specific DNA–nuclease interactions creates new supramolecular systems that mimic complicated and integrated logic operations. This feature has been essential to develop future "computer-like" functions of enhanced complexity[26] since the first AND logic gate was reported.[27]

Experimental Section

Materials and measurements: PFP was synthesized according to the procedure in the literature.[7] All oligonucleotides were purchased from Beijing Sunbiotech Co. Ltd. and purified by PAGE, and their concentrations were determined by measuring the absorbance at 260 nm in 200-μL quartz cuvettes. Equal molar amounts of the three oligonucleotides respectively labeled with fluorescein, Tex Red, and Cy5 were mixed together and annealed at 80 °C for 20 min, and then slowly cooled to room temperature to give the Y-DNA. The Hae III, Pvu II, and EcoRV endonucleases were purchased from New England Biolabs. The nucleases were placed on ice before use. The water used was purified with a Millipore filtration system. Fluorescence measurements were obtained in a 3-mL polystyrene cuvette at room temperature by using a Hitachi F-4500 fluorometer equipped with a xenon-lamp excitation source. The UV/Vis absorption spectra were obtained on a JASCO V-550 spectrophotometer. Electrophoresis analysis experiments were performed as follows. The DNAs before and after cleavage by nucleases were loaded onto a 15% nondenaturing polyacrylamide gel in 1 × TBE buffer (8.9 mM Tris base, 8.9 mM boric acid, 0.2 mM EDTA, pH 7.9) followed by electrophoresis for 125 min. After staining the gel with SYBR Gold, a photograph was taken with a ChemiDoc XRS system.

Assays of DNA cleavage by nucleases: The restriction nucleases (10 U for Hae III, 20 U for Pvu II, 20 U for EcoRV) were added to a solution of total volume 20 μL containing Y-DNA (1 μL, [Y-DNA] = 1.0×10^{-5} M). After incubation at 37 °C for 1 h, the solution was diluted with HEPES buffer (1 mL, 50 mM, pH 7.4). After adding the PFP ([PFP] = 1.0×10^{-6} M), the fluorescence spectra were measured at room temperature at an excitation wavelength of 380 nm.

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