SUPPORTING INFORMATION

Spatially Controlled Activation of Toll-Like Receptor 9 with DNA-Based Nanomaterials

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MATERIALS AND METHODS

Removal of LPS from DNA origami scaffold. To avoid non-specific cell activation, endotoxin contaminants (e.g. LPS) in the scaffold p7560 solution (Tilibit) were removed with Triton X-114 (Sigma Aldrich) following a previously described protocol.[1] Triton X-114 (2% v/v) was mixed with scaffold stock at 4°C for 30 min to solubilize LPS, mixed at 37°C for 5 min at 450 rpm and centrifuged at 37°C for 30 min at 15000 rpm to induce phase separation and recover the purified scaffold in the upper aqueous phase. The procedure was repeated four times and LPS amount was assessed to be lower than 0.5 EU m/L using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript) following the manufacturer's protocol. Purified scaffold was stored at -20°C.

DNA origami self-assembly and CpG- and Cy5- functionalization. Unmodified, Cy5- and CpG-functionalized DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT). CpG-ODN 1826 sequence, specific for murine TLR9 and mouse cell lines like the RAW 264.7, was used both for free and disk-conjugated studies. The DNA origami disk structure[2] self-assembly was performed mixing LPS-purified scaffold p7560 (10 nM, Table S1), core folding staples and 6 Cy5-conjugated staples (100 nM and 30 nM, respectively, Table S2), variable extra folding staples and CpG-functionalized staples if needed (100 nM and 50 nM, respectively), in combinations according to the desired structure (see Table S3 for staple sequences corresponding to each structure type) in 1X folding buffer (FoB: 5 mM Tris, 1 mM EDTA, 5 mM NaCl, 20 mM $MgCl_2$, pH 8.0, stock solutions purchased from Thermo Fisher). Thermal annealing ramp: 80 °C for 5 min, from 60 ˚C to 20 °C in steps of -1 °C per hour and stored at 20 °C.

DNA origami disk purification. Disks were purified from the excess of staples by PEG precipitation, as previously described.[3] DNA origami annealing solution was mixed in ratio 1:1 v/v with PEG precipitation buffer 2X, containing 15% PEG 8000 (VWR), 0.5 M NaCl in 1X disk folding buffer, incubated at room temperature for 30 min and centrifuged at 16000 rcf for 40 min at 20°C. The supernatant was removed, 1X disk folding buffer added, and the solution was incubated at room temperature overnight. Concentration was measured by absorbance at 260 nm using a microvolume spectrophotometer (Quawell Q9000). Purified structures were stored at 4°C.

Analytical agarose gel electrophoresis. Folding and purification quality was assessed by agarose gel electrophoresis (AGE). 10 µl of origami solution at 2.5 nM concentration were mixed with 2 µl of 6X loading dye (Thermo Fisher) and subjected to AGE (2% agarose, 1X TBE, 15 mM $MgCl_2$, 1X SybrSafe) at 70 V for 100 min in ice-water bath. Ladder 1kB (N3232L) was purchased from Biolabs. The gels were imaged using a BioRad ChemiDoc MP.

Stability assays. To assess the stability of the DNA origami in cell medium used for this study, disks were incubated in DMEM+ at 37°C for a total of 5 h in a total volume of 10 µl at 2.5 nM concentration. Similarly, to confirm the stability at pH 5.5 as in the late endosome, disks were incubated in MES Buffer (pH 5.5, Thermo Fisher) at 37°C for a total of 5 h in a total volume of 10 µl at 2.5 nM concentration. To verify the integrity of DNA origami disk, after incubation, 6X loading dye was added and samples were immediately loaded on agarose gel (2% agarose, 1X TBE, 15 mM $MgCl_2$, 1X SybrSafe, run at 70 V for 90 min in ice-water bath). The gels were imaged using a BioRad ChemiDoc MP.

Transmission Electron Microscopy imaging. 3 µl of purified DNA origami at 10 nM concentration were adsorbed on carbon grid and incubated for 2 min. The solution in excess was removed and 5 µl of 2% uranyl formate were added immediately on the grid for 30 seconds. Finally, the solution was removed, and the grid was air dried for 15 min. Images were obtained on a Tecnai T12 transmission electron microscope operated at a voltage of 100 kV.

Cell culture. RAW 264.7 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC). Cells were cultured in DMEM high glucose with L-glutamine and sodium pyruvate (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), and 100 U/ml Penicillin-Streptomycin (BioConcept) and were detached using ROTI-Cell PBS/EDTA (Carl Roth). Cells were kept at maximum 70% confluence and cultured at 37°C in a 5% CO_2 atmosphere. All experiments were performed with cells between passage 20 and 23.

RAW 264.7 TLR9 knock out cell line generation using recombinant CRISPR Cas9. The Tlr9 gene was deleted in RAW 264.7 cells as reported previously for primary T cells[4] with the following alterations: cells were electroporated with pulse DS136, with a complex of Alt-R S.p. Cas9 Nuclease (IDT) and sgRNA (GAUGCUGCCGCAGAGAAACG) targeting Tlr9 (Synthego)

using the SF cell line 4D-Nucleofector X kit S electroporation kit (Lonza) and Lonza 4D-Nucleofector Core Unit. Cells were rested in SAFC media plus 10% FCS at 37°C for 10 min, after which cells were plated out in tissue culture flasks containing 10% FCS SAFC for further expansion. For confirmation of successful Tlr9 deletion, expanded cells were stained intracellularly for TLR9 expression using the eBioscience Foxp3 transcription factor staining kit and anti-mouse TLR9-PE antibody.

