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Supplementary Materials for

Sub–100-nm metafluorophores with digitally tunable optical properties self-assembled from DNA

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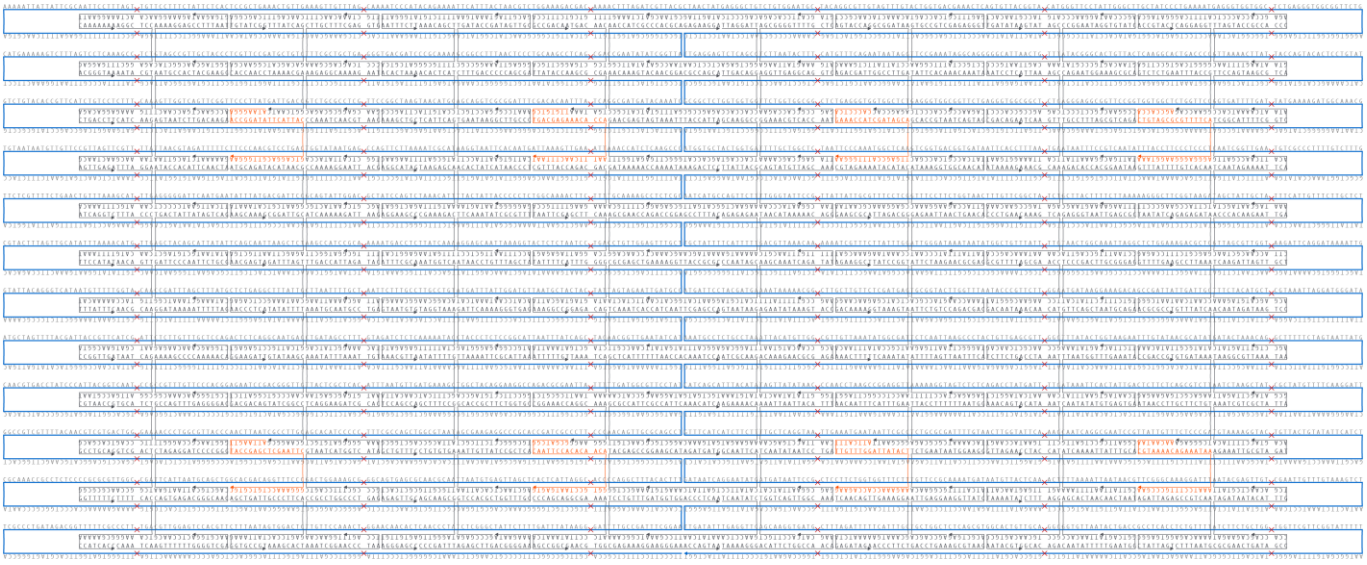

fig. S1. caDNAno DNA origami design. Circular DNA scaffold (blue) is routed in horizontal loops to form 24 parallel helices. Staple strands (gray) connect parts of the scaffold and form the rectangle. Eight strands are biotinylated on the 5'-end (orange). Most gray staples' 3' and 5'-ends are on the same DNA origami face. However, Biotin and dye functionalizations are intended to protrude on opposite faces. With the help of adjacent staples, the orange staples are shifted by one helix. This switches the 3' and 5'-ends to the opposite face. Red crosses define base-skips, which are required to prevent the DNA origami from twisting.

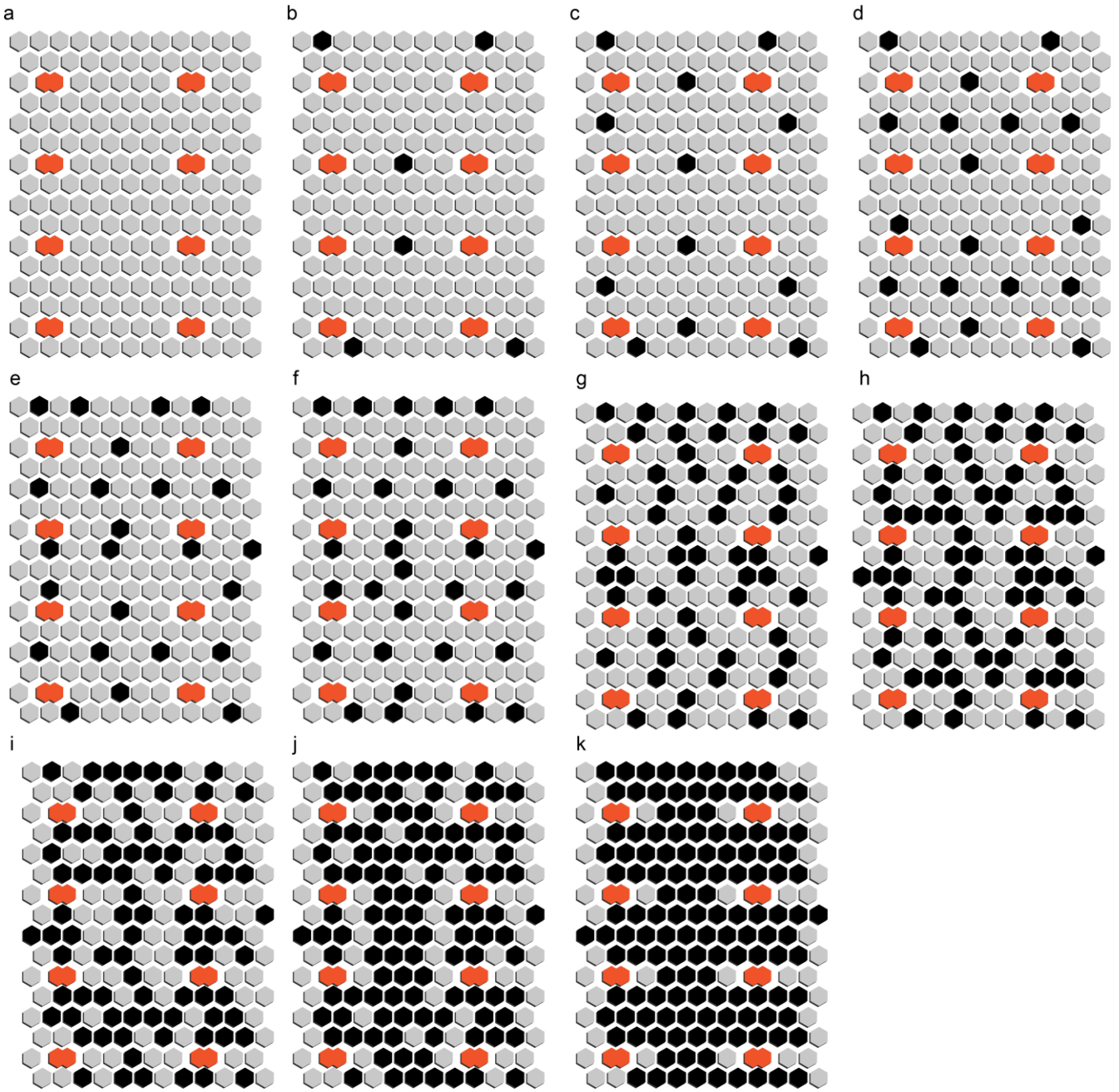

fig. S2. Schematic DNA origami staple layouts of single-color metafluorophores (6 to 132). Hexagons represent 3'-ends of all 176 staples, compare to figure S1. Orange shapes represent biotinylated staple strands, protruding on the opposite face. Black hexagons represent staples with 3'-handle extension, see table S3. Pattern is the same for Atto 647N, Cy3 and Atto 488. (**a**) is a not functionalized structure, corresponding to the caDNAno layout. (**b**) 6, (**c**) 12, (**d**) 18, (**e**) 24, (**f**) 30, (**g**) 54, (**h**) 72, (**i**) 84, (**j**) 108 and (**k**) 132 dyes attached.

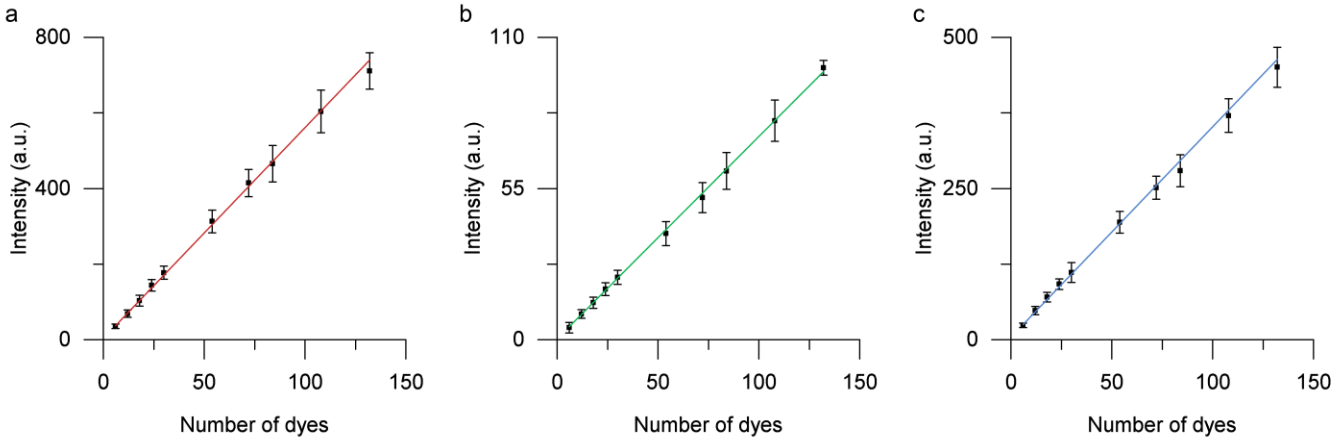

fig. S3. Linear dependence of intensity on the number of dyes per DNA origami (calibrated). From 6 to 132 dyes per DNA origami, the intensity scales linearly for (**a**) Atto 647N, (**b**) Cy3 and (**c**) Atto 488. Investigated samples are identical to those in Figure 1 of the main text. However, samples contained the structure of interest and additionally a second DNA origami with a significantly different dye count as reference. This allows comparison and calibration of measured intensities and thereby reduces sample-to-sample variations. Corresponding data in Figure 1 of the main text is not calibrated.

