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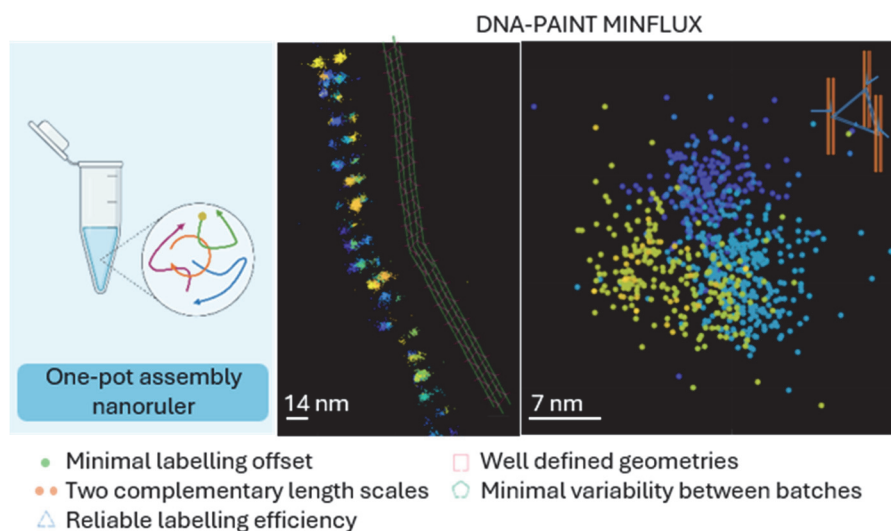
Rigidified and modular wireframe DNA nanotubes as nanorulers to validate super resolution imaging techniques

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ToC. Rigid wireframe DNA nanotube (DxNT) nanorulers are assembled in one pot from only four or five short DNA strands. DNA-PAINT MINFLUX resolves individual binding sites, revealing 13-14 nm inter-subunit and 7-8 nm intra-binding distances. Each color represents localization events assigned to an individual binding site. Their simple assembly and precisely defined nanoscale geometry make DxNTs attractive standards for benchmarking super-resolution fluorescence microscopy.

Abstract

Super resolution microscopy has enabled nanoscale imaging with high spatial and temporal precision. Reliable benchmarking materials are essential for validating performance across instruments and experimental protocols, yet current nanorulers, including protein-, viral-, and DNA origami-based systems, face challenges in stability, labeling efficiency, or fabrication complexity. Here, we present a rigid wireframe DNA nanotube (DxNT) and its subunits as a minimal, well-defined nanoruler assembled from only four or five short DNA strands. Using DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) and DNA-PAINT MINFLUX, we measured precise structural parameters: 13-14 nm inter-subunit spacing and 7-8 nm intra-subunit distances, with a persistence length of approximately 1.6 μm , confirming exceptional rigidity. DxNTs exhibit intrinsic high labeling efficiency, low labeling offset, and broad compatibility with diverse labeling and imaging strategies. Their modular design allows benchmarking across multiple spatial scales, establishing DxNTs as versatile, robust and biologically compatible standards for next-generation super resolution microscopy.

Keywords: super-resolution microscopy; fluorescence microscopy; nanorulers; DNA nanotechnology; DNA nanomaterials; DNA-PAINT; MINFLUX

Main

In recent years, advances in super resolution imaging techniques and instrumentation have enabled unprecedented spatial and temporal resolution, allowing detailed structural characterization and dynamic monitoring of materials and biologically relevant processes.¹⁻⁴ In particular, Single Molecule Localization Microscopy (SMLM) techniques have proven highly versatile across diverse sample types, achieving ever-increasing resolution that can be further enhanced by advanced instrumentation such as MINFLUX.⁵⁻⁸ In both approaches, individual fluorescent molecules are precisely localized through repeated stochastic on–off cycles, with a key difference in how the emitters' positions are determined. In SMLM, single fluorophores are localized by determining the centroid of the fluorescence point spread function (PSF) as captured directly by the camera.⁹ For MINFLUX, each emitter is located by measuring fluorescence intensity relative to a spatially structured excitation pattern, typically a doughnut-shaped laser beam with a central intensity minimum. By iteratively positioning this minimum near the emitter, MINFLUX achieves higher localization precision using far fewer detected photons than conventional localization methods.¹⁰

Within SMLM techniques, DNA-PAINT (DNA Point Accumulation for Imaging in Nanoscale Topography) can be performed on a conventional widefield microscope or integrated with a MINFLUX system for higher spatial precision.¹¹⁻¹³ This method relies on the transient binding kinetics between complementary dye-labeled imager strands freely diffusing in the buffer and docking DNA strands attached to the sample generating the stochastic binding events required for precise emitter localization. As with any other SMLM techniques, this method requires optimization of labeling approaches that maximize labeling efficiency while minimizing linkage error, as low offset values ensure that the fluorophore reports a position that closely matches the intended structural geometry, reducing distance errors caused by linkers.¹⁴

To evaluate the performance and resolution limits of new imaging approaches and emerging instrumentation, several materials have surfaced to facilitate benchmarking. These standards, or nanorulers, provide a well-defined geometric arrangement of fluorophores, enabling correlation between measured emitter localizations and their true spatial position, thereby validating localization accuracy. A number of systems have been used as nanometer-scale benchmarks for MINFLUX imaging, including nuclear pore complexes (NPCs)^{11, 15-18},

microtubules^{19, 20} and DNA origami-based nanostructures.^{10, 15, 21-25} However, these systems have some inherent limitations usually related to their base material (usually a biological substrate or DNA-based scaffolds). Common challenges include difficulty in measuring distances below 20 nm because of structural physical limitations, low labeling efficiency, reliance on specific or costly dyes (such as technique-specific fluorophores, or antibody-based labeling), and the need for cell culture facilities. And even in some cases, distance measurements must also account for structural distortions or warping caused by how or where the material is expressed.²⁶⁻²⁸ With this in consideration, an ideal nanoruler should fulfill several key criteria: (i) a well-defined and precisely spaced arrangement of fluorophores at the nanometer scale, (ii) high structural rigidity and stability to ensure reproducible measurements, (iii) compatibility with commonly used fluorophores, and labeling strategies, ensuring reliable labeling efficiency, while having a low linkage error (iv) compatibility with different imaging modalities, (v) simple and minimal assembly requirements, and (vi) stable surface attachment. Currently, not all these criteria are simultaneously fulfilled by the existing or newly developed reference structures.²⁹⁻³¹

