DNA crossover flexibilities upon discrete spacers revealed by single-molecule FRET

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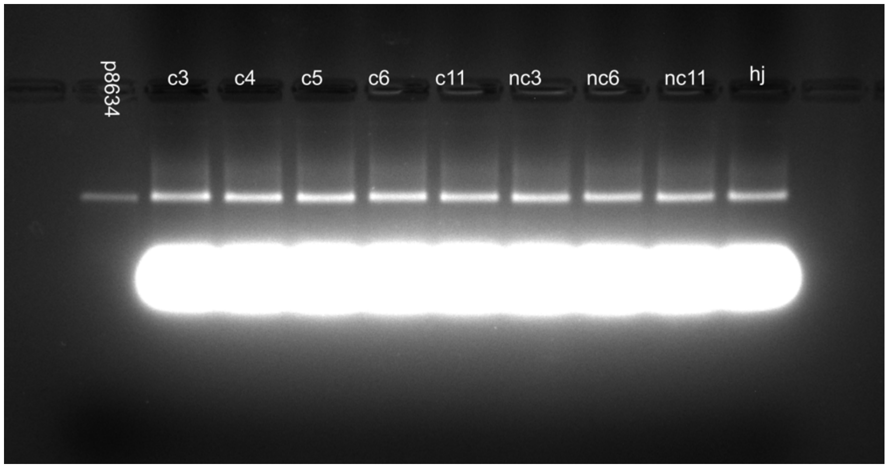
Supplementary Information Note S1: Structure design. The origami was designed using caDNAno. The structure consists of the 14 HB pillar with pedestal 3x 14HBs at the bottom and a minor 6HB structure with all helix packed in honeycomb lattice. The pedestal part is about 30.6 nm high, the upper 14HB is about 66.7 nm (97.3-30.6 nm), and the 6HB is about 49.9 nm. The 6HB is connected with major pillar through DNA scaffold. Two scaffold loops are stored at two ends of 6HB and other two loops are stored at the 14 HB pillar. By shifting scaffold in loop region to the spacer region, it leads to different kinds of spacer type of various length, namely complementary spacer, non-complementary spacer, and the Holliday junction without any bases inserted. This simplified the design procedure, and also minimized the cost for ordering oligonucleotides. The scaffold routing is same for all designs but the starting base is different for complementary or non-complementary spacer. The scaffold intramolecular self-hybridization 11 basepair is addressed with the help of Mfold online server. In order to place the complementary/non-complementary sequences in the spacer region, the overall scaffold sequences are shifted to different starting bases. To anchor DNA structure on glass slide, 19x docking strands are added at the bottom for hybridizing biotin oligonucleotides.

Supplementary Information Note S2: Folding, purification and TEM imaging. DNA origami structures were prepared by mixing core staples and the spacer staples for different kinds (100 nM each), biotin modified oligos (200 nM each), Cy3 and Cy5 modified oligonucleotides (500 nM each), and the circular DNA scaffold strand p8634 (12.5 nM) in 1x TE-Mg^{2+} buffer (10 mM Tris, 1 mM EDTA, 16 mM $MgCl_2$). The mixture was thermally annealed from 65 °C to 4 °C over 35 h (15 min at 65 °C, cooling to 58 °C with a cooling rate of −1 °C per 5 min, 58 °C to 35 °C with rate of −1 °C per 1 h, and from 35 °C to 4 °C with rate of −1 °C per 5 min). Subsequently the folded DNA nanostructures were purified from excess DNA staples by agarose gel electrophoresis without staining, 1 % agarose gels containing 1x Tris-acetate buffer (10 mM Tris, 10 mM acetic acid), 11 mM $MgCl_2$, 65V. All gels were cooled in ice water baths. To avoid contamination during staining, extra samples loaded in the nearby gel pocket were used as control which were excised and stained separately with ethidium bromide. The stained gel pieces were then used as control under gel imager for a blind excision of the desired gel band that were not stained. Structure recovery is accomplished by squeezing the gel between two parafilm-covered glass slides and collecting the resulting liquid droplet with a pipet.

TEM imaging of DNA origami lattices was carried out using a JEM-1400plus transmission electron microscope (JEOL) operating at 100 kV. For sample preparation 10 µL of polymerized DNA origami structures were deposited on plasma cleaned TEM grids for 15 mins and were furthermore quickly washed once with 0.1 ‰ uranyl acetate solution (5 µL) and immediately afterwards stained with 0.1 ‰ uranyl acetate solution (5 µL) for 10 s.



Supplementary Information Figure S1. Agarose gel electrophoresis for designs with different kinds of spacers. 1 % agarose gels containing 1x Tris-acetate buffer (10 mM Tris, 10 mM acetic acid), 11 mM $MgCl_2$, 65V. Full gel stained with ethidium bromide

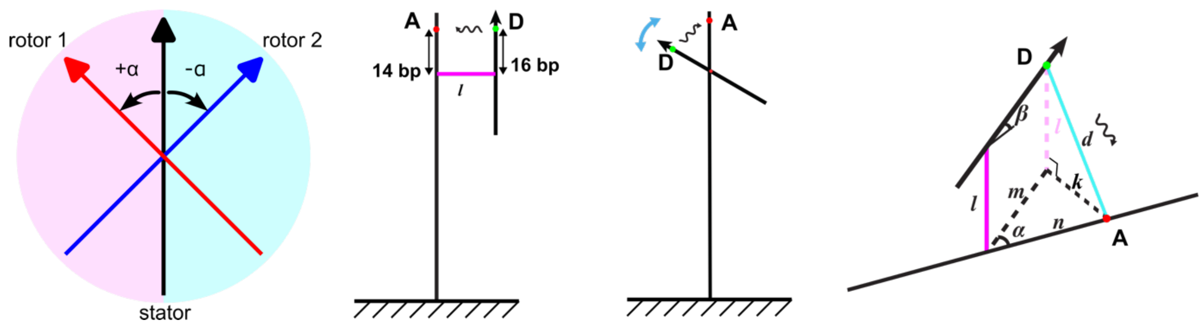
Supplementary Information Note S3: Fluorescence measurement and angle calculations. Glass slides were cleaned by rinsing with acetone, methanol, a mixture of sulfuric acid and hydrogen peroxide with a volume ratio of 7:3, and then sodium ethoxide. The surface was coated with a mixture of 99% mPEG (m-PEG-5000, Laysan Bio, Inc.) and 1% of biotin-PEG (biotin-PEG-5000, Laysan Bio, Inc.). The imaging buffer was composed of 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT and an oxygen scavenging system (0.8% D-glucose; 1 mg/ml glucose oxidase; 0.4 mg/ml catalase; 1 mM Trolox). The experiments were performed on an objective-based total-internal-reflection fluorescence microscope (IX71, Olympus) at room temperature. Cy3 is excited by a 532-nm Sapphire laser (Coherent Inc., U.S.). An oil-immersion objective (100×, N.A. 1.49) was used to generate an evanescent field of illumination. Fluorescence signals from Cy3 and Cy5 are split by a dichroic mirror, and collected by an electron-multiplying charge-coupled device camera (EMCCD) (iXON, Andor Technology). The exposure time was 50 ms.

For the conversion of FRET efficiency to structure angles, we used the FRET equation $E = \frac{1}{1+\left(\frac{r}{r_0}\right)^6}$ (r_0=5.8 nm) and the cosine formula $\alpha = \arccos\left(\frac{m^2+n^2-k^2}{2mn}\right)$. Typically, longer spacer (*l*) will increase the angle α as well as FRET distance (*d*). The 6HB and the spacer (*l*) defines a half-plane, and the 14HB and the spacer (*l*) defines another half-plane, but here we assume that dihedral angle (ß) is zero for the sake of simplicity. For a "correct" angle distribution, the spacer length (*l*) maybe the most significant factor. We first used 0.34 nm/base for all kinds of spacers. Due to the entropic elasticity, we think the unit length 0.34 nm/base is not applicable for nc6 and nc11 spacers. Assuming the interstructural angle to be close to 90˚ can deduce the unit length 0.23nm/base for a 2x ssDNA tether. In addition, if surface diffusion layer merges, the dihedral angle (ß) will be not zero which will complicate the angle calculation.

