

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

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1. Materials

The reagents used for buffer preparation and polyacrylamide gel electrophoresis (PAGE) to assess DNA purity and self-assembly quality were primarily purchased from Bio Basic Inc. These included 40% acrylamide/bis-acrylamide (19:1) solution, boric acid, tris(hydroxymethyl)aminomethane (Tris), EDTA, urea, magnesium chloride, and GelRed stain. Acetic acid was purchased from Fisher Scientific.

All nanomaterial samples were prepared and annealed in TAMg buffer (40 mM Tris, 12.5 mM MgCl₂, pH 8.0), which was prepared from filtered 1 M Tris (pH 8.0) and 1 M MgCl₂ stock solutions (Invitrogen) using molecular biology-grade water (Hyclone, Cytiva).

For glass surface preparation, sulfuric acid, 30% hydrogen peroxide, and streptavidin were purchased from Fisher Scientific. Phosphate-buffered saline (10× PBS) and 5 M NaCl stock solutions were obtained from Cytiva. Poly(ethylene glycol) silane (mPEG-Sil, MW 5000) and biotin-PEG-silane were purchased from Laysan Bio, Inc. Imaging chambers consisted of custom-fabricated components together with adhesive chambers from Grace Bio-Labs, and 150 nm gold nanoparticles were sourced from BBI Solutions.

The imaging buffer contained Tween 20, D-(+)-glucose, glucose oxidase from *Aspergillus niger* (Type VII), and catalase C30 from bovine liver, all purchased from Sigma-Aldrich. Trolox was purchased from Combi-Blocks.

2. Oligonucleotide sequences

All fluorescently labeled DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT). Unlabeled DNA strands were obtained from either IDT or Sigma-Aldrich (Germany). All oligonucleotides were ordered with HPLC purification. Strand concentrations were determined by UV-visible spectrophotometry using a NanoDrop One^C spectrophotometer (Thermo Fisher Scientific) and the sequence-specific extinction coefficients at 260 nm provided by IDT. All sequences have been tested previously^{1,2}, except for the ones containing the docking region.

Table S1. DNA sequences to make all different samples of rungs and DxNT geometries. All sequences are given in the 5' to 3' direction.

Strand name	Length (bp)	Sequence
Ha	70	GTTTCCACTATAGAAGAGCGTAATCTGAGCAGTTGACCATATAGGACGAAGTATGA ATACCAGATGTCGT
Hsc	70	TCAGTCACTATAGAAGAGCGTAATCTGAGCAGTTGACCATATAGGACGAAGTATGA ATACCAGATGTCGT
T1a	60	ACTGATATCTTGGTCCATTTAGGATTACGCTCTTCTATAGTGCATTATCTATGTAAGT AT
T1a-biotin	60	Biotin/ ACTGATATCTTGGTCCATTTAGGATTACGCTCTTCTATAGTGCATTATCTATGT AAGTAT
T2 5'-dock	70	ATACATCTATTCTAAATGGACCAAGATAATCTGGTATTCATACTTCGATACTTACATA GATAATGACGAC
T2 3'-dock	70	CTAAATGGACCAAGATAATCTGGTATTCATACTTCGATACTTACATAGATAATGACG ACTTATACATCTA
C3	63	Cy3orCy5/TCAACTGCTCATCCTATATGGTCAACTGCTCATCCTATATGGTCAACTGCT CATCCTATATGG
C4	84	Cy3orCy5/TCAACTGCTCATCCTATATGGTCAACTGCTCATCCTATATGGTCAACTGCTC ATCCTATATGGTCAACTGCTCATCCTATATGG
C5	105	Cy3orCy5/TCAACTGCTCATCCTATATGGTCAACTGCTCATCCTATATGGTCAACTGCTC ATCCTATATGGTCAACTGCTCATCCTATATGGTCAACTGCTCATCCTATATGG
Imager – P1	9	TAGATGTAT/Cy3B
	9	ATTO633orATTO655/TAGATGTAT

3. Preparation of DNA nanotubes and rungs

3.1. Rungs

The rungs are comprised of a mixture of the Ha, T1a-Biotin, T2 5'-dock, and any of the Cy3-labeled core strands (C3, C4 or C5). Depending on the cross section wanted, either three, four or five equivalents of the Ha, T1a-Biotin and T2 5'-dock strands were mixed with one equivalent of the C3, C4, or C5 strand, respectively. All mixtures were annealed by heating to 95 °C then slowly cooled down to 4 °C over 14 h to give a quantitative yield.

The purity of individual DNA strands was assessed by denaturing PAGE. Denaturing gels containing 4 M urea were prepared in 1x TBE buffer and run at room temperature at a constant voltage of 100 V. Following electrophoresis, the gels were stained with GelRed in 1x TBE before imaging. And after assembly, the quality was assessed by native PAGE. Native gels containing 15% glycerol were prepared in 1x TAMg buffer and run at 4 °C at a constant voltage of 100 V. After electrophoresis, the gels were stained with GelRed in 1x TAMg before imaging (**Fig. S1A and S1B**).

3.2. DxNTs

The DxNTs were assembled using a mixture in which 20% of the T1a strands were biotinylated to enable surface immobilization through streptavidin. Accordingly, each DxNT assembly consisted of Hsc, T1a, T1a-Biotin, either T2 5'-dock or T2 3'-dock, and one of the labeled core strands (C3, C4, or C5). As with the rung assemblies, the strand stoichiometry depended on the desired nanotube cross section. Three, four, or five equivalents of Hsc, T1a/T1a-Biotin (with 20% of the total T1a strands replaced by T1a-Biotin), and either T2 5'-dock or T2 3'-dock were mixed with one equivalent of the C3, C4, or C5 strand, respectively. The mixtures were annealed by heating to 95 °C, followed by slow cooling to 4 °C over 14 h, resulting in quantitative assembly yields.

