Optical and Topological Characterization of Hexagonal DNA Origami Nanotags

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Abstract—DNA origami can be applied as a “ruler” for nanoscale calibration or super-resolution fluorescence microscopy with an ideal structure for defining fluorophore arrangement, allowing the distance between fluorophores to be precisely controlled at the nanometer scale. DNA origami can also be used as a nanotag with arbitrary programmable shapes for topological identification. In this study, we formed a hexagonal origami structure embedded with three different fluorescent dyes on the surface. The distance between each fluorescent block was \(~120\) nm, which is below the diffraction limit of light, allowing for its application as a nano-ruler for super-resolution fluorescence microscopy. The outside edge of the hexagonal structure was redesigned to form three different substructures as topological labels. Atomic and scanning force microscopy demonstrated the consistent nanoscale distance between morphological and fluorescent labels. Therefore, this fluorophore-embedded hexagonal origami platform can be used as a dual nanoruler for optical and topological calibration.

Index Terms—DNA origami, atomic force microscopy, fluorescence labeling, nanoruler.

I. INTRODUCTION

DNA origami has various applications, including DNA computing, nanoarchitecture, and drug design [1]–[9]. A single DNA origami is formed by a long circular scaffold strand with the help of hundreds of staple strands [10], each of which is known in advance and has a unique position in the final assembled structure. The size of a single origami is determined by the length of its scaffold strand, which generally has an area in the nanometer scale. DNA origami also provides an excellent platform for super-resolution fluorescence microscopy due to optical calibration advantages, which allow for precise docking of the fluorophores at predesigned positions. Therefore, DNA origami facilitates the study of fluorescent dye characteristics at different distances [11]–[16]. The development of this technology is aided by various tools for designing and predicting origami structures that help researchers construct DNA origami structures with accuracy and precision [17], [18].

To date, DNA origami monomers have been used to develop several types of fluorescent nanotags, including two-dimensional (2D) and three-dimensional (3D) structures [12], [14]. 2D origami monomer tends to be curved in a solution [19]. 3D compact origami structures have been proven robust and stable. Owing to the precise distance control of fluorophores, the energy transfer between different fluorescent dyes can be calculated [20], allowing for selecting the most suitable fluorescent dye under different conditions of interest [16]. Further, fluorophore-embedded origami structures have been demonstrated to serve as an ideal calibration ruler for various super-resolution microscopy applications, including stimulated emission depletion microscopy (STED), direct stochastic optical reconstruction microscopy, and confocal microscopy [21]–[23]. Lakadamyali et al. demonstrated that DNA origami is a versatile platform for calibrating fluorophore and antibody labeling efficiency to quantify protein copy numbers [24], [25]. With further development of this technology, Jungmann et al. showed that even a single biomolecule (<5 nm) could be identified, such as DNA-PAINT [26]–[28]. Recently, a fluorophore-embedded origami ruler has been commercialized and is being widely applied (Gattaquant, Braunschweig, Germany).

As a single origami structure is in the nanometer size scale, several origamis need to be assembled into a larger shape to enable fluorescence microscopy at different scales, including the microscale, to avoid curvature. For example, cross-shaped origamis were assembled in the front-back pattern to construct flat origami arrays. Different fluorescent dyes were deposited onto the substrate, which enabled optical metrology at precise sites [29]. In addition, polyhedral self-assembled 3D origami structures were formed using DNA origami tripods with fluorophores attached to each vertex, and the 1–60 MD super DNA gridiron was characterized using 3D DNA-PAINT [30].
Fig. 1. Triangular origami was designed by using caDNAno. The length of the edge is 120 nm. As a modification, we loosened the outside edge of the triangle. The two inner edges were used as the linking sites for assembly.

All these previously developed structures were designed as fluorescent nanotags for calibration. However, the origami itself was neglected in these previous designs. As DNA origami can be formed in arbitrary shapes, topological tags are possible [31]. The lack of consideration of the topological characteristics of origami has hampered its broader application in versatile fields, such as genotyping and shape detection [32]. Moreover, an atomic force microscope (AFM) has a markedly higher resolution than a fluorescence microscope in detecting topological features and can be easily calibrated with a standard sample.

