Supporting Information
A reversible light switch for macrocycle mobility in a DNA rotaxane.

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Materials and Methods
Chemicals and reagents
The chemicals used for azobenzene phosphoramidite synthesis were purchased from Alfa Aesar, Sigma Aldrich, and Fluka and were used without further purification. The reagents for DNA synthesis were purchased from J. T.-Baker, Applied Biosystems and Proligo Reagents.

Buffer systems
1x TAE buffer: 40 mM Tris, 20 mM AcOH, 1mM EDTA.
1x DNA storage buffer: 10 mM TrisHCl, 50 mM NaCl, 10 mM MgCl2 at pH 7.5.
1x ligase buffer: 40 mM TrisHCl, 10 mM MgCl2, 10 mM DTT, 5 mM ATP at pH 7.8.
WAX buffer A: 20 mM TrisHCl, pH 9.0.
WAX buffer B: 20 mM TrisHCl, 1 M NaCl, pH 9.0.

Phosphoramidites
The azobenzene phosphoramidites were synthesized according to slightly modified published procedures.1, 2

DNA
The oligodeoxynucleotides (ODNs) consisting only natural bases and fluorophore labels were supplied HPLC purified by METABION. The azobenzene incorporated ODNs were synthesized with an ABI 3400 DNA-Synthesizer and purified by reversed-phase HPLC on an Agilent 1100 Series HPLC system. The modified ODNs were synthesized according to published procedures.1, 2

DNA-Nanostructures
The sequences used for synthesizing the gap rings, the spherical stoppers, the axles and the test system are listed in table S1, together with the release oligodeoxynucleotides used in this study. The purification was achieved by weak anion exchange HPLC: column TSKgel DEAE-NPR 4.6 mm x 35 mm (TOSOH); WAX buffer A-Wax buffer B gradient: 45% - 65% in 20 min. After purification, the product fractions were concentrated using Amicon Ultra-0.5 centrifugal filter devices (YM-30, Millipore), washed twice with 1x DNA storage buffer and elucidated in 100 µl 1x DNA storage buffer. Concentrations were calculated from the OD values obtained from UV measurements.

Assembly of DNA rings
The ODNs (4 µM) and NaCl (40 mM) in 1x ligase buffer were annealed from 60 °C to 15 °C over 75 min. Ligase (1 µl/100 µl, 10 U) was added and ligated over night at 15 °C. The products were purified by HPLC and concentrated using Amicon centrifugal filter devices.

Assembly of spherical stoppers
The spherical stoppers where assembled in a one-pot procedure from the crude precursor DNA rings ring B and ring C’ as described by Ackermann et al.3 Ring B and ring C’ where annealed and ligated as described above. The crude rings were combined and annealed from 60 °C to 15 °C over 75 min. Ligase was added and ligated over night at 15 °C. The products were purified by HPLC and concentrated using Amicon centrifugal filter devices.
Synthesis of pseudorotaxanes
The ODNs (2 µM) used for assembling the DNA axle were annealed in 1x DNA storage buffer at room temperature for 10 min. The gap-ring (2 equiv.), 10x ligase buffer and 1M MgCl₂ were added to obtain a 1x ligase buffer solution containing additional 30 mM MgCl₂ and 50 mM NaCl. The solution was kept at 15 °C over night, before adding the stoppers (2.5 equiv.). After hybridizing for 30 min, ligase (1 µl/100 µl, 10 U) was added and ligated over night at 15 °C. For switching experiments the pseudorotaxane was used without further purification, unless noted otherwise.

Assembly of test system
The ODNs used for the DNA axle and the Cy3 8-mer were mixed together in 1x DNA storage buffer and hybridized for 10 min at room temperature.

Photoregulation of cis-trans isomerization
Photo-irradiation of the samples was carried out in glass cuvettes with an in house fabricated light source, equipped with two UV-LED (NICHIA Corporation) and a Peltier element for temperature control. For cis-isomerization the sample was irradiated 5 min at 365 nm at 40 °C, and for trans-isomerization 2 min at 450 nm at 40 °C.

UV/vis spectroscopy of AB- and DMAB-functionalized ODNs
Thermally induced isomerization from cis to trans azobenzene residues incorporated in DNA was carried out with a Lambda 2S UV/vis spectrometer (PerkinElmer). The azobenzene modified ODNs (10 µM) in TrisHCl buffer (100 mM NaCl, 10 mM TrisHCl, pH 7) were photoisomerized into the trans-form and the absorption was measured from 280 to 500 nm. After photoisomerization into the cis-form, the samples were protected from light, incubated at either 25 or 60 °C, and the absorption spectra were measured after the indicated time (Supp. Figure S5).