Uptake study by confocal microscope. Cells were seeded on ibidi 12-well chamber slides at density of 20'000 cells per well and incubated overnight in the incubator at 37°C with 5% CO_2. Cy5-labelled DNA origami samples were diluted in DMEM supplemented with 20 mM $MgCl_2$ (DMEM+) at final concentration of 5 nM immediately before addition to the cells (100 µl per well) and incubated for 30 min at 37°C. To remove structures on the cell surface, the solution was removed and DNase I diluted in complete cell medium (100 µl at final concentration of 70 U/ml, as previously reported[5]) was added to cells and incubated at 37°C for 30 min. For controls, the solution was changed with fresh complete cell medium. At the end of the incubation, the medium was removed, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS (75 µl, incubating 20 min at room temperature). After washing again with PBS, for intracellular staining, cells were permeabilized incubating with 100 µl of 0.1% Triton X-100 (Thermo Fisher) solution in PBS for 10 min at room temperature. After washing with PBS, 100 µl of blocking buffer (5% BSA in PBS) were added and the slide incubated 1 h at room temperature. After removing the blocking solution, for late endosome staining, anti-RAB7 antibody (ab126712, abcam) was diluted 1:500 in 5% BSA in PBS, 100 µl per well added to the cells and the slide incubated overnight at 4°C. After washing with PBS, the secondary antibody goat anti-rabbit AF488 (ab150077, abcam) was added diluted 1:1000 in PBS at 100 µl per well and the slide incubated 1 h at room temperature. After washing with PBS, cells were stained with DAPI solution (50 µl, 300 nM, Thermo Fisher) for 3 min. After two last washes, all the solution was removed from the slide, slide plastic wells were removed and the glass was left to air dry at room temperature for 15 min. ProLong Glass Antifade Mountant (3 drops, Thermo Fisher) was added on the slide, which was immediately covered with a coverslip and left to air-dry at room temperature for 24 h before analysis. Samples were imaged using the confocal microscope Zeiss LSM700 Upright with an oil-immersion objective (Zeiss, Plan-apochromat, 63X, NA 1.40, oil). 405 nm, 488 nm and 639 nm wavelength lasers were used for DAPI, AF488 and Cy5 signals,

respectively. BP 445/50, 515-565 and 690/50 filters were applied for DAPI, AF488 and Cy5 emissions, respectively, and imaged with an Axiocam MRm (B/W). Image analysis was performed with the software ImageJ.

Flow cytometry. Cells were seeded on a tissue-culture-treated 96 well plate at 30'000 cells per well and incubated overnight at 37°C with 5% CO_2. Cy5-labelled DNA origami samples were tested at 0.5 nM concentration, diluted in DMEM+ immediately before addition to the cells (50 µl per well) and incubated for 30 min at 37°C. Similarly, free CpG (CpG-ODN 1826 sequence, IDT) was diluted at the different concentrations in DMEM+ immediately before addition to the cells (50 µl per well) and incubated for 30 min at 37°C. To remove structures on the cell surface at the end of the incubation, the medium was removed and 50 µl of DNase I diluted in complete cell medium was added (at final concentration of 70 U/ml, as previously reported), and cells were incubated at 37°C for 30 min. Subsequently, the solution was replaced with fresh complete medium, and cells were incubated for additional 4 h to induce the expression of surface markers. At the end of the incubation, cells were detached with detaching buffer, resuspended in Flow Cytometry Staining (FACS) Buffer (R&D), and transferred to V-bottom plates. Cells were then centrifuged at 1500 rpm for 3 min and washed with cold PBS (Thermo Fisher), followed by LiveDead Fixable Blue Dead Cell staining (Thermo Fisher) according to the manufacturer's protocol (diluted 1:500 in PBS, 50 µl per well, incubation 20 min at 4°C). After washing with FACS Buffer, cells were resuspended in 50 µl of anti-mouse CD16/32 antibody solution (Biolegend, cat. 101302) diluted 1:100 in FACS Buffer and incubated 15 min at 4°C for Fc receptor blocking. Cells were then centrifuged at 1500 rpm for 3 min, supernatant discarded, and cells were incubated with the antibody mix for surface marker staining (50 µl per well, incubated 30 min at 4°C). The antibody mix was composed of PE-CD40 (Biolegend, cat. 124610) and BV711-CD83 (BD Reagents, cat. 563136) diluted 1:200 in FACS Buffer. After washing with FACS Buffer, cells were fixed with 4% paraformaldehyde in PBS (Alfa Aesar) and stored at 4°C. Cells were analyzed in LSRII SORP flow cytometer (BD), and data processing was done using FACS Diva (BD) and FlowJo (Tree Star). MFI values were reported normalized to the corresponding controls, as reported in each figure, to compare biological replicates.

ELISA. Cells were seeded on a tissue-culture-treated 96 well plate at 30'000 cells per well and incubated overnight at 37°C with 5% CO_2 and 95% humidity. The same Cy5-labelled DNA

origami samples used for flow cytometry were tested at 0.5 nM concentration, diluted in DMEM+ immediately before addition to the cells (50 µl per well) and incubated for 5 h at 37°C. Similarly, free CpG (CpG-ODN 1826, IDT) was diluted at the different concentrations in DMEM+ immediately before addition to the cells (50 µl per well) and incubated for 5 h at 37°C. At the end of the incubation, cell culture supernatant was collected and stored at -80°C until the analysis. IL-6 was measured and quantified with a standard curve using the commercial kit DuoSet ELISA mouse IL-6 (R&D, cat. DY406) according to manufacturer's instruction. IL-6 values of the control were subtracted, and data reported as increase in pg/ml to compare biological replicates.

Statistical analysis. Results are presented as mean values ± standard deviation of three independent experiments, with three technical triplicates each. Statistical analyses were determined by the one-way ANOVA test or two-tailed Student's t-test, using GraphPad Prism software (*$p \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

SUPPLEMENTARY TEXT

To exclude artifacts, the flow cytometry markers were quantified in the live cell population, gated using a LiveDead stain. Additionally, for disk samples, markers were analyzed in a restricted population of Cy5-positive live cells ("Cy5 gate") to ensure we measured the activation level among samples in cells with a comparable number of disks per cell (gating strategy reported in Figure S3). To further confirm that differential activation was not induced by variance in uptake of different types of CpG-disks, we measured the median fluorescence intensity (MFI) of cells incubated with the Cy5-labelled CpG-disks and we did not observe significant differences in uptake levels among samples (Figure S4). While we did not observe any significant activation induced by the control DNA disk, we further excluded any possible background signal by normalizing CpG-disks data to cells incubated with empty disk. Moreover, APCs can easily be activated by foreign materials regardless of the TLR9 pathway. Therefore, we confirmed the biocompatibility of all DNA structures to exclude the observation of activation differences as a result of different toxicity levels among samples (Figure S5).