3D origami monomers are robust and suitable for binding fluorophores. However, identifying 3D structures requires a transmission electron microscope (TEM), which is not ideal for topological calibration. Additionally, origami must be dry on a copper grid, which will otherwise change its formation (the size of origami is different in solution and air, while cryo-TEM is expensive and time-consuming). Therefore, we assembled a 2D hexagonal DNA origami multimer platform with spectral and topological features by docking three types of fluorescent dyes onto the surface, and three types of substructures were programmed and attached at the edge of the platform. The topological labels were detected using AFM, and optical characteristics were determined using STED.

II. Experiments

A. Design Strategy

The hexagonal origami was assembled using six triangular origamis, which were previously reported by Paul Rothemund [10]. We modified the triangular origamis by loosening the outside edge (Fig. 1), resulting in a tadpole-like shape, i.e., a triangular origami with a loosened tail (Fig. 2b).

The connection strategy followed the originally reported method; the connecting (inner) edges of the triangle were sorted as extended and truncated edges (corresponding staple strands were extended or truncated for connection). By changing the positions of each connecting part, the triangular origami was divided into three groups (Supplementary Fig. S1). Each group had different linking sets, which are illustrated as yellow, green, and red patterns in Fig. 2a. The three extended connection strands link with other truncated strands to bind two groups together. Therefore, the edge of the triangular origami is linked by six connection strands.

The outside edge was loosened (the scaffold was left single-stranded), leaving a region of 845 nucleotides for further topological design.

The DNA strands used in this study were purchased from Sangon Biotech (Shanghai, China) and HAP-purified. The single-strand M13mp18 scaffold was purchased from New England BioLabs (Beijing, China). Staple and scaffold strands were mixed at a 1:10 ratio; the mixed solution was diluted in 1× TAE buffer with 12.5 Mg2+ and then annealed by reducing the temperature from 95°C to 4°C for 13 h.

After forming the single triangular origamis, the samples were purified using ultrafilter tubes (Millipore Ultra-100K, Millipore Shanghai, China) and centrifuging at 9000 rpm for 5 min. The DNA concentrations of the three tubes were measured on a spectrophotometer (Step Thermo Fisher NanoDrop One, Thermo Fisher Scientific, Waltham, MA, USA) at an ultraviolet wavelength (260 nm). The single triangular origamis were then maintained at 4°C until further processing.

The three groups of triangular origamis were assembled into a hexagonal platform while precisely controlling the ratio of the three groups at 1:1:1. Assembly was performed with incubation at 45°C for 2 days (results of different incubation conditions are shown in supplementary fig. S5). The assembled hexagonal samples were then electrophoresed for further purification. The gel harboring the hexagonal platform was cut for purification. The target gel strip was mashed and dipped into 1× TAE buffer (100 µL) at 25°C for 2 days; then, the sample solution was purified and concentrated with ultrafiltration tubes (Millipore Ultra-100K, Millipore Shanghai, China). The result is shown in Fig. 2c. We could only identify two complete hexagonal origamis in the displayed image, indicating that the synthesis process of this origami requires further refinement. Finally, refined hexagonal samples were used (Fig. 2c). The yield of the assembled hexagon
was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA).

B. Gel Electrophoresis

A solution of 100 ml 1% native agarose was prepared and heated until it dissolved. The solution was allowed to cool to 70°C. Ten microliters of 4S GelRed nuclidic acid stain (Sangon Biotech, Shanghai) was added to the solution, following the solution was shaken and vibrated to ensure proper mixing. The gel was then cast in the electrophoresis chamber and run at constant voltage and current (70 V and 100 mA, respectively) for 4 h.

C. AFM Characterization

We used a Bruker Multimode 8 serial AFM (Bruker Corporation, Karlsruhe, Germany) for topological characterization. First, a drop (5 µl) of the hexagonal sample was placed on a fresh mica surface and left for 5 min to allow complete adsorption of the sample onto the mica surface, followed by the addition of 20 µl 1 x TAE buffer. The sample was then placed on the piezoelectric ceramic stage for scanning. We set the AFM to the “ScanAsyst in Fluid” mode using Scan in Fluid tips (model: SCANASYST-FLUID+, silicon tip on nitride lever; cantilever: 150 KHz, 0.7 N/m). The approaching surface set point was 0.075 V; after approaching the mica surface, the AFM functioned at automated set points in the auto-scan mode with a scan rate of 2 Hz per second.