Gel electrophoresis
*Light triggered switch:* The pseudorotaxane (0.6 µM for ring-stopper, 0.1 µM for spherical stopper) was treated with cis-AB6-RO or cis-DMAB5-RO respectively. An aliquot was taken and cooled to 4 °C. Then the sample was irradiated at 450 nm and another aliquot was taken. The sample was irradiated at 365 nm before taking another aliquot. The remaining sample was irradiated at 450 nm once more. All aliquots were incubated for 10 min at 25 °C before analyzing on gel. In the case of the ring stopper rotaxane, 10 equiv. of cis-AB6-RO were added and the experiment was stopped after the first irradiation at 450 nm. A 2.0 % agarose gel was run for 30 min at 180 V. In the experiment with spherical stopper rotaxane, 10 equiv. of cis-AB6-RO (or cis-DMAB5-RO) were added and a 2.4 % agarose gel was run for 2 h at 180 V.
*Toehold triggered switch:* The pseudorotaxane was treated alternating with th-RO and cth, always with 5 equivalents excess with regard to the pseudorotaxane. After each addition, the sample was incubated for 30 min at 25 °C and an aliquot was taken. After two switching cycles, all samples were incubated at 25 °C over night. A 2.4 % agarose gel was run for 2 h at 180 V. All gels were poured from ‘Agarose High Resolution’ (ROTH), run in 0.5 x TAE at 4 °C, stained with ethidium bromide and visualized by UV irradiation.

Fluorescence spectroscopy
*Toehold triggered switch:* The fluorescence of the pseudorotaxane was measured as described above. For the pseudorotaxane/rotaxane switch, the sample was treated alternating with th-RO and cth. In the first switching cycle 2.5 equiv. th-RO and 5 equiv. cth were used to trigger switching. The amount of th-RO and cth added was increased to a final of 20 equiv. of th-RO and 22.5 equiv. of cth in the fourth cycle. After addition of the corresponding ODN, the sample was incubated for 30 min at 25 °C and fluorescence was measured.
*Light triggered switch:* The HPLC purified Cy3-BHQ2-labeled pseudorotaxane with ring stoppers (0.1 µM) in DNA store buffer was excited at 550 nm at 30 °C and the fluorescence emission was measured 5 times at 570 nm for 500 ms in a Varioscan plate reader (Thermo Scientific). The sample was treated with 2.5 equiv. of cis-AB6-RO, incubated 5 min at 30 °C and fluorescence was measured. Following, the sample was irradiated at 450 nm, incubated 5 min at 30 °C and fluorescence was measured.