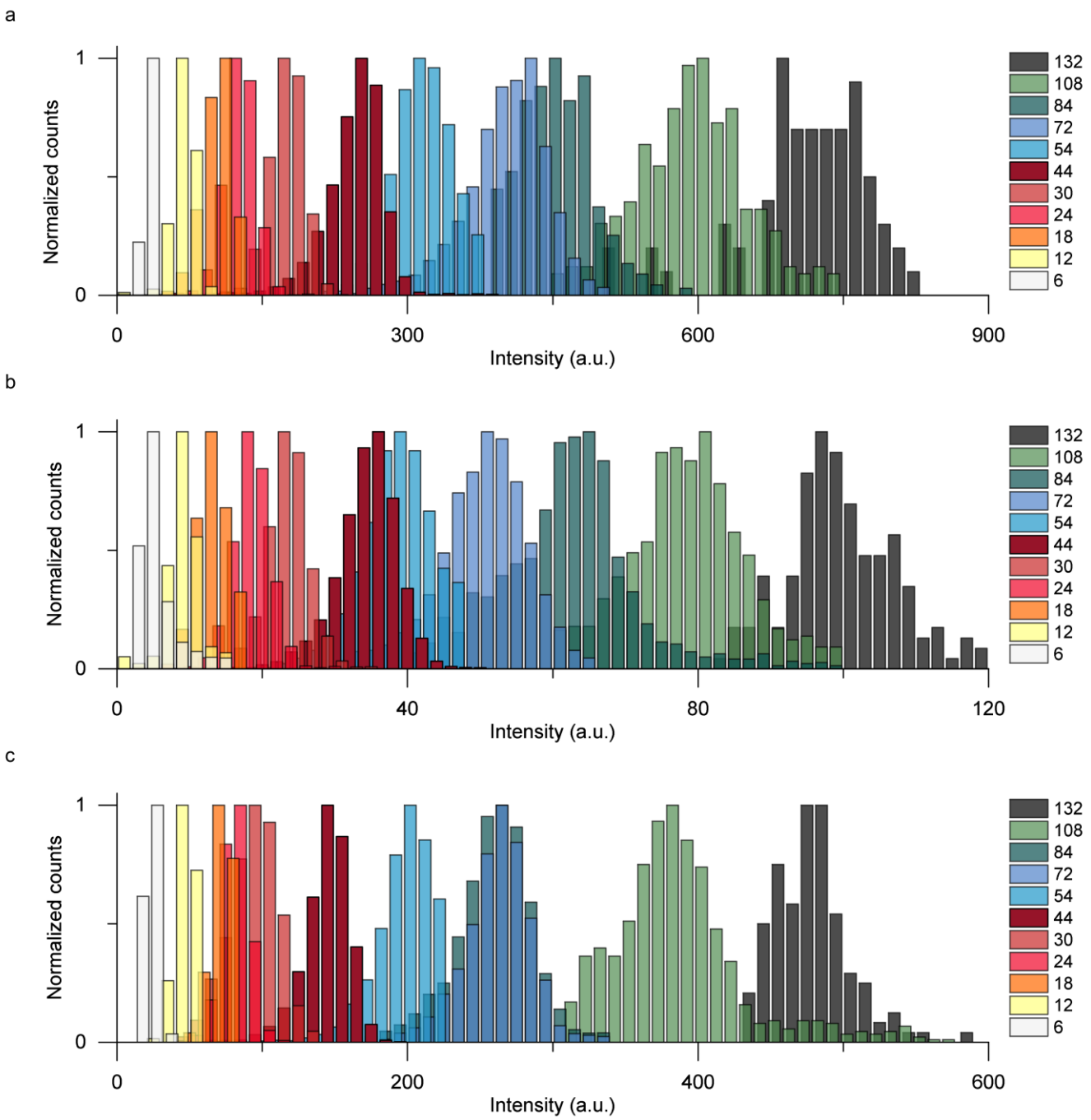

fig. S4. Intensity distributions for 6 to 132 dyes. Data corresponds to figure S3, where mean and standard deviation of the distributions are plotted. (**a**) Atto 647N. (**b**) Cy3. (**c**) Atto 488. Investigated samples contained the structure of interest and a second DNA origami with a significantly different dye count as reference. Reference intensity distributions are not shown.

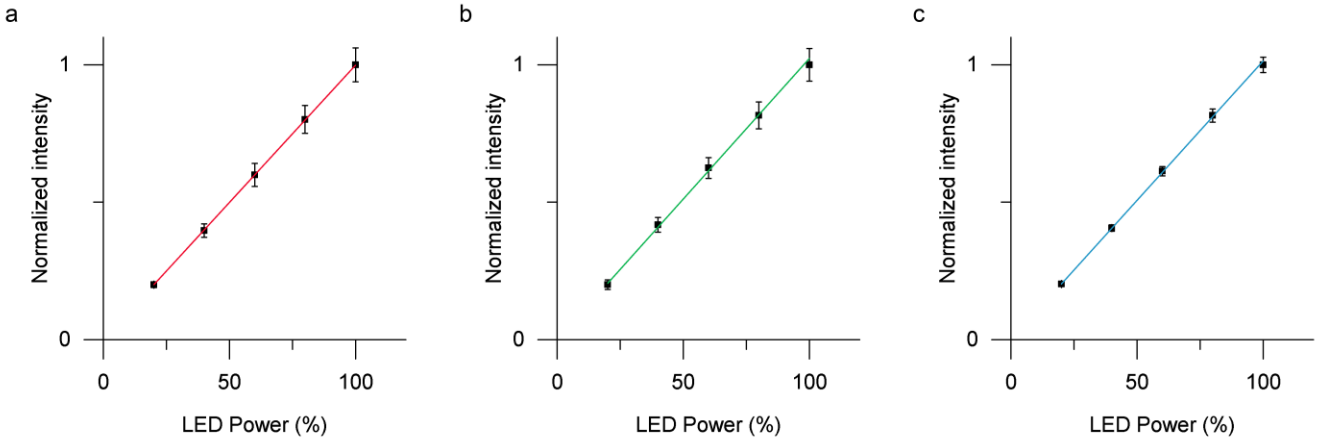

fig. S5. Excitation power variation. The measured intensity of a 30 dye metafluorophore scales linear with the applied excitation intensity for (**a**) Atto 647N, (**b**) Cy3 and (**c**) Atto 488. More than 12,000 metafluorophores were evaluated per data point. Camera integration times were constant at 10 s. All subsequent measurements throughout this study were performed at 60 %.

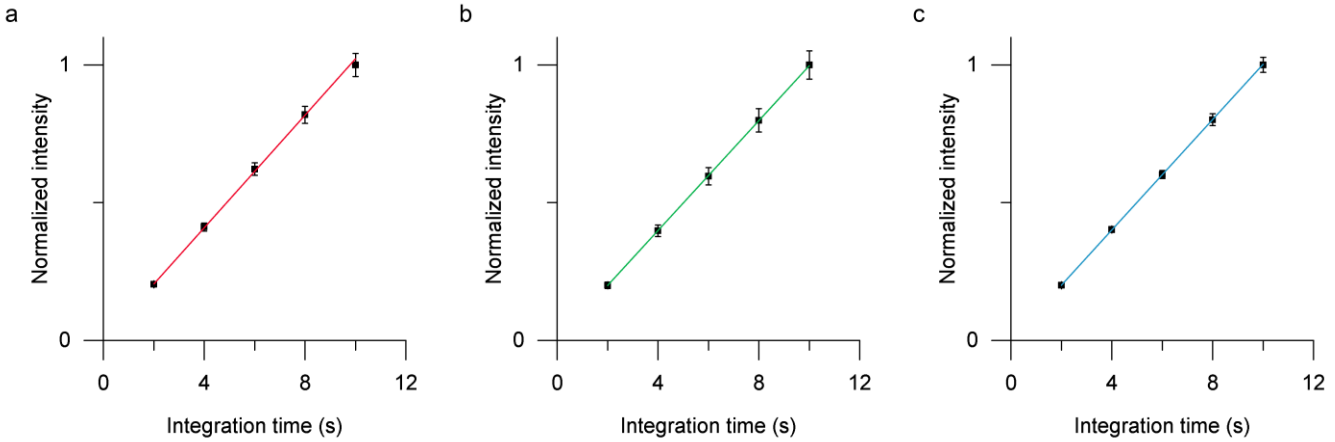

fig. S6. Integration time variation. DNA origami-based metafluorophore recordings were measured using a Hamamatsu ORCA Flash 4.0 sCMOS camera. Integration times were varied from 2 s to 10 s per recording and show a linear increase in intensity of a 30 dye metafluorophore for (**a**) Atto 647N, (**b**) Cy3 and (**c**) Atto 488 at 60 % excitation intensity. More than 12,000 metafluorophores were evaluated per data point.

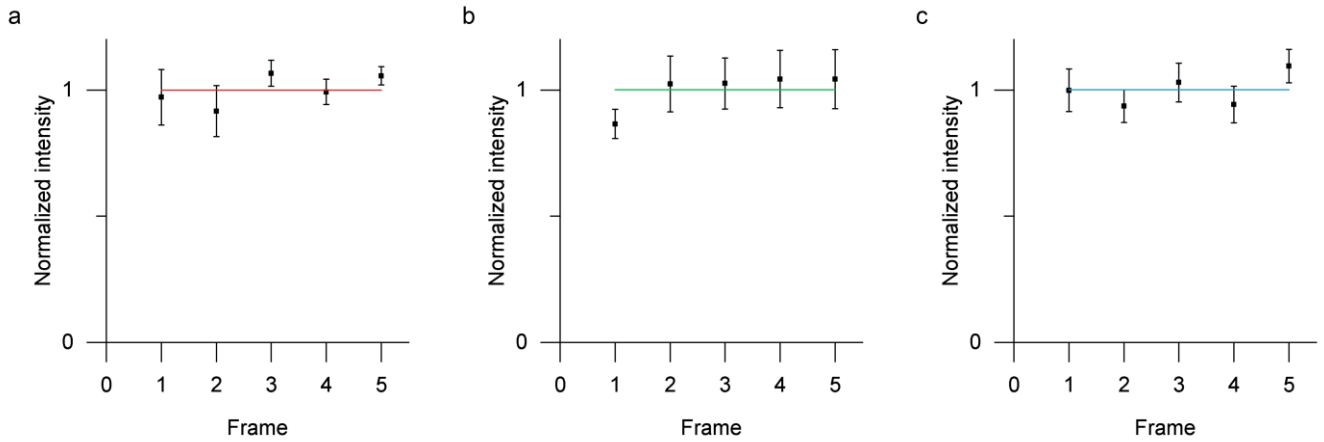

fig. S7. Refocusing performance. While repeated focusing attempts may lead to imaging in different focal planes, different focal planes may yield different intensities of a single target. The same samples, containing DNA origami based metafluorophores with 30 dyes, were imaged and refocused five times for (**a**) Atto 647N, (**b**) Cy3 and (**c**) Atto 488. Plots are normalized to the average value (colored line).

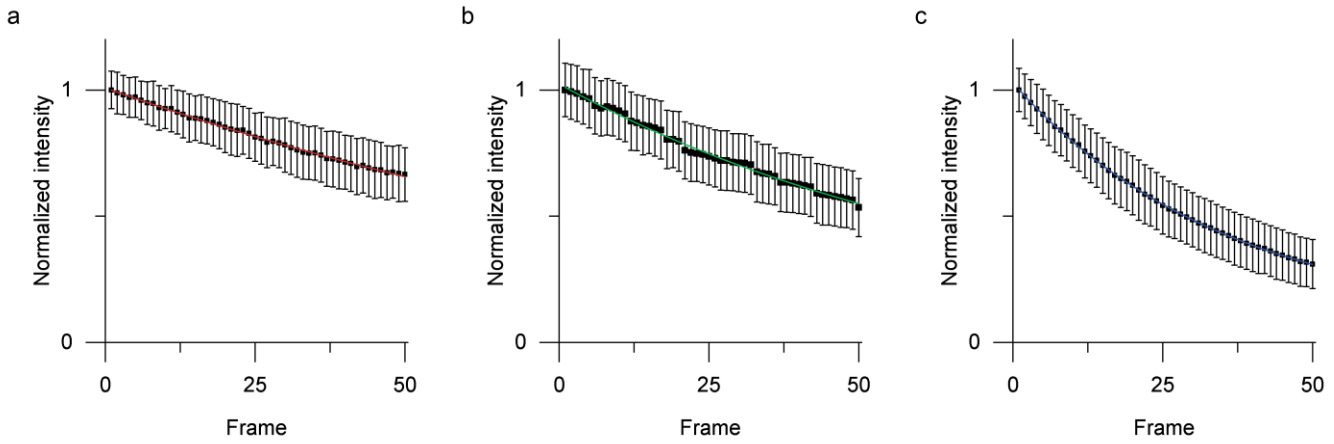

fig. S8. Photostability. Repeated recording of the same area causes photobleaching of the dyes. The measured intensity drops exponentially. Measurements were performed at 60 % excitation power and integration times of 10 s per frame on a 30 dye DNA origami metafluorophore for (**a**) Atto 647N (-0.77 %), (**b**) Cy3 (-1.37 %) and (**c**) Atto 488 (-2.80 % per acquisition) on the Zeiss Axio Observer microscope.

