Methods 67 (2014) 177–184

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Discovering anomalous hybridization kinetics on DNA nanostructures using single-molecule fluorescence microscopy

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ARTICLE INFO

Article history:
Available online 3 March 2014

Keywords:
Single-particle fluorescence resonance energy transfer
Points accumulation for imaging in nanoscale topography
Hybridization kinetics
DNA origami
DNA nanotechnology

ABSTRACT

DNA nanostructures are finding diverse applications as scaffolds for molecular organization. In general, components such as nucleic acids, proteins, and nanoparticles are attached to addressable DNA nanostructures via hybridization, and there is interest in exploiting hybridization for localized computation on DNA nanostructures. This report details two fluorescence microscopy methods, single-particle fluorescence resonance energy transfer (spFRET) and DNA-PAINT (points accumulation for imaging in nanoscale topography), that have been successfully used to detect anomalies of hybridization reactions on individual DNA nanostructures. We compare and contrast the two techniques, highlighting their respective strengths in studying equilibrium and non-equilibrium hybridization as well as assessing the variability of behaviors within individual nanostructures and across a population of nanostructures.

1. Introduction

In the past decade, structural DNA nanotechnology has emerged as a powerful tool for the organization of molecular components with nanometer precision. In particular, the DNA origami technique [1] has been used to build molecular automata [2–4], synthetic multi-enzyme complexes [5], and photonic devices [6–8] and has been proposed as a scaffold for molecular logic circuits [1,9] in the context of DNA computing [10,11]. All of these applications exploit the ability to attach nucleic acids, proteins, or nanoparticles to specific sites on the structural DNA scaffold, usually by hybridization of complementary DNA sequences. Successful execution of these approaches thus hinges on understanding what factors influence hybridization reactions at the surface of DNA nanostructures, especially when the yield and fidelity of intended processes depend on the kinetics of hybridization.

Hybridization to DNA nanostructures has been probed using a variety of techniques, including atomic force microscopy (AFM) [1,4,12], ensemble fluorescence or fluorescence resonance energy transfer (FRET) [13], and native gel electrophoresis. The total internal reflection fluorescence (TIRF) microscopy techniques single-particle FRET (spFRET) [14,15] and DNA-PAINT [16,17] provide high spatiotemporal resolution and low invasiveness that are particularly conducive to detailed studies of hybridization on single nanoscale complexes (Fig. 1). In our implementation of spFRET, a DNA target labeled with a FRET donor is allowed to saturate multiple acceptor-labeled probe sites on a single nanostructure, resulting in a large increase in acceptor fluorescence (Fig. 1A). This implementation of spFRET is well-suited to the non-equilibrium study of binding to many sites on single nanostructures, forgoing super-resolution spatial information in favor of simplicity, greater dynamic range, and less stringent sensitivity requirements than typical single-molecule techniques. In DNA-PAINT, by contrast, the reversible binding of single fluorescently labeled target oligonucleotides to individual probes on nanostructures is monitored over multiple binding cycles, yielding super-resolution spatial information about the kinetics of hybridization to various sites on each nanostructure (Fig. 1A). Unlike spFRET, DNA-PAINT depends on single-fluorophore sensitivity, but requires fluorescent labeling only of extrinsic target strands, thus rendering multicolor imaging more feasible.

Recently, we reported the use of spFRET [15] and DNA-PAINT [17] to characterize the kinetics of hybridization to ssDNA probes on DNA origami, demonstrating several differences from canonical solution behavior when the origami-bound probes are closely spaced. In this work, we present detailed protocols for these two techniques, as well as a comparison of their instrumentation and sample requirements and strengths in assessing different aspects of hybridization to DNA nanostructures (summarized in Fig. 1B).

Abbreviations: spFRET, single-particle FRET; PAINT, points accumulation for imaging in nanoscale topography; OSS, oxygen scavenger system.

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http://dx.doi.org/10.1016/j.ymeth.2014.02.032
1046-2023/