Further confirmation of DxNT assembly with addition of the overhangs for docking of the imager strand were done with AFM imaging. After annealing the samples at a concentration of 500 nM. A 4 μ L aliquot was deposited onto a freshly cleaved mica surface mounted on a steel puck and allowed to adsorb for 30 s. The surface was washed three times with 30 μ L of filtered Milli-Q water. After each wash, excess liquid was removed by gently dabbing the side of the puck with a Kimwipe. After the final wash, residual liquid was removed using compressed air. Samples were dried overnight in a desiccator prior to imaging. AFM imaging was performed in ambient air using a MultiMode8™ SPM equipped with a Veeco NanoScope™ controller. A ScanAsyst-Air triangular silicon nitride probe ($k = 0.4$ N/m, $f_0 = 70$ kHz; Bruker) was used in tapping mode. Images were acquired at a scan rate of 1 Hz with a resolution of 512×512 pixels. AFM images were analysed using NanoScope Analysis (v1.40). Flattening was applied to correct for sample tilt, bow, and scanner drift (**Fig. S1C**).

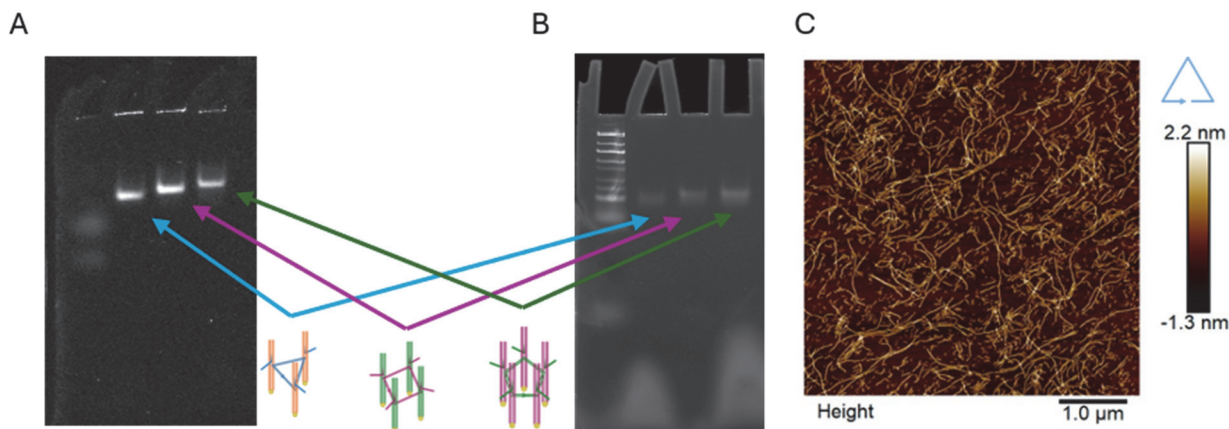


Figure S1. Assembly of each rung for DNA-PAINT. (A) 5% native PAGE in 1xTAMg is shown to illustrate the assembly of the Cy3-labeled triangular (R3x), squared (R4x), and pentagonal (R5x) rungs after annealing. For all, the major product is the fully formed structure. The sample was imaged in the Cy3 channel, (B) and GelRed channel. (C) AFM imaging of NT3x with added docking overhangs was performed to further confirm successful nanotube assembly.

4. Imaging surface preparation

Glass surface preparation was carried out following a modified procedure initially reported by Gidi *et al.*³

Glass coverslips (No. 1 thickness for DNA-PAINT experiments and No. 1.5 thickness for MINFLUX imaging) were cleaned by sonication in piranha solution (30% H₂O₂ and concentrated H₂SO₄, 1:3 v/v) for 90 min. The coverslips were then rinsed three times with molecular biology-grade water (Hyclone, Cytiva) and sonicated for 5 min. To immobilize fiducial markers, the coverslips were rinsed with 10× PBS before incubation with a 1:2 mixture of 150 nm gold nanoparticles (AuNPs) and 5 M NaCl for 10 min. The surfaces were then carefully rinsed with 10× PBS followed by molecular biology-grade water.

The coverslips were dried in an oven at 90 °C for 30 min before a silicone mold was placed on the surface, and continue drying for another 5 minutes. A 99:1 (w/w) mixture of poly(ethylene glycol) silane (mPEG-Sil, MW 5000) and biotin-poly(ethylene glycol) silane (biotin-PEG-Sil, MW 5000) was prepared at 25% (w/w) in anhydrous DMSO and added to the coverslip. Following incubation in the oven for 15 min, the silicone mold was removed, and the coverslips were thoroughly washed with 10× PBS, rinsed with molecular biology-grade water, and dried under a stream of nitrogen.

Imaging chambers were assembled by adhering a Grace Bio-Labs polycarbonate film with an adhesive gasket to the PEG-silane-coated coverslips. Silicone connectors were attached to the pre-drilled inlet and outlet ports using double-sided adhesive tape. Before sample immobilization, 20 µL of a 1 mg/mL streptavidin solution was introduced into the chamber and incubated for 5 min. Excess streptavidin was removed by washing with 20 µL of 1× TAMg buffer prepared with molecular biology-grade water before the DNA sample was introduced.

As a note, for imaging experiments performed at the EMBL Imaging Centre (Heidelberg, Germany), the piranha cleaning step was replaced with oxygen plasma cleaning for 10 minutes, without seeing any major changes.