For calculating the entropic forces, a modified freely-jointed chain (mFJC) model and the force-extension behaviors are adopted from previous researches (references 31-33, specifically *Adv. Mater.,* **2021***, 33,* 2101986). When fixing *d* to be the length of same bases number in duplex form ($d = N \times 0.34\ nm/bp$), the entropic force F is independent of base numbers.

$$d = N \cdot L_B \times \left[\cos(\frac{Fl_k}{k_BT}) - \frac{k_BT}{Fl_k}\right] \times (1 + \frac{F}{S})$$

Parameter	Value
L_B	0.63 nm
l_k	1.5 nm
S	800 pN
k_BT	4.114 pNnm
N	Base number for non-complementary spacer
d	Length of same bases number in duplex form



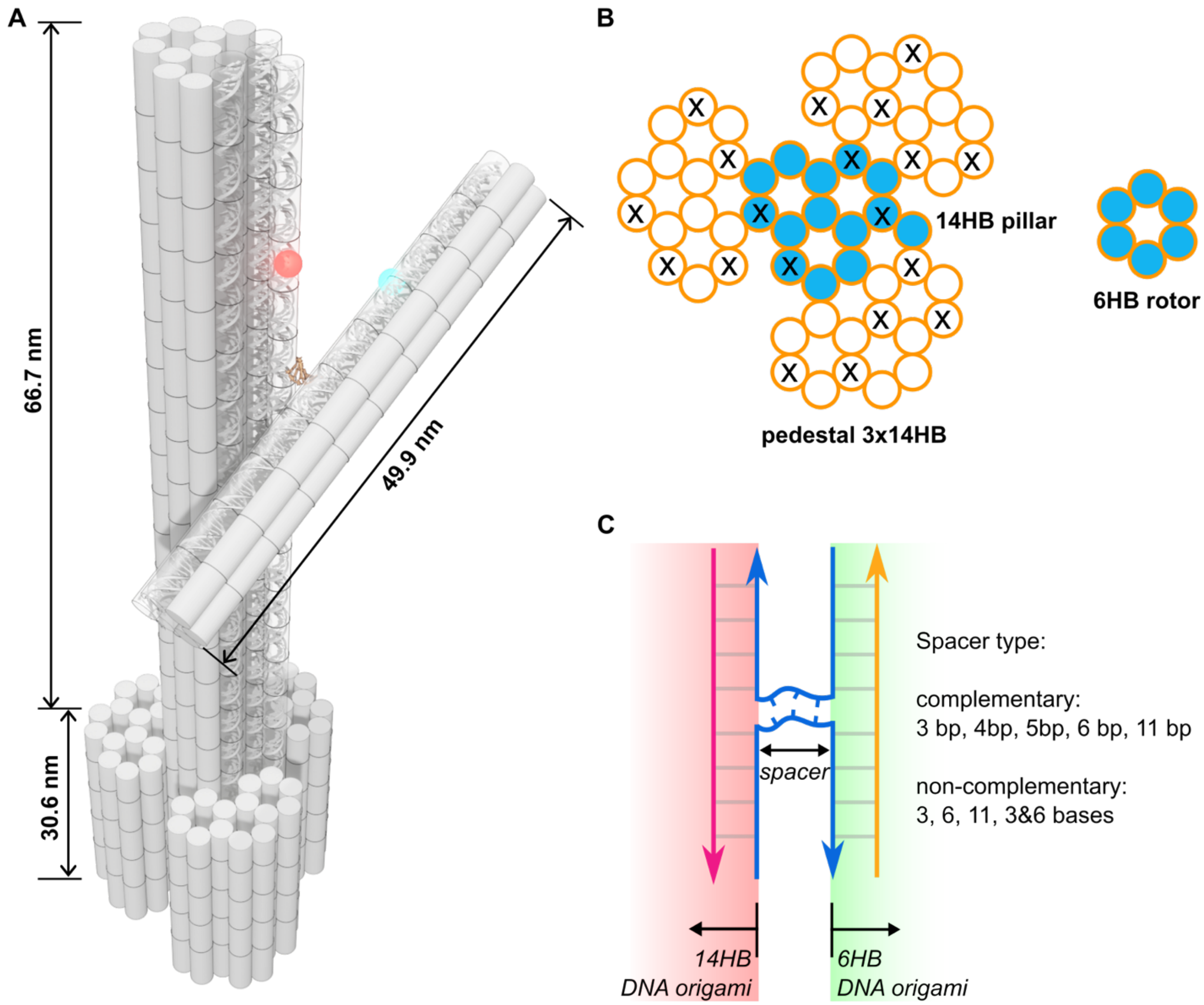
Supplementary Information Figure S2, Rotor 1 and rotor 2 attached to stator with different spacer showing different angles and the dihedral illustration for calculation.

Supplementary Information Table S1, The expected angles, the measured FRET efficiency and calculated angles for rotor and stator connected with spacers of different length.

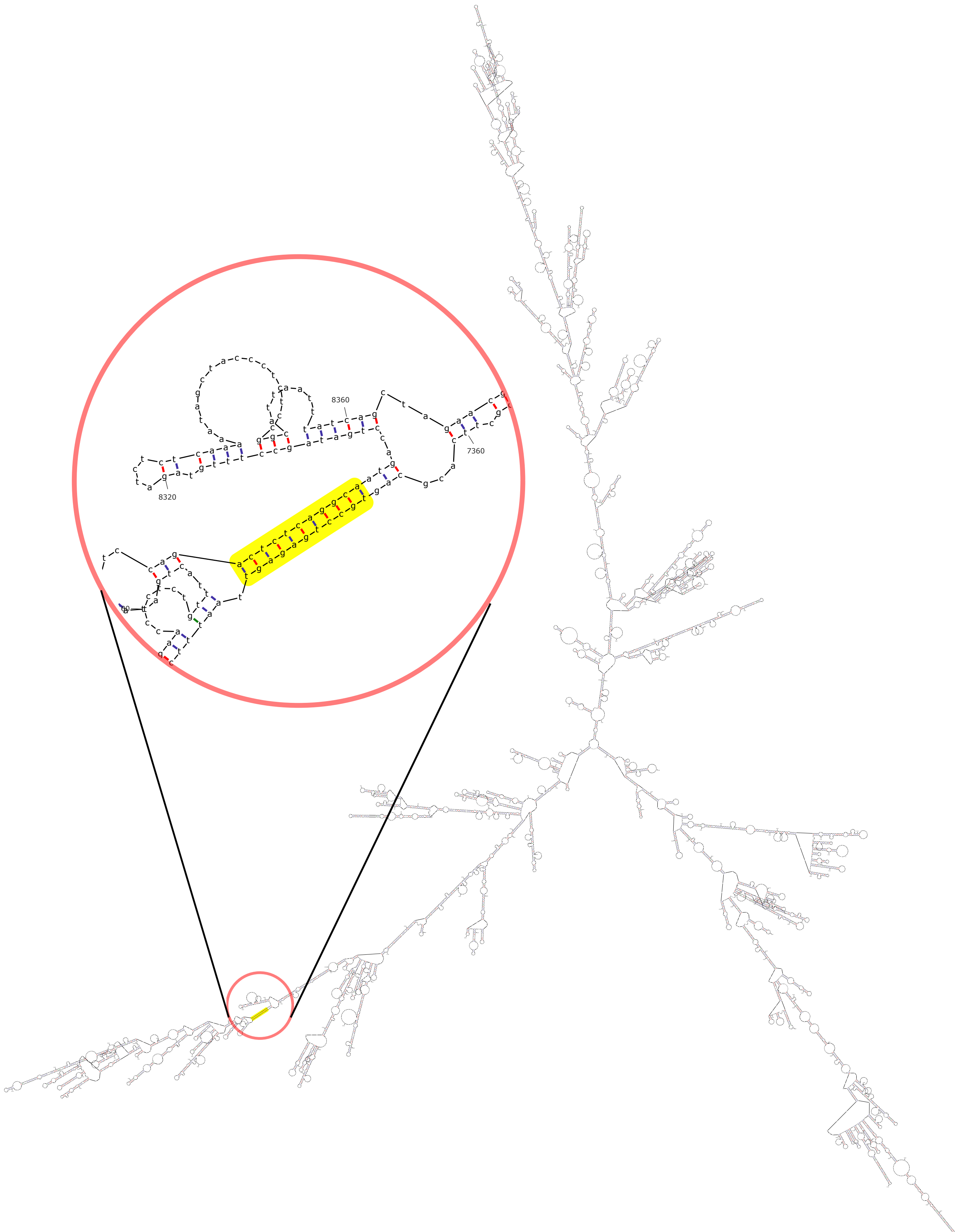
Spacer length (c-type)	Expected angles	FRET efficiency	Calculated angles
0	58.7°	0.70	58.7
3	156.9°	0.36	76.0
4	189.6°	0.20	89.2
5	-137.7°	0.20	88.1
6	-104.9°	0.12	-99.9
11	58.7°	0.28	67.1