In this study, we demonstrate the use of a wireframe DNA nanotube design (DxNT), rigidified through the incorporation of double crossover (DX) tile motifs within each subunit pillar, as a versatile and robust super resolution nanoruler.^{32, 33} The simplicity and modularity of the design allow the formation of either full-length nanotubes (DxNTs) or their discrete subunits (rungs) using only a few unique DNA sequences. By controlling the stoichiometry of these sequences, both nanotubes and rungs can be generated with triangular, square, or pentagonal cross sections (Fig. 1A). We also establish a simple, reproducible sample preparation and imaging workflow that provides ready-to-use nanorulers suitable for routine microscope calibration and benchmarking (Fig. 1B). To ensure that fluorophore positions accurately reflect the intended geometry, we first confirmed the rigidity of the material by calculating the persistence length from DNA PAINT reconstructions (Fig. 1C). DNA-PAINT MINFLUX reconstructions (Fig. 1 D-E) further resolved inter-subunit distances averaging 13-14 nm and inter-binding site separations of 7-8 nm across all cross sections. Importantly, efficient surface immobilization was achieved by incorporating biotin at the end of one DNA sequence, while labeling offset was minimized by incorporating the docking overhang directly into the T2 sequence, eliminating the need for external labeling moieties such as antibodies and ensuring high labeling efficiency through full strand incorporation during assembly. The versatility of the DxNTs was further demonstrated by their

compatibility with two imaging modalities and multiple fluorophores, including ATTO633, ATTO655, and Cy3B, requiring only minimal adjustments, which also highlights its robustness with reproducible assembly and structural stability under diverse experimental conditions.

Together, these measurements confirm that DxNTs and their subunits are structurally reproducible with minimal material complexity³², positioning DxNTs as a highly desirable choice to benchmark super resolution fluorescence imaging setups, such as DNA-PAINT in a conventional widefield microscope, as well as in a MINIFLUX setup.

