Experimental methods

The assembly of the DNA catenane walker, six-helix bundle DNA origami path and operation was performed as previously reported.[1,2] In brief:

Design and assembly of the DNA catenane walker

Rotor and stator rings were assembled as described previously (Figure S1).[1,2] The rotor ring was mixed with 1.1 equivalents of threading ODN in 1x DNA buffer and 1x ligase buffer + 10 mM MgCl₂ and incubated at 4°C for 1 h. In parallel, 1.3 equivalents of the ¾-168-bp stator ring were combined with the Blocker_Rc2c ODN (1.5 equiv.) at 40 °C for 1 h and then cooled down to 4 °C for 5 min (1.5x DNA buffer and 1x ligase buffer solution). The two mixtures were combined and incubated for 15 min prior addition of T4 DNA ligase (2 µl/100 µl). Ligation was performed at 15 °C for 4h. The hybridized catenane (Cat^{hyb}) was converted to the interlocked version (Cat^{inter}) by adding 5 equivalents of RO-rotor ODN and ligase (0.5 µl/100 µl) and incubated at 15 °C for 30 min. Then, 10 equivalents of RO-stator ODN were added and the mixture was incubated at 15 °C for 3h. Addition of 3 equivalents of RO-Blocker_Rc2c and incubation for 30 min, finally releases the Blocker_Rc2c ODN from the stator. The resulting DNA catenane walker was purified by HPLC as previously described[2] and concentrated using Amicon Ultra-30K centrifugal filters.
Scheme S1. Secondary structure of DNA rings used in this study, including the names of the ODNs used for the assembly.

Assembly of the six-helix bundle DNA origami

The DNA M13mp18 scaffold (7429 bp, 20 nM) was mixed with the corresponding DNA staples at 200 nM concentration each, in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8), 5 mM NaCl and 20 mM MgCl₂. The mixture was annealed in a Nexus thermocycler (Eppendorf), from 80 to 65 °C at 1 °C per min and from 65 to 20 in steps of 20 min/°C. The sample was purified following the PEG precipitation protocol described by Dietz and coworkers.[3] mixing the samples 1:1 (v/v) with precipitation buffer (15% PEG 800 (w/v) (Carl Roth), 5 mM Tris, 1 mM EDTA and 505 mM NaCl) and spinning them at 16000 g for 25 min at room temperature using a microcentrifuge (Eppendorf 5424R, Hamburg, Germany). Then, the supernatant was discarded and the pellet containing the DNA origami path dissolved in 1x origami buffer (5 mM Tris HCl, 1 mM EDTA, 18.5 mM MgCl₂ at pH 7.9).
Assembly of the DNA catenane walker to the origami path, walking and regeneration operation and monitoring by bulk fluorescence experiments

The catenane walker (75 nM) was incubated at room temperature for 15 min with 16 µL of the master-mix (2.5x transcription buffer, RNAsin (1:20 v/v), fusion T7RNAP-ZIF (223 nM),\(^{[1,2]}\) 2.5 mM MgCl\(_2\), 18.75% (v/v) DMSO) in order to allow T7RNAP-ZIF (86 nM concentration, 1.15 equivalents with respect to catenane) to complex with the catenane. Simultaneously, the DNA origami nanotube (75 nM) was incubated at 37 ºC with the comp-iStep (BHQ-2) ODN (1.33 equivalents per each iStep) or the Step1_blocker ODN for the corresponding control (2 equivalents) for 15 min. Then, the two solutions were mixed in 2x DNA buffer (20 mM Tris-HCl, 100 mM NaCl, 20 mM MgCl\(_2\) at pH 7.5), adjusting the volume to 40 µL and incubated for 30 min at 37 ºC in order to allow the catenane walker to hybridize to the Step1 position on the DNA origami. The sample was split in two (20 µL each) and loaded in an 8-well PCR tube strip. To each well, the NTPs mixture (25 mM, final concentration 2 mM (1,6 µL)) was added to start transcription. Finally, the fluorescence (TAMRA Ex555 nm, Em: 576 nm; Cy5: Ex: 649 nm, Em: 670 nm) was measured in a qPCR cycler (Bio Rad, IQ5) every minute at 37 ºC for the duration of the assay. Analysis of the kinetic data was performed by exponential fitting (one phase decay for TAMRA fluorophore and one phase association for Cy5 fluorophore) using GraphPad Prism software.