5. Single-molecule TIRF and DNA-PAINT

Single-molecule fluorescence imaging was performed on a total internal reflection fluorescence (TIRF) microscope based on an inverted microscope (Nikon Eclipse Ti) equipped with a motorized illuminator and a Ti-ND6 Perfect Focus System (PFS). Samples were excited using the evanescent field generated by either a 561 nm or 647 nm diode laser from a monolithic laser combiner (MLC-400B, Agilent Technologies). The excitation beam was adjusted to achieve total internal reflection through an oil-immersion objective (Nikon CFI SR Apochromat TIRF 100×, NA 1.49). In all movies, the laser beam was passed through a multiband clean-up filter (ZET405/488/561/647x, Chroma Technology) and directed to the objective using a multiband dichroic beamsplitter (ZT405/488/561/647rpc, Chroma Technology). Fluorescence emission was collected through a multiband emission filter (ZET405/488/561/647m, Chroma Technology). For Cy3 imaging, an additional emission filter (ET600/50m, Chroma Technology) was placed in the detection path.

Images were acquired using a back-illuminated electron-multiplying charge-coupled device (EMCCD) camera (iXon X3 DU-897-CS0-#BV, Andor Technology). Movies were recorded as 16-bit images over a 512 × 512-pixel region of interest with an exposure time of 300 ms. Image acquisition was controlled using Micro-Manager (version 1.4.13), while microscope hardware was operated through Nikon NIS-Elements software.

5.1. Imaging buffer and conditions

For these experiments, three different imager strands were used, each labeled with a different fluorophore. Cy3B was conjugated to the 3' end of the imager strand, whereas ATTO633 and ATTO655 were conjugated to the 5' end, as described in Section 2.

Samples were excited using either the 647 nm laser at an output power of 28.4 mW [423 W/cm²] or the 561 nm laser at an output power of 25.0 mW [373 W/cm²].

The imaging buffer consisted of 1 nM imager strand, 1% (v/v) Tween 20, 3% (w/v) D-(+)-glucose, 165 U/mL glucose oxidase, 2170 U/mL catalase, and 2 mM Trolox in TAMg buffer supplemented with 100 mM NaCl (40 mM Tris, pH 8.0, 12.5 mM MgCl₂).

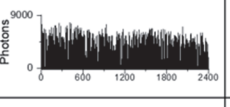
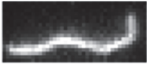
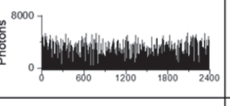
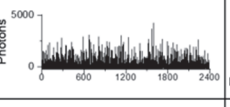
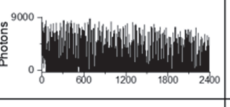
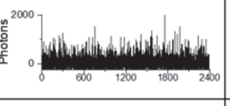
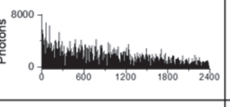
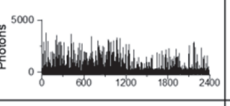
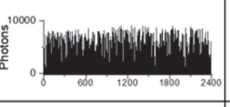
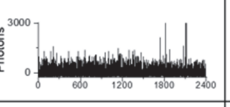
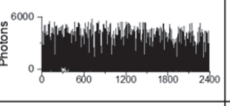
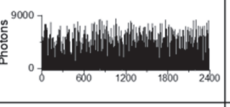
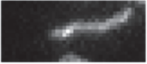
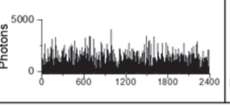
5.2. Data analysis and photophysics

DNA-PAINT analysis. Movies were analyzed with the ThunderSTORM⁴ plugin of Fiji (ImageJ)⁵. The image filtering was performed by applying a Wavelet filter (B-Spline), where the B-Spline order was set to 3 and scale to 2. The approximate localization of individual fluorescence bursts was performed via the local maximum method, with 8-connected neighbourhoods and a peak intensity threshold set to one time the standard deviation of the first wavelet level. The sub-pixel localization of molecules was achieved by fitting a three-pixel radius region with an integrated Gaussian using the weighted least squares fitting method. The initial sigma was chosen to be 1.6 pixels. In the post-processing step, drift correction by tracking fiducial markers (gold nanoparticles) was applied. The lateral tolerance for identification of the marker was set to 40 μm as the maximum distance. Post-processing localization filtering. Localization filtering was performed by analyzing the distributions of key localization parameters, including photon count, spot width (σ), and localization precision (uncertainty_xy). Histograms of these properties were used to identify the primary “true signal” population and exclude outliers, such as shoulders or secondary peaks indicative of multiple populations or poorly fitted localizations. Reconstructed images were rendered as a sum of Normalized Gaussians using ThunderSTORM normalized Gaussian visualization method.

Fluorescence trajectory analysis. Representative fluorescence intensity trajectories were extracted from the raw TIRF image sequences using a custom MATLAB (MathWorks) routine. A 5 × 5 pixel disk-shaped region of interest (ROI) was manually selected along an approximately 800 nm segment of a representative NT5x nanotube, corresponding to roughly 57 pentagonal rungs (five docking overhangs per rung). The fluorescence intensity within the ROI was integrated for every frame to generate photon count versus time trajectories.

The arbitrary EMCCD intensity values were converted to photon counts using a previously established camera calibration obtained under the same analog-to-digital conversion gain and electron multiplication (EM) gain settings. Individual binding events (blinks) were identified using an intensity thresholding algorithm. The background intensity distribution was fitted with a Gaussian function to determine its standard deviation (σ). Fluorescence signals exceeding 5 σ above the background were classified as ON states, whereas signals returning below 1.5 σ were considered transitions to the OFF state. The total number of photons detected per blink was calculated by integrating the photon counts over all frames comprising each ON event. Mean photon counts per blinking event were subsequently determined from all detected binding events within the selected ROI.