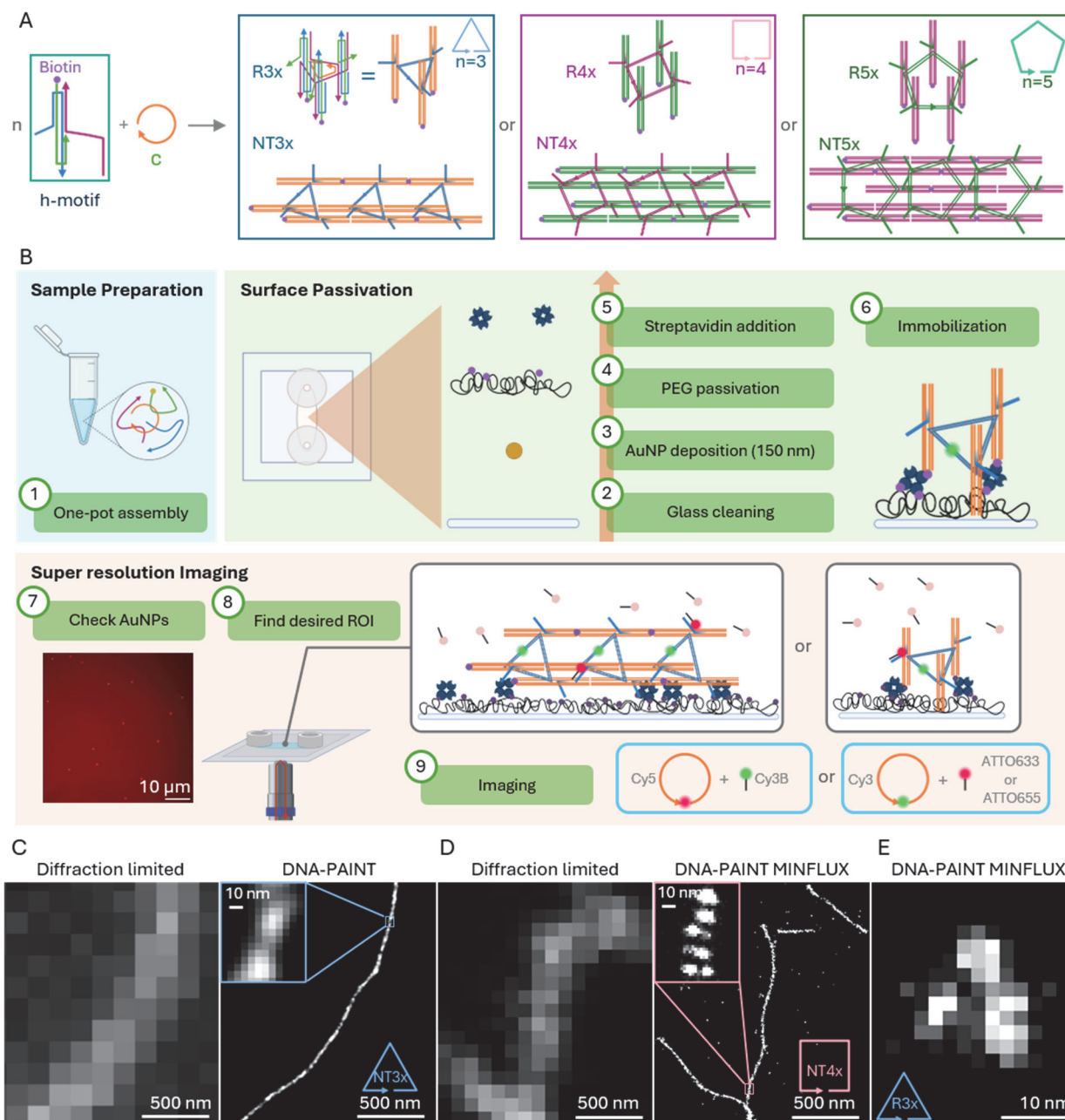


Figure 1. Design, labeling, and imaging of DxNT nanorulers. (A) DxNTs are constructed from n equivalents of the h-motif (teal box), whose pillars are linked by a labeled core strand (C-strand, orange) to form polygonal subunits with triangular, square, or pentagonal cross sections ($n = 3-5$). These subunits can assemble into individual rungs (R3x, R4x, and R5x) or polymerize into full nanotubes (NT3x, NT4x, and NT5x). Biotin sites used for surface immobilization are indicated by purple circles. Short extensions from each pillar represent docking overhangs for DNA-PAINT imaging, resulting in 3, 4, or 5 docking overhangs per subunit for the triangular, square, and pentagonal designs, respectively. (B) Sample preparation and imaging workflow: (i) one-pot assembly of rungs or DxNTs; surface preparation comprising (ii) glass coverslip cleaning, (iii) deposition of 150 nm gold nanoparticles (AuNPs), (iv) PEG passivation, (v) streptavidin functionalization, and (vi) sample immobilization in the imaging chamber; followed by (vii) selection of suitable AuNP fiducials, (viii) identification of regions of interest (ROIs) by localization of the Cy3- or Cy5-labeled nanostructures, with the fluorophore selected according to the imager-strand fluorophore, and (ix) DNA-PAINT data acquisition with Cy3B, ATTO633 or ATTO655 labeled imager strand. (C) Representative DNA-PAINT reconstruction of Cy5-labeled NT3x using Cy3B-labeled imager strands, and its corresponding diffraction-limited total internal reflection fluorescence (TIRF) image. (D) Representative DNA-PAINT MINFLUX reconstruction of Cy3-labeled NT4x with its matched confocal image. (E) Representative DNA-PAINT MINFLUX reconstruction of Cy3-labeled R3x showing the expected triangular cross section. MINFLUX reconstructions in panels D and E were obtained using ATTO655 labeled imagers.

Results

Design, labeling and general imaging workflow of DNA nanoruler

In this study, we characterized six constructs in total, comprising three DxNTs and three rungs, each corresponding to slightly modified versions of previously reported designs from our group and distinguished by their triangular, square, or pentagonal cross sections. Each construct is based on a wireframe DNA nanotube design in which repeating rigid h-motif units form the tube walls (composed of the H, T1, and T2 strands), while a central core strand (C-strand) of variable length defines the polygonal cross section of each subunit. By varying the length and connectivity of the C-strand, we generated triangular ($n = 3$), square ($n = 4$), and pentagonal ($n = 5$) subunits (Fig. 1A, Table S1 with DNA sequences).³²

To adapt the structures for DNA-PAINT, the T2 strand was modified at the 5'-end to introduce one docking overhang per pillar, with positions optimized to prevent interference with nanotube or rung assembly during annealing, as validated by AFM (Fig. S1). As the docking overhangs are integrated into the building block sequence design, the resulting structures exhibit intrinsically high labeling efficiency and minimal labeling offset allowing the imager-bound fluorophore to report positions closer to the true nanotube geometry. An additional modification involved adding biotin at the 5'-end of the T1 strand to enable surface immobilization through biotin-streptavidin

interactions. Samples consisting of individual rungs were fully biotinylated to orient the plane of the rung parallel to the surface. In turn, DxNTs were prepared using a 50:50 mixture of biotinylated and non-biotinylated T1 strands, which allowed stable attachment of the nanotubes to the surface while preserving their ability to polymerize into micron long nanotubes. This configuration ensured that the DxNTs could relax into their straightest conformation, facilitating downstream data analysis.

The general experimental workflow is illustrated in Fig. 1B, and to our knowledge, this represents one of the simplest reported protocols for producing super resolution nanorulers. Assembly was achieved through a one-pot annealing reaction, requiring only a minimal set of synthetic DNA strands: five distinct sequences for DxNT samples, and four for rung samples. The quality of the rung samples was verified by denaturing PAGE, which revealed a single product band bearing the selected fluorophore, Cy3 or Cy5 (Fig. S1).

For imaging, assembled nanotubes or individual rungs were immobilized on PEG-passivated glass coverslips via biotin-streptavidin interactions, to ensure consistent and reproducible measurements over extended periods of time. The imaging protocol included a pre-localization step to identify regions of interest (ROIs) on either the Cy3 or Cy5 channel - whichever was orthogonally labeled relative to the imager strand - before initiating data acquisition. Depending on the imaging modality, diffraction-limited images were collected either in TIRF mode for DNA-PAINT or in confocal scanning mode for DNA-PAINT MINFLUX. Super resolution data were then acquired using a 9-nucleotide imager strand (TAGATGTAT)^{13, 34-36} labeled with Cy3B, ATTO633 or ATTO655, providing wavelength flexibility and enabling multiplexed experiments.

As shown in Fig. 1C-E, DNA-PAINT reconstructions spatially resolved single DxNTs (Fig. 1C), enabling quantitative characterization of their rigidity. Using the same imaging strategy on a MINFLUX microscope, we achieved an additional level of structural detail, resolving longitudinal features along the nanotube axis (Fig. 1D) as well as cross-sectional geometries consistent with the expected three-, four- and five-pillar rung architectures (Fig. 1E). These results demonstrate the versatility, reproducibility, and structural precision of the DxNT platform, which will be explored in greater detail below.

Persistence length analysis of DxNTs based on DNA-PAINT reconstructions

To demonstrate the versatility of our nanoruler design, we first evaluated the DxNTs' performance in DNA-PAINT experiments conducted on a TIRF-modality widefield microscope and using imager strands labeled with different commercially available dyes and targeting two docking overhang positions (Fig. 2A-D). Consistent with previous studies employing concatenated docking sequences (repeating motifs of up to 35 nt)³⁷, we did not observe any significant changes in localization precision arising from positioning of the fluorophore at either end of the imager strand (Table S2).