Similarly, to confirm that differential activation was not induced by uneven uptake among samples with different linkers (Figure 4), we ensured that no significant differences in the Cy5 MFI of cells incubated with CpG-disks were observed (Figure S4) and the previously reported gating strategy was applied (Figure S3).

SUPPLEMENTARY FIGURES

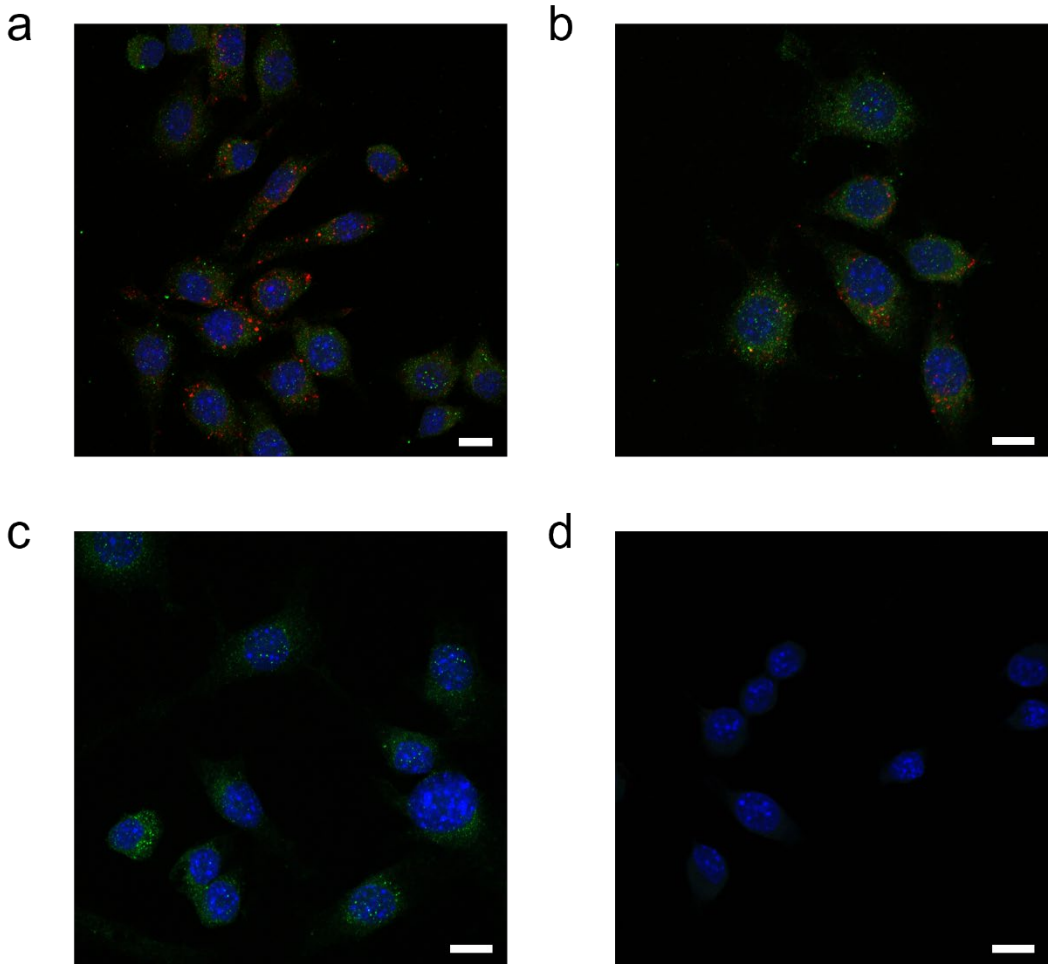

Figure S1. Supplementary confocal microscopy images of the uptake of Cy5-labelled disks by RAW 264.7 cell line. Nuclei stained with DAPI (blue), late endosome marker (RAB7) in green and Cy5-disks in red. Cells were treated with DNase I after uptake to remove DNA structures binding on cell surface and not taken up. Large (a) and small (b) overviews, endosome staining control (c), secondary antibody only control (d). Scale bars: 10 µm.

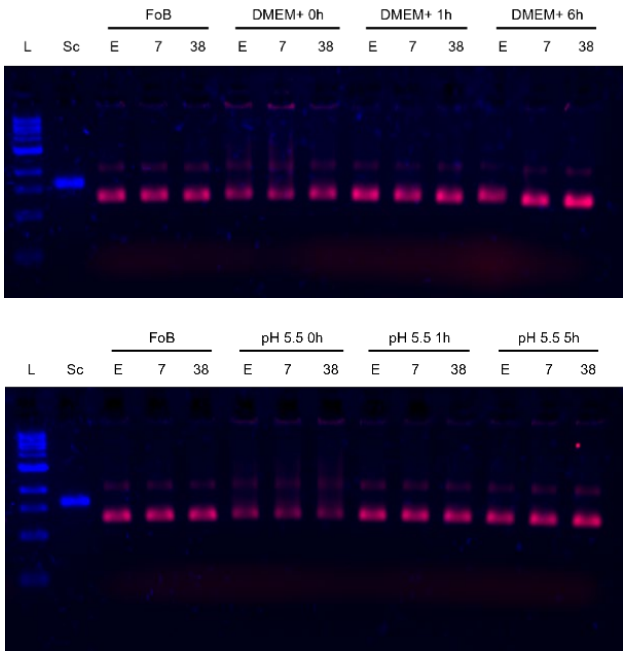

Figure S2. Stability assays by gel electrophoresis of Cy5-labelled DNA origami disks in DMEM+ and pH 5.5. SybrSafe signal in blue and Cy5 signal in red. Details of the protocol are reported in Materials and Methods. L: ladder 1kB, Sc: scaffold p7560; FoB: folding buffer; E: empty disk; 7: disk with CpG-ODNs at 7 nm spacing; 38: disk with CpG-ODNs at 38 nm spacing.

