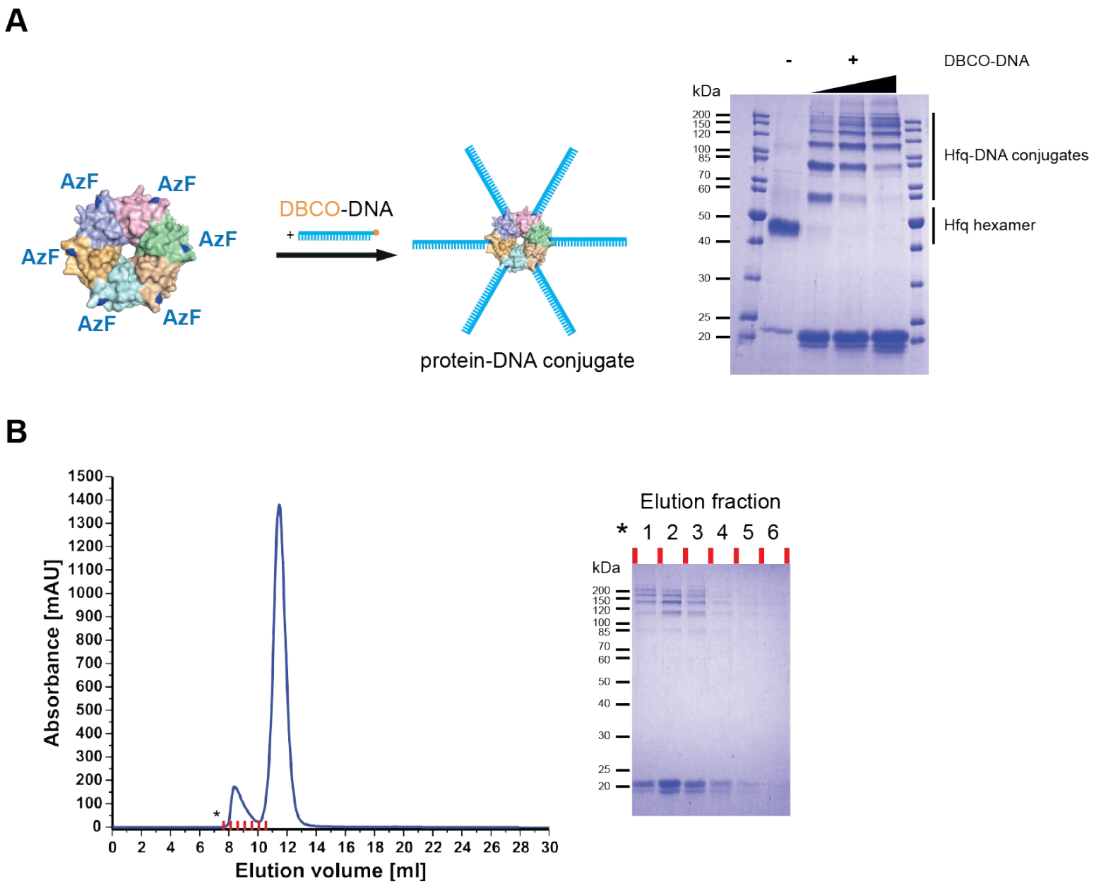
Supplementary Information for

Towards Structural Biology with Super-Resolution Microscopy

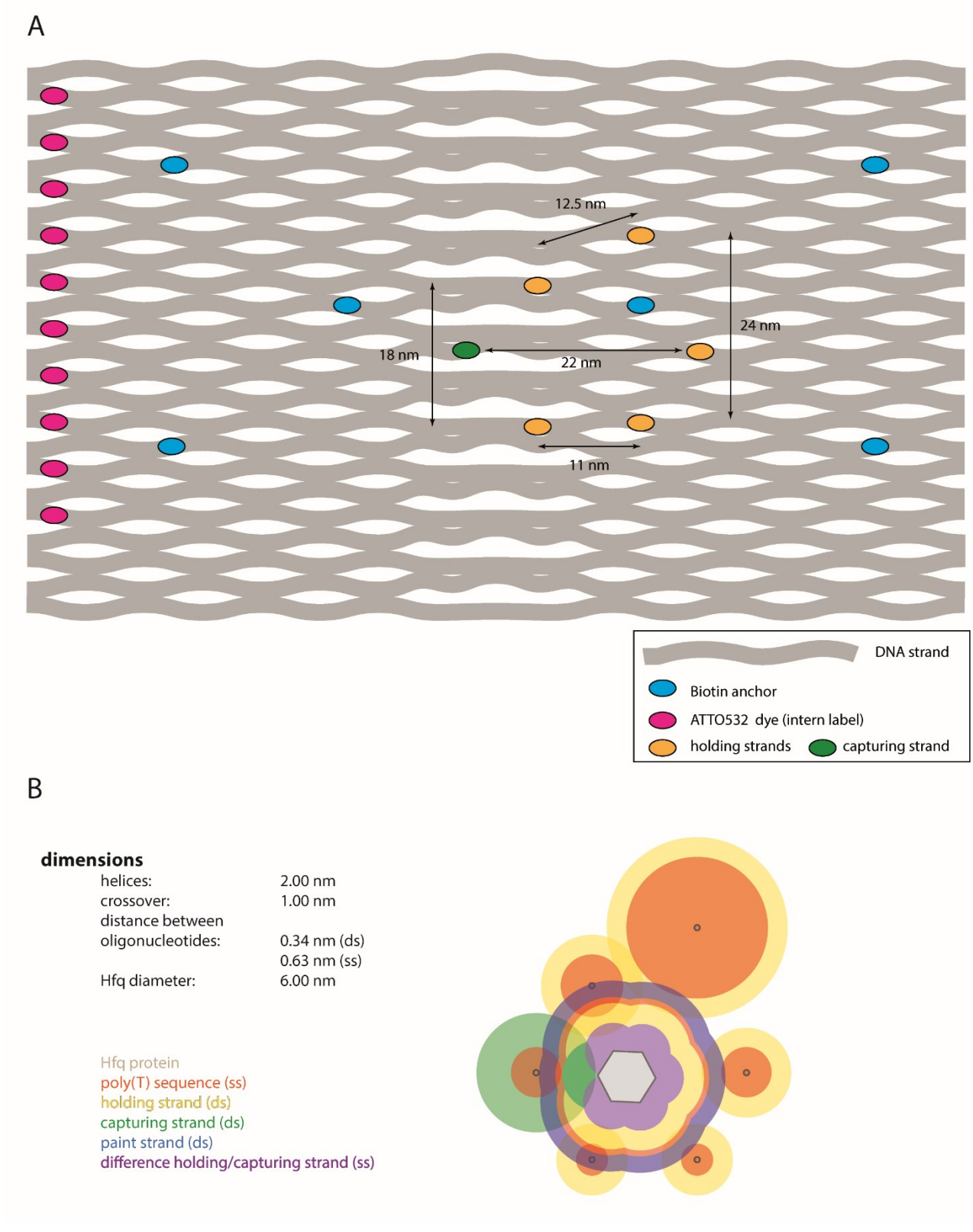
1 Production and purification of MjHfq-DNA conjugates



Supplementary Figure S1 related to Figure 2

Production and purification of MjHfq-DNA conjugates. (A) Site-specific engineering of the unnatural amino acid p-Azido-L-phenylalanine (AzF) into the Hfq-like protein from *Methanocaldococcus jannaschii* (MjHfq, PDB: 2QTX) allows the coupling of a dibenzocycloocytne (DBCO)-modified DNA oligonucleotide via strain-promoted alkyne azide cycloaddition between the azide group of AzF and the cyclooctyne group of DBCO. SDS gel electrophoresis analysis of MjHfq before and after the coupling reaction with the DBCO-DNA. The monomeric MjHfq protein has a molecular weight of 8.3 kDa, the hexameric form has a molecular weight of 49.8 kDa and the DNA oligonucleotide has a nominal molecular weight of 10.6 kDa. Both, MjHfq dimers/trimers (16.6 and 24.9 kDa) and hexamers can be identified in the gel. Even under denaturing conditions, MjHfq adopts its hexameric state. Addition of DBCO-DNA in increasing concentrations leads to the appearance of high molecular weight reaction products that form a step-wise ladder. This indicates the formation of MjHfq-DNA conjugates with a step-wise increase in molecular weight each time a DNA oligonucleotide is added to the protein. The fraction of high molecular weight conjugates increase at higher DNA:protein ratios suggesting that an excess of DBCO-DNA leads to the full saturation of the protein with the six possible conjugated DNA strands. However, even at the maximal DNA concentration, the coupling reaction is not 100% efficient as lower molecular weight bands that correspond to MjHfq coupled to only 1, 2, 3, 4 and 5 DNA oligonucleotides are visible. (B) In order to remove free DNA and to purify MjHfq-DNA conjugates with a high coupling state, the coupling reaction was subjected to size exclusion chromatography using a Superdex 75 column. The elution profile (left) shows two peaks (absorption at 280 nm is shown). The MjHfq-DNA conjugates after 8-10 ml while the second peak at 12 ml corresponds to free DNA. The MjHfq-DNA fractions were analysed on a SDS gel (right). Elution fraction 1 contains mainly MjHfq proteins conjugated with five or six DNA oligonucleotides with a small amount of MjHfq-DNA_3 and MjHfq-DNA_4. Elution fraction 1 was used for the formation of the DNA origami – Hfq nanostructure.