Supplementary Information Table S2: The dihedral angle distributions for all kinds of spacers:

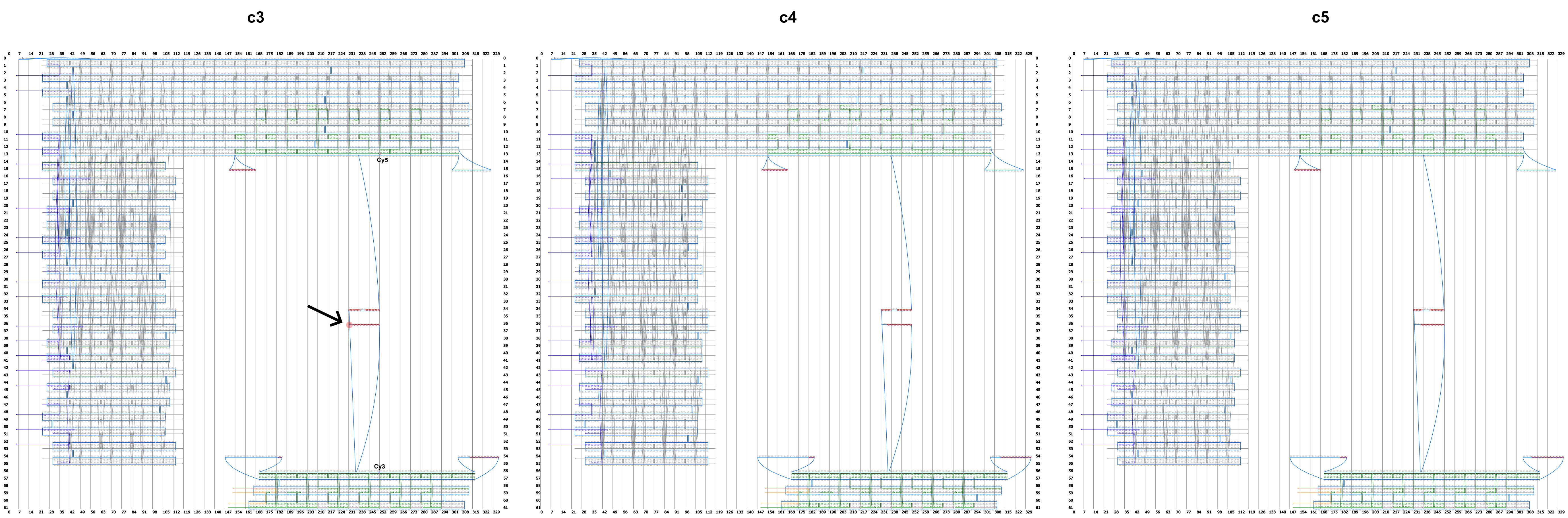
Spacer	hj	c3	c4	c5	c6	c11	nc3	nc6	nc3&6	nc11
Angle/°	58.7	76.0	89.2	88.1	-99.9	67.1	75.3	82.6	81.2	89.4