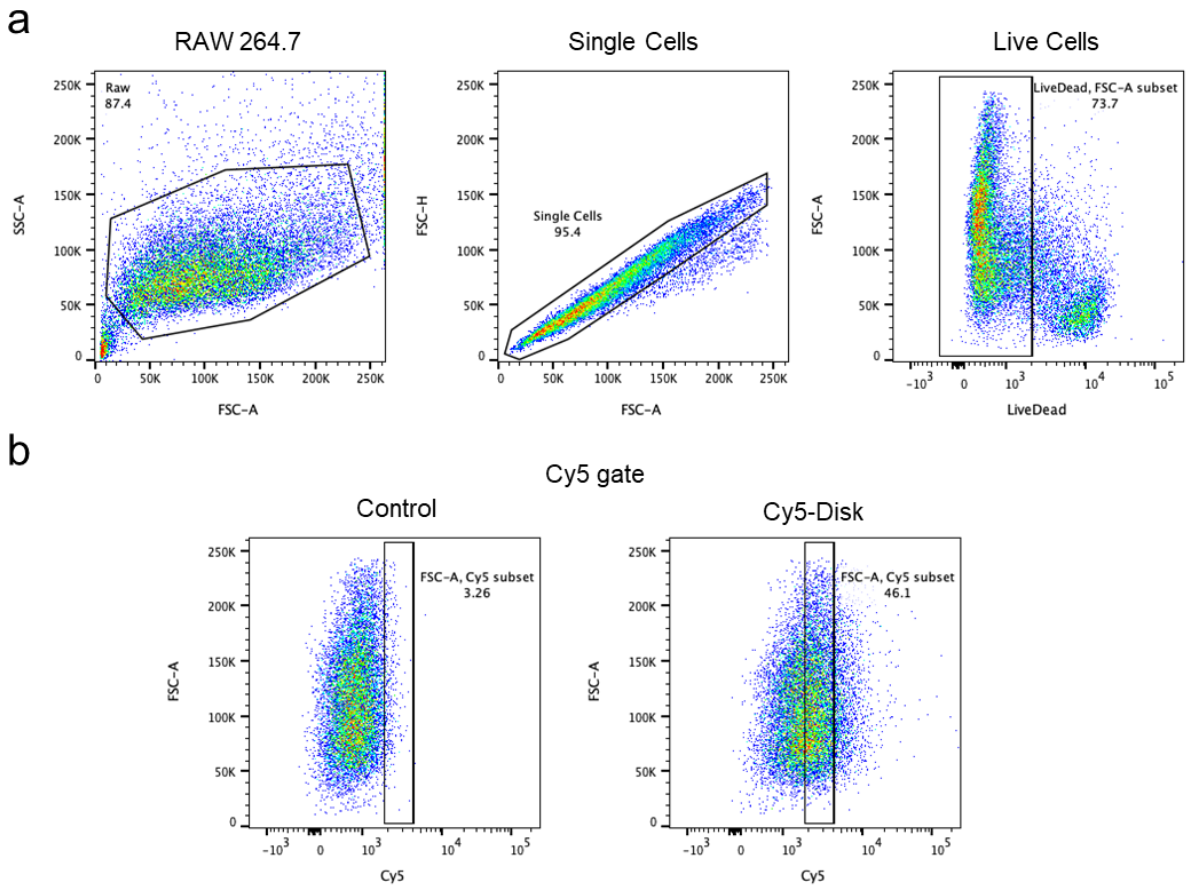

Figure S3. Gating strategy for flow cytometry activation assays. RAW 264.7 cells and single cells were gated at first. Subsequently, live cells were gated as negative for LiveDead staining. MFI values were calculated for this population when CD83 or CD40 is indicated. To normalize samples with DNA origami disk, MFI values were calculated in a population gated as represented here, to compare only cells with an intermediate restrict similar range of number of disks per cell. In this case, CD83 or CD40 in Cy5 gate is indicated.

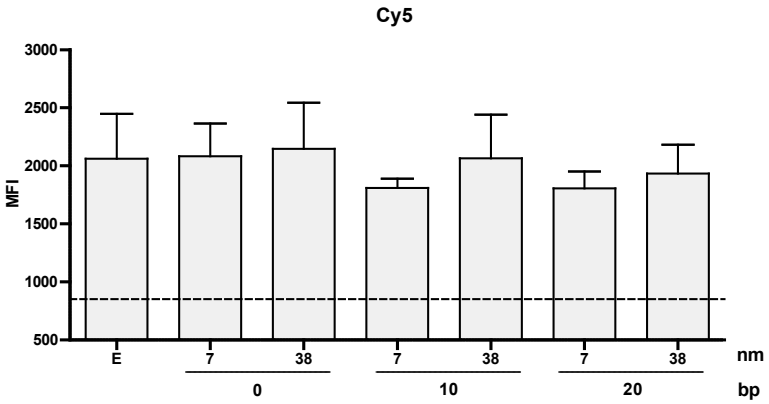

Figure S4. By flow cytometry, Cy5 MFI values for cells in the Live gate were measured to ensure no significant differences in uptake among disk samples. The dotted line indicates the background Cy5 level for unstained cells. Data represent the average and the standard deviation of the three biological replicates. Empty: empty disk; 7: disk with CpG-ODNs at 7 nm spacing; 38: disk with CpG-ODNs at 38 nm spacing; 0, 10 or 20 base pairs (bp) linkers.