Resolution was assessed by measuring the full width at half maximum (FWHM) of ~30 DxNTs in the field of view using the image correlation spectroscopy (ICS) analysis tool. FWHM values of (37.3 ± 0.7) , (45.4 ± 0.5) , and (59 ± 1) nm were obtained for Cy3B, ATTO633 and ATTO655, respectively (resolution information found in Table S2). The observed image resolution was consistent with the number of detected photons per blink obtained from the fluorescence trajectories. Values ranged from (5500 ± 400) for Cy3B, (4000 ± 500) for ATTO633, and (2000 ± 400) for ATTO655, corresponding to localization precision of (9 ± 2) , (11 ± 2) , and (19 ± 5) nm respectively. As expected, the higher photon yield of Cy3B produced the best localization precision and the highest reconstructed image resolution. In terms of localization density, the fluorescence trajectories exhibited different numbers of detected binding events, with 1026, 778, 262 peaks for Cy3B, ATTO633 and ATTO655 respectively. Despite using the same imager concentration, docking sequence, and acquisition time for all experiments, the number of binding events differed among fluorophores. This difference likely reflects fluorophore-dependent experimental factors rather than differences in DNA hybridization kinetics. A potential contributing factor is increased non-specific adsorption ("sticking") of imager strands (containing ATTO dyes) to the sample or surface, which would reduce the effective concentration of freely diffusing imagers available for productive binding. Although Tween-20 was included in the imaging buffer to minimize non-specific interactions, this effect could still contribute to the reduced localization density. Fluorescence intensity profiles along the nanotube centerline revealed a recurring signal approximately every 30-40 nm and, occasionally, pairs of signals separated by about 50-60 nm (Fig. S2). The latter, in a physical sense, could signify a missing localization corresponding to one rung along the nanotube axis. The consistent 30-40 nm

periodicity demonstrates the nanoruler capability of DxNTs and confirms that they can be used to benchmark imaging performance.

To evaluate the reproducibility of reconstructions across multiple fluorophores we conducted experiments labeling the DxNT core with Cy3 and then re-imaging the same region of interest after washing and exchanging the imaging buffer to sequentially introduce ATTO633- and ATTO655-labeled imager strands. Each imaging cycle yielded consistent structural reconstructions (Fig. 2B-D), demonstrating that the DxNT architecture is inherently robust to labeling variations and tolerant of multiple rounds of imaging.

After confirming the multi-labeling reproducibility and resolution of the reconstructions, we next quantified the DxNTs' rigidity, an essential property for any nanoruler, as it ensures that fluorophore positions remain fixed and reproducible, providing a stable reference. For this, we measured the contour length and end-to-end distance for multiple DxNTs representing all cross sections and imager dyes. An example zoom-in for a single NT4x is shown in Fig. 2E. The data were fitted using the Worm-like Chain (WLC) model to determine the persistence length as a measure of mechanical rigidity (Fig. 2F).³⁸ Persistence length measurements were consistent across all imager dyes and independent of docking strand orientation relative to the nanotube center, confirming robustness of the results (Table S3). Using the top-performing Cy3B imager, population-level WLC fits for DxNTs with triangular (NT3x, $N = 275$), square (NT4x, $N = 202$), and pentagonal (NT5x, $N = 84$) cross-sections yielded persistence lengths of $(1.7 \pm 0.1) \mu\text{m}$, $(1.6 \pm 0.1) \mu\text{m}$, and $(1.6 \pm 0.1) \mu\text{m}$, respectively (Fig. 2F-G). These values indicate a substantial enhancement in rigidity compared to double-stranded DNA, corresponding to a 30-40-fold increase, establishing DxNTs among the most rigid DNA wireframe materials reported to date.³⁹ Compared with other DNA nanotubes, DxNTs, particularly NT3x, achieve high rigidity with minimal material requirements and persistence lengths comparable to larger DNA tile and RNA origami nanotubes (Fig. 2G). The use of high-resolution DNA-PAINT reconstructions further increases confidence in the calculated values by enabling precise contour tracing and persistence length determination. Collectively, these findings demonstrate that DxNTs are exceptionally rigid, reproducible nanomaterials that maintain consistent mechanical and imaging performance across multiple fluorophores and DNA PAINT conditions.

