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Viewpoint

DNA-FRET Constructs Enable Multiplexed Fluorescence Detection at the Single-Molecule Level

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Multiplexed fluorescence measurement at the single-molecule level provides unique insight into the molecular composition and interaction within a complex system.[1,2] However, fluorescence-based detection is typically restricted to 3−4 colors at a time, due to a low signal-to-noise ratio, high spectral overlap, and the need to maintain chemical compatibility of dyes. Although chemically diverse fluorophores offer a potentially broad spectroscopic palette, further multiplexing is ultimately constrained by the chemical compatibility and varying labeling performance of different fluorophores.

Förster resonance energy transfer (FRET) represents a well-established strategy to generate a plethora of distinct spectroscopic signals from a limited number of chemical compounds. FRET is influenced by not only the photophysical properties of fluorophores per se but also the geometry of the donor and acceptor. DNA nanotechnology offers a powerful platform to precisely position and orient covalently linked fluorophores.[3] The photophysical property of a fluorophore on DNA is influenced by the local physicochemical environment including the dye attachment chemistry and neighboring base sequence.[4] These photophysical changes, combined with the capability of DNA nanotechnology to control donor−acceptor geometry with subnanometer precision,[5] allow construction of DNA-FRET nanostructures with tunable spectroscopic features such as fluorescence emission and lifetime.[6]

In a recent report in *Nature Nanotechnology*, Squires and colleagues reported dozens of DNA nanostructure-based FRET labels called FRETfluors, which enabled multiplexed fluorescence detection at the single-molecule level.[7] FRETfluors are facilely constructed from merely three chemical components (DNA, Cy3, and Cy5). Figure 1a illustrates the FRETfluor design of AB*N* constructs, where Cy3 and Cy5 are site-specifically incorporated into two complementary DNA strands ("A" and "B"), respectively, separated by *N* base pairs ($6 \leq N \leq 20$). More FRETfluor variants ($AB_{sk}N$, A_cBN, and $AB_{in}N$) with unique spectroscopic signatures were created by varying the DNA sequence and introducing an additional Cy3 fluorophore (Figure 1b). Within each FRETfluor construct, an additional "bridge" strand facilitates sequence-specific labeling of nucleic acid targets or the introduction of functional groups for general protein labeling. With meticulously designed local sequence and attachment chemistry, the FRET efficiency and donor lifetime of FRETfluor could be precisely modulated (Figure 1c,d).

The authors employed a custom-built Anti-Brownian ELectrokinetic (ABEL) trap, which enables precise characterization of each FRETfluor across multiple parameters such as emission brightness and fluorescence lifetime. Moreover, ABEL trap measurement allows monitoring the size of a FRETfluor (or FRETfluor-labeled molecule) and analysis of a FRETfluor-labeled sample at a concentration down to tens of femtomolar. The single-molecule emission properties (e.g., green and red channel brightness and donor lifetime) of most FRETfluors produce characteristic tight clusters in the detection parameter space. Excluding those prone to misclassification, the authors identified a subset of 27 FRETfluors that were suitable to use in a single mixture (Figure 2a). Changes in environmental conditions such as salt concentration and pH within a physiological range have a minor effect on the FRETfluor signal, underscoring the stability and reliability of FRETfluor performance and providing a basis for its practical application.

Importantly, FRETfluors can be programmed to specifically label biomolecules. For example, FRETfluor targets nucleic acids via sequence complementarity of the "bridge" strand. FRETfluor is also capable of covalently labeling proteins via a conventional bifunctional linker (Figure 2b). Consequently, FRETfluors allow multiplexed detection of complex mixtures of low-abundance biomolecules including ssDNA, dsDNA, mRNA, and protein. By separately labeling three mRNAs, six dsDNAs, and two proteins with a subset of the 27-FRETfluor combination, together with two off-target free FRETfluors, the authors demonstrated that all 13 FRETfluors showed their distinct spectroscopic signatures in the detection parameter space (Figure 2c). Another advantage of ABEL trap-based detection is that it can distinguish a FRETfluor bound to the target from an unbound FRETfluor, thereby enabling wash-free sensing.

Overall, the FRETfluor design exploits the nanoscale addressability of DNA nanotechnology to precisely finetune the dye photophysics, markedly expanding the multiplexing capability of fluorescence detection. Moreover, this study