Table S2. DNA-PAINT photon statistics and localization performance. Representative fluorescence intensity trajectories (photon counts versus time) extracted from a 5 pixel in diameter disk-shaped region of interest (ROI), corresponding to an ~800 nm segment of a DxNT containing approximately 57 pentagonal rungs (five docking overhangs per rung). Mean photon counts per blinking event, image resolution (FWHM) determined by image correlation spectroscopy (ICS), localization uncertainty and number of blinking events are summarized for Cy3B-, ATTO633-, and ATTO655-labeled imager strands.

TIRF	DNA-PAINT	Photons vs time trace	Sample	Photons/blink	FWHM (ICS)	Localization uncertainty	# Blinks	Exposure Time (ms)
			 Near to core	5500 ± 400	37.3 ± 0.7 nm	9 ± 2 nm	1026	300
			 Re-used region	4000 ± 500	45.4 ± 0.5 nm	11 ± 2 nm	778	300
			 Re-used region	2000 ± 400	59 ± 1 nm	19 ± 5 nm	262	300
			 Away to core	6500 ± 500	38.9 ± 0.5 nm	8 ± 2 nm	859	300
			 Re-used region	693 ± 42	60 ± 1 nm	29 ± 6 nm	96	300
			 Re-used region	2700 ± 160	49 ± 2 nm	11 ± 2 nm	930	100
			 Re-used region	2200 ± 500	52 ± 2 nm	13 ± 3 nm	282	300
			 Away to core	7450 ± 660	42 ± 1 nm	9 ± 2 nm	1090	300
			 Re-used region	1640 ± 90	55 ± 1 nm	28 ± 6 nm	449	100
			 Re-used region	3290 ± 300	53.3 ± 0.5 nm	13 ± 3 nm	855	300
			 Away to core	4600 ± 165	34.3 ± 0.6 nm	8 ± 2 nm	1043	300
			 Re-used region	1800 ± 400	53.8 ± 0.3 nm	18 ± 4 nm	485	300

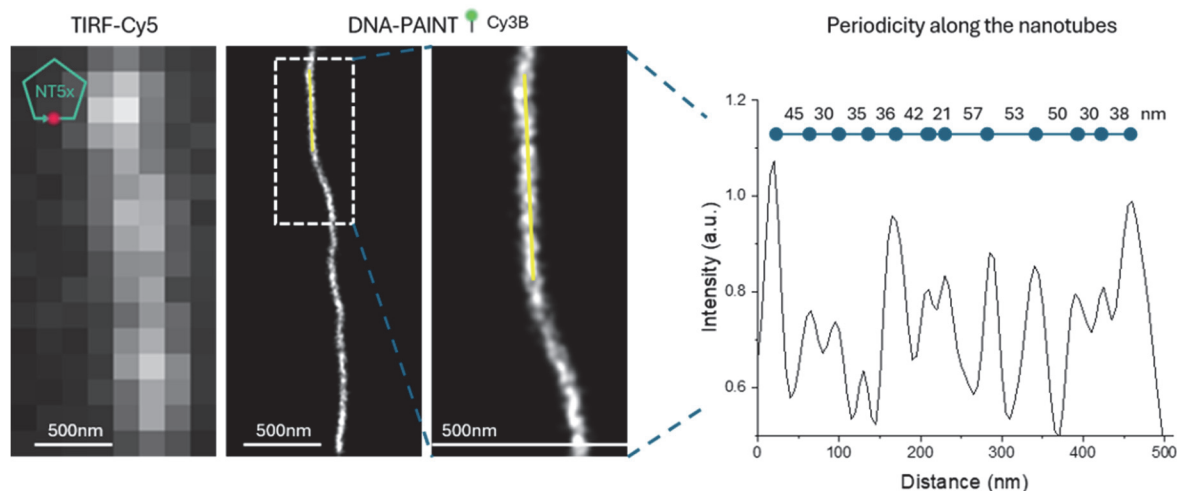


Figure S2. Periodicity of DxNTs shown by DNA-PAINT. Fluorescence intensity profile extracted along the centerline of a representative NT5x reconstruction acquired using Cy3B-labeled imager strands, revealing the periodic arrangement of the DxNT architecture. The corresponding diffraction-limited TIRF image is shown for comparison.

5.3. Persistence length calculations

To gain insight into the rigidity of the newly designed nanotubes, the worm-like chain (WLC) model was used to estimate the persistence length (p), a measure of bending stiffness. The persistence length was calculated from the end-to-end distance (R) and contour length (L) measured from the DNA-PAINT reconstructions using Equation 1.⁶

$$\langle R^2 \rangle = 4pL \left\{ 1 - \frac{2p}{L} \left[1 - e^{\left(-\frac{L}{2p} \right)} \right] \right\} \quad \text{Equation 1}$$

Following data analysis and image reconstruction, as described in Section 5.2, all reconstructed images (32 nm pixel size) were processed in Fiji with a protocol described elsewhere. In brief, the images were first smoothed using the built-in *Smooth* filter, after which reconstruction artifacts arising from moving DxNTs were manually removed. The processed nanotubes were then skeletonized, and their contour and end-to-end distances were extracted using a Fiji macro available at: <https://github.com/GCosaLab/Persistence-Length-Analysis>.⁷

The image processing generated a CSV file containing the measured contour (L) and end-to-end (R) distances for each nanotube. These measurements were subsequently fit using Equation 1 to estimate the persistence length. The resulting persistence lengths for each fluorophore and labeling configuration are summarized below.