2 DNA origami structure

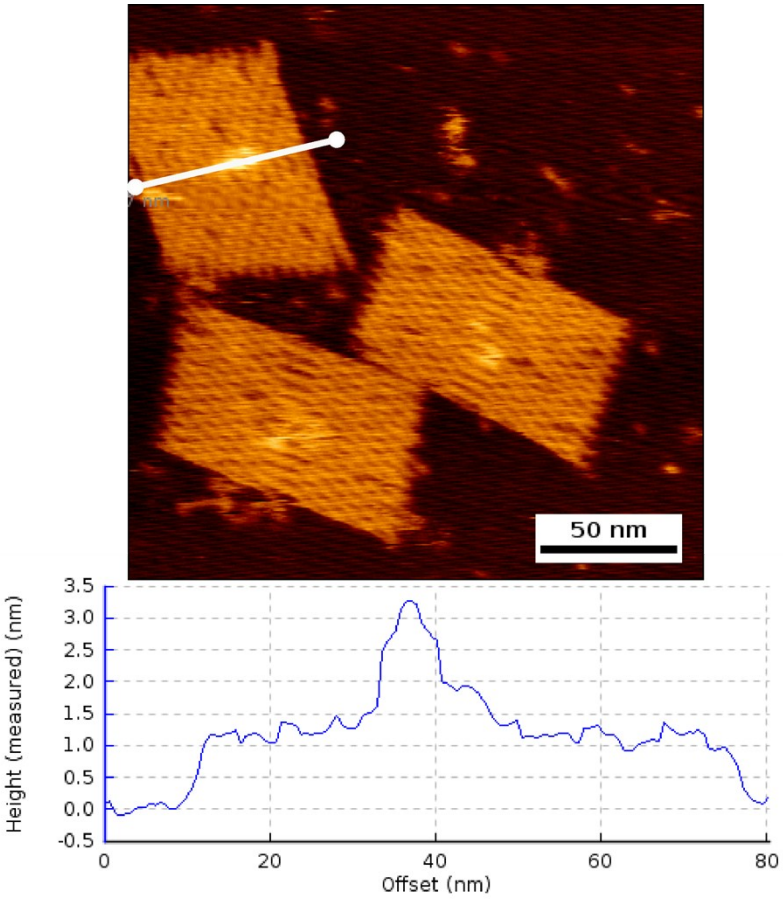


Supplementary Figure S2 related to Figure 1**.**

(A) A schematic view of a rectangular origami used in this study [1]. The origami is shown in top view. Grey wave-like lines represented the DNA strands of the origami itself. Six staples were replaced by a modified biotinylated ssDNA

(indicated by a blue oval). The biotins are orientated towards the bottom. The capturing (green oval) and five holding strands (orange oval) are orientated upwards to allow immobilization of the DNA-conjugated Hfq protein on top of the origami. The capture and holding strands are placed between the two central biotin modifications to ensure that the mounting of the protein is not influenced by bending and tilting of the nanostructure. Magenta ovals indicate the positon of ten Atto532-labelled staple strands that were used for DNA origami identification in fluorescence miscroscopy images. Distances between origami helices and anchoring positons are given.
(B) Dimensions of the Hfq-DNA conjugate relative to the anchoring points on the DNA origami. The centre of the six red circles with yellow or green rings illustrate the coordinates of the capturing (green ring) and holding strands (yellow ring) oligonucleotides that protrude from DNA origami nanostructure. These coordinates were directly converted from the caDNAno (https://cadnano.org/) file. The red circle indicates the single stranded (ss) poly(T) sequence that is part of the respective holding strands (see paragraph 5 in the Supplementary Information). The poly(T) sequence varies in length to adjust the length of the respective strand to ensure that the strand is long enough to hybridize to the complementary DNA sequence in the DNA strands coupled to the Hfq protein. Hence, the dimension of the red circles varies depending on the position of the holding strand relative to Hfq (grey hexagon). The yellow and green ring reflects the dimensions of the part of the holding strand and capturing strands, respectively, that is able to form a double strand (ds) with the complementary DNA sequence in the DNA strands coupled to the Hfq protein. The following layers (from inside to the outside) surround the Hfq hexagon (grey): purple – single-stranded part of the DNA strand conjugated to Hfq that does not hybridise to capturing or holding strands; yellow – part of the DNA strand conjugated to Hfq that is complementary to capturing or holding strands; red – short poly(T) stretch; light blue – DNA sequence complementary to the DNA PAINT sequence. Same colors were chosen for complementary sequences (yellow part of the holding strands can bind to yellow part of the Hfq-conjugated strand). This illustration does not indicate any higher order structures of the DNA that might result in smaller spheres/circles. Nevertheless, this figure illustrates how whether or not the dimensions of the capturing and holdings strands overlap with the dimension of the DNA-conjugated Hfq. If one assumes that the capturing stand is always bound to Hfq (due to the longer complementary DNA sequence this interaction should be more stable as compared to the interaction with the holding strands), it is easy to imagine that the protein can associate with up to four holding strands if the flexibility of the DNA origami nanostructure is taken into account. However, hybridization of the Hfq-coupled DNA strands with the capturing strand and all holding strands seems to be impossible. Please note that the binding of the PAINT strands is nevertheless possible. These paint binding events most likely lead to a shortened distance as compared to the theoretical distance and a more broad distribution of the distances measured via super-resolution microscopy.