D. Super-Resolution Characterization

Two illuminations are required with a STED system: one for excitation and the other for light loss. In this study, we used the Leica STED 3X serial system for super-resolution microscopy to acquire fluorescent images. This is a high-resolution microscope based on the confocal microscopy system; its x/y plane resolution is ≤50 nm, and the z-space resolution is ≤130 nm.

We chose three dyes for analysis with this platform: Alex 488, Cy3, and Cy5. Alex 488 is a green fluorescent dye with an excitation spectrum at the 488-nanometer laser line. Cy3 is an orange dye with an excitation wavelength at 600 nm, and Cy5 is a red dye with an excitation wavelength at 700 nm. All three dyes are highly fluorescence-sensitive, photostable, and resistant to light bleaching. These characteristics facilitate the identification of the dyes on the platform using STED.

The hexagonal origami platform was attached to a coverslip surface via a sandwich linking strategy using bovine serum albumin (BSA)-biotin, streptavidin, and a BSA-biotin bridge. All the triangular origamis were functionalized with biotin anchors at the backs of their surfaces. We set eight docking positions for each triangular origami, and six staple strands at the brim of its outer edge. The loosened tile is 845 nucleotides long. The blue dots indicate the docking positions for fluorophores.

The samples were embedded onto the prepared and dried coverslips; a 5-microliter sample was uniformly pipetted onto the surface and dried at room temperature in a dark environment. It is important to store these samples carefully while avoiding light.

The sample was placed on the Leica TCS Sp8 STED 3X microscope stage, and an appropriate field of view was selected under the bright field. The loading settings were excitation at 491 nm and 592 nm STED wavelengths for Alexa 488, 561-nanometer laser for Cy3, and 670-nanometer laser for Cy5. The laser wavelengths were chosen to cover the excitation peak spectrum of each dye. To prevent cross-coloring of the three fluorescent dyes, we used one laser at a time to detect each dye. The intensity of the STED laser was set to its maximum power to obtain the best resolution ratio.

III. RESULTS AND DISCUSSION

A. Design and Assembly

As shown in Fig. 2c, our assembly strategy resulted in various subunits, including dual-, triple, quadra-, and penta-assemblies. This reflects inefficient connections due to the increasing difficulty in forming connections with a larger platform. Even when we maintained the sample at 45°C for 2 days, the hexagon yield was markedly low (Supplementary Figure S3).

B. Super-Resolution Fluorescent Tag

STED is the first method that was applied to overcome the diffraction barrier of light [31]. In STED, reduction in the effective fluorescent luminescence area is achieved by stimulated emission effects.

We designed six docking sites at the outer edge of the triangular origami, including one of the three dyes: Alex 488 embedded on a single triangular origami for group one, Cy3 on group two, and Cy5 on group three. The blue dots in Fig. 3 indicate the docking positions for each fluorophore.

After forming the single triangular origami, a 20-fold volume of fluorescent staple strands was added to each corresponding group and annealed at 45°C for 6 h followed by ultrafiltration purification, as described for forming the hexagonal platform. After annealing, the samples were purified through gel electrophoresis (Fig. S3). Representative super-resolution microscopy images are shown in Figure 4.

In addition, we verified the effectiveness of the monochromatic origami platform, displaying clear orange halos for Cy3 (Fig. 4g), red halos for Cy5 (Fig. 4h), and green halos for Alexa 488 (Fig. 4i), demonstrating the attachment of the dyes.
Fig. 4. STED super-resolution microscopy images. (a–c) Tricolored origami platform with Alex 488, Cy3, and Cy5 attached, shown at three scales: (a) scale bar = 6 µm, (b) scale bar = 3 µm, and (c) scale bar = 1 µm. (d) Cy5 attached (red); scale bar = 500 nm. (e) Alexa 488 attached (green); scale bar = 500 nm. (f) Bicolored origami platform combining Cy5 and Alexa 488; scale bar = 500 nm. (g–i) Monochromatic platforms with only (g) Cy3 (scale bar = 6 µm), (h) Cy5 (scale bar = 2 µm), and (i) Alexa 488 (scale bar = 2 µm) attached.