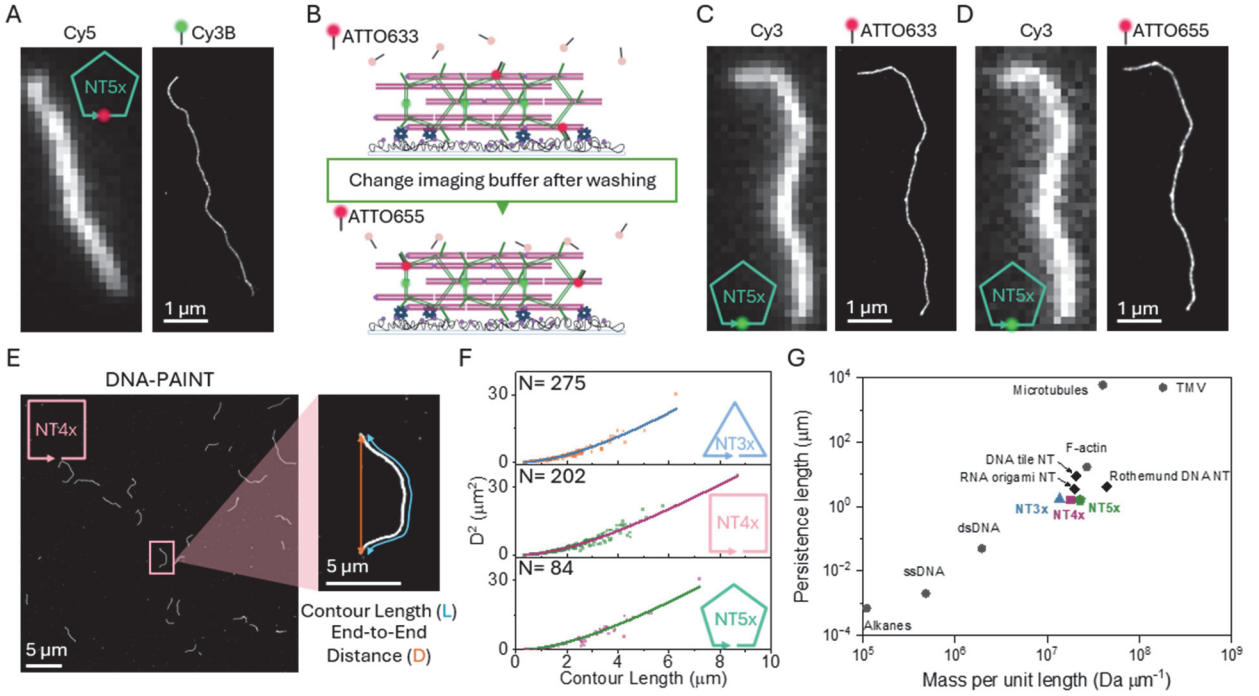


Figure 2. Characterization of DxNTs using DNA-PAINT reconstructions for persistence length measurements. (A) NT5x DNA-PAINT reconstruction with corresponding diffraction-limited TIRF image acquired using a Cy3B-labeled imager strand. (B) Schematic of the experimental workflow showing imaging of a given ROI in panels C and D, where the imager dye is switched from ATTO633 to ATTO655. (C) NT5x DNA-PAINT reconstruction with corresponding diffraction-limited TIRF image employing ATTO633 as the imager fluorophore. (D) DNA-PAINT reconstruction of NT5x with corresponding diffraction-limited TIRF image using ATTO655 after washing and exchanging the imaging buffer in the same ROI. (E) Schematic illustrating the extraction of contour length (in light blue) and end-to-end distance (in orange) from a DNA-PAINT reconstruction, shown with a zoom-in of a single NT4x sample. (F) Worm-like chain (WLC) model fits used to calculate persistence length for nanotubes with triangular (NT3x), square (NT4x), and pentagonal (NT5x) cross sections, with N indicating the number of nanotubes analyzed for each fit. (G) Calculated persistence length (~ 1.6 - $1.7 \mu\text{m}$) for each cross section DxNT compared to several materials including alkane chains⁴⁰, ssDNA⁴¹, dsDNA⁴², F-actin and microtubulin⁴³, and tobacco mosaic virus (TMV)⁴⁴. More directly comparable nucleic acid nanostructures include the Rothemund DNA nanotube⁴⁵, DNA tile nanotubes⁴⁶, and an RNA origami nanotube⁴⁷. The plot was adapted from a similar comparison by Bharti *et al.*⁴⁸

DNA-PAINT MINFLUX validation of DxNTs as nanorulers

To demonstrate the utility of DxNTs as nanorulers with nanometer reporting capability, we employed MINFLUX microscopy coupled with the DNA-PAINT protocol, as the spatial resolution achieved in widefield experiments was insufficient to fully resolve the structures. MINFLUX allowed us to investigate whether individual subunits within the DxNTs could be distinctly visualized and, more importantly, whether the inter-subunit distances could be measured

with high accuracy. Previous cryo-EM studies reported this spacing to be between 14.2-14.4 nm across all cross sections, providing a reference for our measurements.³²

Compared to the DNA-PAINT imaging from the previous section, DNA-PAINT MINFLUX required longer acquisition times. To maintain sample integrity, it was therefore essential to fully seal the imaging chambers to prevent buffer evaporation and to ensure robust attachment and stabilization of AuNP fiducials, as detailed in the *Methods* (notably, once sealed with imaging buffer, DxNT samples remained stable for at least two weeks at 4 °C (Fig. S3)). DNA-PAINT MINFLUX reconstructions of DxNTs revealed a striking increase in spatial resolution (Fig. 3A). Individual rungs were clearly resolved along the nanotube axis, and occasional discontinuities (“missing” rungs) became apparent.

To enable systematic analysis of the MINFLUX data, we developed an automated script that identifies individual nanotubes within the full field of view and extracts rung-to-rung distances along the structure (Fig. 3B, SI-section 6.4). For each identified nanotube structure, the algorithm fits a spline through the corresponding skeleton points to define the centerline. Manual adjustments can be applied to correct any misidentified regions without the need to exclude larger regions of the dataset. Once the centerline is defined, the nanotube is linearized by projecting all localizations onto the fitted spline. A moving sum is then computed over the projected localizations to generate a localization-density profile along the tube axis. Localization density peaks associated with detected rung positions were next detected, and inter-peak distances were measured, providing a quantitative readout of nanotube periodicity (Fig. 3C).

The dominant population of inter-rung distances, centered at 13-14 nm, accounted for approximately 78-86% of all measured distances and corresponded to the expected spacing between adjacent rungs. These values are consistent with, although not identical to, those obtained by cryo-EM. This difference may arise from the distinct measurement conditions: cryo-EM distances represent an average across multiple unbound frozen structures, whereas our measurements reflect individual DxNTs interacting with the imaging surface and may therefore capture surface-induced conformations or structural variations (Table S6). A secondary population, centered at approximately 26-28 nm, represented 14-22% of measurements and corresponded to distances spanning one missing subunit.

Analysis of localization data from multiple DxNTs imaged on different days suggests that the larger 26–28 nm spacing does not arise from an intrinsic structural feature of the nanotubes. For example, a representative NT4x imaged for over 8 h (Fig. 3D) shows a relatively constant accumulation of localization events throughout the acquisition, progressively resolving the full structure. Furthermore, the number of consecutive rungs observed before a missing rung follows an exponential distribution (Fig. S5), consistent with a stochastic process rather than a defined structural pattern. Based on these observations, and on the higher relative abundance of the 26–28 nm population in NT3x compared with NT4x and NT5x, we propose the structural model shown in Fig. 3E. The ~13 nm population corresponds to the expected rung-to-rung spacing, whereas the 26–28 nm population is consistent with a missing localization event between adjacent detected rungs. This may result from a combination of insufficient imaging time, as unfilled regions remain after ~5.5 h of acquisition, photodamage or depletion of docking overhangs during prolonged high power imaging, and reduced accessibility of docking overhangs due to strain associated with the intrinsic helicity of the DxNTs and their strong interactions with the streptavidin functionalized surface, as 50% of the T1 strands are biotinylated.