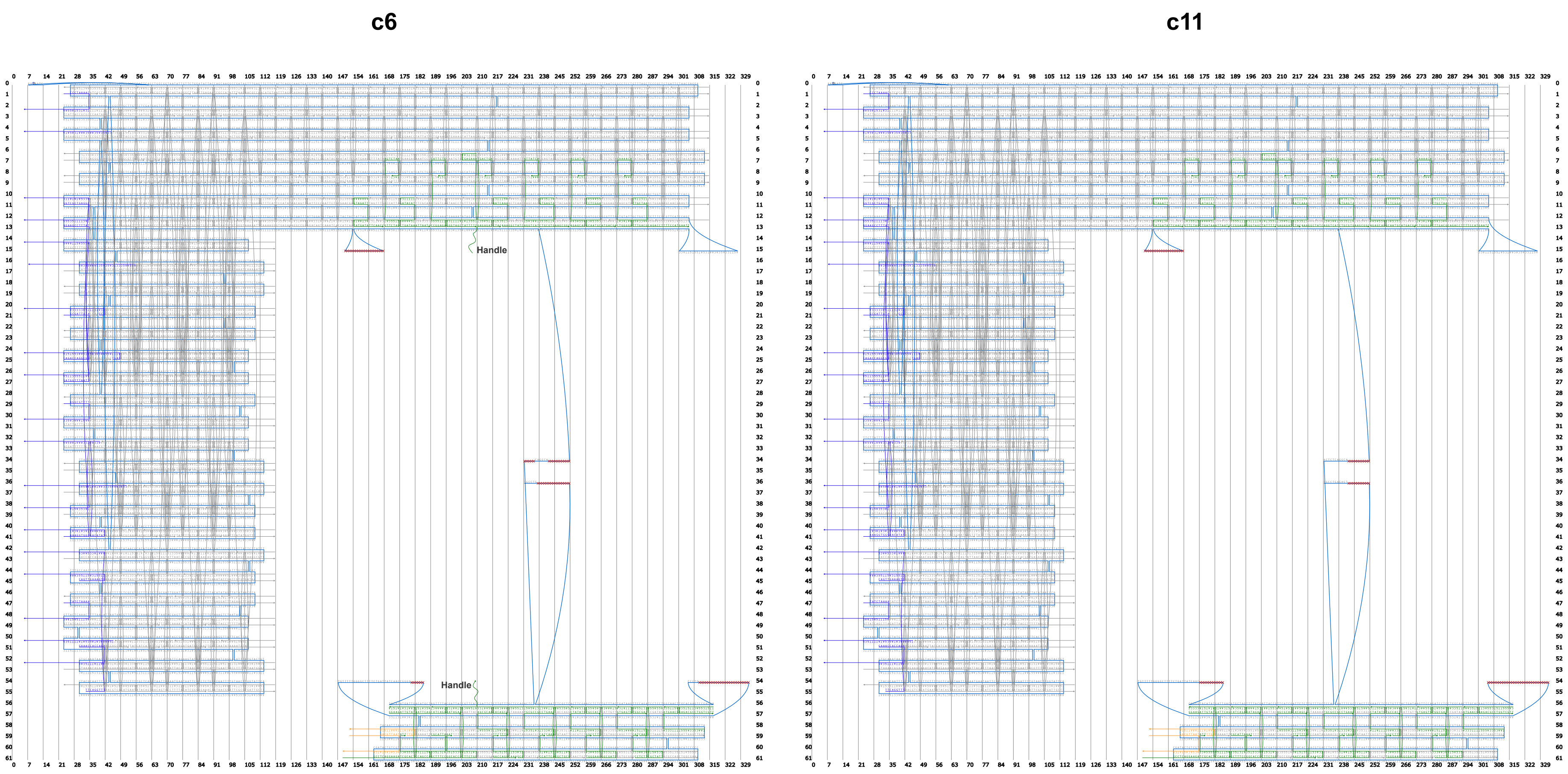


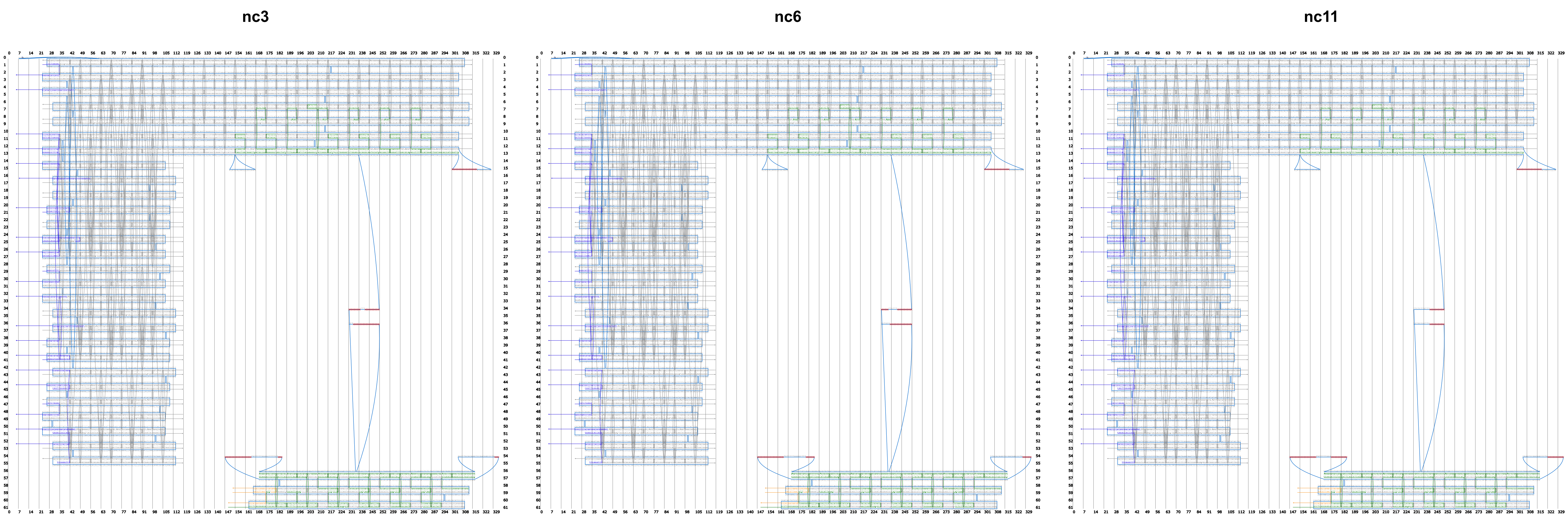
Supplementary Information Figure S3: The structural design principles. A, The 3D model showing the stator containing pedestal section and the 14HB pillar, the rotor 6HB. B, The cross-sectional image of the design. The blue circles present 14HB pillar and 6HB rotor respectively. X labels the overhangs for hybridizing biotin-strands. C, The spacer region is via scaffold only.

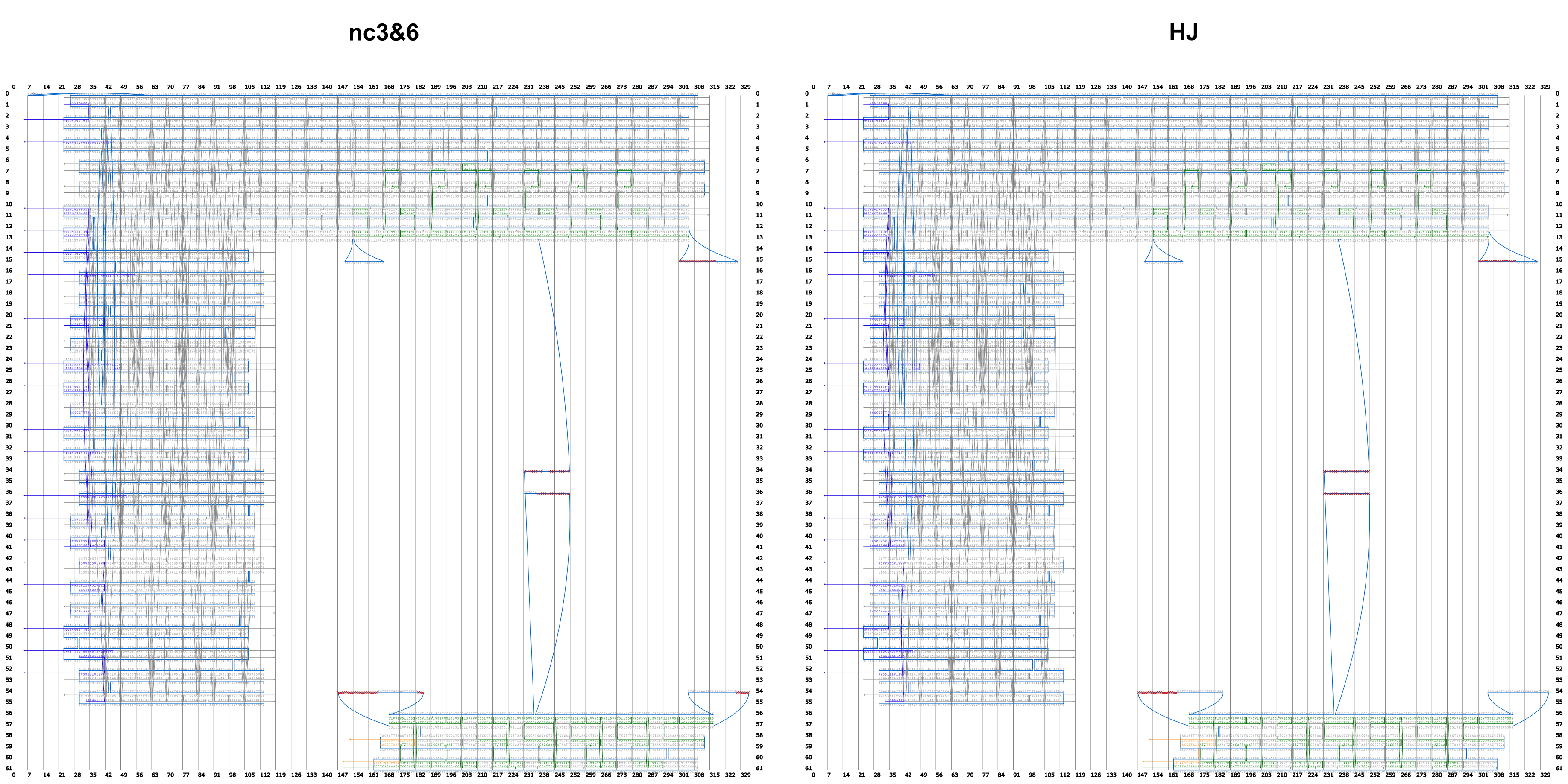


Supplementary Information Figure S4: The secondary structure of p8634 scaffold calculated by Mfold. The highlighted duplex is for the complementary spacers.