To regenerate the burnt-bridges path, 1 µL of different solutions of RNase A, H or A+Ti (between 1 and 2 µg; Thermo Scientific) were added when the walker reached Step2 (TAMRA fluorescence completely quenched, usually after 100 min).

Further walking cycles were achieved after addition of 6 µL of RNasin Ribonuclease Inhibitor Recombinant (Promega) and 1,6 µL of a mixture containing (1 µL NTPs (25 mM mixture), 1.1 1xDA and 0.4 µL of T7RNAP-ZIF (223 nM)).
**Fitting of the data kinetic analysis**

Fluorescence data were normalized and fitted using the exponential functions (GraphPad Prism software) below.

One phase decay:

\[ FL = (F_{max} - F_{min}) \times e^{(-K \times X(time))} \]

One phase association:

\[ FL = F_{min} + (F_{max} - F_{min}) \times (1 - e^{(-K \times X(time))}) \]
Table S1. List of ODNs used for the assembly of DNA rings and catenanes and origami.

| Steps used in this study | ODNs for the catenane walker/Steps on DNA origami path and comp-iStep |
|--------------------------|---------------------------------------------------------------------|

**210-bp rotor ring**

| ODN | Sequence |
|-----|----------|
| JV210_r | 5’-Phos-TCTTTTTTGGTCCTTTTTTGCAGTCTTTTTTTCGCAGTTTTTGGC |
| JV210_1f | 5’-Phos-AAAGCTGCAAAAAAAAAAGGCCAAAAAATGCAGAAAAAAA |
| JV210_2f | 5’-Phos-CGCGAAAAAGTGCAGAAAAAGACCAAAAAAGGCCGAAAAAGGC |
| ALgP_f | 5’-Phos-ACCGGGGGTTTGGGCGCTTTCTCGCTTTTTTCGCAGTTTTTGGC |
| JVgmblong | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| ALgP_r | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| JVR2short_f | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| RO_rotor | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |

**168-bp ring stator**

| ODN | Sequence |
|-----|----------|
| Alfa-b1zif | 5’-Phos-CGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| 168Alpha2_r | 5’-Phos-CGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| Alfa-a1zif2 | 5’-Phos-CGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| Threading ODN | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| Beta f short | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| Alfa b3long | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| RO CAT2 | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| RO_stator | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| 168beta_re_1short | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| 168beta_re2short | 5’-Phos-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| Blocker rc2c | 5’-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| RO_Blocker rc2c | 5’-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |
| Step1_6HB_JV | 5’-ACGCGCAAAAAACGTGCAAAAAAGAGCCAAAAACTGCGGAAAAAA |

**ODNs for the catenane walker/Steps on DNA origami path and comp-iStep**

| Steps used in this study | ODNs for the catenane walker/Steps on DNA origami path and comp-iStep |
|--------------------------|---------------------------------------------------------------------|
### Table S2. ODNs for the catenane walker/DNA origami path