The datasets achieved an average localization precision of 2.6-2.8 nm prior to any data filtering or aggregation, reflecting the overall high quality of data extracted from the DxNTs constructs. Additional data quality metrics are provided in Table S5. Together, these results underscore the ability of DxNTs to serve as reliable nanorulers across multiple fluorescence imaging modalities for benchmarking sub-20-nanometer spatial resolution.

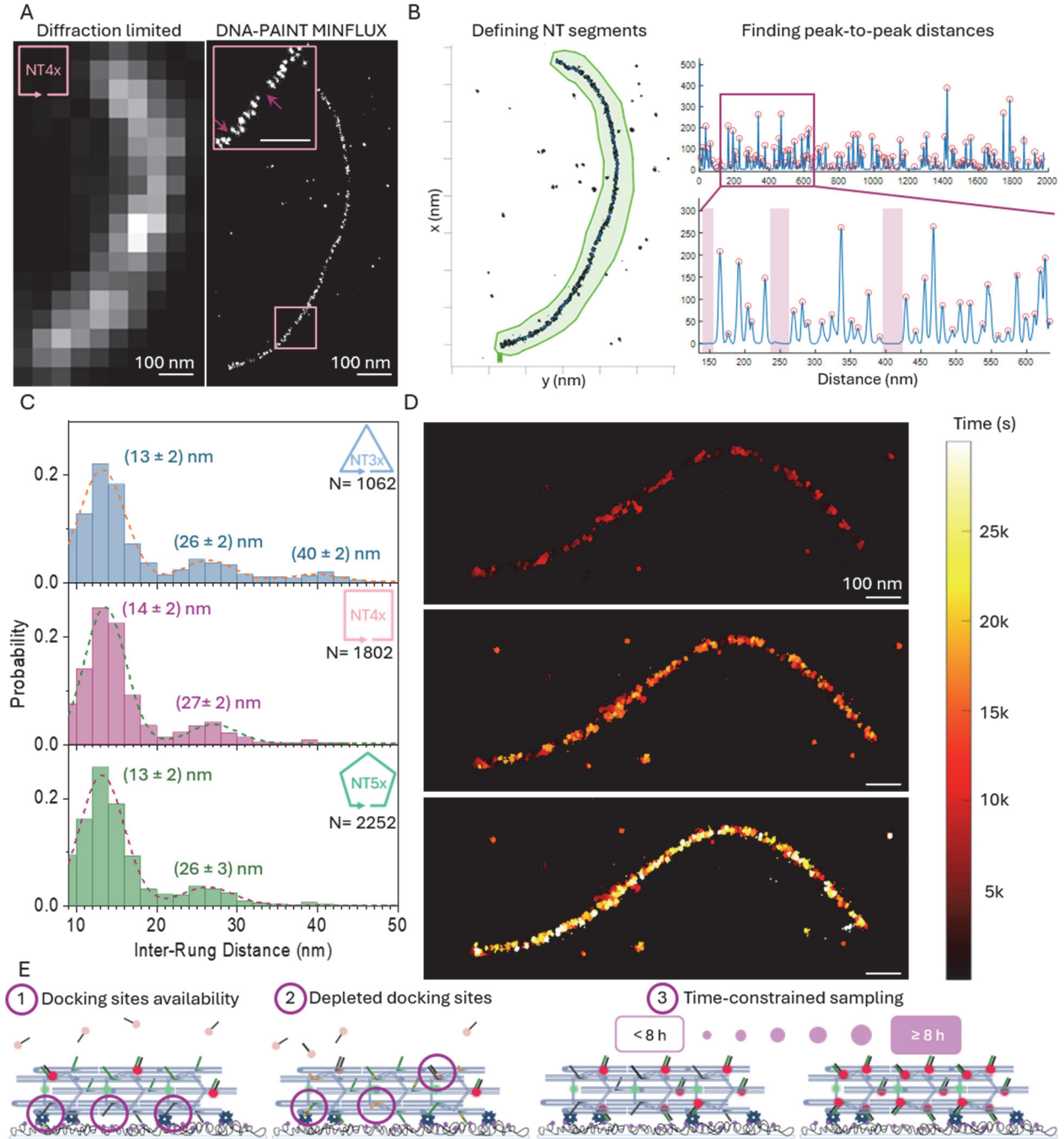


Figure 3. DNA-PAINT MINFLUX analysis of DxNTs as nanorulers. (A) Representative NT4x DNA-PAINT MINFLUX reconstruction with corresponding confocal image. (B) Workflow for inter rung distance analysis along DxNTs. Left: defining the ROIs: tube-like regions are automatically detected but manual ROI definition can also be applied to split nanotubes into sections or exclude regions. Skeleton points defining the nanotube centerline are placed automatically, with the option for manual adjustment or addition. Right: automated visualization of detected peaks along the nanotube, used for peak-to-peak distance calculations. Each peak represents a single rung and regions skipping a rung (peak) are shadowed in pink. (C) Histograms

of inter-rung distances from DNA-PAINT MINFLUX reconstructions showing main populations at 13-14 nm, with a secondary peak at twice that distance across all cross-section geometries studied. The total number of measured distances (N) included in each histogram is indicated on the corresponding plot. **(D)** Time-resolved plotting of individual localizations of a representative NT4x at 10,000 s (top), 20,000 s (middle), and 30,000 s (bottom), displayed using the same time scale (right). Localizations are observed throughout a representative nanotube over more than 8 h of imaging in a region of interest that captured multiple nanotubes, demonstrating its stability and the progressive accumulation of localizations that resolves the structure. **(E)** Schematic illustrating three factors that may contribute to gaps or "missing rungs" along the DxNT. (1) Limited docking-site accessibility due to the intrinsic helicity of DxNTs³² and steric hindrance at the surface can restrict imager strand binding. (2) Progressive photodamage of docking strands during prolonged imaging can permanently eliminate binding sites, decreasing the number of detectable localizations over time. (3) Insufficient acquisition times may prevent all docking sites from being sampled before photodamage occurs.