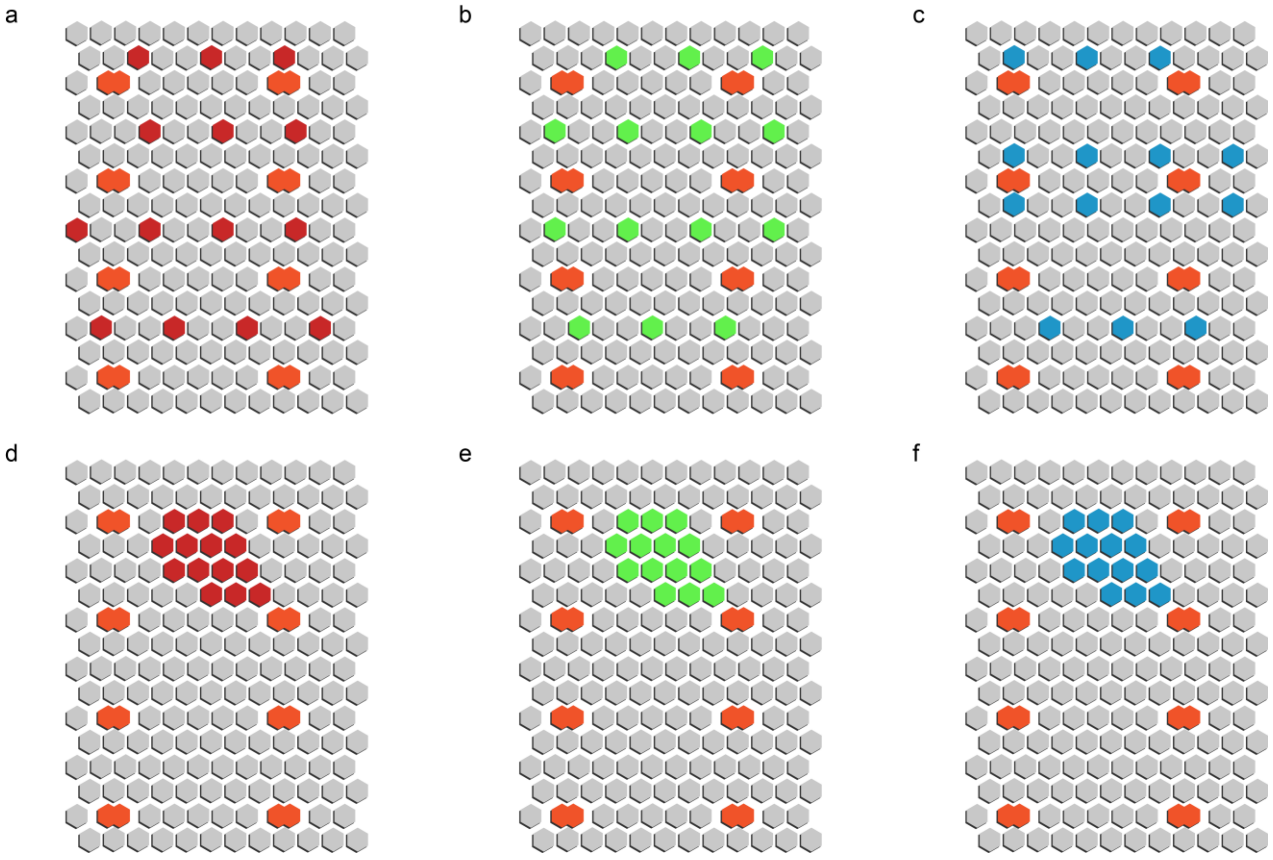

fig. S9. Schematic DNA origami staple layouts of self-quenching study. (**a** to **c**) Sparse dye patterning on DNA origami with ~15 nm dye-to-dye distance, for Atto 647N (red), Cy3 (green) and Atto 488 (blue). (**d** to **f**) Dense dye patterning on DNA origami with ~5 nm dye-to-dye distance, for Atto 647N (red), Cy3 (green) and Atto 488 (blue).

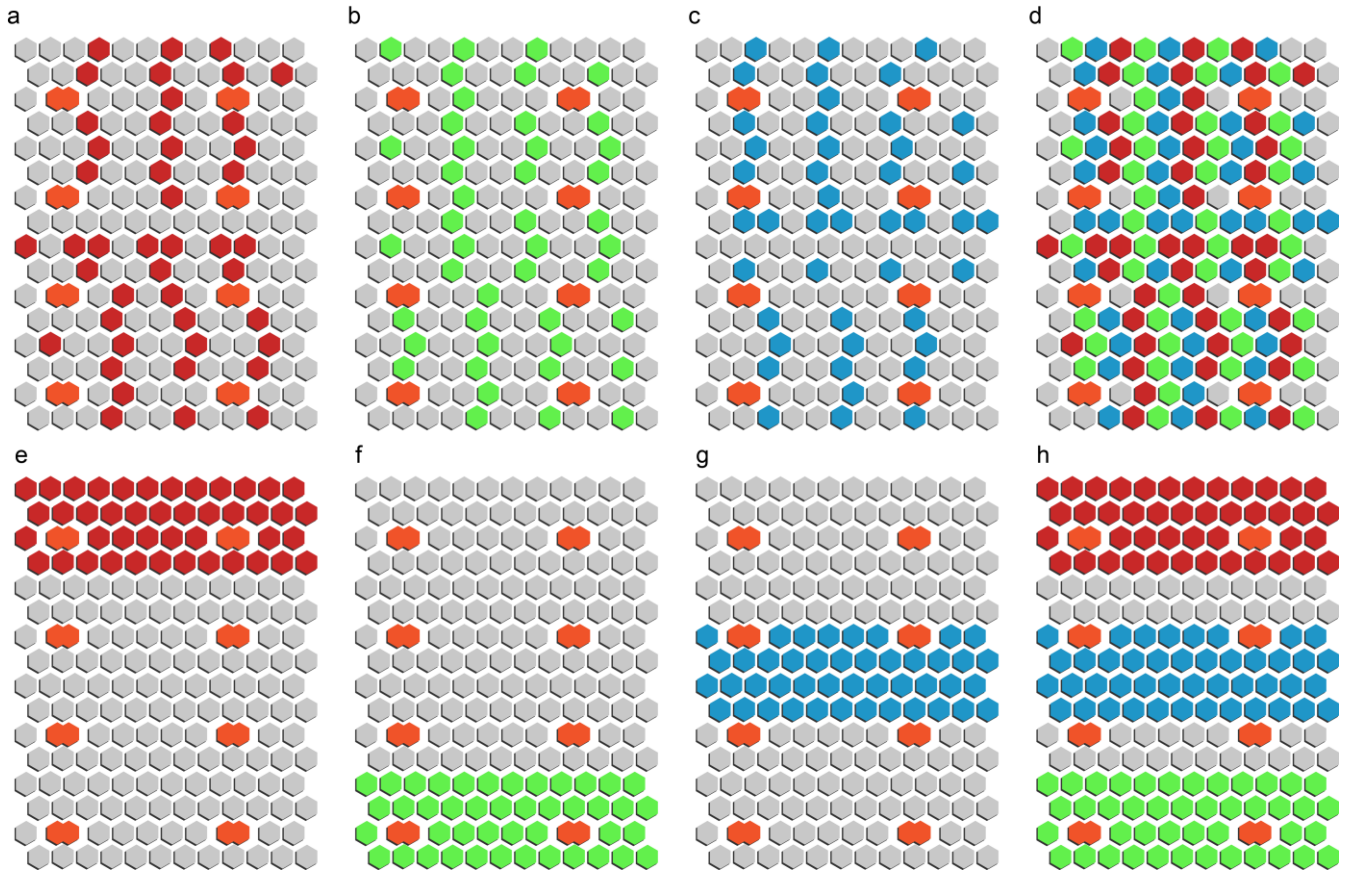

fig. S10. FRET investigation dye patterning (random and column-wise). (**a** to **d**) Mixed dye patterns, corresponding to Figure 2 (a-c) of the main text. (**e** to **h**) Column-wise dye pattern with inter-color spacing > 10 nm, corresponding to figure 2 (d-f) of the main text.

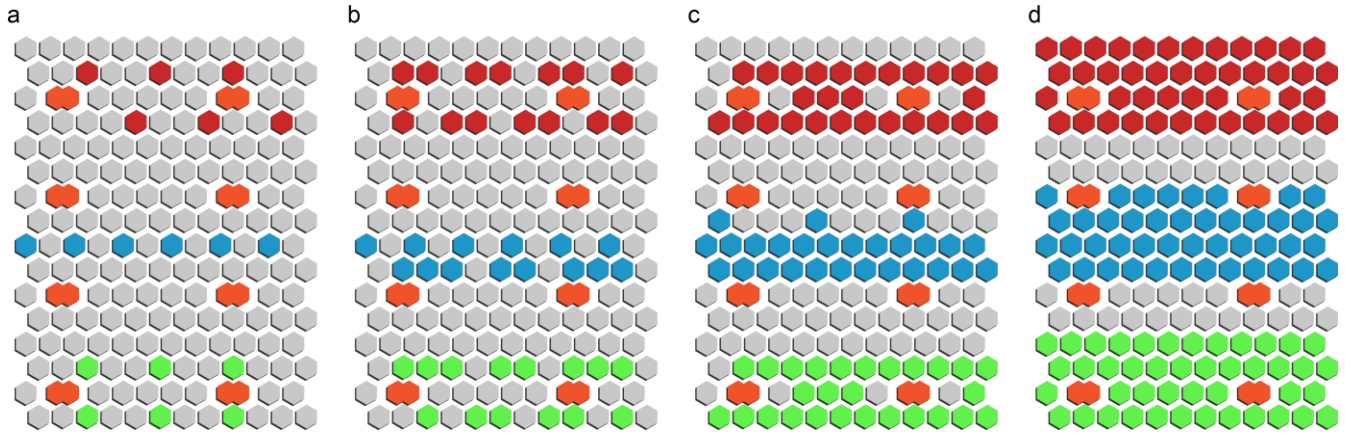

fig. S11. Intensity barcode dye patterns. The column-wise dye pattern separates distinct dyes > 10 nm and thus prevents FRET. (**a**) 6, (**b**) 14, (**c**) 27 and (**d**) 44 dyes attached per color. These layouts were used to independently control brightness levels for all three colors in the barcode studies.