| Name | Sequence |
|------|----------|
| 6HB_JV1 | TACATTTTTCAGCTATCTTACCAGAAGCCCGCTAAATATCAGAG |
| 6HB_JV2 | ACCCGCCAGCCCATTTGAACAAAGTTACAGAGAACTGACAGGAGACC |
| 6HB_JV3 | ACTCAAACATATCGGGGATACCCAAAAGCGAGAGAATAACA |
| 6HB_JV4 | ACTTCTTTGATTAGGCAGTATGTTAGCACGATTTTTGTTTA |
| 6HB_JV5 | TAAAAGAGTCTGTCCAACATATAAAAGAAAAAAATTAAAACAGCA |
| 6HB_JV6 | AAGAATCCTCTAGAAGTTTTTTGTCACATTTACACGCTACAGAGC |
| 6HB_JV7 | CAGGAGCGCCATTACAAAGACAAAGGTTGCTATTITTCGACC |
| 6HB_JV8 | GTATAACGTTGCTTTGTAATATTGGCAGCCGAGCTGGCGAGGA |
| 6HB_JV9 | GCGCCCGCTACAGGCGTCCAGGACTTATTCAGTACATT |
| 6HB_JV10 | GCGGTCAGCAGCTGCACCGACCAGTAAGACCAATACCCGACAGG |
| 6HB_JV11 | AAGAAAGCGAAAGCAATGAAACCATCGCTCATCGAGAACAA |
| 6HB_JV12 | GCTTGACGGGGAAAAATCAAGTTTGCCTTTCCTTATCC |
| 6HB_JV13 | AAGAATCCTCTAATGCGGCCAATTTATCA |
| 6HB_JV14 | GGTTTTCCCAGTCACGACGTTGTAAAACGACGGCAGGGATAGCAAGCCAACGCCTAGCA |
| 6HB_JV15 | TGCAGCATACAGCCAAAATCAATTTATCTT |
| 6HB_JV16 | CAGTTTGGAACAAAGGAGCCACCACCCTCGAAGACCCACAC |
| 6HB_JV17 | ATATAACAAAGACAGCCCGCAGATGCGAGGAGCAATTTTCAAT |
| 6HB_JV18 | AAAATCGTGTGTTGAGCTTGTGATGTTG |
| 6HB_JV19 | AGTTGCAGCAACGAATGAAAAGCGAGTCAGTGACCCCTACG |
| 6HB_JV20 | ACGGGCAACAGCTGCATACTGATTGCTTTTGTGCTAGAGCTTTG |
| 6HB_JV21 | TGCAGTAATCGAGGTCCATAGTTTGCTAGAGGAGCAGG |
| 6HB_JV22 | TGCAGTAGTAATGACAGGTGATGTAATTTTCTT |
| 6HB_JV23 | CGACTCTAGAGATCACCCCTAGCAGAACAGAAGTACCTAAG |
| 6HB_JV24 | CCTGGGTTGCTTAATAGGAATTAGCGGAGGATAATATTTCG |
| 6HB_JV25 | CAATTTCACAACCCGAGCGTCGAGAAGGTGTTCTAGAGAA |
| 6HB_JV26 | CATGGTCATAAGCTGCACCGTACTCCAGAAATTTTCTGTATAG |
| 6HB_JV27 | CGACTCTAGAGATCACCCCTAGCAGAACAGAAGTACCTAAG |
| 6HB_JV28 | GGTATCTCCAGTCAGCATCTGTAAGAGCAGGAGGATAGCAAGCAACCGCCTCTAGAC |
| 6HB_JV29 | AGTCACACGACAAATTTATCA |
| 6HB_JV74 | TGTACCAAAAAACATCATATAGTACCCCGGTTGAGTGGTTGTC  |
| 6HB_JV75 | AAAATTAAGCAATAAGAGATTGTATAAAGCGCAAAAATCCCTT  |
| 6HB_JV76 | ATTAACATCTCAATACAAAACCTGAGGCGGAGAGGCGGAG  |
| 6HB_JV77 | GCGAGCTGAAAAAGGTTTAAACCAATAGGCTGCGGCTGAGAG  |
| 6HB_JV78 | CAAAAGGATCAAATACCTGAGTACCCCGGTTGAGTGGTTGTC  |
| 6HB_JV79 | CTGGGCAACCGAGATGACTAAACACACCGTGCGGGGAGAGGCGG  |
| 6HB_JV80 | GTGTCTGCGTTGTTTACCGGAATGGGAGAGGCGGAGGTTCTGAGACGCTCA  |
| 6HB_JV81 | TGTACCAAAAAACATCATATAGTACCCCGGTTGAGTGGTTGTC  |