Individual DxNT rungs as complementary sub-10 nm nanorulers

While examining the MINFLUX reconstructions of DxNTs, we observed that some NT4x and NT5x structures occasionally displayed distinct individual signals within each identified rung. To further explore the potential of individual subunits to function as nanorulers in their own right (Fig. 4), we employed a fully biotinylated rung design that orients the plane of the structures parallel to the imaging surface. This configuration enables precise assessment of fluorophore positioning within the expected triangular, square, and pentagonal geometries of each subunit, expected to be separated from each other by ~ 7-8 nm along the perimeter.

The quality of these datasets was comparable to those obtained from DxNT MINFLUX reconstructions, with an average localization precision between 2.2-2.6 nm. Additional data quality metrics for the rung datasets, in addition to those for the DxNTs, are provided in Table S5, as these datasets were acquired using a different MINFLUX setup. Representative MINFLUX reconstructions of clearly resolved triangular and square rungs are shown in Fig. 4A. Because of the smaller expected inter-binding site distances, a dedicated analysis algorithm was developed to first identify large subclusters within the full field of view, presumably individual rungs, and subsequently detect individual binding sites and determine their centroids, enabling precise extraction of inter-binding distances (Fig. 4B).

Whereas rung-to-rung spacing yielded average distances of approximately 13 nm (Fig. 3C), binding sites within a single rung were positioned in each side, providing an opportunity to

benchmark performance in a more demanding regime. Analysis of all three rung geometries revealed three distinct classes of observations (Fig. 4C). First, a subset of rungs exhibited the expected triangular or square geometries with inter-binding site distances of approximately 6-10 nm, in excellent agreement with the designed structure (Fig. 4A-B). These measurements likely represent optimal immobilization on the surface, in which the rung pillars retain their intended orientation and geometry. Second, distortions of the expected geometry were frequently observed, particularly for R4x and R5x, resulting in deviations from the designed inter-binding site distances. These distortions are consistent with surface-induced tilting of the pillars following immobilization, which alters the apparent geometry of the rung (Fig. 4C and Fig. S6). Finally, some rungs exhibited an incomplete number of resolved binding sites due to one or more missing localization clusters. This behavior was observed across all geometries and most likely results from incomplete sampling or depletion of individual docking sites during extended imaging.

Despite these limitations, the results demonstrate that individual rungs provide a promising complementary nanoruler for benchmarking sub-10 nm distances in super-resolution microscopy.