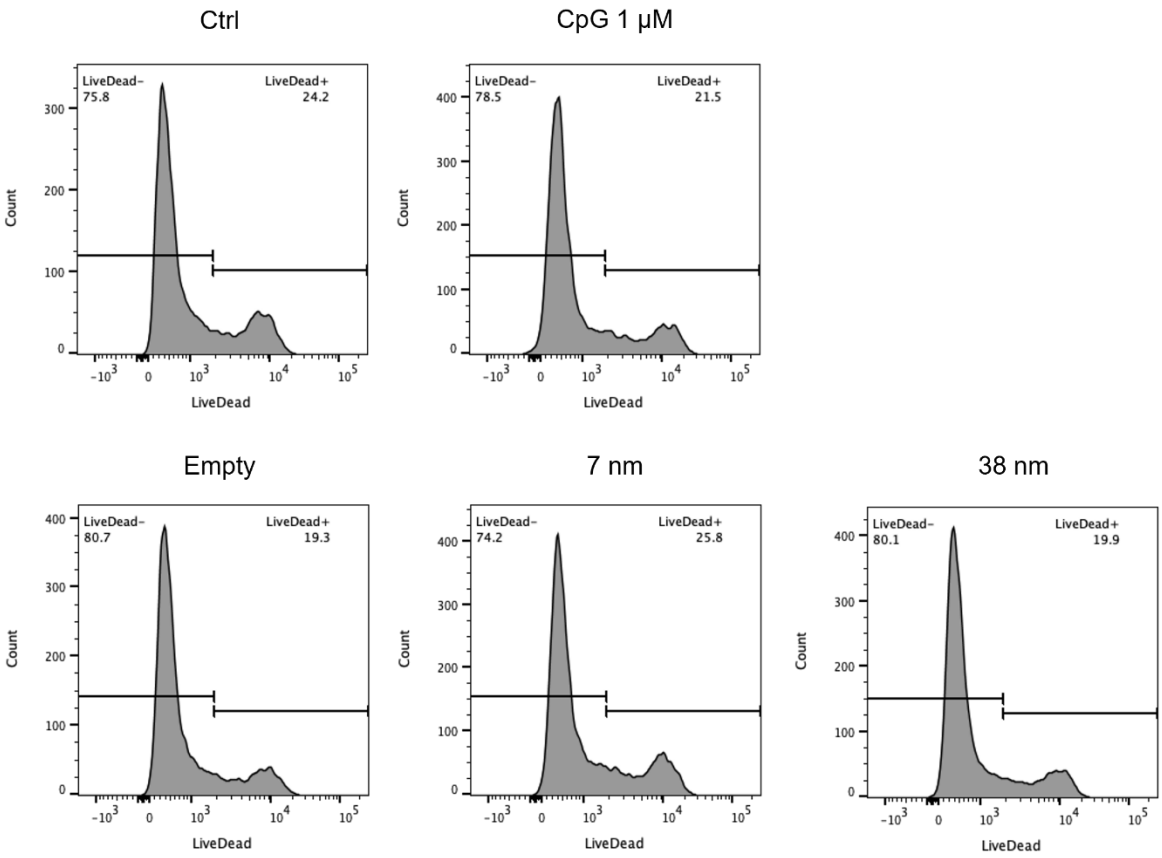

Figure S5. Histograms for representative samples of the LiveDead staining measured by flow cytometry. Percentages of negative and positive cells for LiveDead are reported in each plot. No significant differences in cell viability were observed among samples compared to the control. Empty: empty disk; 7 nm: disk with CpG-ODNs at 7 nm spacing; 38 nm: disk with CpG-ODNs at 38 nm spacing.

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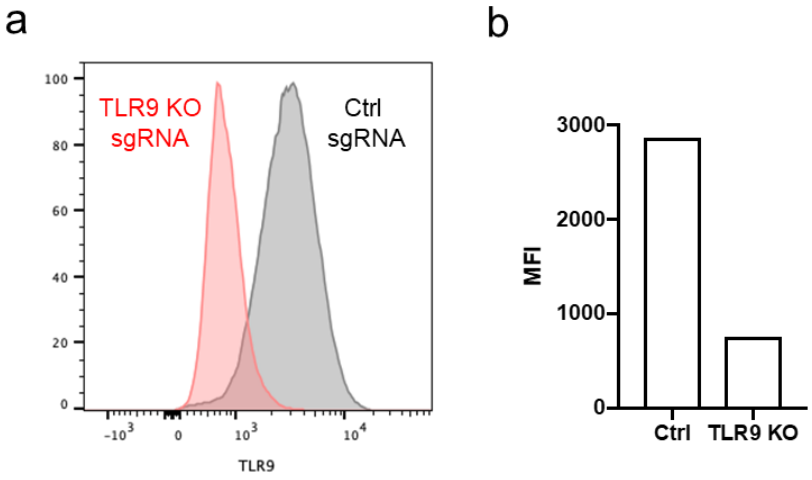

Figure S6. Characterization of TLR-9 knock-out (KO) RAW 264.7. Histograms (a) and MFI values (b) of TLR9 expression measured by flow cytometry. Details in Materials and Methods section.

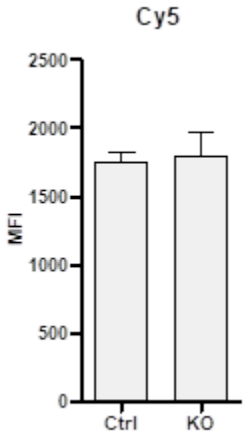

Figure S7. Quantitative analysis of uptake of Cy5-labelled disks (CpG at 7 nm) in standard (Ctrl) and TLR9-knock out (KO) RAW 264.7 cells. Cy5 MFI values for cells in the Live gate were measured by flow cytometry. Data represent the average and the standard deviation of the three biological replicates.

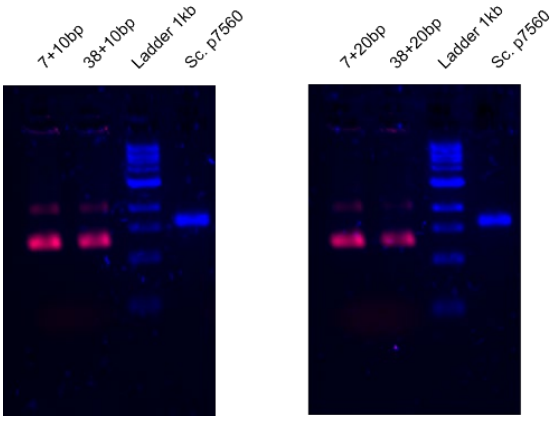

Figure S8. Analysis by agarose gel electrophoresis of purified Cy5-labelled DNA origami disks in folding buffer. SybrSafe signal in blue and Cy5 signal in red. 7+10bp(or 20bp): disk with CpG-ODNs at 7 nm spacing and 10(or 20) base pairs linker; 38+10bp(or 20bp): disk with CpG-ODNs at 38 nm spacing and 10(or 20) base pairs linker.

SUPPLEMENTARY TABLES

Table S1. Scaffold p7560 sequence.