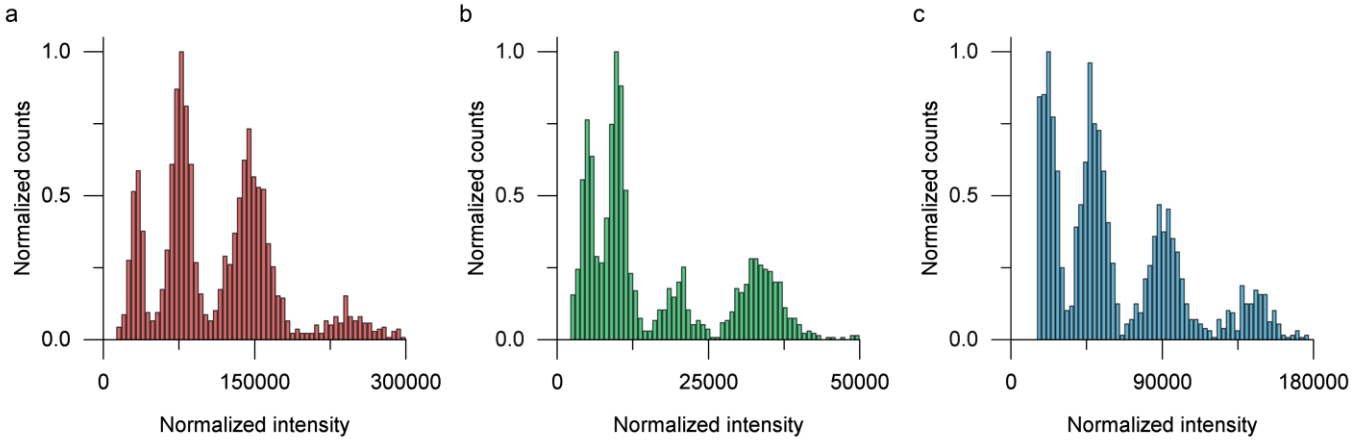

fig. S12. Intensity distributions for 124 barcodes in one sample. Exemplary intensity distributions of 25 distinct metafluorophores combined in one sample for (**a**) Atto 647N, (**b**) Cy3 and (**c**) Atto 488. Four levels (corresponding to 6, 14, 27 and 44 dyes) are clearly distinguishable. Overlapping regions in between peaks were identified (see software section) and barcodes displaying corresponding intensities were classified as unqualified.

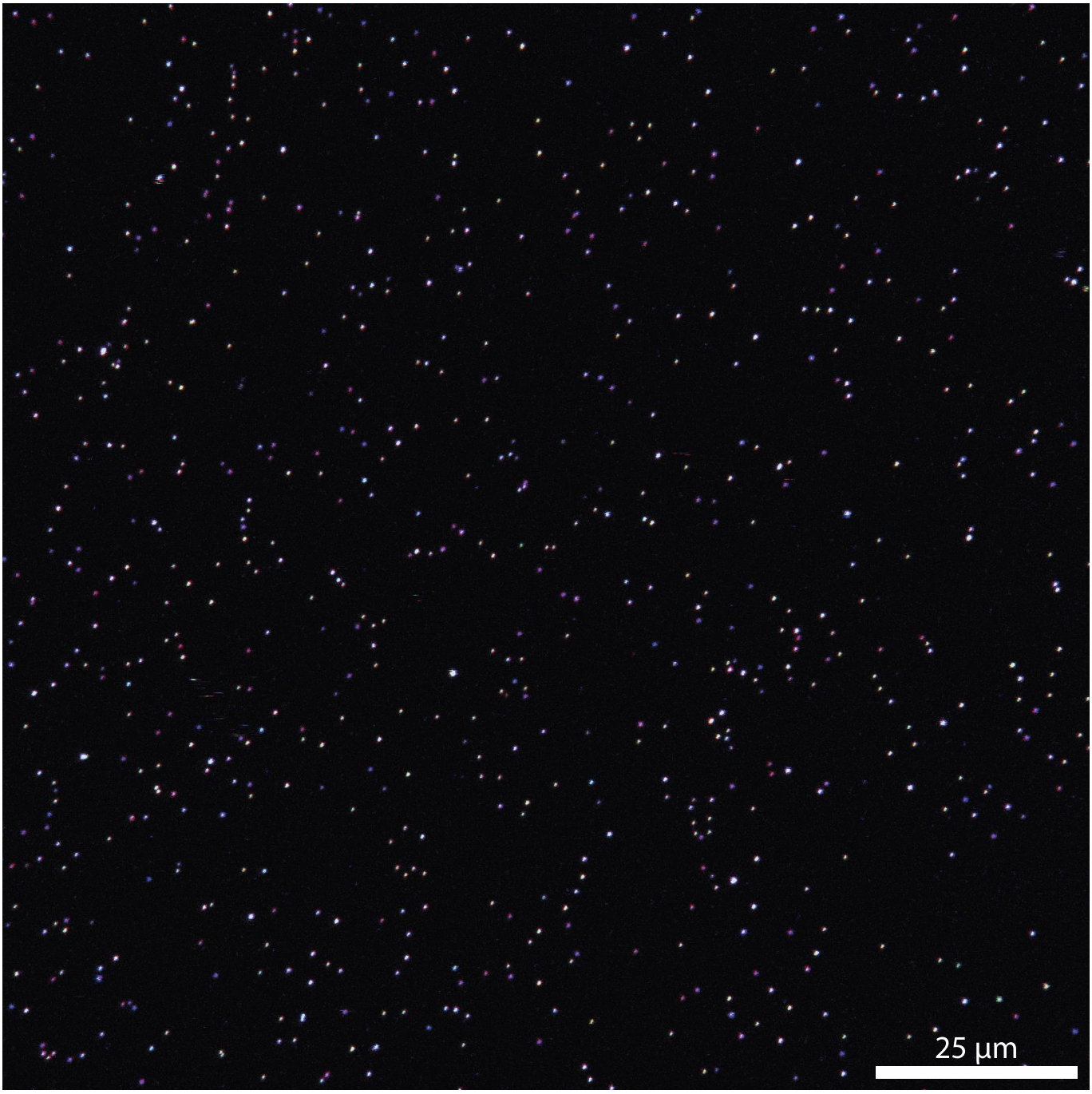

fig. S13. Exemplary fluorescent image of nucleic acid detection. Three-color confocal image of the nucleic acid detection experiment. Eight different barcodes were used (see table S7), six of them specifically tethered to the glass surface by synthetic DNA targets. Note that the fluorescent image (and thus the data analysis) is comparable to the image in Fig. 3b. Identifying all barcodes yields the first data set (i.e. the green bars) in figure S14b.

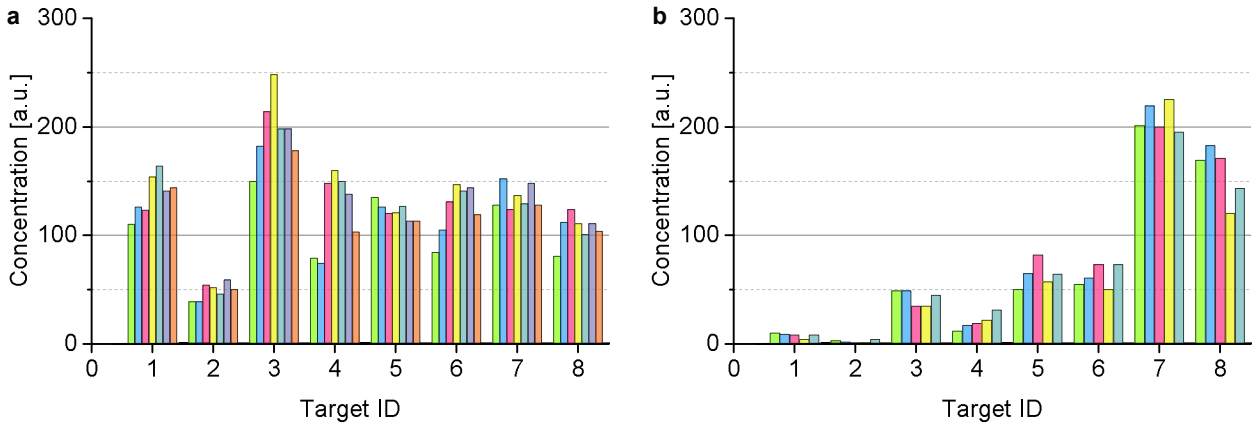

fig. S14. DNA detection calibration. (**a**) Eight DNA targets (See table S7) have been added to a detection assay at the same designed concentration (12.5 pM). Due to imperfect initial determination of target concentration and subsequent pipetting errors, the different targets are not detected with the same frequency. However, this target-to-target variation is constant between samples. By normalizing the average of each target to a reference target (here Target ID '3'), a calibration table can be produced. This allows the normalization of future measurements with the same targets. (**b**) Counted metafluorophores from the same experiment as shown in Fig. 4c, before normalization. In contrast to (a), targets have been added at different concentrations: 0 pM (targets 1 and 2), 1.5 pM (targets 3 and 4), 4.5 pM (targets 5 and 6) and 13.5 pM (targets 7 and 8). Since targets 1 and 2 have been omitted, the corresponding detections represent non-specifically bound metafluorophores and false-positives.

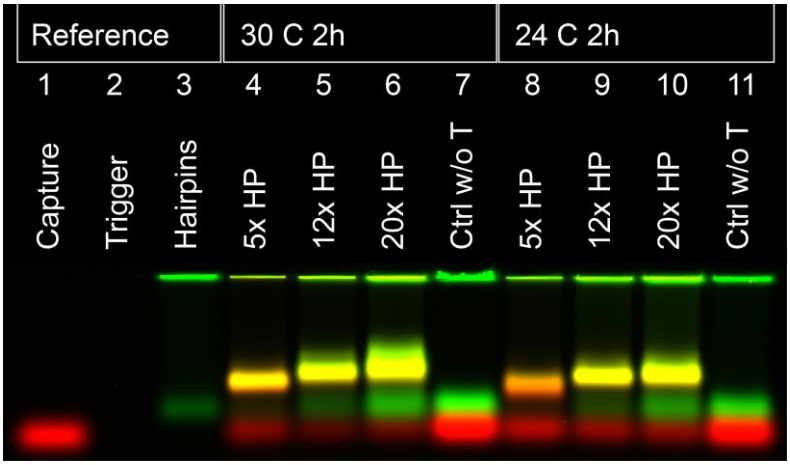

fig. S15. Triggered assembly formation gel assay. See 'triggered assembly gel assay protocol' for details. Capture strands (CAP) are labeled with Alexa 647 (red), hairpins (HP) with Cy3 (green). Trigger strands (T) are unlabeled. Lane 1 (1 pmol CAP) and 3 (12 pmol) serve as reference for CAP and HP migration speeds. Lanes 4 - 7 show reactions performed at 30 C, lanes 8 - 11 at 24 C, respectively (1 pmol CAP each).
Control lanes 7 and 11 are missing the (T) strand, thereby inhibiting triangle formation. Lanes only show CAP and HP bands, in agreement with the reference bands.
Assembly reactions in lanes 5 and 9 had 12 fold excess of HP strands over CAP strands (10.9 over T) and triangles (10 HP per triangle) are formed as indicated by the strong band migrating slower than the reference bands. The presence of a CAP reference band indicates that not all CAP strands formed a triangle. Since HP strands are in slight stoichiometric excess in regards to the triangles, a weak HP band is notable.
Lanes 6 and 10 contain reactions with higher HP excess. Product bands appear to migrate slightly slower than the product bands in lane 5 and 9, indicating only marginally increased triangle size.
Reactions in lanes 4 and 8 were had insufficient HP to fully assemble a triangle (<5 of 10 strands). Lanes show a faster product band than the corresponding 12x and 20x lanes, implying only partly assembled triangles. The green HP band is very weak, indicating complete usage of HP strands.

table S1. DNA origami staple sequences. The colors match those in the caDNAno layout shown in fig. S1. The first column denotes the staples position according to the caDNAno layout. The first digit indicates the helix the 5'-end is located on (y-coordinate), the succeeding number in brackets marks the number of base pairs the 5'-end is away from the boundary (x-coordinate). The second pair of numbers corresponds to the 3'-end in similar fashion.