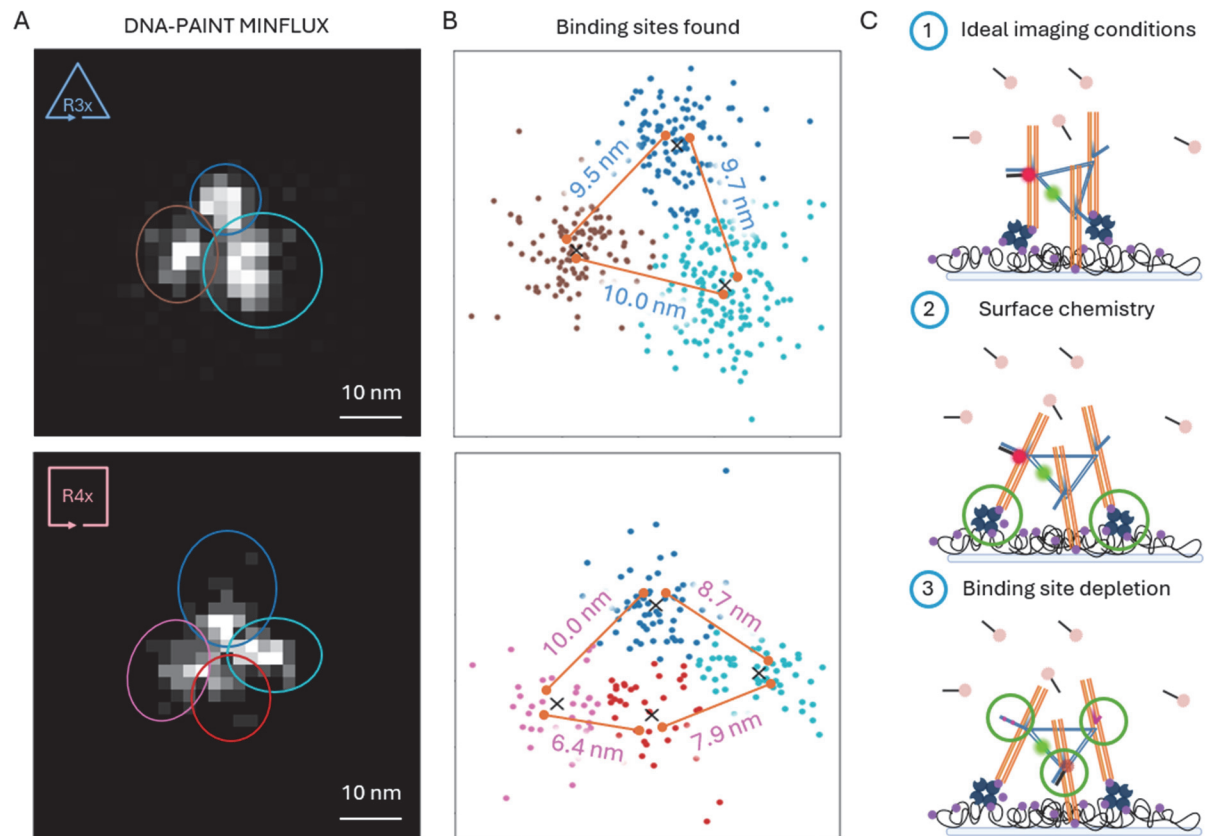


Figure 4. DNA-PAINT MINFLUX approach to validate label positioning accuracy within DxNT subunits. (A) Representative subunit reconstructions for triangular (R3x, top) and square (R4x, bottom) designs. (B) Visualization of localization clusters corresponding to a single rung. Within each rung, individual binding sites are identified, centroid coordinates are calculated, and inter-binding site distances are measured from these centroids. Top, and bottom panels, correspond to triangular (R3x) and square (R4x) designs, with binding sites displayed in distinct colors (3 or 4 per rung). (C) Proposed causes that may contribute to variations in the measured intra-binding site distances. Top: Ideal surface immobilization, in which the nanopillars retain their designed geometry, resulting in inter-binding site distances that closely match the expected values (7-9 nm) and reconstructed images displaying the predicted triangular, square, or pentagonal arrangement of localization clusters. Middle: Distortion arising from surface-induced tilting of the pillars after immobilization, which may alter the apparent geometry and alter the measured inter-binding site distances. Bottom: Depletion of docking sites, resulting in missing localization clusters and incomplete reconstruction of the designed rung geometry.

Discussion

As fluorescence imaging techniques continue to evolve through advances in instrumentation, software, labeling chemistry, and fluorophore photophysics, reliable benchmarks for spatial resolution are increasingly important. Existing standards, such as DNA-origami assemblies, fluorescent protein aggregates, and cell-expressed NPCs, have significantly advanced the field, but remain limited by sub-20-nm resolution, assembly or expression complexity, and batch-to-batch variability, limiting their use for routine and statistically robust benchmarking.

In this work, we designed a system that fulfills many of the essential characteristics of an ideal nanoruler. Automated image analysis of hundreds of nanotubes established a dominant periodicity of about 13-14 nm corresponding to the rung-to-rung spacing. Complementary analysis of individual rungs further resolved inter-binding site separations of approximately 7-8 nm under favorable surface immobilization conditions. The DxNT platform offers an experimentally accessible, structurally rigid, and geometrically well-defined alternative for the calibration and validation of emerging super resolution microscopy methods capable of spanning two spatial regimes, tens of nanometers along the nanotube axis and sub-10 nm scales within single rungs.

DxNTs are wireframe DNA nanotubes assembled in a single one-pot annealing step using only four or five short synthetic DNA sequences, each under 70 base pairs in length, making the process highly straightforward and user-friendly. The synthesis requires only basic laboratory equipment such as a pipette and a thermocycler (PCR), making it broadly accessible without the need for specialized infrastructure. The length of the oligonucleotides makes them compatible with standard commercial modifications, such as biotin for controlled surface immobilization or fluorophore conjugation. This design eliminates the need for bulky targeting molecules such as antibodies and allows flexible placement of docking sequences within the structural framework, reducing labeling offset and improving reconstruction fidelity. In addition, by incorporating the docking overhangs directly into the T2 strand, it ensures a naturally high labeling efficiency.

An important requirement for any nanoruler is structural rigidity, ensuring that measured fluorophore positions accurately represent the designed geometry. The mechanical characterization of DxNTs through DNA-PAINT reconstructions demonstrated a persistence length between 1.6-2.0 μm , placing these structures among the most rigid DNA-based assemblies reported to date, comparable to systems that require significantly more material and complex

sequence designs.⁴⁹ Although many DNA-based assemblies depend on high magnesium concentrations for stability, previous work from our group has shown that DxNTs remain stable under biologically relevant magnesium chloride concentrations (provided they are first generated in 12.5mM Mg²⁺, followed by buffer exchange once they are assembled). Even greater rigidity and chemical stability could be achieved, if required, through UV crosslinking or disulfide bond formation, depending on the specific imaging conditions.^{50, 51}

The simplicity of the DxNT design also translates into practical advantages for routine microscopy calibration. We developed a fully assembled imaging chamber containing immobilized DxNTs, imaging buffer with imager strands, and fiducial gold nanoparticles, providing a ready to use calibration sample that can be repeatedly imaged without additional sample preparation. When properly sealed, samples remained structurally stable and retained their fluorescence performance for at least two weeks when stored at 4 °C and for up to one week at room temperature without noticeable degradation. Despite these advantages, prolonged illumination progressively reduced the number of active docking sites, likely through photodamage. Imaging sessions shorter than 8 h therefore provide sufficient localization density while minimizing docking site depletion. Importantly, properly sealed samples can be reused over multiple shorter imaging sessions of approximately 2 h without noticeable loss of performance.

An important advantage of DNA-PAINT MINFLUX was its ability to distinguish true missing localization events from apparent gaps caused by insufficient localization density. While conventional DNA-PAINT revealed enlarged inter-rung spacings, MINFLUX demonstrated that these gaps arise from the complete absence of localization clusters. The stochastic distribution of these events argues against an intrinsic structural feature of the DxNTs and instead suggests that they primarily originate from experimental factors, such as limited docking-site accessibility following surface immobilization, progressive photodamage during prolonged imaging, or incomplete sampling during insufficient acquisition times. Post-processing artifacts and photophysical effects, including HOMO- and hetero-FRET, are unlikely to account for the observed missing localization events. Further improvements in surface immobilization, such as pre-orienting the rungs using a dimer configuration to better preserve the designed geometry around the docking sites, together with the use of faster-binding imager strands to improve

docking-site sampling and reduce acquisition times, may further enhance the performance of DxNTs as quantitative nanorulers.

Together, our results position DxNTs and its subunits as a new class of DNA-based nanorulers that combine ease of assembly, biological compatibility, and mechanical robustness with exceptional versatility across super resolution modalities. Beyond their demonstrated compatibility with DNA-PAINT and DNA-PAINT MINFLUX, DxNTs have the potential to serve as calibration standards for emerging nanoscopy approaches.⁵²⁻⁵⁷

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon reasonable request. Additional experimental data and supporting analyses are provided in the Supporting Information accompanying this article.

The custom analysis code and example datasets required to reproduce the reported analysis workflows are available at <https://github.com/GCosaLab?tab=repositories>.

Conflict of interest

The authors declare no competing interests.

Author Approval

All authors have reviewed and approved the submitted version of the manuscript and agree to its submission for publication.

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