Table S3. Summary of the persistence lengths measured from DNA-PAINT reconstructions of DxNTs labeled with Cy3B, ATTO633, and ATTO655 at different labeling positions and for all DxNT geometries.

Imager Fluorophore	DxNT Geometry	Dye position relative to core	DxNTs analyzed	Persistence length (μm)
Cy3B	Triangular	Away	278	1.7 ± 0.1
	Squared	Away	202	1.6 ± 0.1
	Pentagonal	Away	84	1.6 ± 0.1
	Pentagonal	Near	82	1.7 ± 0.1
ATTO633	Triangular	Near	189	1.7 ± 0.1
	Squared	Near	127	2.0 ± 0.1

	Pentagonal	Near	49	2.0 ± 0.2
ATTO655	Triangular	Near	219	1.5 ± 0.1
	Squared	Near	161	2.0 ± 0.1
	Pentagonal	Near	79	1.6 ± 0.1

6. MINFLUX Imaging

MINFLUX measurements were performed using two different instruments. Imaging of full DNA nanotubes was carried out at the EMBL Imaging Centre in Heidelberg, Germany, whereas all rung measurements were acquired using the MINFLUX instrument at The Hospital for Sick Children (SickKids) in Toronto, Canada. To ensure consistency between datasets, imaging conditions and acquisition parameters were matched as closely as possible across both instruments, none of the datasets analyzed were aggregated.

6.1. Imaging buffer and conditions

The sample preparation for the MINFLUX experiments differed from that used for the Nikon Eclipse Ti TIRF microscope in two key aspects. First, all experiments employed an ATTO655-labeled imager strand. Second, the DNA structures (rungs and DxNTs) were labeled with Cy3 to allow their visualization in the confocal channel before MINFLUX acquisition, enabling verification of both sample distribution and successful surface immobilization.

Prior to sample introduction, the immobilized gold nanoparticles (AuNPs) were evaluated to ensure they provided stable fiducial markers for drift correction and exhibited no detectable motion. Once adequate AuNP stability was confirmed, the Cy3-labeled sample was introduced into the imaging chamber. The confocal signal was used to verify an appropriate surface density and stable attachment of the DNA structures. The imaging buffer was then added, and the sample was briefly assessed to confirm the presence of DNA-PAINT binding events. Finally, the imaging chamber was sealed with dental glue (Twinsil, picodent, Germany) before data acquisition. The sealed samples remained stable for at least 2 weeks, if kept at 4°C (**Fig. S3**)

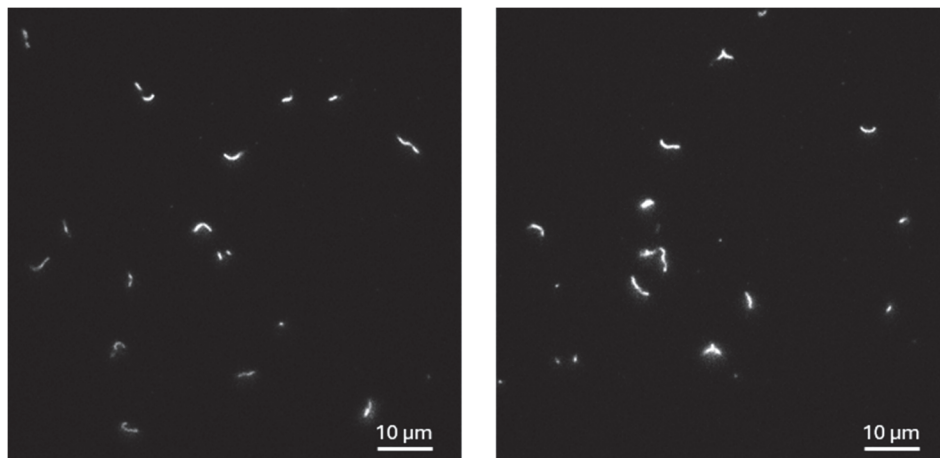


Figure S3. Long term stability of NT5x samples. TIRF images of NT5x acquired from the same imaging chamber 14 days apart. The chamber was sealed with dental silicone and stored at 4 °C between measurements.

All measurements were acquired using the default Imaging_2D sequence provided by Abberior (See Table S4). The MINFLUX 640 nm laser was operated at 3% power on the instrument at The Hospital for Sick Children (SickKids) and 183 μ W power on the instrument at the EMBL Imaging Centre measure at the back focal plane of the objective lens. At least four immobilized AuNPs were tracked throughout each acquisition for drift correction. Typical acquisition times ranged from approximately 4 to 8 h for DxNT samples, and up to 12 h for rung samples.

The imaging buffer consisted of 0.1% (v/v) Tween 20 in 1× TAMg buffer supplemented with 100 mM NaCl and did not contain an oxygen-scavenging system or triplet-state quenchers. For all DxNT experiments, the ATTO655-labeled imager strand was used at a concentration of 200 pM. Rung samples were imaged using imager concentrations between 200 and 400 pM, depending on the nanotube cross-section.

6.2. MINFLUX imaging parameters

DNA-PAINT MINFLUX experiments were performed using a commercial MINFLUX microscope (Abberior Instruments) equipped with a 100× oil immersion objective (UPLSAPO100XO, NA 1.40, Olympus, Tokyo, Japan). Fluorophores were excited using a 642 nm continuous-wave laser with a reference excitation power of 183 μ W at the first MINFLUX iteration. Data acquisition was controlled using Abberior Inspector (version 16.3.13924-m2112). Localizations were reconstructed as histogram images using a 2 nm pixel size without additional filtering or post-processing. A typical dataset required approximately 12 h of acquisition and generated ~1 GB of raw data.