Position	Sequence	Color	Description
0[111]1[95]	TAAATGAATTTTCTGTATGGGATTAATTTCTT		Structure strand
0[143]1[127]	TCTAAAGTTTTGTCGTCTTTCCAGCCGACAA		Structure strand
0[175]0[144]	TCCACAGACAGCCCTCATAGTTAGCGTAACGA		Structure strand
0[207]1[191]	TCACCAGTACAAACTACAACGCCTAGTACCAG		Structure strand
0[239]1[223]	AGGAACCCATGTACCGTAACACTTGATATAA		Structure strand
0[271]1[255]	CCACCCTCATTTTCAGGGATAGCAACCGTACT		Structure strand
0[47]1[31]	AGAAAGGAACAACTAAAGGAATTCAAAAAAA		Structure strand
0[79]1[63]	ACAACTTTCAACAGTTTCAGCGGATGTATCGG		Structure strand
1[128]4[128]	TGACAACTCGCTGAGGCTTGCATTATACCAAGCGCGATGATAAA		Structure strand
1[160]2[144]	TTAGGATTGGCTGAGACTCCTCAATAACCGAT		Structure strand
1[192]4[192]	GCGGATAACCTATTATTCTGAAACAGACGATTGGCCTTGAAGAGCCAC		Structure strand
1[224]3[223]	GTATAGCAAACAGTTAATGCCCAATCCTCA		Structure strand
1[256]4[256]	CAGGAGGTGGGGTCAGTGCCTTGAGTCTCTGAATTTACCGGGAACCAG		Structure strand
1[32]3[31]	AGGCTCCAGAGGCTTTGAGGACACGGGTAA		Structure strand
1[64]4[64	TTTATCAGGACAGCATCGGAACGACACCAACCTAAAACGAGGTCAATC		Structure strand
1[96]3[95]	AAACAGCTTTTTGCGGGATCGTCAACACTAAA		Structure strand
10[111]8[112]	TTGCTCCTTTCAAATATCGCGTTTGAGGGGGT		Structure strand
10[143]9[159]	CCAACAGGAGCGAACCAGACCGGAGCCTTTAC		Structure strand
10[175]8[176]	TTAACGTCTAACATAAAAACAGGTAACGGA		Structure strand
10[207]8[208]	ATCCCAATGAGAATTAACTGAACAGTTACCAG		Structure strand
10[239]8[240]	GCCAGTTAGAGGGTAATTGAGCGCTTTAAGAA		Structure strand
10[271]8[272]	ACGCTAACACCCACAAGAATTGAAAATAGC		Structure strand
10[47]8[48]	CTGTAGCTTGACTATTATAGTCAGTTCATTGA		Structure strand
10[79]8[80]	GATGGCTTATCAAAAAGATTAAGAGCGTCC		Structure strand
11[128]13[127]	TTTGGGGATAGTAGTAGCATTAAAAGGCCG		Structure strand
11[160]12[144]	CCAATAGCTCATCGTAGGAATCATGGCATCAA		Structure strand
11[192]13[191]	TATCCGGTCTCATCGAGAACAAGCGACAAAAG		Structure strand
11[224]13[223]	GCGAACCTCCAAGAACGGGTATGACAATAA		Structure strand
11[256]13[255]	GCCTTAAACCAATCAATAATCGGCACGCGCCT		Structure strand
11[32]13[31]	AACAGTTTTGTACCAAAAACATTTTATTTC		Structure strand
11[64]13[63]	GATTTAGTCAATAAAGCCTCAGAGAACCCTCA		Structure strand
11[96]13[95]	AATGGTCAACAGGCAAGGCAAAGAGTAATGTG		Structure strand
12[111]10[112]	TAAATCATATAACCTGTTTAGCTAACCTTTAA		Structure strand
12[143]11[159]	TTCTACTACGCGAGCTGAAAAGGTTACCGCGC		Structure strand
12[175]10[176]	TTTTATTTAAGCAAATCAGATATTTTTTGT		Structure strand
12[207]10[208]	GTACCGCAATTCTAAGAACGCGAGTATTATTT		Structure strand
12[239]10[240]	CTTATCATTCCCGACTTGCGGGAGCCTAATTT		Structure strand

12[271]10[272]	TGTAGAAATCAAGATTAGTTGCTCTTACCA		Structure strand
12[47]10[48]	TAAATCGGGATTCCCAATTCTGCGATATAATG		Structure strand
12[79]10[80]	AAATTAAGTTGACCATTAGATACTTTTGCG		Structure strand
13[128]15[127]	GAGACAGCTAGCTGATAAATTAATTTTTGT		Structure strand
13[160]14[144]	GTAATAAGTTAGGCAGAGGCATTTATGATATT		Structure strand
13[192]15[191]	GTAAAGTAATCGCCATATTTAACAAAACTTTT		Structure strand
13[224]15[223]	ACAACATGCCAACGCTCAACAGTCTTCTGA		Structure strand
13[256]15[255]	GTTTATCAATATGCGTTATACAAACCGACCGT		Structure strand
13[32]15[31]	AACGCAAAATCGATGAACGGTACCGGTTGA		Structure strand
13[64]15[63]	TATATTTTGTCATTGCCTGAGAGTGGAAGATT		Structure strand
13[96]15[95]	TAGGTAAACTATTTTTGAGAGATCAAACGTTA		Structure strand
14[111]12[112]	GAGGGTAGGATTCAAAAGGGTGAGACATCCAA		Structure strand
14[143]13[159]	CAACCGTTTCAAATCACCATCAATTCGAGCCA		Structure strand
14[175]12[176]	CATGTAATAGAATATAAAGTACCAAGCCGT		Structure strand
14[207]12[208]	AATTGAGAATTCTGTCCAGACGACTAAACCAA		Structure strand
14[239]12[240]	AGTATAAAGTTCAGCTAATGCAGATGTCTTTC		Structure strand
14[271]12[272]	TTAGTATCACAATAGATAAGTCCACGAGCA		Structure strand
14[47]12[48]	AACAAGAGGGATAAAAATTTTTAGCATAAAGC		Structure strand
14[79]12[80]	GCTATCAGAAATGCAATGCCTGAATTAGCA		Structure strand
15[128]18[128]	TAAATCAAAATAATTCGCGTCTCGGAAACCAGGCAAAGGGAAGG		Structure strand
15[160]16[144]	ATCGCAAGTATGTAAATGCTGATGATAGGAAC		Structure strand
15[192]18[192]	TCAAATATAACCTCCGGCTTAGGTAACAATTTCATTTGAAGGCGAATT		Structure strand
15[224]17[223]	CCTAAATCAAAATCATAGGTCTAAACAGTA		Structure strand
15[256]18[256]	GTGATAAAAAGACGCTGAGAAGAGATAACCTTGCTTCTGTTCGGGAGA		Structure strand
15[32]17[31]	TAATCAGCGGATTGACCGTAATCGTAACCG		Structure strand
15[64]18[64]	GTATAAGCCAACCCGTCGGATTCTGACGACAGTATCGGCCGCAAGGCG		Structure strand
15[96]17[95]	ATATTTTGGCTTTCATCAACATTATCCAGCCA		Structure strand
16[111]14[112]	TGTAGCCATTAAAATTCGCATTAAATGCCGGA		Structure strand
16[143]15[159]	GCCATCAAGCTCATTTTTTAACCACAAATCCA		Structure strand
16[175]14[176]	TATAACTAACAAAGAACGCGAGAACGCCAA		Structure strand
16[207]14[208]	ACCTTTTTATTTTAGTTAATTTCATAGGGCTT		Structure strand
16[239]14[240]	GAATTTATTTAATGGTTTGAAATATTCTTACC		Structure strand
16[271]14[272]	CTTAGATTTAAGGCGTTAAATAAAGCCTGT		Structure strand
16[47]14[48]	ACAAACGGAAAAGCCCCAAAAACACTGGAGCA		Structure strand
16[79]14[80]	GCGAGTAAAAATATTTAAATTGTTACAAAG		Structure strand
17[160]18[144]	AGAAAACAAAGAAGATGATGAAACAGGCTGCG		Structure strand
17[224]19[223]	CATAAATCTTTGAATACCAAGTGTTAGAAC		Structure strand
17[32]19[31]	TGCATCTTTCCCAGTCACGACGGCCTGCAG		Structure strand
17[96]19[95]	GCTTTCCGATTACGCCAGCTGGCGGCTGTTTC		Structure strand
18[111]16[112]	TCTTCGCTGCACCGCTTCTGGTGCGGCCTTCC		Structure strand
18[143]17[159]	CAACTGTTGCGCCATTCGCCATTCAAACATCA		Structure strand
18[175]16[176]	CTGAGCAAAAATTAATTACATTTTGGGTTA		Structure strand
18[207]16[208]	CGCGCAGATTACCTTTTTTAATGGGAGAGACT		Structure strand
18[239]16[240]	CCTGATTGCAATATATGTGAGTGATCAATAGT		Structure strand