Sequence of scaffold p7560
AGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCA
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TGTTGCAATGGCTGGCGGTAATATTGTTCTGGATATTACCAGCAAGGCCGATAGTTTGAGTTCTTCTACTCAGGC
AAGTGATGTTATTACTAATCAAAGAAGTATTGCTACAACGGTTAATTTGCGTGATGGACAGACTCTTTTACTCGG
TGGCCTCACTGATTATAAAAACACTTCTCAGGATTCTGGCGTACCGTTCCTGTCTAAAATCCCTTTAATCGGCCT
CCTGTTTAGCTCCCGCTCTGATTCTAACGAGGAAAGCACGTTATACGTGCTCGTCAAAGCAACCATAGTACGCG
CCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCT
AGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGG
GGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTTGGGTGATGGTTCA
CGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTC
TTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCG
GAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGC
CAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGC
AAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGG
CAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGC
TCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCG
AGCTCGGTACCCGGGGATCCTCCGTCTTTATCGAGGTAACAAGCACCACGTAGCTTAAGCCCTGTTTACTCATTA
CACCAACCAGGAGGTCAGAGTTCGGAGAAATGATTTATGTGAAATGCGTCAGCCGATTCAAGGCCCCTATATTC
GTGCCCACCGACGAGTTGCTTACAGATGGCAGGGCCGCACTGTCGGTATCATAGAGTCACTCCAGGGCGAGCGT
AAATAGATTAGAAGCGGGGTTATTTTGGCGGGACATTGTCATAAGGTTGACAATTCAGCACTAAGGACACTTAA
GTCGTGCGCATGAATTCACAACCACTTAGAAGAACATCCACCCTGGCTTCTCCTGAGAA

Table S2. Core folding staples. Cy5-functionalized sequences are reported in red. Sequence names correspond to the position of the staple in the caDNAno file.

Name	*Sequence*
13[140]46[140]	ACAATGTCTTCTAAATTACGCGCGACCT
17[56]14[66]	CCCGAACGTTATCATTGAGGAATATCAAGCAA
53[44]8[41]	AAAATCAGTAGCCCATCACGCAAAAAA
48[118]15[118]	GAAACGCAAAAGAACGACTTAAAGGAAT

15[161]44[161]	GGGTTTTCTCTTCGTGAAAGAAGAGTAA
15[88]48[77]	GTCCGGTCAGAAAATACAATAGAACCCTTCTTCGGAACTCACAAT
36[107]23[118]	ATTATTAAGAATAAATCAAATAATATCCCGCTAATCGTATCAATA
5[130]56[119]	TGTCGCTGGCAAGTGTAATAGCCCCTCAGAG
11[182]49[196]	CCCCGGGGTGACTCCCAACCTAAAACAAA
36[118]27[118]	GCCAACAACGCTCAGTCTGAGGCTAAAT
18[107]14[108]	CACGATCGCACACTAACCAGTTGAAGAG
44[76]49[66]	CACCCAATCCAAATAGCGCATAATAATACAAAGTTAAATTCAAGCG
28[151]22[150]	AAATATAATACTAAGCCTTCCTCATAGTAAAGATAATGCCGAGA
60[114]1[119]	GATGATACAGGTGCCTTGAGTAACACCCAAATCCAACGT
17[140]42[140]	GGTGCCGCGTGGGATATGCGAAAGATTC
18[128]44[119]	ATTCCGGCACGTTCTAAAATATCTGGCGCAGACGTAAC
1[120]58[119]	CAAAGGCTATTAATCTGAAAGGGTTTT
9[119]5[129]	TTGCTGGTAATAACCCCGCTTGGGCGCCCAGCAAGATCC
11[67]6[51]	GGGTTTGACGTTCTTTGGTAAAAGCGCCAGAAAAGGGATTTTAGACAAAA
23[77]36[55]	TTCATTTAACCTTGCAGAAGCCAGTAATAAGAGAATATAAAGTACCGAAA
48[170]53[186]	AAACCACTACTCTTAAAAGCCTTTTCCAGACTAACGATCTAAAGTTTAAA
48[160]52[150]	CTAACGTAGAAAATAAACGGGTAGTTGCCAAAAAATTTC
27[140]33[154]	GACCCTGGTAGTAGTTGATTCCCAATAAA
15[30]44[22]	AAAAATCTAAAGAAACAATTACAGAGCGTCAAAAATGAAAATAAAA
52[97]55[87]	AATAGCAAGGCCGGGACTGTAACCGCCTAGCC
39[130]21[139]	AAGACCAAAAACGCGAGGCGTTCAATCAAAATATAAGC
29[72]28[72]	AAAGCGAGAAAACTTTTTCAAGCAAGACAAAGAACAAA
39[44]22[41]	AAATGTTCAGCTGATGAAACAAACAAA
46[97]17[97]	GATAACCCACCCTGAGATTAGATCGGCC
21[140]38[140]	AAATATTTGGAGCAAGAGGGGAAATGCT
25[140]34[140]	TTAGAACTATTTCATAATTGCCTTAGAG
46[139]17[139]	GCTCCATATCATAAGGGGGATCGCTTCT
5[98]2[105]	TAACGTGCTTTCGCCGCTGCGAAGCGAAGGAGGGA
21[56]38[44]	GAATACCAGAAGATAATGCAGACGACAATAAACAACAAAA
46[128]50[129]	CTTGATTAAGGGCAACATTTCATGCAAC
43[46]46[45]	GTTTGTTTAAAGAATAATGAA
42[65]19[76]	ACACATATTATTTATCCTGCATCTCATATTC
55[54]54[54]	AAAAGCCACGAACCAAAA
40[160]23[160]	AACCCTCTAAAATGATTGCCTGGAGAGG
42[197]43[197]	AAAATCTACAAAAAAAAA
23[161]37[175]	GTAGCTATGAGTAAGAACCAGACCGGAAA
6[172]55[175]	AAAGCTGATTGCTACCGTAACACTAAA
9[140]50[140]	CACAACAAAATTGTAAATCTCGCCGACA
11[77]8[84]	GGCCAACAGAGAATCGCTCATCACTTGCTGTAATC