| 6HB_JV82 | GGGTCACCGGATCGAGTAAACACACCGTGCGGGGAGAGGCGGAG  |
| 6HB_JV83 | GCACTGGATTAGAGATCTGGTGCCGGAAATTGTTGAGGCGGACTCA  |
| 6HB_JV84 | TCGAGCTTCAATACCGCAGAAGCGGATCGTCGACATTAGGGAATTC  |
| 6HB_JV85 | ATAGGAGGAAGCCCGAAATACCTGAGTACCCCGGTTGAGTGGTTGTC  |
| 6HB_JV86 | TGTGATAAATAAGGATCCAAATAAGAAAAACGTAGAAAATACGAG  |
| 6HB_JV87 | AAATCAATAGGTCGAGAGACTACAAATAGTGGAGGACCGCAATA  |
| 6HB_JV88 | ATATAACTATATATGAGAGGGTAATTGAGCTTTTTAAGAAAAGAAA  |
| 6HB_JV89 | CAAAGAACGCGAGACGCATTAGACGGGAAAGGAAACCGAGGAG  |
| 6HB_JV90 | TTCATCTTCTGACCAATAGCAGCCTTTTAACCTGGCAGATGATTA  |
| 6HB_JV91 | TGTGATAAATAAGGATCCAAATAAGAAAAACGTAGAAAATACGAG  |
| 6HB_JV92 | TAATTTCTAAGAAAATTTGCCAGTTCAACGCAAAGCACCAC  |
| 6HB_JV93 | AAATTTCTTACGCTTCTTCTGAGTATGAGGACCGCAATACATGAGGAGT  |
| 6HB_JV94 | ATAGGAGGAAGCCCGAAATACCTGAGTACCCCGGTTGAGTGGTTGTC  |
| 6HB_JV95 | ATAGGAGGAAGCCCGAAATACCTGAGTACCCCGGTTGAGTGGTTGTC  |
| 6HB_JV96 | CCGACAAAGGTTAATCAGATAATAGAAGGGCGCATTTGGAATT  |
| 6HB_JV97 | ACAACATGTGGTACGTTTTTTCGTTAGATTACGATTACAGAAG  |
| 6HB_JV98 | ATAGGAGGAAGCCCGAAATACCTGAGTACCCCGGTTGAGTGGTTGTC  |
| 6HB_JV99 | TTACGAGCATGAGATAATCGGCTGCTTTAGCGTACAGACTG  |
| 6HB_JV100 | CAGACCGTCAATACCGCAGGATTATAATGTGATAGGCCCCTTTAT  |
| 6HB_JV101 | AGAGGACAGATGAAATACCAAGCGCGAATCACCGGAACCAG  |
| 6HB_JV102 | CTTACGACCTTCAAAAAAGAATACACTACCGCCACCCTCAGA  |
| 6HB_JV103 | ATTACCCAAAATCAGTAAGCTCAGATGACGGCAGCCACCAG  |
| 6HB_JV104 | AGGTGGGCTCTCTCTAGACAGATCCTTTTCATGCAGAGGTGTGGGAG  |
| 6HB_JV105 | AGGTGGGCTCTCTCTAGACAGATCCTTTTCATGCAGAGGTGTGGGAG  |
| 6HB_JV106 | CTTATCGAGTTTTTCCGCTTTTCCGCGGATCTTCGAATTTACA  |
| 6HB_JV107 | CGTTGGAAGAAGAAAACCGGATATTTCGGAATACAGAGGATGTGAGT  |
| 6HB_JV108 | ACAACATTACTACGTAAGTCTGGCGGCTTCTGAGTACAC  |
| 6HB_JV109 | GGAATACACACATTACGCTCTTCTGCTTTCTGCTATTTCGGAAG  |
| 6HB_JV110 | GAATTACGAGGCATCAAAGAAAGGCTCAGAGGCTGAGACTC  |
| 6HB_JV111 | TTTACGACCGAGCTAAAGAAGGAGTTTCGAGTTTCTCAGATACC  |
| 6HB_JV112 | GCAAAGAAGTTTTACTTTCAACAGTATTATAAGTAGTACAGCCC  |
| 6HB_JV113 | GACTTTGATAGCGCTGACGTCTTACTGACATGGAATGTGGGAGTTGGA  |
| 6HB_JV114 | ATTTCAACCCCTCTCCTCTGAGAAGTCTGAGTCTGCTCTGAG  |
| 6HB_JV115 | AATGACCATAAATCCACAGTACAAACTACATACATAGGGAATAGCCCA  |