Fluorescence spectroscopy of Cy3-labeled macrocycle/rotaxane in the presence of DMAB5-RO: The Cy3 labeled samples (HPLC purified macrocycle (0.2 µM) and crude rotaxane (0.14 µM) in DNA store buffer were excited at 550 nm at 25 °C and the fluorescence was measured form 500 to 650 nm in a fluorescence spectrometer (PerkinElmer). After addition of trans-DMAB5-RO the fluorescence was measured. In the case of the macrocycle, the sample was irradiated at 365 nm to obtain the cis-DMAB5-RO and fluorescence was measured again.
Supporting Figure S1. Sequences and secondary structures of DNA axle (a), gap ring (b) and the setup for the fluorescence quenching experiments (c). In (c), the ODNs R1u1 of the gap ring and EFCT-2 of the axle are replaced by the fluorophor/quencher labelled ODN Cy3-R1u1 and BHQ2-EFCT-2. (d) Synthesis scheme for the 2’,6’-dimethylazobenzene Phosphoramidite. e) Unsubstituted azobenzene molecule with the threoninol backbone, as it is introduced into a DNA strand. f) 2’,6’-dimethylazobenzene as it is introduced into a DNA strand.
| name (number of bases) | sequence |
|------------------------|----------|
| **gap ring**            |          |
| R1o1 (43)              | 5'phos-<br>CAGTTTTTGCCCTTTTTCGCTTTTTGCCTTTTTCCG |
| R1o2 (44)              | 5'phos-<br>TCTTTTTGGCACTTTTTTCTTCGCAGCGGTACGTTTTTTACCGC |
| R1o3 (39)              | 5'phos-<br>TTTTTGAACATTTTTTGACAGTTTTTCCGTCTTTTTTGC |
| R1u1 (53)              | 5'phos-<br>AGAAAAAAGTGCCAAAAAGACGGAAAAAACGCGCAAAAAGCGCGAAAAAAGGG |
| R1u2 (63)              | 5'phos-<br>CCAAAAACTGGCAAAAAAGACGGAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| Cy3-R1u1 (53)          | 5'-Cy3-<br>AGAAAAAAGTGCCAAAAAGACGGAAAAAACGCGCAAAAAGCGCGAAAAAAGGG |
| **spherical stopper (ring B)** |          |
| HJalpha b (53)         | 5'phos-<br>AAAGTGCCAAAAAGACCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| HJalpha c (61)         | 5'phos-<br>AAAAGTGCCAAAAAGACCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| HJbeta a (56)          | 5'phos-<br>AAAAGTGCCAAAAAGACCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| HJbeta k (60)          | 5'phos-<br>AAAAGTGCCAAAAAGACCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| Bogen f (47)           | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| Bogen r (47)           | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| **spherical stopper (ring C')** |          |
| Ring1 r (56)           | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| RingSE a (54)          | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| RingSE b (52)          | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| GE-1 (39)              | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| GE-2 (42)              | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| GE-3 (45)              | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| GE-4 (42)              | 5'phos-<br>AAAAGCGCGAAAAAAGGGCCAAAAACTGTCAAAAAATGTTCAAAAAGCGGTAAAAAACGT |
| **DNA axle**           |          |
| A1u1 (57)              | 5'phos-<br>CACGACTGTTATCCCTGTGAACTAAGTCCGCTGCGTATGACAGTTACTGTACCTGGA |
| EFCT-1 (20)            | 5'-<br>TTAGTTCAAGGGATAACAG |
| EFCT-2 (25)            | 5'phos-<br>CAGGATCCAGGTACAGTAACTGTCA |
| BHQ2-EFCT2 (25)        | 5'phos-<br>CAGGATCCAGGTACAGTAACTGTCA-<br>BHQ2 |
| **release oligos**     |          |
| um-RO (12)             | 5'-<br>TACGCGAGCGGAC |
| AB2-RO (12)            | 5'-<br>TACGCGAGCGGAC |
| AB3-RO (12)            | 5'-<br>TACGCGAGCGGAC |
| AB4-RO (12)            | 5'-<br>TACGCGAGCGGAC |
| AB5-RO (12)            | 5'-<br>TACGCGAGCGGAC |
| AB6-RO (12)            | 5'-<br>TACGCGAGCGGAC |
| AB8-RO (12)            | 5'-<br>TACGCGAGCGGAC |
| DMAB5-RO (12)          | 5'-<br>TAYCGYCAAYCGYAC |
| th-RO (19)             | 5'-<br>TACGCGAGCGGAC |
| th (19)                | 5'-<br>TACGCGAGCGGAC |
| **test system**        |          |
| Cy3-8mer (8)           | 5'-<br>Cy3-CGACGCGG |

**Supporting Table S1.** Sequences used in rotaxane assembly and for switching experiments. The X in the AB-ROs stands for one unsubstituted azobenzene residue according to Figure S1e, the Y in the DMAB5-RO stands for one 2',6'-dimethylazobenzene residue according to Figure S1f.
**Supporting Figure S2.** Pilot study for a light induced, reversible strand displacement switch (test system). In the upper part, the sequences and the setup used in this study are shown, exemplarily with the AB6-RO. The sequences of the DNA axle are identical to the sequences of the rotaxane axle. The sequence of the Cy3 8-mer is identical to the single-stranded gap sequence of the macrocycle used in the rotaxane assembly. Due to the fluorophor/quencher labels attached to the 8-mer ODN and the axle, hybridization of the 8-mer with the gap on the axle could be detected by fluorescence measurements. The efficiency of strand displacement by the AB-RO (in cis- and in trans-form, respectively) was determined by measuring the fluorescence quenching/dequenching.

Fluorescence was measured after addition of 2 equiv. of the cis-AB-RO to the axle/8-mer-hybrid (1 µM). The sample was then repetitively irradiated first at 450 nm (2 min, vis) and then 365 nm (5 min, UV). After each irradiation step the fluorescence was measured. This experiment was performed at different temperatures and with six different AB-RO’s having 2, 3, 4, 5, 6, or 8 azobenzene residues incorporated. It was found, that the highest switching efficiency was achieved with AB6-RO at 30 °C.