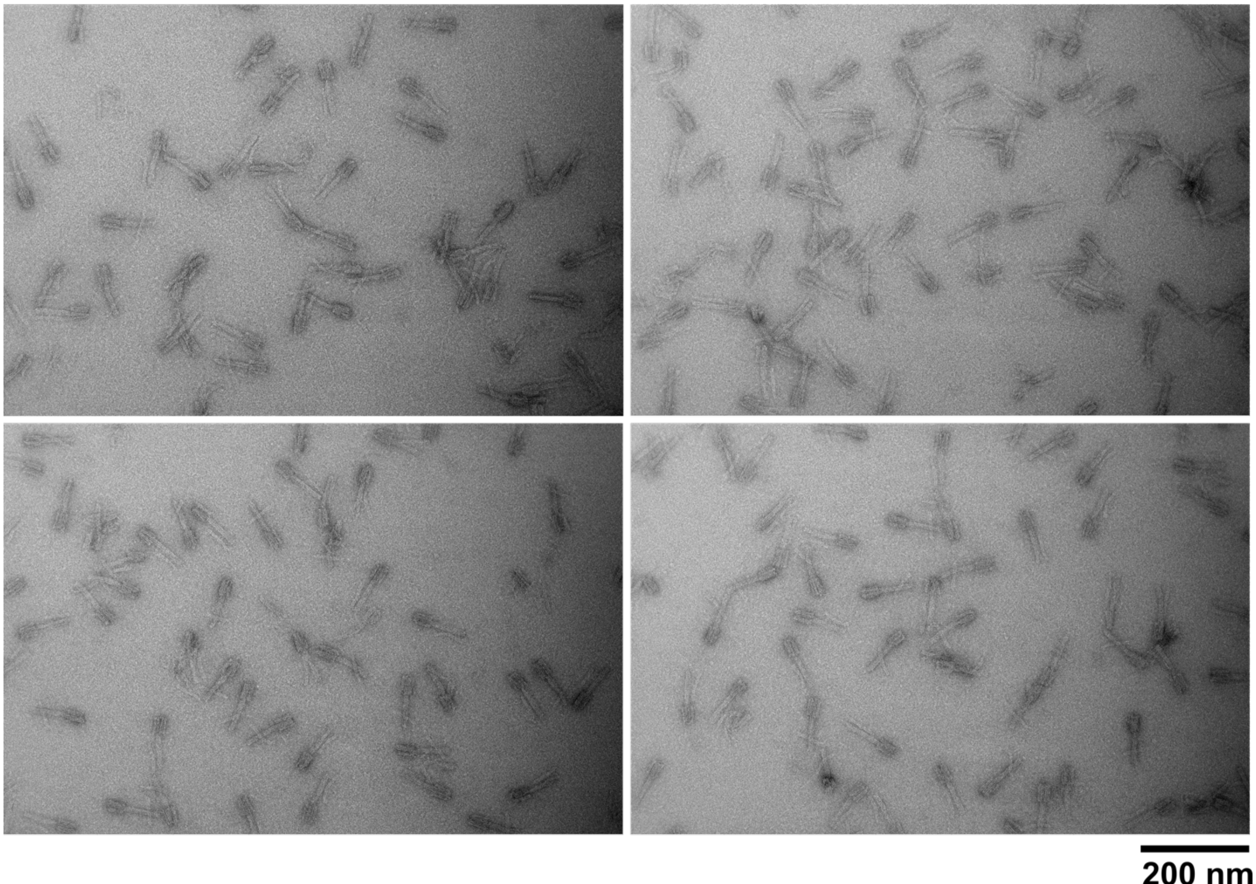




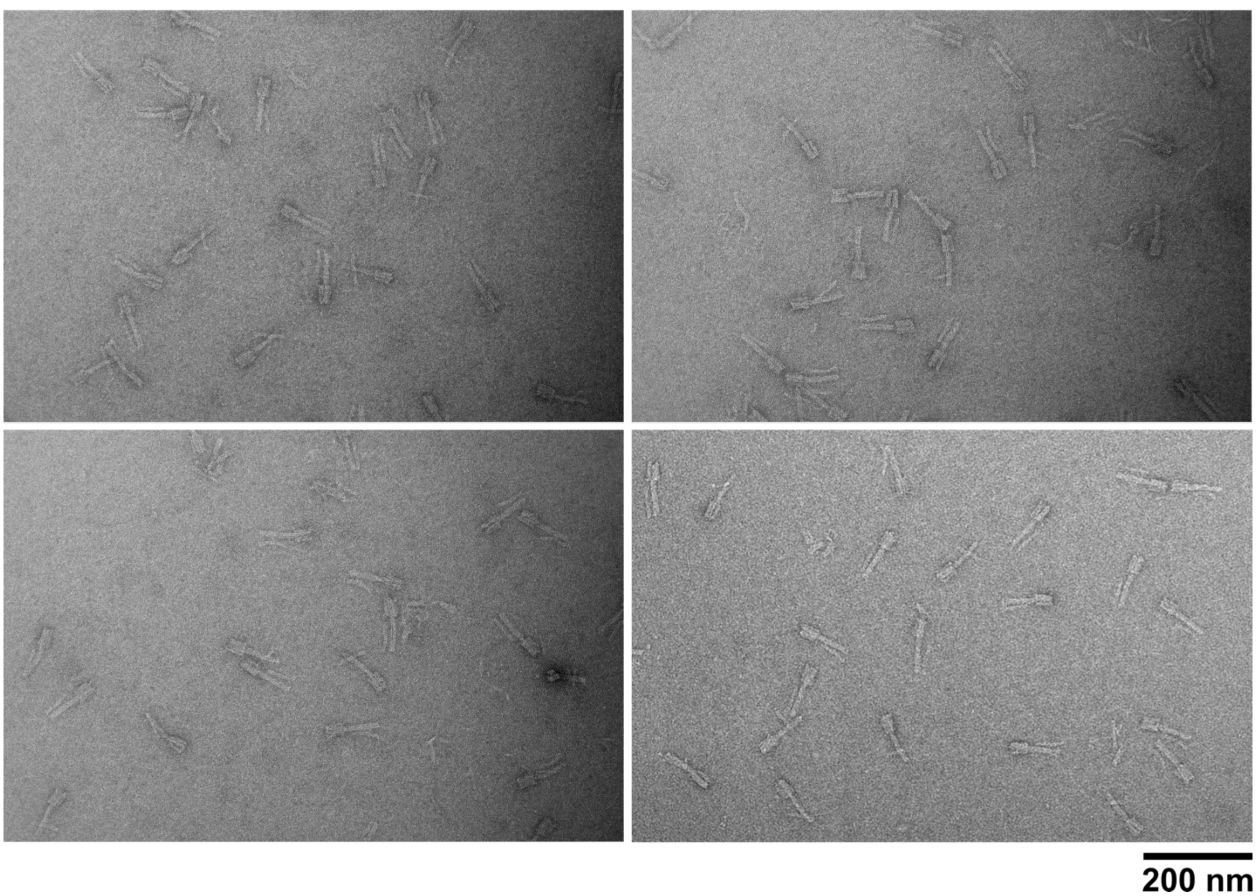


Supplementary Information Figure S5: The caDNAno layout of the DNA origami design. The staple oligonucleotides are sorted into different groups: core oligonucleotides (grey), biotin docking oligonucleotides (blue), and oligonucleotides to adjust spacers (green). Handle positions are marked in the c6 spacer design. Scaffold added position (arrow), dyes labeling position are same as indicated in c3 for all designs.