| 6HB_JV116 | AGATAACCCACAAAGCTTTTTAACCCTCGATATAAGACGCTGAAG |
| 6HB_JV161 | ACCACCACCAGAGCTAGCCCGAGATAGGTTGATAATCAGAAA |
|-----------|-------------------------------------------|
| 6HB_JV162 | CAGGTCAGACGATTGGTGTTCCGAAATCAAATTTAAATT   |
| 6HB_JV163 | CTCATTAAGCCAGGTCCACCGTGTTTCATATTAAATT    |
| 6HB_JV164 | GTTCCAGTAAGCGTATTGCCTTCACCAGACGCCATCAAAAA |
| 6HB_JV165 | GTACTGTAATAAGCAGGGTGGATTATTTGCTTTATCAACAT |
| 6HB_JV166 | AGTGCCCCTATAAAATCGGCAAACGGCACTTCTCCGTTGGAA |
| 6HB_JV167 | CCTATTATTTCTGAAGCTTTCCAGTCGGGGTTACGTTTGTTG |
| 6HB_JV168 | CTCAAGAGAAGGATTTGAGTGAGCTAACTCCAGTTTGAGGGGA |
| 6HB_JV169 | AGGCGGATAAGTCGATCGAGCCGAAAGGCACTCCAGCCACG |
| 6HB_JV170 | CGGAATAAGGTGTATTTCTGTGTAACCCAGGCAAAGCGCC |
| 6HB_JV171 | ACCCTCAAGACGCCCGGGTACCGAGGGGAAGGGCGATCGG |
| 6HB_JV172 | ACCACCCTCATTTTCAGTGCAAAGCTTGGCGAAAGGGGAT |
Figure S1. Normalized TAMRA fluorescence signal (F1) of the catenane walker after a walking cycle and addition of RNase A (1µg) at t=105 min (red line, n=2). No transcription control (green line) with the same experimental conditions but lacking NTPs, shows that the catenane remains stable in Step 1 during the experiment. Step1 blocked control (blue line) shows that the catenane efficiently hybridizes to Step2 of the track when Step1 is blocked by a blocking ODN (signal quenched) and remains attached to Step2 throughout the experiment. In these control experiments RNase A (1 µg at t=105 min) is also added.
Figure S2. Normalized TAMRA fluorescence signal (F1) of the catenane walker after a walking cycle and addition of RNase H (1 µg), RNase A and RNase A+Ti (10 µg) at t=110 min. Step2→1 transition half-lives (τ) were 21.9 ±0.6 min, 15.7 ±0.5 min and 14.0 ±0.4 min, respectively (mean ±s.d.).
Figure S3. Normalized TAMRA (A) and Cy5 (B) fluorescence signal (F1 and F2 respectively) of the catenane walker after a walking cycle, addition of RNase H (1 µg) at t=120 min and RNasin addition (t=300 min). As expected, these data shows that RNasin is not able to inhibit RNase H, and allow performing a second walking cycle (mean ±s.d.).
References:

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