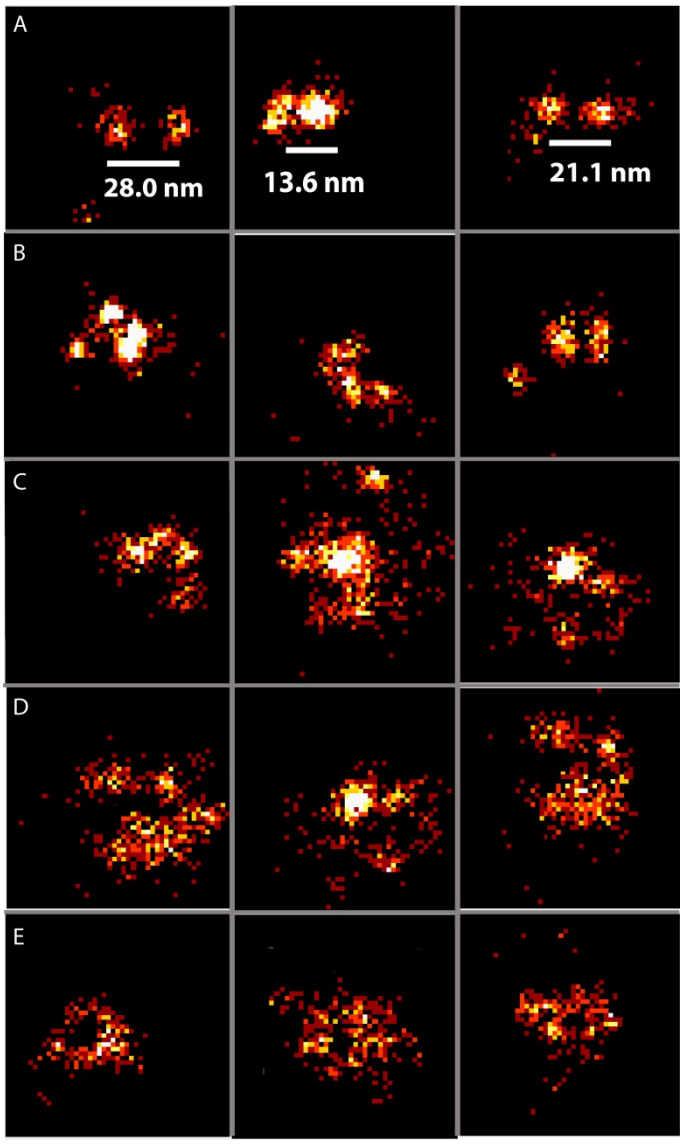
3 Height profile of Hfq-DNA origami nanostructures



Supplementary Figure S3 related to Figure 2**.**

Height profile of a DNA origami-Hfq nanostructure. Atomic force microscope image of DNA-Origami-Hfq nanostructures. Rectangular DNA origamis (dimensions of 82 nm x 62 nm) show a circular spot at the designed MjHfq-immobilisation site at the centre of the DNA origami. Height analysis shows that this additional structure differs in height from the DNA origami rectangle (1.5 nm). In the crystal structure, MjHfq is approximately 2-3 nm in height in 6 nm in width [2] matching the dimensions measured using AFM (height: 2.2 nm, width: 10 nm).

4 Selection of super-resolved DNA PAINT images of the DNA origami-Hfq nanostructures



Supplementary Figure S4 related to Figure 3**.**

Selection super-resolved images of the DNA origami-Hfq nanostructures based on DNA PAINT measurements. Overview of super-resolved images of the DNA origami-Hfq nanostructure with increasing numbers of single-molecule localisations (magnified view (50x50 pixels) with 1 nm/pixel) imaged via DNA-PAINT. All spots co-localise with green ATTO532 dyes as identifier for the presence of a DNA origami (fluorescence of ATTO532 not shown for clarity). Panel A-E shows images with 2, 3, 4, 5 and most likely 6 single Atto655 localisations. Please note that images with more than three seperated spots were not used for distance analysis as they do not appear at separate spots.