46[44]13[55]	ATAAAAAGTAGCCAAAGGGAAGGTAAATAACGTGGCACATGCGCG
55[88]5[97]	GCCTCAGACGTCATTAAAGCCACCACCACACGCACGTA
0[140]60[115]	AAACTATCATACGTGAACCATCAGTGCCCGAACCTATTTT
14[65]11[66]	CAGAGCCCTAAAGCGTAAAAA
16[45]12[45]	TTACCATCACCCCAGCAGGTCTTTAAGAC
40[55]43[45]	AGAACGGGTATTAACCGTTTTCTTACCAGCCA
30[137]27[129]	AAAATAACCTGTAAAGGTGATCCAATACCA
34[165]38[150]	AAATCATTTTTGCGGATGGTCCTTTTAGGATTATCGAGCTCCCC
49[109]52[108]	TTGCGATATATAAAGGACGGA
9[98]50[98]	GAAGAACTGCAACAGAACAACTTCGGTC
38[139]25[139]	TTAAACATATCGCGGGGTGAGAAAATTT
58[128]61[144]	GCGCATGAAATATTTCGGTATAAACAGTTAATGAAA
18[86]15[87]	ATCCGACAGTAGCCGTCTCTG
17[182]42[178]	CGCAACTATCAACATGGGAAGGTTAATAAAAC
40[118]45[108]	AGATCCTAATTTACGGCTTATGTTTTGAGGTTTAACTCATTCTCAG
10[193]51[196]	AAACCGAGCTCGTCGGTTTATCAGAAA
19[172]14[171]	GCCTGAGCGACGCCATTTGCGGGCCCCAGTCTTTC
36[139]39[129]	CCAATCGCCATATTCTTCAAAGTTCAGAAAAG
46[196]17[181]	AAAGTACAACGGAGATTTGTATTGAACGGCGATCGGCAGGCTG
25[77]34[65]	AAAACATTTGGGTTTATGCGTACTAGAAAAAGCCTGTAAA
56[165]7[160]	AAACGCCACCCTACCCATGCCTTCACAGTGAGA
54[139]58[129]	TAGCCATCTTTTCAATTTTCACGCCACCGGAATAGATTA
21[98]38[98]	TGATAATGCATGTCACAAGAAAAAAATC
50[97]13[97]	GCTGAGGGCAACGGTTCACATCGAACGA
38[86]44[87]	CTGTAGATAACAATCAAGCAAGCAATTAGTTGTAGTAAGCCC
24[172]19[171]	AAACCTTTTTGAAAGGCTATTGTTAATTTTTTATCTG
52[118]11[118]	GTTTCAGATTGCGATTACCGCCCTGGTT
10[149]3[151]	GTGTACGAGCTTAATTGTTTCACCCGCCTGGGAAATCGTTATAAATGAAA
15[77]44[77]	ATCAATAAATAGATGAGAATTCGAGAAA
23[41]24[51]	AAAATCAAGAAAACAAAATTAACGCTATTAAAAA
46[118]49[108]	ACTGAGCGCTAATAAATACCCAAAGACAGGCT
43[151]47[151]	AAGAAGAACCGAACTGAGAAATCCAGTAT
50[196]13[181]	AAACTTGCTTTCGAGGTGAATTGAAGGCATATGATAATTTACG
8[107]54[98]	CGTGGGAGAGTTCTCGTTAGAATCCCACCACGAGCCAC
38[186]42[171]	AAACTGCGGAATCGTCATATGGATAGAGCAACATAATGCACTAA
50[128]9[139]	CATTTTTTTCAACAACTTTATTAGCGTTTGCCTCACTGAATTCCA
1[83]0[104]	AAATAAAGCACTAAATCGGAACCCTAAACTCAAGTTT
11[161]48[161]	ACCTCGATGCGGCCCGTAATGCACTCAT
36[175]35[165]	AAAAAGCAAACTCCAACAGGTCGATAAGAGGAAA
10[58]52[44]	ATCGAATTAGGGGATCGTCTTGACGGAAATTCTTGAGCTAAAA

15[151]10[150]	GGTGGATGTTCCCGCCACTGTAAGACGTGGTCTGT
2[151]59[154]	AAATTCCAGTTTGAGGCTGAGACTAAA
47[152]15[160]	GTTAGCATTCCAGGGTAACGCCA
33[76]29[104]	AAAAAATAAGAATAAACACCTGCAAAATTAAATATATTTTAGT
44[118]19[118]	AAAGCTGTTTCAACATGGGATCCTGATT
27[98]32[76]	CCTCAGAGAATTAGCAACTAAACCGACCGTGTGATAAATAAGGCGTTAAA
42[55]21[55]	CCTGAATTATTTTCACCTTTTTTGCTTT
5[140]54[140]	TGGTTCCCCCTGAGCAAGCCCACGCCTG
8[183]9[183]	AAAAGTGCCCTGGGAAAA
25[98]34[98]	TTAAGACCTTTTTACCAGTATTTTTAAA
23[98]18[108]	AATGGAAACAGTCCAAAACTACAGAAAAATAAAGAATATAATAGGT
11[119]48[119]	GGTGTAATATAGGGAAGACTTTATAAAA
19[182]41[196]	AGCCAGCGTTAAATAACGCCAAAAGGAAA
44[160]19[160]	TCTTGACAACTGGCCCCGTCGAAAAATA
42[170]48[171]	CGGACCAGTCTTCATCAGGACAGACATCGCCAGCGATTACTA
44[86]51[87]	TGAAACTGAACACAAGAGAGGAAATATTTTGGAGGGTACTTGCAGCACC
22[183]23[183]	AAAACTACAGAGATAAAA
32[154]31[137]	AAATCTGCGAACGAGTAGATTTCAAAA
27[119]32[111]	CGGTTGTAAATCATAAGTTTCCCATTAGATATGGTT
3[72]56[77]	AAACGGCGAACGGCTTAATTAAATCCATTGGCC
56[76]7[76]	TTGATATGCCACCAGCCGATTATCCTGA
46[55]17[55]	GAAACAACATAAAAGTATTAGTCCTTTG
7[77]5[87]	GAAGTGTTTTTAAAGAGCTAATTGA
27[62]24[87]	AAAGTAAATGCTGATGCAAATCCAATCGCGCTTAGGAGCGATAAGTG
42[177]21[193]	GAAGATACATCAGCTCAAATTCGCATTAAATTTAAA
38[97]25[97]	AGGTCTTATTGCATATATGTGGCTTAGA
52[107]7[118]	GTGGCATTTTGGAACCACAGAGCCGCCGCACCGCTGGTGCGGTTT
19[30]40[22]	AAAGGAACAAAGGAGAAACAAGCAAGACCAAGTACCGCACTCAAAA
5[88]2[72]	CGACCGCCGCTGGCGAGGCTTGACGGGGAAAGCAAA
21[30]16[46]	AAATAACGGATTCGCCTGAACATCGGAAACCACAACATTATATTAAAACT
20[66]42[56]	ACAGTTATCATGCTAATTTTAAAAGAAACAGCATTTTAT
14[107]46[98]	ATAAAACAGAATAACGGTCAGAGA
8[83]52[77]	AGTGAGGAGCGTCAAAACGTC
19[161]40[161]	ATTCGCGACCAATAATTCAACCTATCAT
58[107]3[118]	GGCCATACATGGCTTATAGAACGTAGGAGCG
19[77]40[77]	CTGATTATAACGTCCCCAATATAATCGG
29[105]26[108]	TAATTTTAGGCAAAGCATAAAAGAC
7[119]52[119]	GCGTATTTCCAGTCAGCCCCCTTCAACA
21[77]18[87]	CGCAGAGGCGAATATCAGGTTTCAGATGGCGC
15[182]45[196]	TGTAAAAGGAAGGGTGTACAGACCAGAAA