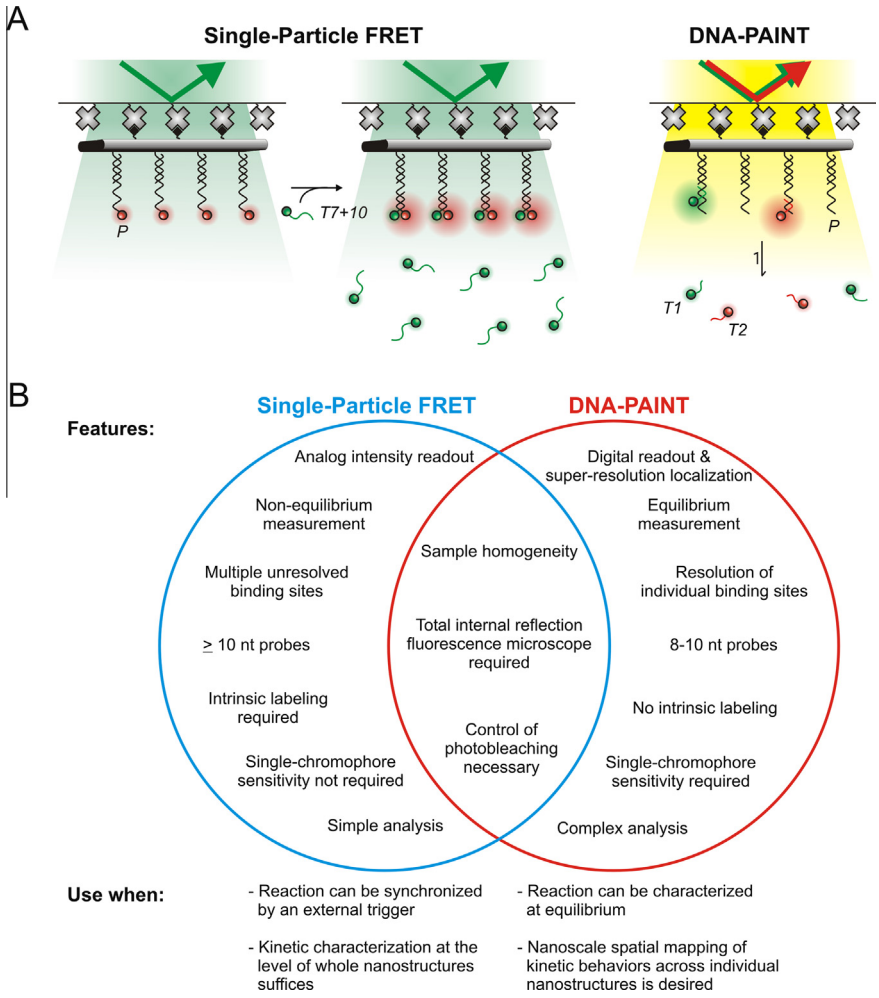

Fig. 1. Overview of techniques. (A) Schematic illustration of studying hybridization to single DNA origami by spFRET and DNA-PAINT. Nanostructures are labeled with biotin (black diamonds) to permit immobilization on a glass surface coated with streptavidin or NeutrAvidin (gray crosses). (B) Comparison of the features and capabilities of spFRET and DNA-PAINT.

2. Materials and methods

2.1. Microscope

Both spFRET and DNA-PAINT require a microscope capable of TIRF or HILO [18] illumination to reject background signal from unbound fluorescent probes. A schematic of the microscope used in this study is shown in Fig. 2A. Excitation is provided by two diode lasers: a CrystaLaser CL532-050-L (532 nm, 50 mW) for Cy3 excitation, and an Olympus LAS/640/100-D (640 nm, 100 mW) for Cy5 excitation. In the case of spFRET measurements, only the green (532 nm) laser is used, and is attenuated using a neutral-density filter (optical density of 1.0) to minimize photobleaching, which would interfere with kinetics measurements. Using a series of mirrors and a focusing lens, the excitation beams are directed to a prism lying on top of the microscope slide containing the sample, where they undergo total internal reflection at the slide-solution interface (Fig. 1A), exciting fluorophores within ~100 nm of the slide surface. The fluorescence emission is passed through a 60× objective and an optional 1.6× magnifier (used in DNA-PAINT for increased spatial resolution). The image is cropped by passing through a narrow slit, then split into two frequency bands, or channels, using a dichroic mirror with a cutoff wavelength of 610 nm (Chroma). Using a series of mirrors and lenses, each of the two image channels is focused onto one half of an intensified CCD camera chip (iPentamax HQ Gen III, Roper Scientific, Inc.). A Newport ST-UT2 vibration isolation table is used in all experiments to reduce instrument interference.

2.2. Fluorescent target design

DNA-PAINT and spFRET impose somewhat different constraints on the design of single-stranded DNA (ssDNA) probe–target interactions. For non-equilibrium spFRET measurements, targets must be long enough (typically $\geqslant$ 10 nt) to ensure a small dissociation constant (preferably < 10 nM), which allows the binding sites to be highly populated without increasing target concentrations beyond ~100 nM, at which point background signal from unbound target may become problematic. For the spFRET assays described here, we use an 8–17 deoxyribozyme target *T7+10-Cy3* (5′-/5Cy3/TCT CTT CTC CGA GCC GGT CGA AAT AGT GAA AA) and a probe sequence *P* (5′-GAT GAA TGG TGG GTG AGA GGT TTT TCA CTA TrAG GAA GAG), which is a substrate for the 8–17 deoxyribozyme [19]. Importantly, since our conditions contain no divalent metal cations, no substrate cleavage occurs on the timescale of our experiments. For spFRET, *P* is modified with Alexa Fluor 647 (AF647) at its 3′-end to act as a FRET acceptor upon binding. For dissociation reactions, we use an unlabeled *P* strand to sequester target as it dissociates from the nanostructures. We also investigate the dissociation of a second target, *T11O*-Cy3 (5′-/5Cy3/TCT CTT CCT ATA