5 DNA origami and DNA oligonucleotides

The DNA origami used is a modification of Rothemund's rectangular DNA origami [3]. A caDNAno-file for this DNA Origami can be found in Schmied et al [4]. The DNA Origami is based on the M13 phage p7249 scaffold and the following unmodified staple strands were used:

Oligo number	Sequence (5' to 3' orientation)
1	ATCCCAATGAGAATTAACTGAACAGTTACCAG
2	TGGAACAACCGCCTGGCCCTGAGGCCCGCT
3	GCACAGACAATATTTTTGAATGGGGTCAGTA
4	CATGTAATAGAATATAAAGTACCAAGCCGT
5	TTTATCAGGACAGCATCGGAACGACACCAACCTAAAACGA
6	TAAATCAAAATAATTCGCGTCTCGGAAACC
7	CTGTGTGATTGCGTTGCGCTCACTAGAGTTGC
8	TAAATCATATAACCTGTTTAGCTAACCTTTAA
9	CTCGTATTAGAAATTGCGTAGATACAGTAC
10	CAGAAGATTAGATAATACATTTGTCGACAA
11	ACAACTTTCAACAGTTTCAGCGGATGTATCGG
12	TAGGTAAACTATTTTTGAGAGATCAAACGTTA
13	GCCAGTTAGAGGGTAATTGAGCGCTTTAAGAA
14	TCTAAAGTTTTGTCGTCTTTCCAGCCGACAA
15	CAACTGTTGCGCCATTCGCCATTCAAACATCA
16	TCAATATCGAACCTCAAATATCAATTCCGAAA
17	CTTATCATTCCCGACTTGCGGGAGCCTAATTT
18	ATATTTTGGCTTTCATCAACATTATCCAGCCA
19	TCATCGCCAACAAAGTACAACGGACGCCAGCA
20	GCCCGTATCCGGAATAGGTGTATCAGCCCAAT
21	GCAAGGCCTCACCAGTAGCACCATGGGCTTGA
22	CACATTAAAATTGTTATCCGCTCATGCGGGCC
23	GCGAGTAAAAATATTTAAATTGTTACAAAG
24	AAATTAAGTTGACCATTAGATACTTTTGCG
25	CTTTAGGGCCTGCAACAGTGCCAATACGTG
26	GATGGTTTGAACGAGTAGTAAATTTACCATTA
27	AGACGACAAAGAAGTTTTGCCATAATTCGAGCTTCAA
28	ATTTTAAAATCAAAATTATTTGCACGGATTCG
29	CGAAAGACTTTGATAAGAGGTCATATTTCGCA
30	GATTTAGTCAATAAAGCCTCAGAGAACCCTCA
31	TTTAGGACAAATGCTTTAAACAATCAGGTC
32	TTAAAGCCAGAGCCGCCACCCTCGACAGAA
33	AAGTAAGCAGACACCACGGAATAATATTGACG
34	TATATTTTGTCATTGCCTGAGAGTGGAAGATTGTATAAGC
35	TTATTACGAAGAACTGGCATGATTGCGAGAGG
36	TGTAGCCATTAAAATTCGCATTAAATGCCGGA
37	ATATTCGGAACCATCGCCCACGCAGAGAAGGA
38	ACCCTTCTGACCTGAAAGCGTAAGACGCTGAG
39	GCGAAAAATCCCTTATAAATCAAGCCGGCG