We performed iterative MINFLUX in which the fluorophore position is progressively refined through successive iterations. During each iteration, the fluorophore is probed using a hexagonal targeted coordinate pattern (TCP), where the donut-shaped excitation beam is sequentially positioned at six locations surrounding the estimated emitter position. After each iteration, the estimated fluorophore position is updated and the TCP diameter (L) is reduced, increasing the localization precision from an initial L= 300 nm to L=40 nm in the final iteration.

The 2D MINFLUX sequence consisted of five refinement iterations, with the final iteration (L = 40 nm) providing the highest localization precision. A localization corresponds to the fluorophore coordinates calculated after successful completion of the entire iterative sequence. Localizations were accepted only when all iteration criteria summarized in [Table S4](#) were satisfied. Therefore, the localization sequence was terminated whenever the fluorophore switched to the off state, the minimum photon threshold was not reached, the background exceeded the predefined threshold, or the central frequency ratio (CFR) criterion was not fulfilled.

During a single DNA-PAINT binding event, the fluorophore often remained in the ON state long enough to complete multiple localization sequences. The first localization required approximately 5 ms, corresponding to the complete pre-localization and four refinement iterations. If the fluorophore remained bound, subsequent localizations required approximately 1 ms each because only the final iteration (L = 40 nm) was repeated until an abort criterion was reached. A trace therefore consists of a series of successive localizations assigned to the same molecular binding event (tid). The parameters used for each MINFLUX iteration are summarized in [Table S4](#).

Table S4. MINFLUX iteration parameters.

MINFLUX Iteration parameters	Pre-localization	Iteration 1	Iteration 2	Iteration 3	Iteration 4
L size [nm]	300	170	150	75	40
Targeted coordinate pattern (TCP)	Hexagon	Hexagon	Hexagon	Hexagon	Hexagon
How many times the complete TCP is repeated before proceeding to the next iteration.	1	5	5	5	5
Minimum number of collected photons	160	150	100	100	150
Laser power factor*	1x	1x	2x	4x	6x
TCP dwell time [ms]	1	1	1	1	1
Central frequency ratio (CFR)	x	x	0.8	0.8	2.0
Background threshold [kHz]	15	10	10	10	10

Excitation wavelength (nm)	642	642	642	642	642
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*This is a multiplier on the reference laser power to achieve an actual power used in each iteration

6.3. MINFLUX data processing and quality assessment

Raw MINFLUX datasets were exported from *Inspector* to MATLAB for post-processing. The temporal window containing the main localization events was first identified using *ParaView*. Localizations were subsequently filtered using the *DataViewer* MATLAB routine (available at <https://github.com/embl-ic/minflux-viewer-legacy>) based on acquisition time, a center frequency ratio (CFR) threshold of 0.8, and, when required, the estimated fluorescence occupancy (EFO) to remove secondary localization populations arising from multi-emitter events.

Filtered localizations were saved in both JSON and MATLAB formats. Super-resolution images were reconstructed using the *Interactive_render_MINFLUX* routine with a pixel size of 2 nm, selected to match the localization precision. The reconstructed images were exported as TIFF files for subsequent quantitative analysis. To evaluate data quality and identify potential issues related to sample preparation or image acquisition, several quality metrics were extracted from *ParaFLUX* and are summarized in [Table S5](#). The software can be found here: <https://github.com/GCosaLab/MINFLUX-DxNT-Analysis>

Total catching events correspond to all fluorescence bursts detected during the initial scouting phase, in which the microscope searches for candidate fluorophores before initiating the iterative MINFLUX localization sequence. This value therefore represents 100% of all detected fluorescent signals or burst.

Passed events (%) indicate the fraction of total catching events that successfully completed the entire MINFLUX iteration sequence and became valid localizations.

Dark (%) represents the fraction of events that were aborted because the detected signal fell below the minimum brightness threshold, typically due to fluorophore photobleaching, or transition to a dark state before completion of the full iteration process.

Number of photons (all iterations, valid bursts only) is the total number of photons collected across all MINFLUX iterations for bursts that resulted in valid localizations. This value represents the total photon budget available for determining the molecular position.

Localization uncertainty (Iteration 4) corresponds to the localization precision estimated from the final MINFLUX refinement iteration ($L = 40$ nm), which provides the highest localization precision.

Localizations per burst (Loc/burst) is the average number of valid localizations obtained from a single DNA-PAINT binding event. A burst consists of all successive localizations recorded while the same imager strand remains bound to its docking site.

Center frequency ratio (CFR med, Iteration 4) is the ratio of photons detected at the pattern (TCP) center versus the outer positions. Lower CFR values (general threshold value is 0.8) indicate well-centered, isolated fluorophores, whereas higher values are commonly associated with elevated background or simultaneous emission from multiple fluorophores.

Emission frequency at offset (EFO med, Iteration 4) is the median photon emission rate (Hz) measured during the final iteration (average frequency in Hz of the photons collected at the outer points of the TCP pattern corrected for background) and reflects the brightness of the fluorophore above the background. Besides serving as a measure of signal intensity, EFO provides an effective diagnostic for identifying multi-emitter events. Single fluorophores typically produce a peak around 40 kHz, whereas events near 80 kHz indicate the simultaneous emission of two fluorophores within the same diffraction-limited region. Localizations associated with these higher EFO values were excluded during post-processing.

Table S5. Summary of MINFLUX quality parameters extracted from the ParaFLUX for DNA-PAINT MINFLUX datasets before any filtering or localization aggregation.