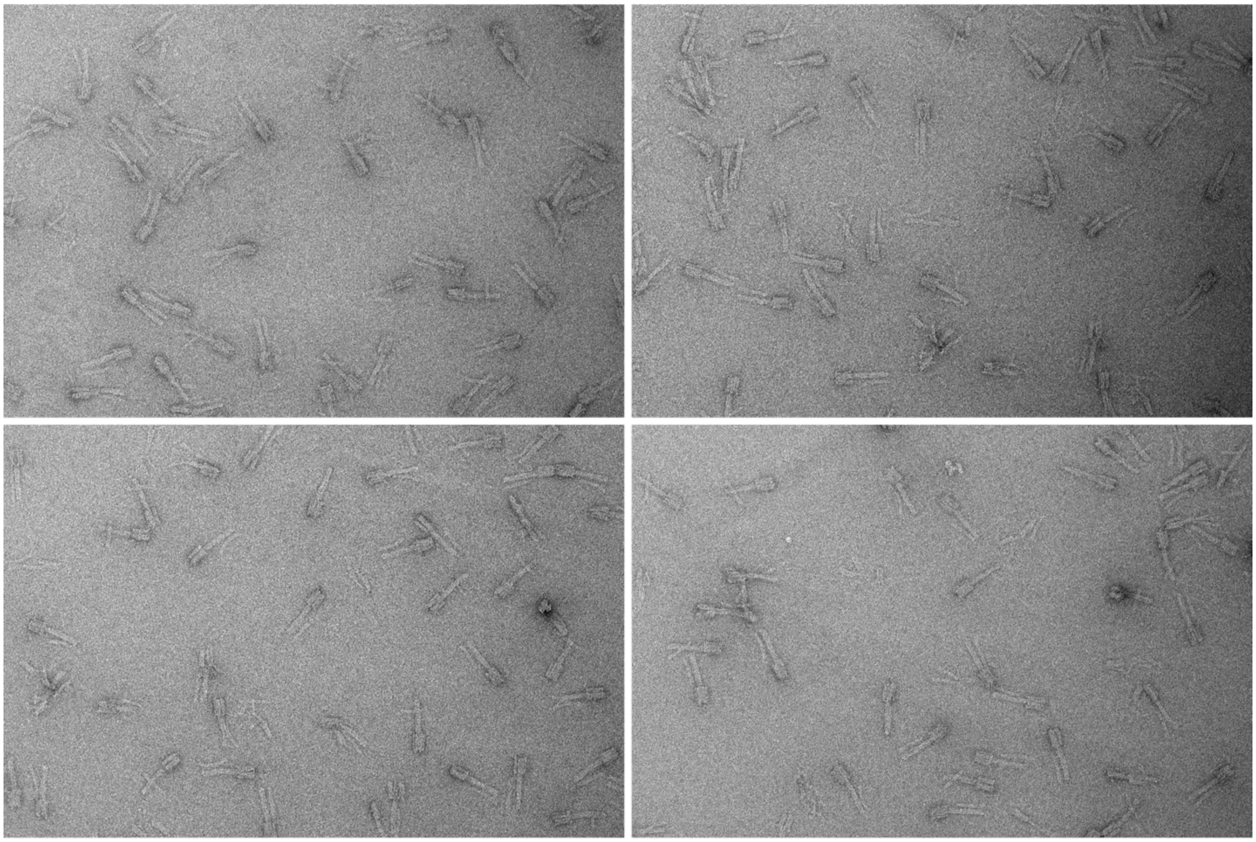
c3



c6

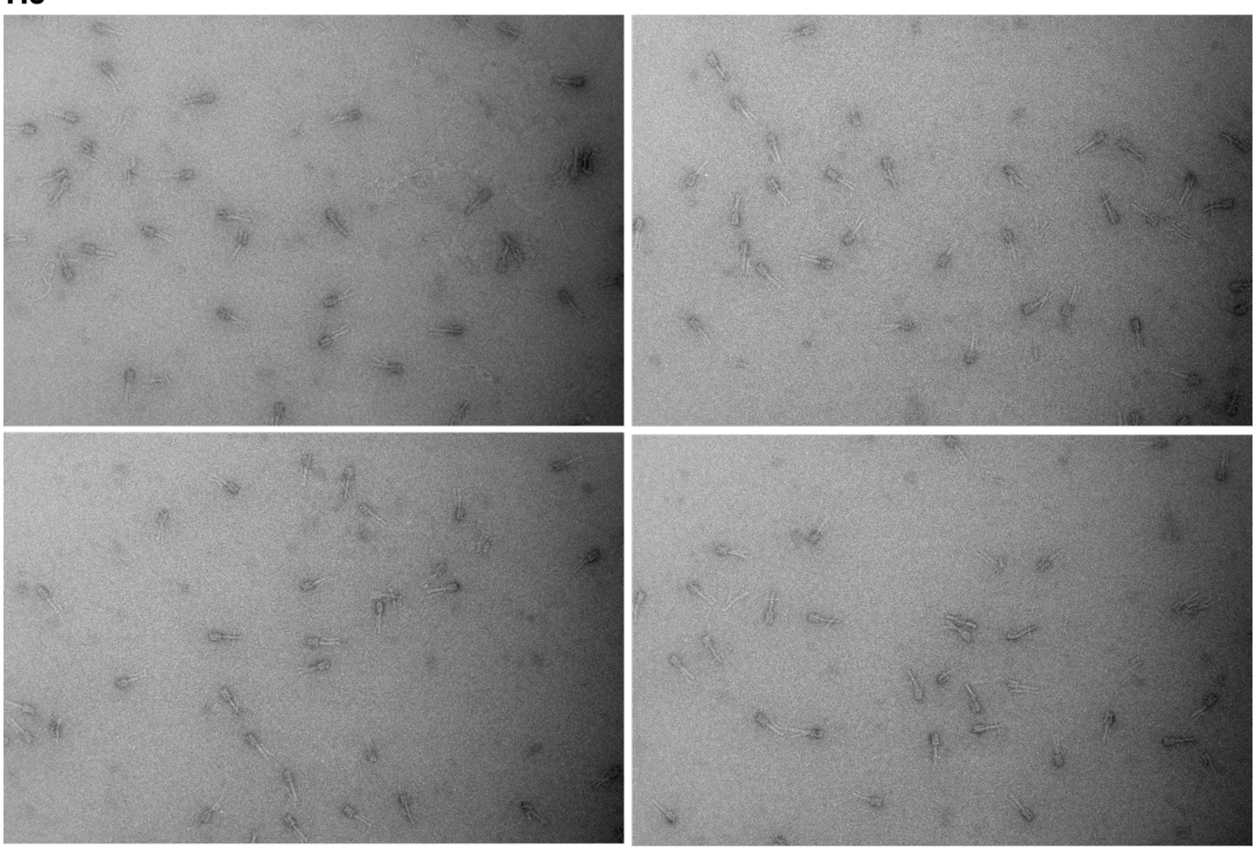


c11



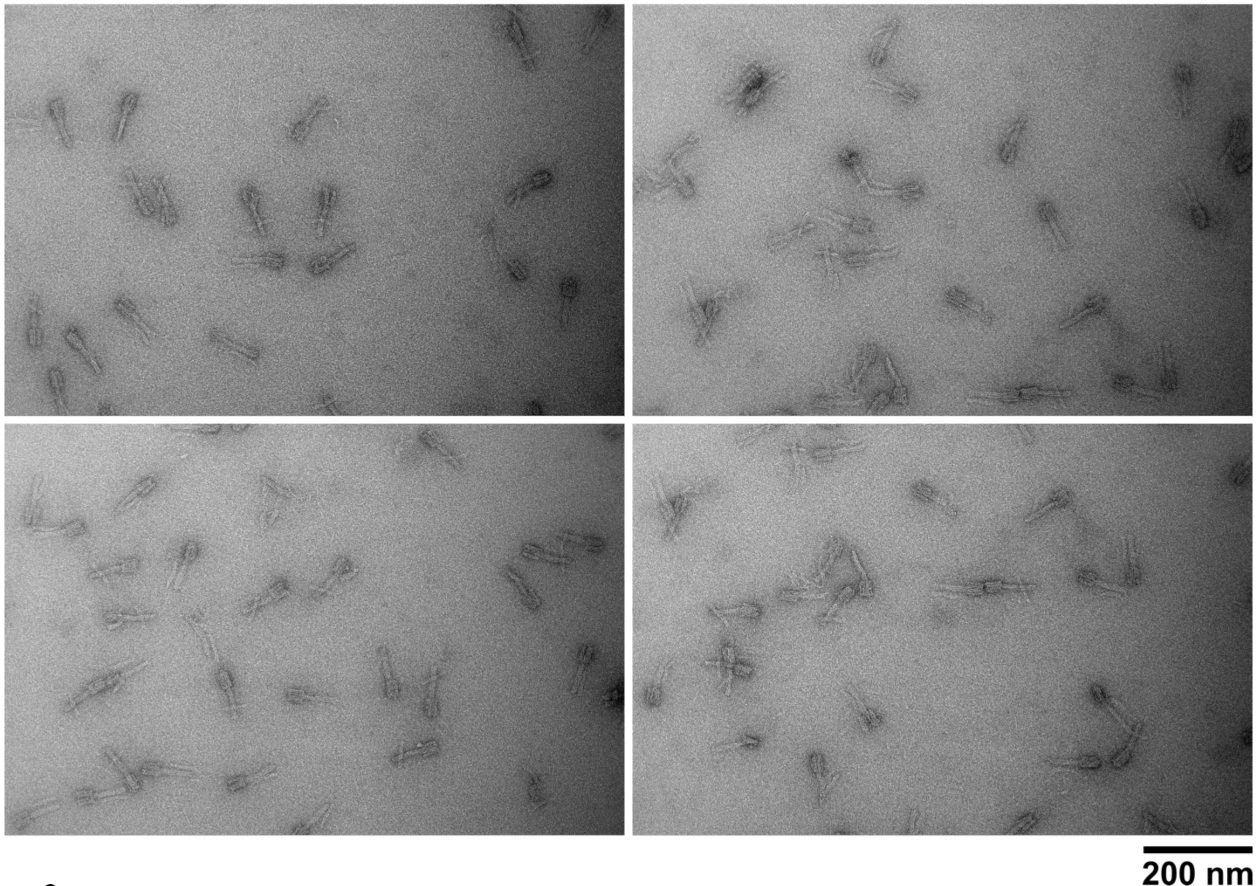
200 nm

HJ

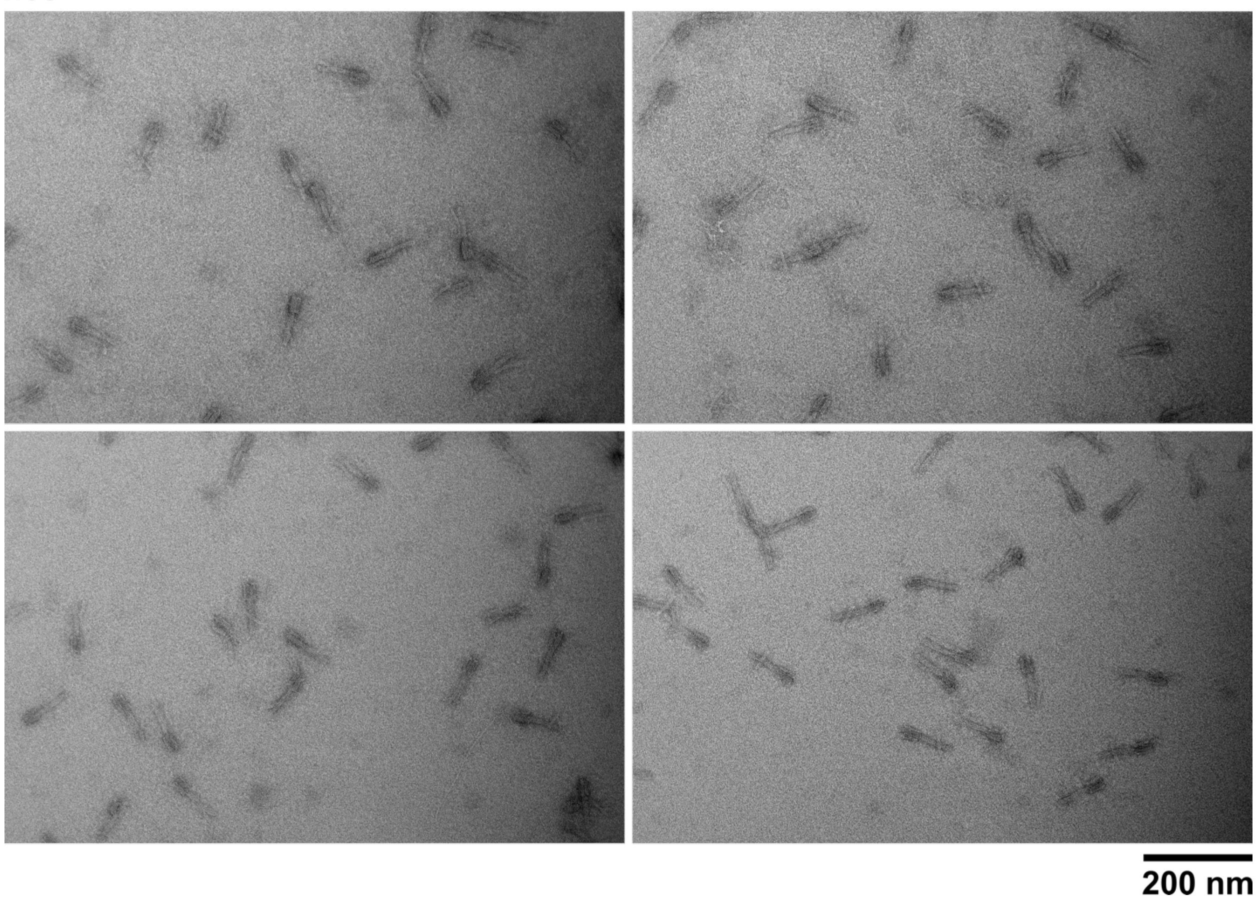


200 nm

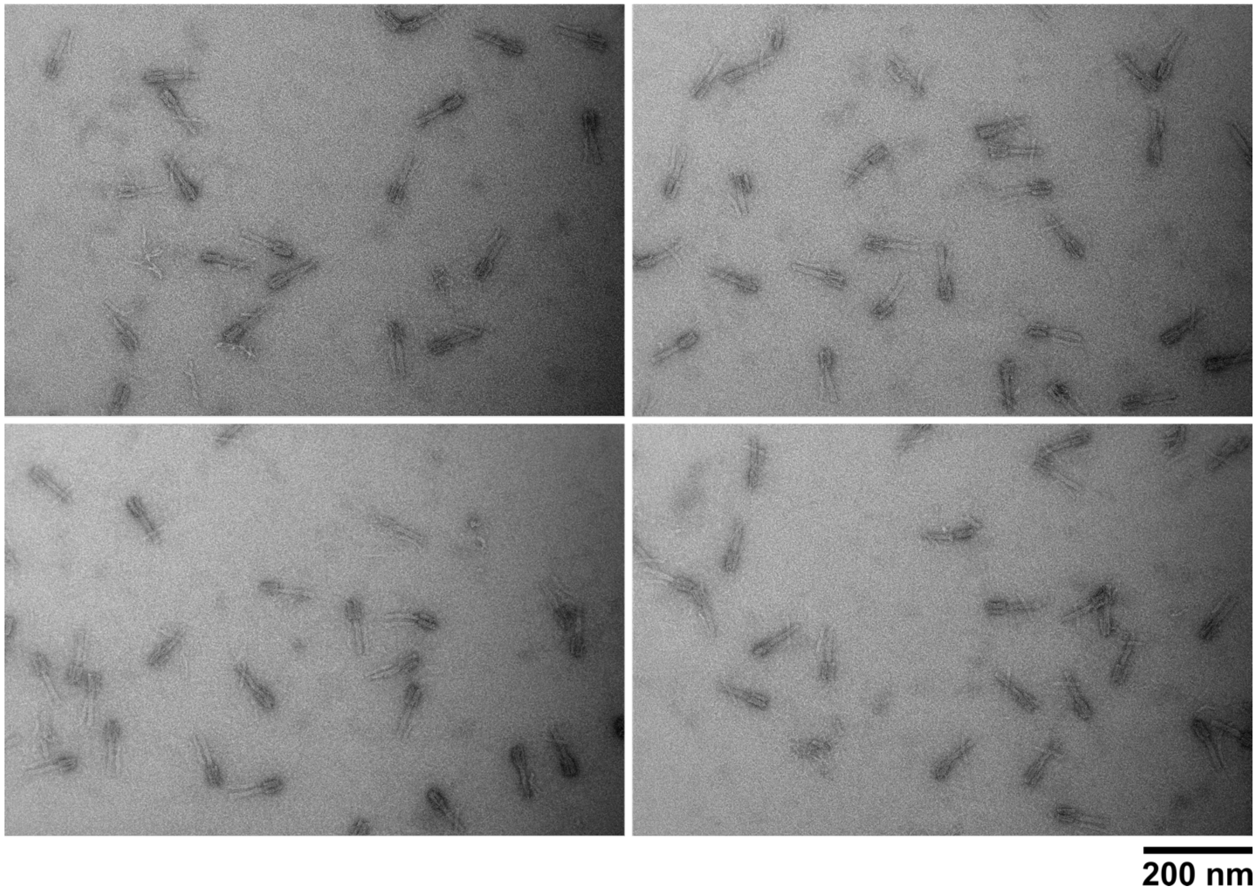
nc3



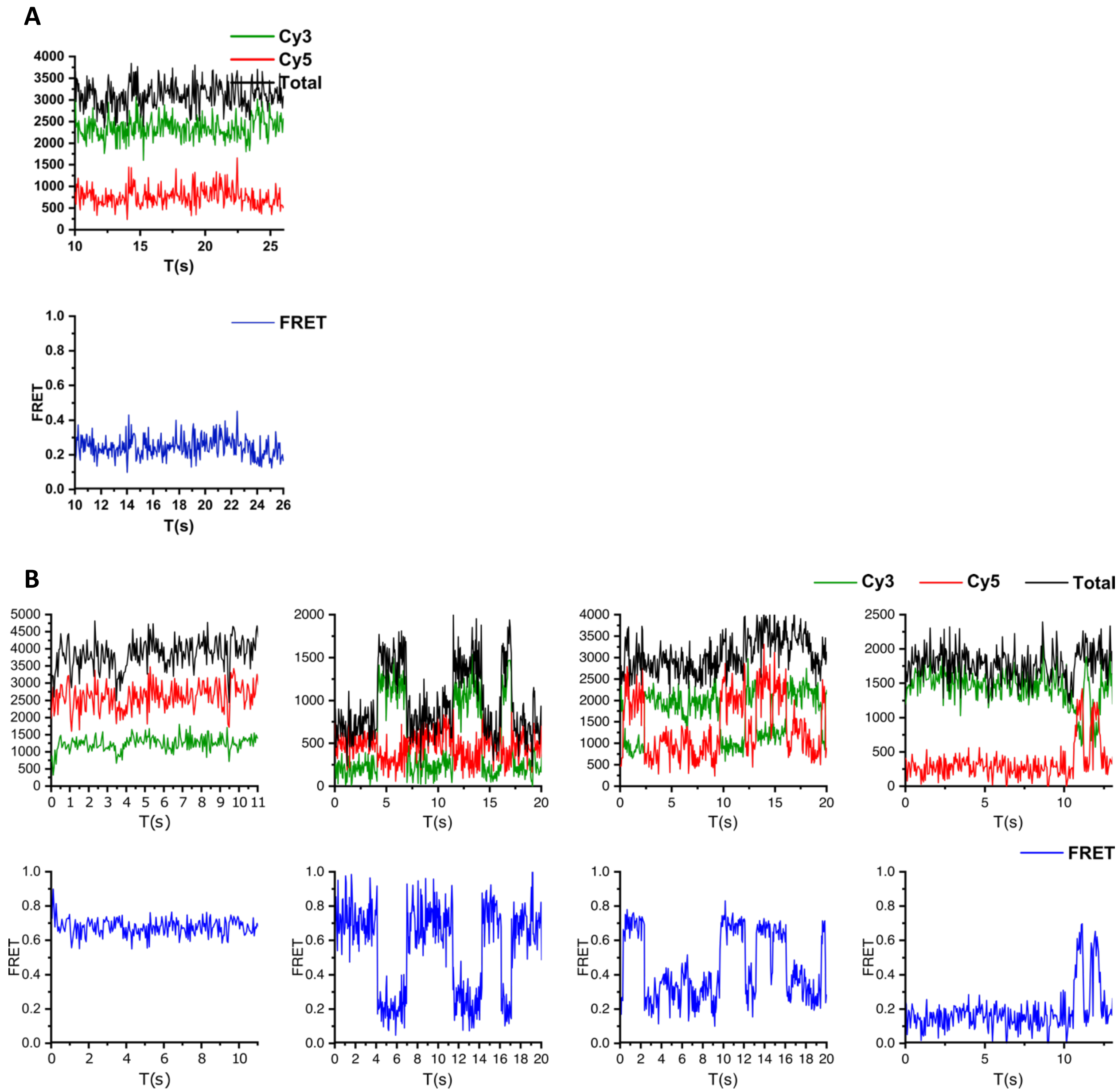
nc6



nc11



Supplementary Information Figure S6: TEM images of origami structures containing different kinds of spacer. Due to the negatively staining and drying effect, samples didn't show any angle distribution.