40	GAGAGATAGAGCGTCTTTCCAGAGGTTTTGAA
41	AAATCACCTTCCAGTAAGCGTCAGTAATAA
42	AACGCAAAGATAGCCGAACAAACCCTGAAC
43	GACAAAAGGTAAAGTAATCGCCATATTTAACAAAACTTTT
44	CCACCCTCATTTTCAGGGATAGCAACCGTACT
45	ACAACATGCCAACGCTCAACAGTCTTCTGA
46	CGCGCAGATTACCTTTTTTAATGGGAGAGACT
47	ACACTCATCCATGTTACTTAGCCGAAAGCTGC
48	TAAGAGCAAATGTTTAGACTGGATAGGAAGCC
49	GCTTTCCGATTACGCCAGCTGGCGGCTGTTTC
50	TACCGAGCTCGAATTCGGGAAACCTGTCGTGCAGCTGATT
51	GAGGGTAGGATTCAAAAGGGTGAGACATCCAA
52	AAGGCCGCTGATACCGATAGTTGCGACGTTAG
52	GTTTTAACTTAGTACCGCCACCCAGAGCCA
54	CCAACAGGAGCGAACCAGACCGGAGCCTTTAC
55	CAGCGAAACTTGCTTTCGAGGTGTTGCTAA
56	ACGGCTACAAAAGGAGCCTTTAATGTGAGAAT
57	GCCCTTCAGAGTCCACTATTAAAGGGTGCCGT
58	GATGTGCTTCAGGAAGATCGCACAATGTGA
59	ATTATCATTCAATATAATCCTGACAATTAC
60	TAATCAGCGGATTGACCGTAATCGTAACCG
61	CCACCCTCTATTCACAAACAAATACCTGCCTA
62	AACGTGGCGAGAAAGGAAGGGAAACCAGTAA
63	CCTGATTGCAATATATGTGAGTGATCAATAGT
64	GCTATCAGAAATGCAATGCCTGAATTAGCA
65	AATACTGCCCAAAAGGAATTACGTGGCTCA
66	CGGATTGCAGAGCTTAATTGCTGAAACGAGTA
67	TTGACAGGCCACCACCAGAGCCGCGATTTGTA
68	ACGCTAACACCCACAAGAATTGAAAATAGC
69	CTGAGCAAAAATTAATTACATTTTGGGTTA
70	CACAACAGGTGCCTAATGAGTGCCCAGCAG
71	CATTTGAAGGCGAATTATTCATTTTTGTTTGG
72	GCCCGAGAGTCCACGCTGGTTTGCAGCTAACT
73	GTATAGCAAACAGTTAATGCCCAATCCTCA
74	AATTGAGAATTCTGTCCAGACGACTAAACCAA
75	TTAGTATCACAATAGATAAGTCCACGAGCA
76	ACCGATTGTCGGCATTTTCGGTCATAATCA
77	GCGAACCTCCAAGAACGGGTATGACAATAA
78	GCGGAACATCTGAATAATGGAAGGTACAAAAT
79	GTAATAAGTTAGGCAGAGGCATTTATGATATT
80	AAACAGCTTTTTGCGGGATCGTCAACACTAAA
81	CAAATCAAGTTTTTTGGGGTCGAAACGTGGA
82	TTCCAGTCGTAATCATGGTCATAAAAGGGG
83	TTATACCACCAAATCAACGTAACGAACGAG
84	CCTAAATCAAAATCATAGGTCTAAACAGTA
85	AATGGTCAACAGGCAAGGCAAAGAGTAATGTG
86	GCGGATAACCTATTATTCTGAAACAGACGATT

87	TGCATCTTTCCCAGTCACGACGGCCTGCAG
88	CTACCATAGTTTGAGTAACATTTAAAATAT
89	AGAAAACAAAGAAGATGATGAAACAGGCTGCG
90	GCCTCCCTCAGAATGGAAAGCGCAGTAACAGT
91	AGCCAGCAATTGAGGAAGGTTATCATCATTTT
92	TTAACGTCTAACATAAAAACAGGTAACGGA
93	CAGCAAAAGGAAACGTCACCAATGAGCCGC
94	AAAGTCACAAAATAAACAGCCAGCGTTTTA
95	AAAGCACTAAATCGGAACCCTAATCCAGTT
96	AACAGTTTTGTACCAAAAACATTTTATTTC
97	AACACCAAATTTCAACTTTAATCGTTTACC
98	AATACGTTTGAAAGAGGACAGACTGACCTT
99	CAACCGTTTCAAATCACCATCAATTCGAGCCA
100	GCCATCAAGCTCATTTTTTAACCACAAATCCA
101	CTTTTACAAAATCGTCGCTATTAGCGATAG
102	AGCGCGATGATAAATTGTGTCGTGACGAGA
103	TCATTCAGATGCGATTTTAAGAACAGGCATAG
104	TTTCGGAAGTGCCGTCGAGAGGGTGAGTTTCG
105	GCCTTAAACCAATCAATAATCGGCACGCGCCT
106	GTTTATCAATATGCGTTATACAAACCGACCGTGTGATAAA
107	GGCCTTGAAGAGCCACCACCCTCAGAAACCAT
108	TGTAGAAATCAAGATTAGTTGCTCTTACCA
109	TATTAAGAAGCGGGGTTTTGCTCGTAGCAT
110	TCTTCGCTGCACCGCTTCTGGTGCGGCCTTCC
111	AATAGTAAACACTATCATAACCCTCATTGTGA
112	AGCAAGCGTAGGGTTGAGTGTTGTAGGGAGCC
113	TCACCGACGCACCGTAATCAGTAGCAGAACCG
114	TTAACACCAGCACTAACAACTAATCGTTATTA
115	GCCGTCAAAAAACAGAGGTGAGGCCTATTAGT
116	CGTAAAACAGAAATAAAAATCCTTTGCCCGAAAGATTAGA
117	TCACCAGTACAAACTACAACGCCTAGTACCAG
118	GTTTATTTTGTCACAATCTTACCGAAGCCCTTTAATATCA
119	TGAAAGGAGCAAATGAAAAATCTAGAGATAGA
120	GATGGCTTATCAAAAAGATTAAGAGCGTCC
121	CCCGATTTAGAGCTTGACGGGGAAAAAGAATA
122	AAGCCTGGTACGAGCCGGAAGCATAGATGATG
123	ATACCCAACAGTATGTTAGCAAATTAGAGC
124	TTTTATTTAAGCAAATCAGATATTTTTTGT
125	AGTATAAAGTTCAGCTAATGCAGATGTCTTTC
126	TCGGCAAATCCTGTTTGATGGTGGACCCTCAA
127	CATCAAGTAAAACGAACTAACGAGTTGAGA
128	CATAAATCTTTGAATACCAAGTGTTAGAAC
129	CTTTAATGCGCGAACTGATAGCCCCACCAG
130	GACCTGCTCTTTGACCCCCAGCGAGGGAGTTA
131	CGATAGCATTGAGCCATTTGGGAACGTAGAAA
132	ATGCAGATACATAACGGGAATCGTCATAAATAAAGCAAAG
133	CAGGAGGTGGGGTCAGTGCCTTGAGTCTCTGAATTTACCG