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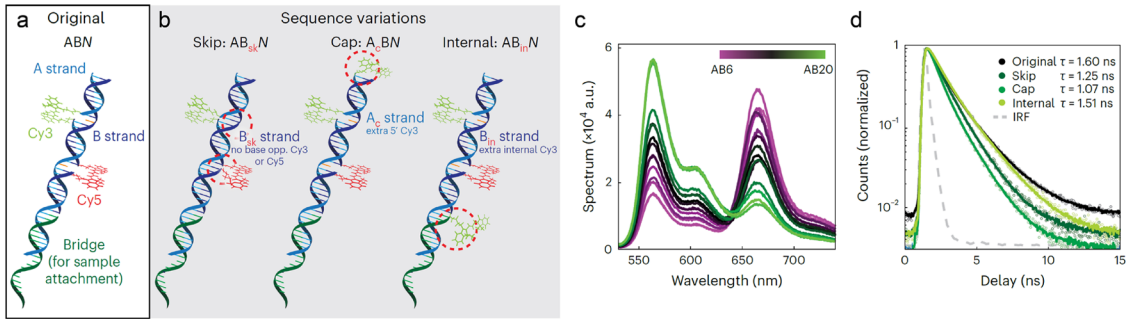

Figure 1. FRETfluor design. a, FRETfluor design for ABN constructs. **b**, FRETfluor design variations AB$_{sk}$N, A$_c$BN, and AB$_{in}$N, which create additional unique spectroscopic signatures. **c**, Emission spectra of ABN constructs demonstrate that donor−acceptor geometry modulates FRET efficiency, leading to varying Cy3 and Cy5 brightness. **d**, Fluorescence lifetime curves show that the Cy3 lifetime depends on the local DNA sequence and attachment chemistry. Reproduced with permission from ref 7. Copyright 2024 Springer Nature Ltd.

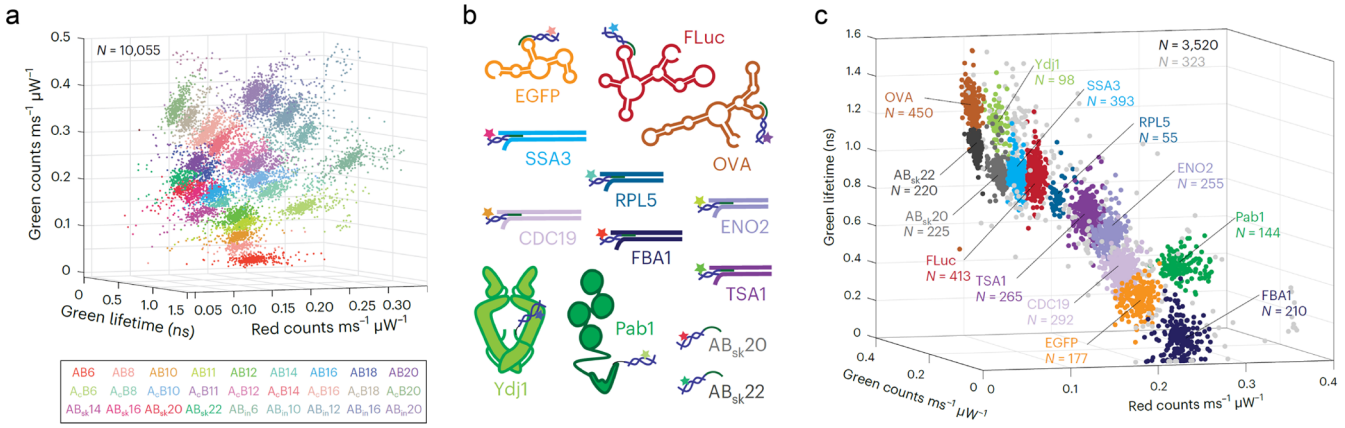

Figure 2. FRETfluor application to detect complex mixtures of biomolecules at low concentration. a, 3D projection of 27 FRETfluor labels colored according to the most likely identity (bottom). **b**, Identities of three mRNAs, six dsDNAs, two proteins, and two targetless FRETfluor samples. **c**, 3D projection of spectroscopic data for a complex mixture of FRETfluor-labeled mRNA, dsDNA, and proteins. Reproduced with permission from ref 7. Copyright 2024 Springer Nature Ltd.

provides a proof of concept for using dozens of FRETfluors for multiplexed sensing of low-abundance biomolecules in a highly heterogeneous mixture. This approach is compatible with other multiplexing strategies such as additional excitation lasers,[8] novel fluorophore types, orientational control to influence dye polarization, and DNA-PAINT.[9,10] Alternative molecular scaffolds could also be used to create FRETfluors with appealing properties. Particularly, xeno-nucleic acids (XNAs) with synthetic backbones are more resistant to nuclease digestion and thus suitable for use in complex biological mixtures.[11−13] Additionally, an XNA scaffold offers a platform to delicately modulate donor−acceptor geometry and the local environment of a fluorophore, in a new chemical space that is intrinsically inaccessible to DNA. A limitation of the current approach is that a FRETfluor is functionalized to react with an accessible cysteine residue on the protein surface, which necessitates separate conjugation and precludes specific labeling in a complex mixture. The FRETfluor specificity for proteins of interest might be programmed using targeting moieties such as antibodies and aptamers.

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Notes

The authors declare no competing financial interest.

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