Supplementary Information Figure S7: Selected fluorescence traces for c6 complementary spacer at the initiate state (A) and after adding block strand (B).

Handles and Lock/Antilock sequences:

Handles on 13th helix	5’-GTC CTT CGT TGC G - Structure
Handles on 56th helix	Structure-TCT GGT TCA CTC GC-3’
Lock	5’- ATT ATT ATT GCG AGT GAA CCA GA CGC AAC GAA GGA C
Antilock	5’- GTC CTT CGT TGC GTC TGG TTC ACT CGC AAT AAT AAT

The shifted P8634 scaffold for complementary spacers:

TGCCTGAGAGTTAATTTCGCTCACTTCGAACCTCTCTGTTTACTGATAAGTTCCAGATCCTCCTGGCAACT
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TCCCGTTCATCCAGCAGTTCCAGCACAATCGATGGTGTTACCAATTCATGGAAAAGGTCTGCGTCAAATC
CCCAGTCGTCATGCATTGCCTGCTCTGCCGCTTCACGCAG

The shifted P8634 scaffold for non-complementary spacers:

TCTGCCGCTTCACGCAGTGCCTGAGAGTTAATTTCGCTCACTTCGAACCTCTCTGTTTACTGATAAGTTCC
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GTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCATCTACACCAACGTGACCTATCCCATTA
CGGTCAATCCGCCGTTTGTTCCCACGGAGAATCCGACGGGTTGTTACTCGCTCACATTTAATGTTGATGA
AAGCTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTCCTATTGGTTAAAAAATGAGCTG
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