134	TTTTCACTCAAAGGGCGAAAAACCATCACC
135	ACCTTTTTATTTTAGTTAATTTCATAGGGCTT
136	AGGCTCCAGAGGCTTTGAGGACACGGGTAA
137	ACCTTGCTTGGTCAGTTGGCAAAGAGCGGA
138	AAGGAAACATAAAGGTGGCAACATTATCACCG
139	CTTAGATTTAAGGCGTTAAATAAAGCCTGT
140	ATTATACTAAGAAACCACCAGAAGTCAACAGT
141	AACGCAAAATCGATGAACGGTACCGGTTGA
142	TAAAAGGGACATTCTGGCCAACAAAGCATC
143	CCAATAGCTCATCGTAGGAATCATGGCATCAA
144	GAAATTATTGCCTTTAGCGTCAGACCGGAACC
145	TTAGGATTGGCTGAGACTCCTCAATAACCGAT
146	GCGCAGACAAGAGGCAAAAGAATCCCTCAG
147	TGACAACTCGCTGAGGCTTGCATTATACCA
148	AGGCAAAGGGAAGGGCGATCGGCAATTCCA
149	ATTACCTTTGAATAAGGCTTGCCCAAATCCGC
150	TTTACCCCAACATGTTTTAAATTTCCATAT
151	AGAAAGGAACAACTAAAGGAATTCAAAAAAA
152	TCCACAGACAGCCCTCATAGTTAGCGTAACGA
153	TCAAGTTTCATTAAAGGTGAATATAAAAGA
154	GTCGACTTCGGCCAACGCGCGGGGTTTTTC
155	AATAGCTATCAATAGAAAATTCAACATTCA
156	AAAGGCCGGAGACAGCTAGCTGATAAATTAATTTTTGT
157	CACCAGAAAGGTTGAGGCAGGTCATGAAAG
158	AGGAACCCATGTACCGTAACACTTGATATAA
159	TATAACTAACAAAGAACGCGAGAACGCCAA
160	TTGCTCCTTTCAAATATCGCGTTTGAGGGGGT
161	TAAATGAATTTTCTGTATGGGATTAATTTCTT
162	GAATTTATTTAATGGTTTGAAATATTCTTACC
163	CTTTTGCAGATAAAAACCAAAATAAAGACTCC
164	GCAATTCACATATTCCTGATTATCAAAGTGTA

Additionally, the following biotin-modified oligonucleotide strands were used:

Oligo number	**Sequence (5’ to 3’ orientation)**
165	Biotin-CGGATTCTGACGACAGTATCGGCCGCAAGGCGATTAAGTT
166	Biotin-AGCCACCACTGTAGCGCGTTTTCAAGGGAGGGAAGGTAAA
167	Biotin-GAGAAGAGATAACCTTGCTTCTGTTCGGGAGAAACAATAA
168	Biotin-ATAAGGGAACCGGATATTCATTACGTCAGGACGTTGGGAA
169	Biotin-TAGAGAGTTATTTTCATTTGGGGATAGTAGTAGCATTA
170	Biotin-GAAACGATAGAAGGCTTATCCGGTCTCATCGAGAACAAGC

For co-localization purposes, the NRO is also modified with 10 DNA oligonucleotides that extends from the NRO. These DNA strands are complementary to additionally added ATTO532-labeled DNA oligonucleotides. The sequence highlighted in bold represents the docking site for the ATTO532-labeled DNA.