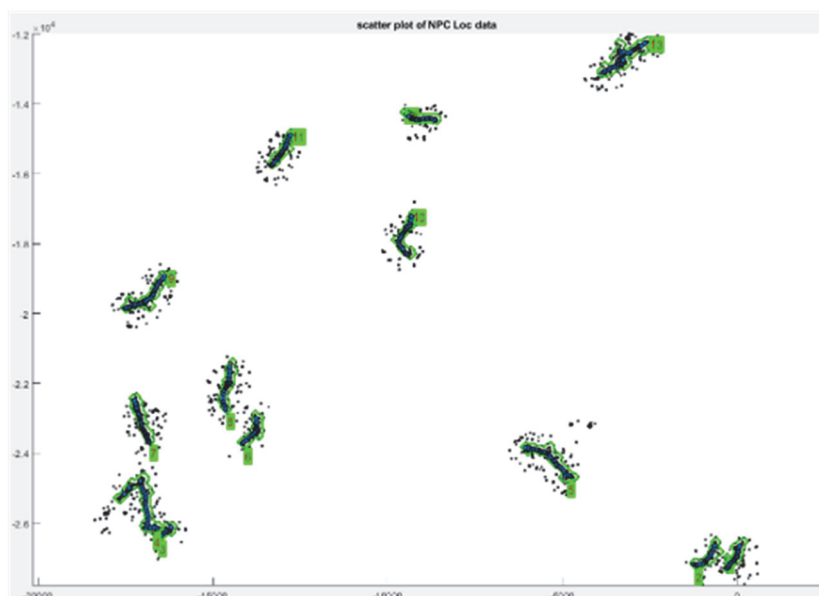
DxNT	Imaging time (hours)	Total catching events	% valid events	% dark	No. photons all iteration only valid events	Loc Uncertainty (nm) Itr 4	loc/burst (mean)	cfr med Itr4	efo med It4 (Hz)
NT3x	15.2	55235	45.6	2	37169	2.66	53.2	0.37	175043
NT4x	12.0	37676	51.6	2.3	30983	2.44	46.3	0.44	158039
NT5x	2.5	9025	54.8	1.5	34866	2.75	56.5	0.59	119529
R3x	8.27	9972	78.9	11	7080	2.26	16.5	0.42	26847
R4x	14.54	15868	80	4.1	9492	2.54	21.1	0.5	34158
R5x	18.85	22930	79.5	4.9	7289	2.58	15.8	0.5	33640

6.4. Data analysis for rung-to-rung distance measurements in DxNTs

For the DxNTs, filtered MINFLUX localization data stored in a MATLAB .mat file were analyzed using the spline-fitting based script `fit_splines_minflux.m`, to identify individual nanotubes and measure the rung-to-rung distances. The script first displays the full field of view and automatically detects regions of interest (ROIs) corresponding to individual DxNTs. The automatically generated ROIs at this stage can be manually modified before downstream analysis.

Several analysis parameters can be modified by the user, including the distance from the nanotube centerline used for the intensity profile, the order of the peak-to-peak distance measurements, and the window size used for peak detection. For the analyzed data presented, we used a 25 nm distance to center, and a peak-to-peak distance order of 1, as shown in [Figure S4](#). Then based on the selected parameters, the script will process the ROIs sequentially, compute peaks along the central line, measure peak positions and inter-peak distances. Finally, it creates the localization density profile plot and overlay the detected peaks onto it, export the measurement result to CSV files, for subsequent analysis. The code can be found in the same repository as the last section: <https://github.com/embl-ic/minflux-viewer-legacy>

Open full field of view imaged, with automatic ROIs added



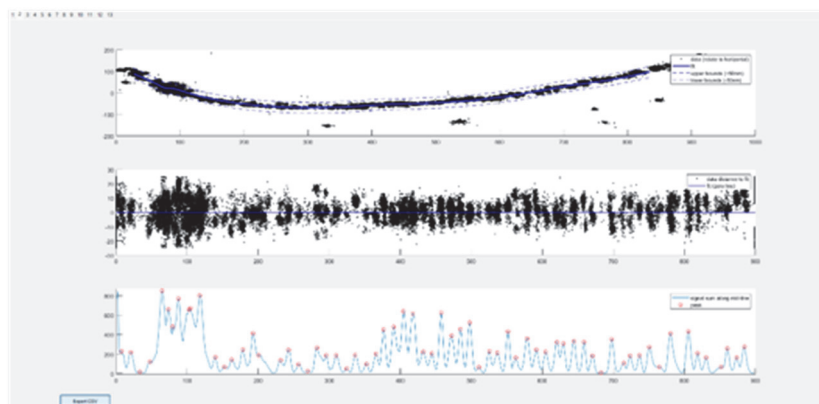
Modify ROIs as needed or add manually NTs

☒ data
 ☒ ROIs
 ☒ points

1-sided distance to center (nm)
 window size to compute peak (nm)

 order of peak-peak distances

Select appropriate distance to center, minimum distance between peaks, and the order. Press *Fit Spline*.



Revise NT data, per ROI selected. To save, press *Export CSV* to get inter-rung distances and rungs identified along each NT.

Figure S4. Protocol for extracting inter-rung distances from localization data acquired by MINFLUX microscopy.

Table S6. Gaussian fitting parameters for the inter-rung distance distributions obtained from the DNA-PAINT MINFLUX reconstructions histograms shown in Fig. 3C. The table summarizes the center position and relative weight of each Gaussian population.