Fig. 5. Three different topological labels. (a) Striped-shaped label. (b) Rectangular-shaped label. (c) Triangular-shaped label.

to the platform. Figure 4(d) shows three Cy5 fluorophores separately docked on the platform, appearing as three separate red dots. Figure 4(h) shows six Cy5 fluorophores appearing as a red circle. The arrangement is the same for Alex 488, shown in Figs. 4(e) and (i).

C. Topological Labeling

As mentioned above, the outside edge of the triangular origami was loosened. We programmed three different topological labels for the platform: triangular, rectangular, and stripe-shaped labels. Figure 5 shows a schematic of each design.

In the topological labeling experiment, the three different substructures were assigned to each group, which were annealed, purified separately, and mixed at a 1:1:1 ratio. The last step involved refining the topological labels by refining the hexagonal origami from the gel brick. The AFM image of the refined sample is shown in Fig. 6 and Supplementary Fig. S4.

As shown in Fig. 6, some subunits remain in the sample, indicating structures that are not successfully purified. This could lead to a negative effect on the calibration. Nonetheless, in the STED images, all the platforms appear as circular halos with different colors docked separately, and the hexagonal platform could still be distinguished from the subunits in the AFM images.

Additionally, purification with gel electrophoresis demonstrated that most of the structures were hexagonal platforms. In a larger scan image, the topological figures appeared as six dots around the platform. These topological labels were linked at the center of the edges with a precise distance. As shown in Figure 6(c), the green lines indicate the distances, which range between 110 and 120 nm (Supplementary figure. S6).

Notably, there were some distortions in the substructures. The stripe-shaped label was folded into a “V” shape because of the instability of this structure, whereas the triangular substructure was distorted as a circle. These distortions resulted from thermodynamic randomness and the instability of the substructures assembled from a few staple strands (<500 nucleotides).

IV. CONCLUSION

In this study, we combined fluorescent nanotags with topological labels to form a dual-nanotag platform. The fluorescent characteristics were detected using a STED super-resolution microscope, and the topological labels were identified using AFM. The separation of origami multimers of different orders was the primary concern in this study. As observed in the gel extraction results, single origami and misfolded structures remain in the sample. Nondestructive purification methods, such as the gel gradient method [34] and size-exclusion chromatography [35], may be helpful.

These DNA origamis were observed in solution using the liquid-phase mode, which will cause a certain degree of flexibility and deformation in the assembled hexagonal structure. Since each component of the hexagonal structure was based on the same triangular origami scheme, the basic topological calibration of triangular origami was obtained both with the analysis of statistical data of experimental samples and theoretical calculation of the designed triangular origami scheme, thus minimizing the influence of flexibility and deformation on the measurement accuracy of the assembled hexagonal structure. Further, the calibration system used in the present study was based on the large-scale self-assembly of DNA...
origami with high precision detection (∼1 nm) using AFM. Therefore, the calibration accuracy of fluorescence microscopy images could be guaranteed.

This strategy offers a dual method for calibrating nanoscale samples. Recently, dual in situ calibration has emerged as a research hotspot for the design of nanorulers [36], [37], and many researchers and companies are actively engaged in manufacturing in situ microscopes. Although our experiments had some limitations, most notably due to the remnant sub-units indicating imperfect purification, we believe this strategy offers the possibility of realizing spectrum- and topology-based nanotag calibration. Further studies can be conducted based on our results to achieve improvements.

DATA AVAILABILITY STATEMENT
All data generated and analysed during this study are included in the published article and Supplementary Information files.

COMPETING INTERESTS
There are no conflicts to declare.

AUTHOR CONTRIBUTION
Xiaolong Shi, Jin Xu, and Xin Li designed the experiments. Congzhou Chen, Yihao Zhang, and Xiaolong Shi wrote the manuscript.

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