Oligo number	Sequence (5’ to 3’ orientation)
171	GACCAACTAATGCCACTACGAAGGGGGTAGCA **TTTTCCTCTACCACCTACATCAC**
172	CTCCAACGCAGTGAGACGGGCAACCAGCTGCA **TTTTCCTCTACCACCTACATCAC**
173	TTAATGAACTAGAGGATCCCCGGGGGGTAACG **TTTTCCTCTACCACCTACATCAC**
174	ATCCCCCTATACCACATTCAACTAGAAAAATC **TTTTCCTCTACCACCTACATCAC**
175	ACAAACGGAAAAGCCCCAAAAACACTGGAGCA **TTTTCCTCTACCACCTACATCAC**
176	AACAAGAGGGATAAAAATTTTTAGCATAAAGC **TTTTCCTCTACCACCTACATCAC**
177	CCAGGGTTGCCAGTTTGAGGGGACCCGTGGGA **TTTTCCTCTACCACCTACATCAC**
178	TACGTTAAAGTAATCTTGACAAGAACCGAAC **TTTTTCCTCTACCACCTACATCAC**
179	TAAATCGGGATTCCCAATTCTGCGATATAATG **TTTTCCTCTACCACCTACATCAC**
180	CTGTAGCTTGACTATTATAGTCAGTTCATTGA **TTTTCCTCTACCACCTACATCAC**

Sequence of the permanently bound DNA origami **identification strand** labeled with the dye ATTO532:

5’-GTG ATG TAG GTG GTA GAG G -ATTO532

Additionally, six strands protrude from the DNA origami to capture the Hfq-DNA conjugate. One of these strands acts as “capturing strand”. 21 nucleotides of this strand are complementary to the Hfq-DNA strand leading to a permanent binding of the complementary strand coupled to the HFQ protein. The remaining five strands (called “holding strands”) are only complementary to the Hfq-DNA strand over a region of 12 nucleotides. This way, a stable anchoring of a single Hfq-DNA conjugate via the “capturing strand” is possible. After capturing of the protein, the hexameric protein is further anchored and orientated in a planar fashion via the “holding strands”. This way, the protein is not only captured and orientated on the DNA origami but binding of two Hfq molecules is thermodynamically disfavoured. Additionally, both types of strands exhibit a poly T-stretch and at the 3’-end follows the sequence that is incorporated into the NRO.

Oligo number	**Sequence (5’ to 3’ orientation)**
181	GCACATTATAAATTTTTTTTTTTTTTTTTTTTTTTATACATACC GAGGAAACGCAATAAGAAGCGCATTAGACGG
182	GCACATTATAAATTTTTTTTTTAGAGAGAAAAAAATGAAAAT AGCAAGCAAACT
183	GCACATTATAAATTTTTTCAAATATAACCTCCGGCTTAGGTA ACAATTT

184	TTTCGGACTGCACATTATAAATTTTTTTTTTCTACTACGCGAG CTGAAAAGGTTACCGCGC
185	GCACATTATAAATTTTTATCGCAAGTATGTAAATGCTGATGA TAGGAAC
186	GCACATTATAAATTTTTTTTGTACCGCAATTCTAAGAACGCG AGTATTATTT

The **DNA oligonucleotide** used for the **conjugation** reaction with Hfq^{AzF} is the following:

5'-ATA CAT CTA GTT TTT ATA ATG TGC AGT CCG AAA - **DBCO**

Sequence of the **imager strand**:

5’- CTA GAT GTA T -ATTO655

6 References

1. M. Raab, J. J. Schmied, I. Jusuk, C. Forthmann and P. Tinnefeld, *Chemphyschem*, 2014, 15, 2431-2435.

2. J. S. Nielsen, A. Boggild, C. B. Andersen, G. Nielsen, A. Boysen, D. E. Brodersen and P. Valentin-Hansen, *Rna*, 2007, 13, 2213-2223.

3. P. W. Rothemund, *Nature*, 2006, 440, 297-302.

4. J. J. Schmied, A. Gietl, P. Holzmeister, C. Forthmann, C. Steinhauer, T. Dammeyer and P. Tinnefeld, *Nat Methods*, 2012, 9, 1133-1134.