NT geometry	1 st population (nm)	Relative weight (%)	2 nd population (nm)	Relative weight	3 rd population (nm)	Relative weight (%)
NT3x	13 ± 2	77 ± 4	26 ± 2	16.6 ± 3.5	40 ± 2	5.8 ± 4.9
NT4x	14 ± 2	86 ± 2	27 ± 2	14.0 ± 2.3	—	—
NT5x	13 ± 2	86 ± 2	26 ± 3	13.9 ± 2.1	—	—

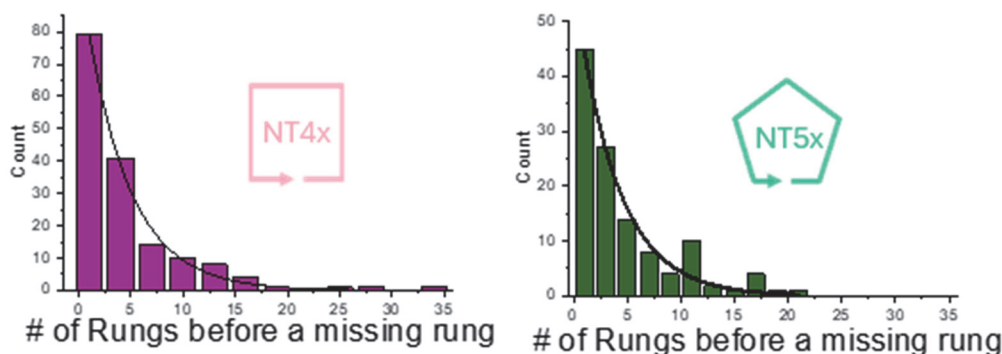


Figure S5. Histograms showing the distribution of the number of rungs preceding a missing rung/gap for NT4x and NT5x, constructed from across all ~20 nanotubes analyzed for each geometry. The distributions follow an exponential decay, indicating that missing localization events occur stochastically rather than following a periodic or structurally defined pattern intrinsic to the DxNT architecture.

6.5. Data analysis for rungs

The analysis consists of three sequential steps: (i) identification of individual rungs, (ii) identification of binding sites within each rung, and (iii) calculation of the inter-binding site distances.

This code is available here: <https://github.com/GCosaLab/MINFLUX-DxNT-Rungs>

Identification of individual rungs

Individual rungs were identified using the Density-Based Spatial Clustering of Applications with Noise (DBSCAN) algorithm. DBSCAN groups localizations that lie within a user-defined maximum distance (ϵ) set to 9nm and contain at least a user-defined minimum number of localizations (minPts) set to 3. Each detected cluster was assigned a unique rung ID, while isolated localizations that did not satisfy the clustering criteria were classified as noise (label = -1) and excluded from further analysis.

Identification of binding sites within each rung

Each identified rung was subsequently partitioned into a user-defined number of subclusters using the K-means clustering algorithm. The number of subclusters corresponded to the expected number of binding sites for the selected geometry (three for R3x, four for R4x, and five for R5x). The centroid of each subcluster was calculated and taken as the estimated position of an individual binding site. Only subclusters containing at least the user-defined minimum number (10) of localizations were retained for further analysis.

Measurement of inter-binding site distances

To preserve the spatial arrangement of the rung, the centroid positions were ordered according to their angular position around the geometric center of the rung. This procedure identified neighboring binding sites along the perimeter of the structure. Inter-binding site distances were then calculated as the Euclidean distance between successive centroid pairs.

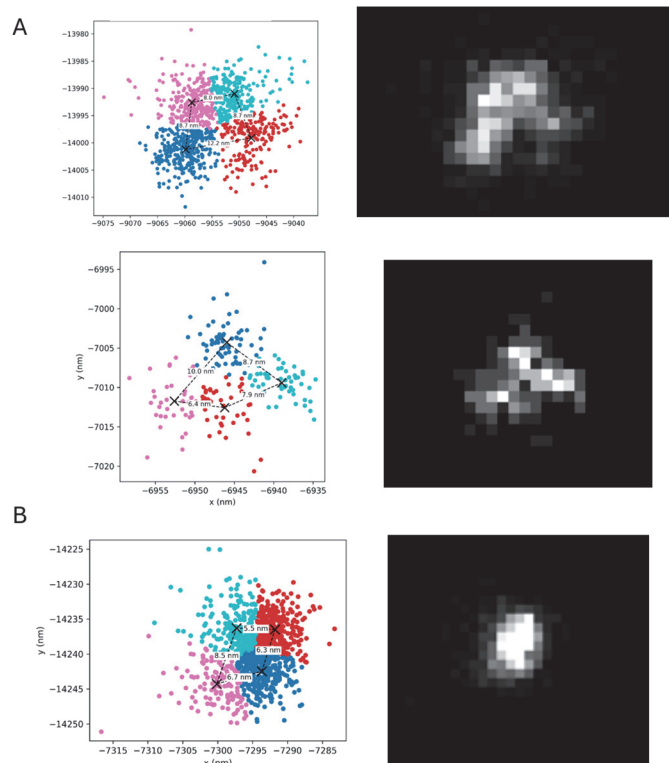


Figure S6. Representative DNA-PAINT MINFLUX reconstructions of R4x designs and corresponding analysis workflow used to identify localization clusters within individual rungs. For each rung, the analysis script identifies individual localization clusters, calculates their centroid coordinates, and determines the inter-binding site distances from the centroid positions. **(A)** Representative R4x reconstruction exhibiting distortion of the expected square geometry, consistent with surface-induced tilting of the pillars following immobilization. This distortion results in unequal inter-binding site distances (e.g., ~12 nm and ~6 nm) relative to the designed geometry. **(B)** Representative R4x reconstruction appearing as a single localization cluster. Because the analysis algorithm is constrained to identify four binding sites for the square geometry, it partitions the localization into four subclusters despite the absence of clearly resolved individual binding sites.

7. References

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