The data obtained for this system (AB6-RO) are shown at the bottom.
Supporting Figure S3. Schematic for the macrocycle displacement and slippage mechanism and architecture of the analyzed pseudorotaxane/rotaxane switch. Grey/yellow sphere: quenched/unquenched Cy3-fluorophor. Black sphere: BHQ2-quencher. Green: AB6-RO. b) Fluorescence spectroscopy of the HPLC purified Cy3-BHQ2-labeled pseudorotaxane with ring stoppers. c) Analytical agarose gel electrophoresis of the DNA dumbbell (lane 1), the DNA pseudorotaxane before (lane 2) and after addition of the cis-AB6-RO (lane 3). Lane 4 belongs to the sample after photoisomerisation of the AB6-RO into the trans-form. The bands on lane 3 indicate, that the cis-AB6-RO does not release the macrocycle from the dumbbell (ratio pseudorotaxane : dumbbell 9 : 1), while the bands on lane 4 are evidence for the macrocycle displacement by the trans-AB6-RO (ratio pseudorotaxane : dumbbell 3 : 7).
**Supporting Figure S4.** Cy3 quenching by dimethylazobenzene. a) The HPLC purified Cy3 labeled macrocycle (0.2 µM) was excited at 550 nm and fluorescence emission was measured from 500 to 650 nm (blue curve). After addition of 10 equivalents of DMAB5-RO (trans) the fluorescence measured was significantly decreased (black curve). When the DMAB5-RO was photoisomerized to the cis-form, a small increase in fluorescence was obtained (red curve). b) The fluorescence emission of the Cy3 labeled rotaxane (0.14 µM, crude product) was measured before (blue curve) and after addition of 2, 4 and 10 equivalents of trans-DMAB5-RO (purple, brown and black curve). Even tough the trans-DMAB5-RO displaces the macrocycle from the axle and the Cy3-dye is no more quenched by BHQ2, increasing amounts of trans-DMAB5-RO resulted in a decrease in fluorescence emission. This quenching effect is due to the DMAB-residues. Because this quenching effect strongly interferes with the desired fluorescence emission of the mobile macrocycle when an excess of DMAB5-RO is used, switching of the macrocycle mobility in the rotaxane cannot be followed by fluorescence spectroscopy.
Thermally induced isomerization of the cis-azobenzene-ROs (AB6-RO and DMAB5-RO) to the trans-form analyzed by UV/vis spectroscopy. a) AB6-RO in its trans-form as a reference sample (red curve). Starting from its cis-form (blue curve) the AB6-RO isomerizes back to the trans-form (black curve) within 4 h at 60 °C, whereas at room temperature nearly no conversion is observed, not even after 12 h (purple curve). b) DMAB5-RO in its trans-form (red) and its cis-form (blue) as reference samples. Neither at room temperature (brown) nor at 60 °C any substantial isomerization to the trans-form within 15 h (black) or within 24 h (purple) occurs. However, irradiation of the DMAB5-RO at 450 nm resulted in nearly quantitative trans-isomerization (green). These observations agree with the results from Asanuma et al.\textsuperscript{5} and document the improved photochemical properties of the DMAB5-RO as compared to the AB6-RO.

The improved switching behaviour of 2',6'-dimethylazobenzene- as compared to the azobenzene-functionalized ODNs in the rotaxane architecture, however, can be explained on the basis of differences in the melting temperature rather than on the photochemical properties. The more bulky methyl groups favor DNA double strand formation in the trans-form through better stacking interactions on one hand, while destabilizing the DNA duplex more effectively due to increased steric hindrance in the cis-form on the other hand. The crucial physicochemical property that determines whether base pairing is switched on or off is the difference in the melting temperature of the two isomeric forms. The higher this difference the better the switch can be operated. This has been unambiguously demonstrated on the level of DNA melting temperatures.\textsuperscript{1-3,5}
Supporting Figure S6: Representation of the entire gels form different switching experiments. a) toehold triggered switch as shown in Figure 2c. b) Light-induced switch mediated by AB6-RO using purified pseudorotaxane as in Figure 3b. c) Light-induced switch mediated by DMAB5-RO starting from crude pseudorotaxane as in Figure 3c.)
Supporting Information References.

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