18[271]16[272]	CTTTTACAAAATCGTCGCTATTAGCGATAG		Structure strand
18[47]16[48]	CCAGGGTTGCCAGTTTGAGGGGACCCGTGGGA		Structure strand
18[79]16[80]	GATGTGCTTCAGGAAGATCGCACAATGTGA		Structure strand
19[160]20[144]	GCAATTCACATATTCCTGATTATCAAAGTGTA		Structure strand
19[224]21[223]	CTACCATAGTTTGAGTAACATTTAAAATAT		Structure strand
19[32]21[31]	GTCGACTTCGGCCAACGCGCGGGGTTTTTC		Structure strand
19[96]21[95]	CTGTGTGATTGCGTTGCGCTCACTAGAGTTGC		Structure strand
2[111]0[112]	AAGGCCGCTGATACCGATAGTTGCGACGTTAG		Structure strand
2[143]1[159]	ATATTCGGAACCATCGCCCACGCAGAGAAGGA		Structure strand
2[175]0[176]	TATTAAGAAGCGGGGTTTTGCTCGTAGCAT		Structure strand
2[207]0[208]	TTTCGGAAGTGCCGTCGAGAGGGTGAGTTTCG		Structure strand
2[239]0[240]	GCCCGTATCCGGAATAGGTGTATCAGCCCAAT		Structure strand
2[271]0[272]	GTTTTAACTTAGTACCGCCACCCAGAGCCA		Structure strand
2[47]0[48]	ACGGCTACAAAAGGAGCCTTTAATGTGAGAAT		Structure strand
2[79]0[80]	CAGCGAAACTTGCTTTCGAGGTGTTGCTAA		Structure strand
20[111]18[112]	CACATTAAAATTGTTATCCGCTCATGCGGGCC		Structure strand
20[143]19[159]	AAGCCTGGTACGAGCCGGAAGCATAGATGATG		Structure strand
20[175]18[176]	ATTATCATTCAATATAATCCTGACAATTAC		Structure strand
20[207]18[208]	GCGGAACATCTGAATAATGGAAGGTACAAAAT		Structure strand
20[239]18[240]	ATTTTAAAATCAAAATTATTTGCACGGATTCG		Structure strand
20[271]18[272]	CTCGTATTAGAAATTGCGTAGATACAGTAC		Structure strand
20[47]18[48]	TTAATGAACTAGAGGATCCCCGGGGGGTAACG		Structure strand
20[79]18[80]	TTCCAGTCGTAATCATGGTCATAAAAGGGG		Structure strand
21[120]23[127]	CCCAGCAGGCGAAAAATCCCTTATAAATCAAGCCGGCG		Structure strand
21[160]22[144]	TCAATATCGAACCTCAAATATCAATTCCGAAA		Structure strand
21[184]23[191]	TCAACAGTTGAAAGGAGCAAATGAAAAATCTAGAGATAGA		Structure strand
21[224]23[223]	CTTTAGGGCCTGCAACAGTGCCAATACGTG		Structure strand
21[248]23[255]	AGATTAGAGCCGTCAAAAAACAGAGGTGAGGCCTATTAGT		Structure strand
21[32]23[31]	TTTTCACTCAAAGGGCGAAAAACCATCACC		Structure strand
21[56]23[63	AGCTGATTGCCCTTCAGAGTCCACTATTAAAGGGTGCCGT		Structure strand
21[96]23[95]	AGCAAGCGTAGGGTTGAGTGTTGTAGGGAGCC		Structure strand
22[111]20[112]	GCCCGAGAGTCCACGCTGGTTTGCAGCTAACT		Structure strand
22[143]21[159]	TCGGCAAATCCTGTTTGATGGTGGACCCTCAA		Structure strand
22[175]20[176]	ACCTTGCTTGGTCAGTTGGCAAAGAGCGGA		Structure strand
22[207]20[208]	AGCCAGCAATTGAGGAAGGTTATCATCATTTT		Structure strand
22[239]20[240]	TTAACACCAGCACTAACAACTAATCGTTATTA		Structure strand
22[271]20[272]	CAGAAGATTAGATAATACATTTGTCGACAA		Structure strand
22[47]20[48]	CTCCAACGCAGTGAGACGGGCAACCAGCTGCA		Structure strand
22[79]20[80]	TGGAACAACCGCCTGGCCCTGAGGCCCGCT		Structure strand
23[128]23[159]	AACGTGGCGAGAAAGGAAGGGAAACCAGTAA		Structure strand
23[160]22[176]	TAAAAGGGACATTCTGGCCAACAAAGCATC		Structure strand
23[192]22[208]	ACCCTTCTGACCTGAAAGCGTAAGACGCTGAG		Structure strand
23[224]22[240]	GCACAGACAATATTTTTGAATGGGGTCAGTA		Structure strand
23[256]22[272]	CTTTAATGCGCGAACTGATAGCCCCACCAG		Structure strand

23[32]22[48]	CAAATCAAGTTTTTTGGGGTCGAAACGTGGA		Structure strand
23[64]22[80]	AAAGCACTAAATCGGAACCCTAATCCAGTT		Structure strand
23[96]22[112]	CCCGATTTAGAGCTTGACGGGGAAAAAGAATA		Structure strand
3[160]4[144]	TTGACAGGCCACCACCAGAGCCGCGATTTGTA		Structure strand
3[224]5[223]	TTAAAGCCAGAGCCGCCACCCTCGACAGAA		Structure strand
3[32]5[31]	AATACGTTTGAAAGAGGACAGACTGACCTT		Structure strand
3[96]5[95]	ACACTCATCCATGTTACTTAGCCGAAAGCTGC		Structure strand
4[111]2[112]	GACCTGCTCTTTGACCCCCAGCGAGGGAGTTA		Structure strand
4[143]3[159]	TCATCGCCAACAAAGTACAACGGACGCCAGCA		Structure strand
4[175]2[176]	CACCAGAAAGGTTGAGGCAGGTCATGAAAG		Structure strand
4[207]2[208]	CCACCCTCTATTCACAAACAAATACCTGCCTA		Structure strand
4[239]2[240]	GCCTCCCTCAGAATGGAAAGCGCAGTAACAGT		Structure strand
4[271]2[272]	AAATCACCTTCCAGTAAGCGTCAGTAATAA		Structure strand
4[47]2[48]	GACCAACTAATGCCACTACGAAGGGGGTAGCA		Structure strand
4[79]2[80]	GCGCAGACAAGAGGCAAAAGAATCCCTCAG		Structure strand
5[160]6[144]	GCAAGGCCTCACCAGTAGCACCATGGGCTTGA		Structure strand
5[224]7[223]	TCAAGTTTCATTAAAGGTGAATATAAAAGA		Structure strand
5[32]7[31]	CATCAAGTAAAACGAACTAACGAGTTGAGA		Structure strand
5[96]7[95]	TCATTCAGATGCGATTTTAAGAACAGGCATAG		Structure strand
6[111]4[112]	ATTACCTTTGAATAAGGCTTGCCCAAATCCGC		Structure strand
6[143]5[159]	GATGGTTTGAACGAGTAGTAAATTTACCATTA		Structure strand
6[175]4[176]	CAGCAAAAGGAAACGTCACCAATGAGCCGC		Structure strand
6[207]4[208]	TCACCGACGCACCGTAATCAGTAGCAGAACCG		Structure strand
6[239]4[240]	GAAATTATTGCCTTTAGCGTCAGACCGGAACC		Structure strand
6[271]4[272]	ACCGATTGTCGGCATTTTCGGTCATAATCA		Structure strand
6[47]4[48]	TACGTTAAAGTAATCTTGACAAGAACCGAACT		Structure strand
6[79]4[80]	TTATACCACCAAATCAACGTAACGAACGAG		Structure strand
7[120]9[127]	CGTTTACCAGACGACAAAGAAGTTTTGCCATAATTCGA		Structure strand
7[160]8[144]	TTATTACGAAGAACTGGCATGATTGCGAGAGG		Structure strand
7[184]9[191]	CGTAGAAAATACATACCGAGGAAACGCAATAAGAAGCGCA		Structure strand
7[224]9[223]	AACGCAAAGATAGCCGAACAAACCCTGAAC		Structure strand
7[248]9[255]	GTTTATTTTGTCACAATCTTACCGAAGCCCTTTAATATCA		Structure strand
7[32]9[31]	TTTAGGACAAATGCTTTAAACAATCAGGTC		Structure strand
7[56]9[63]	ATGCAGATACATAACGGGAATCGTCATAAATAAAGCAAAG		Structure strand
7[96]9[95]	TAAGAGCAAATGTTTAGACTGGATAGGAAGCC		Structure strand
8[111]6[112]	AATAGTAAACACTATCATAACCCTCATTGTGA		Structure strand
8[143]7[159]	CTTTTGCAGATAAAAACCAAAATAAAGACTCC		Structure strand
8[175]6[176]	ATACCCAACAGTATGTTAGCAAATTAGAGC		Structure strand
8[207]6[208]	AAGGAAACATAAAGGTGGCAACATTATCACCG		Structure strand
8[239]6[240]	AAGTAAGCAGACACCACGGAATAATATTGACG		Structure strand
8[271]6[272]	AATAGCTATCAATAGAAAATTCAACATTCA		Structure strand
8[47]6[48]	ATCCCCCTATACCACATTCAACTAGAAAAATC		Structure strand
8[79]6[80]	AATACTGCCCAAAAGGAATTACGTGGCTCA		Structure strand
9[128]11[127]	GCTTCAATCAGGATTAGAGAGTTATTTTCA		Structure strand

9[160]10[144]	AGAGAGAAAAAAATGAAAATAGCAAGCAAACT		Structure strand
9[192]11[191]	TTAGACGGCCAAATAAGAAACGATAGAAGGCT		Structure strand
9[224]11[223]	AAAGTCACAAAATAAACAGCCAGCGTTTTA		Structure strand
9[256]11[255]	GAGAGATAGAGCGTCTTTCCAGAGGTTTTGAA		Structure strand
9[32]11[31]	TTTACCCCAACATGTTTTAAATTTCCATAT		Structure strand
9[64]11[63]	CGGATTGCAGAGCTTAATTGCTGAAACGAGTA		Structure strand
9[96]11[95]	CGAAAGACTTTGATAAGAGGTCATATTTCGCA		Structure strand
4[63]6[56]	Biotin – ATAAGGGAACCGGATATTCATTACGTCAGGACGTTGGGAA – 3’		5’-Biotin modification
4[127]6[120]	Biotin – TTGTGTCGTGACGAGAAACACCAAATTTCAACTTTAAT – 3’		5’-Biotin modification
4[191]6[184]	Biotin – CACCCTCAGAAACCATCGATAGCATTGAGCCATTTGGGAA – 3’		5’-Biotin modification
4[255]6[248]	Biotin – AGCCACCACTGTAGCGCGTTTTCAAGGGAGGGAAGGTAAA – 3’		5’-Biotin modification
18[63]20[56]	Biotin – ATTAAGTTTACCGAGCTCGAATTCGGGAAACCTGTCGTGC – 3’		5’-Biotin modification
18[127]20[120]	Biotin – GCGATCGGCAATTCCACACAACAGGTGCCTAATGAGTG – 3’		5’-Biotin modification
18[191]20[184]	Biotin – ATTCATTTTTGTTTGGATTATACTAAGAAACCACCAGAAG – 3’		5’-Biotin modification
18[255]20[248]	Biotin – AACAATAACGTAAAACAGAAATAAAAATCCTTTGCCCGAA – 3’		5’-Biotin modification

table S2. M13mp18 scaffold sequence.

TTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAAC
TTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGG
AACAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGG
ACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTC
TCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGC
ACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTC
GGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGC
CTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGT
TTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGAGGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCAT
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GAAGGCCAGACGCGAATTATTTTTGATGGCGTTCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAATGCGAATTTTAACAAAATATTAACGTTTACAATTTA
AATATTTGCTTATACAATCTTCCTGTTTTTGGGGCTTTTCTGATTATCAACCGGGGTACATATGATTGACATGCTAGTTTTACGATTACCGTTCATCGATTCTCTTGTT
TGCTCCAGACTCTCAGGCAATGACCTGATAGCCTTTGTAGATCTCTCAAAAATAGCTACCCTCTCCGGCATTAATTTATCAGCTAGAACGGTTGAATATCATATTGATG
GTGATTTGACTGTCTCCGGCCTTTCTCACCCTTTTGAATCTTTACCTACACATTACTCAGGCATTGCATTTAAAATATATGAGGGTTCTAAAAATTTTTATCCTTGCGT
TGAAATAAAGGCTTCTCCCGCAAAAGTATTACAGGGTCATAATGTTTTTGGTACAACCGATTTAGCTTTATGCTCTGAGGCTTTATTGCTTAATTTTGCTAATTCTTTG
CCTTGCCTGTATGATTTATTGGATGTTAATGCTACTACTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATGAAAATATAGCTAAACAGGTTATTGACC
ATTTGCGAAATGTATCTAATGGTCAAACTAAATCTACTCGTTCGCAGAATTGGGAATCAACTGTTATATGGAATGAAACTTCCAGACACCGTACTTTAGTTGCATATTT
AAAACATGTTGAGCTACAGCATTATATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGAGCAATTAAAGGTACTCTCTAATCCTGACCTG
TTGGAGTTTGCTTCCGGTCTGGTTCGCTTTGAAGCTCGAATTAAAACGCGATATTTGAAGTCTTTCGGGCTTCCTCTTAATCTTTTTGATGCAATCCGCTTTGCTTCTG
ACTATAATAGTCAGGGTAAAGACCTGATTTTTGATTTATGGTCATTCTCGTTTTCTGAACTGTTTAAAGCATTTGAGGGGGATTCAATGAATATTTATGACGATTCCGC
AGTATTGGACGCTATCCAGTCTAAACATTTTACTATTACCCCCTCTGGCAAAACTTCTTTTGCAAAAGCCTCTCGCTATTTTGGTTTTTATCGTCGTCTGGTAAACGAG
GGTTATGATAGTGTTGCTCTTACTATGCCTCGTAATTCCTTTTGGCGTTATGTATCTGCATTAGTTGAATGTGGTATTCCTAAATCTCAACTGATGAATCTTTCTACCT
GTAATAATGTTGTTCCGTTAGTTCGTTTTATTAACGTAGATTTTTCTTCCCAACGTCCTGACTGGTATAATGAGCCAGTTCTTAAAATCGCATAAGGTAATTCACAATG
ATTAAAGTTGAAATTAAACCATCTCAAGCCCAATTTACTACTCGTTCTGGTGTTTCTCGTCAGGGCAAGCCTTATTCACTGAATGAGCAGCTTTGTTACGTTGATTTGG
GTAATGAATATCCGGTTCTTGTCAAGATTACTCTTGATGAAGGTCAGCCAGCCTATGCGCCTGGTCTGTACACCGTTCATCTGTCCTCTTTCAAAGTTGGTCAGTTCGG
TTCCCTTATGATTGACCGTCTGCGCCTCGTTCCGGCTAAGTAACATGGAGCAGGTCGCGGATTTCGACACAATTTATCAGGCGATGATACAAATCTCCGTTGTACTTTG
TTTCGCGCTTGGTATAATCGCTGGGGGTCAAAGATGAGTGTTTTAGTGTATTCTTTTGCCTCTTTCGTTTTAGGTTGGTGCCTTCGTAGTGGCATTACGTATTTTACCC
GTTTAATGGAAACTTCCTCATGAAAAAGTCTTTAGTCCTCAAAGCCTCTGTAGCCGTTGCTACCCTCGTTCCGATGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCA
AAAGCGGCCTTTAACTCCCTGCAAGCCTCAGCGACCGAATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCGCAACTATCGGTATCAAGCTGTTTAAGA
AATTCACCTCGAAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTTGGAGCCTTTTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAG
TTGTTCCTTTCTATTCTCACTCCGCTGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCTGGAAAGACGACAAAACTTTAGATCG
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ATATCAACCCTCTCGACGGCACTTATCCGCCTGGTACTGAGCAAAACCCCGCTAATCCTAATCCTTCTCTTGAGGAGTCTCAGCCTCTTAATACTTTCATGTTTCAGAA
TAATAGGTTCCGAAATAGGCAGGGGGCATTAACTGTTTATACGGGCACTGTTACTCAAGGCACTGACCCCGTTAAAACTTATTACCAGTACACTCCTGTATCATCAAAA
GCCATGTATGACGCTTACTGGAACGGTAAATTCAGAGACTGCGCTTTCCATTCTGGCTTTAATGAGGATTTATTTGTTTGTGAATATCAAGGCCAATCGTCTGACCTGC
CTCAACCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCTGAGGGTGGTGGCTCTGAGGGTGGCGGTCTGAGGGTGGCGGCTCTGAGGGAGG
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GCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACGGTGCTGCTATCGATGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATTTTG
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TTTTGTCTTTGGCGCTGGTAAACCATATGAATTTTCTATTGATTGTGACAAAATAAACTTATTCCGTGGTGTCTTTGCGTTTCTTTTATATGTTGCCACCTTTATGTAT
GTATTTTCTACGTTTGCTAACATACTGCGTAATAAGGAGTCTTAATCATGCCAGTTCTTTTGGGTATTCCGTTATTATTGCGTTTCCTCGGTTTCCTTCTGGTAACTTT
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TCTCAGCGTGGCACTGTTGCAGGCGGTGTTAATACTGACCGCCTCACCTCTGTTTTATCTTCTGCTGGTGGTTCGTTCGGTATTTTTAATGGCGATGTTTTAGGGCTAT
CAGTTCGCGCATTAAAGACTAATAGCCATTCAAAAATATTGTCTGTGCCACGTATTCTTACGCTTTCAGGTCAGAAGGGTTCTATCTCTGTTGGCCAGAATGTCCCTTT
TATTACTGGTCGTGTGACTGGTGAATCTGCCAATGTAAATAATCCATTTCAGACGATTGAGCGTCAAAATGTAGGTATTTCCATGAGCGTTTTTCCTGTTGCAATGGCT
GGCGGTAATATTGTTCTGGATATTACCAGCAAGGCCGATAGTTTG

table S3. Fluorescently labeled DNA sequences.

Label	Handle
5' – GTGATGTAGGTGGTAGAGGAA – Atto 647N	staple – TTCCTCTACCACCTACATCAC – 3′
5' – TATGAGAAGTTAGGAATGTTA – Cy3	staple – TAACATTCCTAACTTCTCATA – 3′
5' – CGAGTTTAGGAGAGATGGTAA – Atto 488	staple – TTACCATCTCTCCTAAACTCG – 3′

table S4. Intensity barcode subset (25 of 124).

Barcode-No	RED	GRN	BLU	Subset No
1	6	0	0	
2	14	0	0	
3	27	0	0	
4	44	0	0	
5	0	6	0	
6	6	6	0	1
7	14	6	0	
8	27	6	0	
9	44	6	0	
10	0	14	0	
11	6	14	0	
12	14	14	0	
13	27	14	0	2
14	44	14	0	
15	0	27	0	
16	6	27	0	3
17	14	27	0	
18	27	27	0	
19	44	27	0	
20	0	44	0	
21	6	44	0	
22	14	44	0	
23	27	44	0	4
24	44	44	0	5
25	0	0	6	
26	6	0	6	
27	14	0	6	
28	27	0	6	6
29	44	0	6	7

30	0	6	6	
31	6	6	6	
32	14	6	6	
33	27	6	6	
34	44	6	6	
35	0	14	6	
36	6	14	6	
37	14	14	6	
38	27	14	6	
39	44	14	6	
40	0	27	6	
41	6	27	6	
42	14	27	6	
43	27	27	6	
44	44	27	6	
45	0	44	6	
46	6	44	6	
47	14	44	6	
48	27	44	6	
49	44	44	6	
50	0	0	14	
51	6	0	14	
52	14	0	14	
53	27	0	14	
54	44	0	14	
55	0	6	14	
56	6	6	14	
57	14	6	14	
58	27	6	14	8
59	44	6	14	
60	0	14	14	9
61	6	14	14	10
62	14	14	14	
63	27	14	14	
64	44	14	14	11
65	0	27	14	
66	6	27	14	
67	14	27	14	12
68	27	27	14	
69	44	27	14	13
70	0	44	14	

71	6	44	14	
72	14	44	14	
73	27	44	14	14
74	44	44	14	15
75	0	0	27	
76	6	0	27	
77	14	0	27	
78	27	0	27	
79	44	0	27	16
80	0	6	27	
81	6	6	27	
82	14	6	27	17
83	27	6	27	
84	44	6	27	
85	0	14	27	
86	6	14	27	
87	14	14	27	18
88	27	14	27	
89	44	14	27	19
90	0	27	27	20
91	6	27	27	
92	14	27	27	
93	27	27	27	
94	44	27	27	
95	0	44	27	
96	6	44	27	
97	14	44	27	21
98	27	44	27	
99	44	44	27	
100	0	0	44	
101	6	0	44	
102	14	0	44	
103	27	0	44	22
104	44	0	44	
105	0	6	44	23
106	6	6	44	
107	14	6	44	
108	27	6	44	
109	44	6	44	
110	0	14	44	
111	6	14	44	

112	14	14	44	
113	27	14	44	
114	44	14	44	
115	0	27	44	
116	6	27	44	
117	14	27	44	
118	27	27	44	
119	44	27	44	
120	0	44	44	24
121	6	44	44	25
122	14	44	44	
123	27	44	44	
124	44	44	44	

table S5. Intensity barcode subset (12 of 64).

Barcode-No	RED	GRN	BLU	Subset No
1	6	6	6	
2	14	6	6	
3	27	6	6	
4	44	6	6	
5	6	14	6	1
6	14	14	6	
7	27	14	6	
8	44	14	6	
9	6	27	6	
10	14	27	6	
11	27	27	6	2
12	44	27	6	
13	6	44	6	
14	14	44	6	3
15	27	44	6	
16	44	44	6	
17	6	6	14	
18	14	6	14	
19	27	6	14	4
20	44	6	14	
21	6	14	14	
22	14	14	14	5
23	27	14	14	
24	44	14	14	6
25	6	27	14	
26	14	27	14	
27	27	27	14	
28	44	27	14	
29	6	44	14	
30	14	44	14	
31	27	44	14	
32	44	44	14	
33	6	6	27	
34	14	6	27	
35	27	6	27	
36	44	6	27	
37	6	14	27	
38	14	14	27	

39	27	14	27	
40	44	14	27	
41	6	27	27	
42	14	27	27	
43	27	27	27	7
44	44	27	27	
45	6	44	27	8
46	14	44	27	
47	27	44	27	
48	44	44	27	9
49	6	6	44	
50	14	6	44	
51	27	6	44	
52	44	6	44	
53	6	14	44	
54	14	14	44	
55	27	14	44	
56	44	14	44	10
57	6	27	44	
58	14	27	44	11
59	27	27	44	
60	44	27	44	
61	6	44	44	12
62	14	44	44	
63	27	44	44	
64	44	44	44	

table S6. Intensity barcode subset (5 of 20).

