Sensors and probes

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A closer look at FluoroCubes

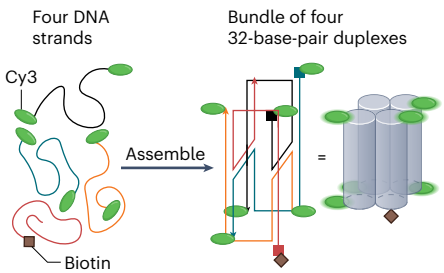
Researchers explore the unique and puzzling photostability of DNA FluoroCubes. Moreover, they improve the probes' performance and highlight their diverse applicability.

DNA FluoroCubes are ~6 nm DNA nanostructures that can harbor one or more fluorophores at controlled spacings. In their 2020 study, Niekamp et al. discovered that DNA FluoroCubes with six Cy3 fluorophores have a greater than 50-fold increase in photobleaching lifetime and emit over 40-fold more photons than individual organic dyes. In that work, the team showed that these probes could enable extended single-molecule tracking, following the movements of a single kinesin for over 6 µm.

While the original study included a thorough characterization of the FluoroCubes' photophysical properties and performance benefits, it did not pin down a mechanism underlying the improved photostability. This open door led to an exciting follow-up study spearheaded by postdoctoral fellow Aaron Blanchard (now at Duke University) in the lab of Nils Walter at the University of Michigan, drawing on their combined expertise in nucleic acids, spectroscopy and microscopy.

The path to the project was fortuitous. Blanchard was impressed with the FluoroCubes and particularly intrigued by what caused the increased photostability. "But after reading the paper, having discussions with lab mates, and poring over the literature, it seemed clear that nobody knew exactly why." A subsequent discussion about the probes on social media made him curious about testing different hypotheses. Blanchard recalls, "Once I started thinking about how I would run these experiments, I kind of got sucked into the project's gravitational orbit." Walter shared his enthusiasm, saying "FluoroCubes were fascinating from the moment they were published, but needed more mechanistic characterization for their potential to be fully realized. When Aaron proposed to study them in depth, I became excited."

Based on the original study's evidence, the team thought both dye–dye and dye–DNA



A schematic depiction of the formation of labeled DNA FluoroCubes.

interactions might contribute to increased photostability in the FluoroCubes. To test this, they created 14 different FluoroCube variants with one to six Cy3 dyes at different positions and immobilized them on glass coverslips for single-molecule photobleaching assays.

Their results surprised even themselves, according to Blanchard. "We initially hypothesized that photostability was improved by interactions between fluorophores. Not only was that not the case, but we found that such interactions often hindered FluoroCube performance." Indeed, the takeaway from their experiments was that the photostability of the dyes is primarily caused by interactions between the dyes and the DNA nanostructures to which they are tethered. They carried out further studies that effectively ruled out a role for dye–dye interactions in improving photostability. Indeed, the finding that the four-dye FluoroCubes emitted more photons than the six-dye indicated that dye–dye quenching may actually be detrimental to FluoroCube performance. These results were corroborated by a detailed analysis of the impact of face-to-face dimerization (H-dimerization) on the emissive properties of the dyes in various constructs.

In further work, the researchers created smaller 'mini' FluoroCubes and found that they had comparable photophysical properties. The reduced size makes the mini FluoroCubes similar in size to monomeric fluorescent proteins, which are commonly used as tags. Next, the team showed that the FluoroCubes are compatible with dark quenching applications, where proximity to a quenching moiety decreases fluorescence, and optimized FluoroCubes for maximal quenching. Finally, the team demonstrated that FluoroCubes labeled with Cy3 could be used as FRET donors to FluoroCubes labeled with Cy5.

Though FluoroCubes seem more promising than ever in terms of brightness, photostability and versatility, the researchers still note caveats. "The size of FluoroCubes is comparable to fluorescent proteins, but for some applications may still be a little too large," says Walter. Beyond size, FluoroCubes have a few other downsides, according to Blanchard. Beyond their cost, which can be "quite expensive" because they require up to three dual-labeled DNA strands, Blanchard explains that "the FluoroCubes exhibit substantial temporal fluctuations in brightness, which might make FluoroCubes difficult to adapt for applications that typically require more consistent fluorescence intensity such as obtained from single dyes." He says, "the four-dye constructs that we present in our work partially address these issues, though not completely."

Although the project came together in the end, Blanchard shares that it wasn't without hiccups. The team had to balance a ticking clock associated with a cross-country move to complete the project. Walter credits Blanchard and the lab team for the success, saying "Aaron engaged in extensive teamwork to get all aspects of the mechanistic characterization covered, which accelerated the project and became key to its success," while Blanchard credits his teammates and the probes themselves. "This was possible because the assembly and purification of the FluoroCubes and the experiments themselves were straightforward. In my opinion this is a testament to why FluoroCubes are so great to work with."

The team notes that there are still open questions regarding DNA–fluorophore interactions and many more potential applications to be explored for FluoroCubes, such as in environmental sensing.

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Original references: *Nat. Methods* **17**, 437–441 (2020); *Nano Lett.* **22**, 6235–6244 (2022)