Barcode-No	RED	GRN	BLU	Subset No
1	44	14	14	
2	14	44	14	1
3	14	14	44	
4	44	44	14	
5	44	14	44	2
6	14	44	44	
7	44	44	44	
8	14	14	14	
9	44	44	0	
10	44	0	44	3
11	0	44	44	
12	14	14	0	
13	14	0	14	
14	0	14	14	4
15	44	14	0	
16	44	0	14	
17	0	44	14	
18	14	44	0	5
19	14	0	44	
20	0	14	44	

table S7. DNA detection sequences and corresponding barcodes.

Target	Sequence	Barcode (RED-GRN-BLU)
1	5′ – GCAGTTTCCGACCGATATAGT TTT CGGTTGTACTGTGACCGATTC – 3′	14-44-14
2	5′ – TAGAGTCCAAGAGTCCTCGTT TTT CGGTTGTACTGTGACCGATTC – 3′	14-44-44
3	5′ – GGTTAAGGTCAACATCGTCTC TTT CGGTTGTACTGTGACCGATTC – 3′	14-14-14
4	5′ – CATGTCAGGAGATTTTCAGCC TTT CGGTTGTACTGTGACCGATTC – 3′	14-14-44
5	5′ – TACCCTATCTGAGTGAGTAGC TTT CGGTTGTACTGTGACCGATTC – 3′	44-14-14
6	5′ – CTTCCCGTTATGACAAGATGG TTT CGGTTGTACTGTGACCGATTC – 3′	44-14-44
7	5′ – CCCTAGTGCTTTTGGAGAAAC TTT CGGTTGTACTGTGACCGATTC – 3′	44-44-14
8	5′ – GGACACGGTATTCCTAATTCG TTT CGGTTGTACTGTGACCGATTC – 3′	44-44-44

table S8. Triggered assembly sequences.

Description	Sequence
Capture	Alexa 647 – CTCCTCGCCCTTGCTCACCAT - Biotin
Trigger	5’ - ATGGTGAGCAAGGGCGAGGAG … CCTCACCTCTACTCCCACCCACACGCACCCTC CCTCACCTCTACTCCCACCCACACGCACCCTC … CCTCACCTCTACTCCCACCCACACGCACCCTC CCTCACCTCTACTCCCACCCACACGCACCCTC – 3’
Hairpin	Cy3 - TCCCACCCACACGCACCCTC CCTCACCTCTAC … GAGGGTGCGTGTGGGTGGGA GTAGAGGTGAGG – 3’

protocol S1. DNA origami self-assembly.

Self-assembly was performed in a one-pot reaction with 20 µl total volume containing 10 nM scaffold strand (M13mp18), 100 nM folding staples and 150 nM biotinylated strands, 100 nM strands with dye-handle extension and 225 nM fluorescently-labeled anti-handles in folding buffer (1×TAE Buffer with 12.5 mM $MgCl_2$). The solution was heatshocked at 65 °C for 5 min to remove eventual secondary structures and subsequently cooled to 4 °C over the course of 1 h. DNA origami were purified by agarose gel electrophoresis (1.5 % agarose, 1×TAE Buffer with 12.5 mM $MgCl_2$) at 4.5 V/cm for 1.5 h, on ice. Gel bands were cut, crushed and filled into a 'Freeze 'N Squeeze' column and spun for 5 min at 1000×g at 4 °C.

protocol S2. Microscopy sample preparation.

Clean coverslip (No. 1.5, 18×18 mm^2, ≈0.17 mm thick) and microscopy slide (3×1 $inch^2$, 1 mm thick) with Isopropanol.
Build flow chamber by sandwiching two strips of sticky tape between coverslip and glass slide, ~ 20 µl volume.

Incubate 2 min with 20 µl 1 mg/ml BSA-Biotin in Buffer A (see Materials).
Rinse with 40 µl Buffer A.
Incubate 2 min with 20 µl 0.5 mg/ml Streptavidin in Buffer A.
Rinse with 40 µl Buffer A.
Rinse with 40 µl Buffer B (see Materials).
Incubate for 2 min with 20 µl ~300 pM DNA origami based metafluorophores.
Rinse with 40 µl Buffer B.

Seal chamber with epoxy.

protocol S3. Triggered assembly on surface.

Capture (CAP) and trigger (T) strands were annealed in a thermocycler directly before adding to the sample at 1 µM in 1x TAE with 12.5 mM $MgCl_2$ with 0.05% Tween20 (85 °C for 5 min, gradient from 85 °C to 10 °C in 15 min).
Hairpin (HP) strands were annealed in a thermocycler directly before adding to the sample at 1 µM in 1x TAE with 12.5 mM $MgCl_2$ (85 °C for 5 min, gradient from 85 °C to 10 °C in 15 min).

Build flow chamber with 3x sticky tape height, ~ 60 µl volume.
Incubate 2 min with 60 µl 1 mg/ml BSA-Biotin in Buffer A.
Rinse with 120 µl Buffer A.
Incubate 2 min with 60 µl 0.5 mg/ml Streptavidin in Buffer A.
Rinse with 120 µl Buffer A.
Rinse with 120 µl 1x TAE with 12.5mM $MgCl_2$ with 0.05% Tween-20.
Incubate 1 min with 60 µl 25 pM annealed CAP-T duplexes in 12.5mM $MgCl_2$ with 0.05% Tween-20.
Rinse with 120 µl 1x TAE with 12.5mM $MgCl_2$ with 0.05% Tween-20.
Incubate for 2 min with 60 µl 100 pM DNA origami standards.
Rinse with 120 µl 1x TAE with 12.5mM $MgCl_2$ with 0.05% Tween-20.

Incubate for 20 min with 60 µl 30 nM annealed HP.
Rinse with 120 µl 1x TAE with 12.5mM $MgCl_2$ with 0.05% Tween-20.
Repeat HP incubation 3 times.

Seal chamber.

protocol S4. Triggered assembly in solution and gel assay.

Triggered assembly of triangles for the gel assay was performed in a one-pot reaction. Capture strands (CAP), trigger strands (T) and fluorescently labeled hairpins (HP) were added in varying stoichiometric ratios to a total volume of 40 µl. CAP strands were at a final concentration of 100 nM, T strands at 110 nM and HP strands at 550 nM (5x), 1.325 µM (12x) or 2.2 µM (20x). Strands were diluted in 1x TAE with 12.5 mM MgCl2. HP strands were annealed in a thermocycler directly before adding to the triggered assembly reaction at 10 µM in 1x TAE with 12.5 mM $MgCl_2$ (85 C for 5 min, gradient from 85 C to 10 C in 15 min). The control sample did not contain the T strand but HP strands at 1.325 µM (12x). Assembly was performed in low retention PCR tubes at either 30 C or at 24 C for 2 h each.
Gel electrophoresis was performed using a 2% agarose gel in 1x TAE with 12.5 mM $MgCl_2$, with 4.5 V/cm for 3 h on ice.
Gel was scanned with a Typhoon scanner.

Materials

Unmodified DNA oligonucleotides were purchased from Integrated DNA Technologies. Fluorescently modified DNA oligonucleotides were purchased from Biosynthesis. Streptavidin was purchased from Invitrogen (Catalog number: S-888). Albumin, biotin labeled bovine (BSA-biotin) was obtained from Sigma Aldrich (Catalog Number: A8549). Glass slides and coverslips were purchased from VWR. M13mp18 scaffold was obtained from New England Biolabs. 'Freeze N Squeeze' columns were ordered from Bio-Rad.

Two buffers were used for sample preparation and imaging:

Buffer A (10 mM Tris-HCl, 100 mM NaCl, 0.05 % Tween-20, pH 8).

Buffer B (5 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.05 % Tween-20, pH 8).

Optical setup

DNA origami-based metafluorophore imaging was performed on a Zeiss Axio Observer Z1 Inverted Fluorescence Microscope with Definite Focus and a Zeiss Colibri LED illumination system (ATTO 488: 470 nm, Cy3: 555 nm, ATTO 647N: 625 nm). We used a Zeiss Plan-apochromat (63x/1.40 Oil) oil-immersion objective and a Hamamatsu Orca-Flash 4.0 sCMOS camera.

ATTO 488: Zeiss filter set 38: (BP 470/40, FT 495, BP 525/50).

Cy3: Zeiss Filter Set 43 (BP 545/25, FT 570, BP 605/70).

ATTO 647N: Zeiss filter set 50 (BP 640/30, FT 660, BP 690/50).

Triggered assembly imaging was carried out on an inverted Nikon Eclipse Ti microscope using a Nikon TIRF illuminator with an oil-immersion objective (CFI Apo TIRF 100×, numerical aperture (NA) 1.49, oil).

Lasers: 488 nm (200 mW nominal, Coherent Sapphire), 561 nm (200 mW nominal, Coherent Sapphire) and 647 nm (300 mW nominal, MBP Communications).

Camera: iXon X3 DU-897 EMCCD (Andor Technologies)

Excitation filters: (ZT488/10, ZET561/10 and ZET640/20, Chroma Technology)

Multiband beam splitter: (ZT488rdc/ZT561rdc/ZT640rdc, Chroma Technology)

Emission filters: (ET525/50m, ET600/50m and ET700/75m, Chroma Technology)

Multiplexed nucleic acid detection was performed on a Zeiss LSM 780 confocal laser scanning microscope equipped with a ZEISS Plan-APO 63x/NA1.46 oil immersion objective and a GaAsP array for detection.

Lasers: 458 nm (Argon), 561 nm (DPSS), 647 nm (HeNe).

Software section

Spot detection, intensity analysis

After image acquisition, spot-detection was performed using a custom LabVIEW script. Here, 2D Gaussians were fitted within a 10x10 px^2 area around the center of the spots. The volume of the 2D Gaussian is proportional to the photon count and is thereby defined as intensity. Finally, one obtains a molecule-list with both, spatial coordinates and corresponding intensity values.

Barcode identification

All intensity values were plotted as a histogram and the local maxima (peaks) were fitted with Gaussians. Based on the intersections of these fits, the distinct intensity-level intervals could be determined.
Non-overlapping regions in between two peaks had to be identified and barcodes with a corresponding intensity had to be classified as qualified. To identify the overlapping interval between two peaks, the height of the intersection (x counts) of the corresponding fits was determined. By determining the intersections of the two Gaussians with half the height of their intersection ($x/2$ counts), the overlapping interval and boundaries for barcode levels were defined. Additionally, spots that did not meet the criteria of the robust subsets were removed (e.g. removal of barcodes that do not have three colors for the 64-subset.).

After removing the spots that did not qualify, the intensity values in the molecule-list were replaced with barcode-level indicators. Individual barcodes were identified by combining spots from the three molecule-lists (corresponding to the three recorded colors), which are in close proximity (i.e. < 500 nm).

Triggered assembly

Triggered assembly evaluation was performed by determining spot coordinates and spot intensities as described above. Colocalizations of red (Alexa 647) and green (Cy3) spots were grouped as triangles and blue (Atto 488) and green (Cy3) colocalizations as DNA origamis. Plotting the two groups together resulted in Figure 6c of the main test.