27[130]34[119]	AAAGATCAAAATCATAGACAGTAGATAATGC
52[149]57[165]	TGTAGACAGCAACTACAAATAGGACAGAACCTCAGGAGGTTTAGTACAAA
52[76]11[76]	ACCAATGAAAATCACCTACATACATTCT
7[161]52[161]	CGGGCAATGAGCTAGTTAGCGGTTAGTA
15[119]12[129]	TGAGGAAGGTTAGCTGAATTCTTGTCAAGCAC
58[97]60[86]	TGGAATGGAAAGCGATTTACCTAAAA
54[97]9[97]	CACCGGAGCGCGTTGCATTAACTGAGTA
40[196]39[186]	AAAAATTACGAGGCATAGTAAGCGTCCAATAAAA
4[162]5[162]	AAAAATCCCGCAAAAAAA
38[149]43[150]	CTCGTAATAGGTTTACCTTTAGGAAGGTAGATTTT
58[154]5[139]	AAACCTCAAGAGAAGGATTAGGGTGTATCAATAGCCTTGATGG
29[120]33[129]	AGCTGATTAGCTAATTTCGCAAATGGTAGTTTGAATTC
5[62]6[81]	AAAGGCGCGTACTATGGTTGCTACA
57[65]4[62]	AAAACAAACAAAGCGCCGCTACAGAAA
56[118]58[108]	CCCAGCATTGACAGGGGTTGAACCA
19[119]40[119]	GTTTGGACACGTAATCTAAGATAGCGAG
14[170]7[172]	TCACTAATCTCCGACAGTAAAGACAATCATGGTAAAGCTAATGAGCAAAA
34[118]29[119]	TGTAGCTTGTCTGGACAGGCAGGGGCGCG
25[51]20[67]	AAATTAATTTTCCCTTAGAAAATCGTTTACATTGAGCAAAAAGTTACTAT
34[97]38[87]	TAGGAATCATAATTTATACAATTCGAGCAAAGCGGTACC
13[182]47[196]	CTCGCCCGGCCAGTGCGCGAAACAAAAAA
2[104]58[98]	GCCCCCGTAAGCGTGGATAAG
9[41]10[59]	AAATTAACCGTTGTAGCAATACCTCA
32[110]36[108]	TGAAATAGTACGGCAACATGAAAGCCATGTA
34[139]29[151]	CTTAATTATAACAGCATTAACGCATCAATTCTACTAAAAA
38[76]42[66]	TATCTGTCCAGACGAACGCGCTCCTTATGAATCATAGCT
13[56]46[56]	AACTGATTGCCACGAGCCGAAAGAGCAA
42[97]21[97]	AATCAAGAATCAGATAGATTTCCCCGGT
61[86]0[83]	AAAAGTTTTAACGTCGAGGTGCCGAAA
19[140]15[150]	AATGGAAGGGTTTCGATTCTCGAAACCAGCCAGCTGTTG
49[67]56[65]	AAACTTTTGCAGCCAGCAAACCATTCAAGTTACCCTCACCTCAGATCAAA
26[162]27[162]	AAAAGGGAGTTTGCAAAA
0[103]60[100]	TTTGGGGGGGTCAGAGTG
23[119]36[119]	TGATATTGGAGACACGAAAGATAACAAC
40[76]23[76]	CTGTCTTCTGTTTAATTACCTTAACAAT
48[76]15[76]	CAATAGAACCAGAAACCGCCTACCCTCA
31[100]30[100]	AAACTGACCTAAATTTAACTATTTTCATTCATCTTAAA
35[65]26[62]	AAATTAGTATCAATATAACTATATAAA
50[139]13[139]	ATGACAAAGGAAGTTCGGTGGCCTTATG
22[128]18[129]	CGAAAGATTGTTATTTGTTATACTGCGG

42[139]46[129]	ATTTAGCGAACCTCATTGTGACAAATCAACGGTCAGTTA
52[160]11[160]	AATGAATAAGGCTCCTGTTTCGCTTGTT
11[30]48[22]	AAAGGCAGATTCATTTTTGGAGGGAGACAAAAGGGCGACATTAAAA
59[75]58[75]	AAAATCTGACAGTCAAAA
42[160]68[149]	TTATTACATACCACGGAACGCTAAACGT
46[76]72[65]	TAAGCCCTAGACGGAATACATGTTTGAG
52[186]78[171]	AAATGTCGTCTTAATTGTAAATTCGTGGAGGAT
50[160]76[149]	ATACCGATAAAATACTGCCATAAATAAC
60[99]86[87]	TACTGGTAAGTTCCAGATTTAGAAAAGGAA
54[160]80[149]	CAGTACACCTCATAACTCACACGGAAGC
44[97]70[87]	AAGGCTTATTGGGCTAGATGGATGGCAA
50[118]76[108]	GCATAACAGGACTAGCCTTGATCCTTAG
51[88]78[87]	ATTACCATAGGGAAAAACATTTCT
56[97]82[87]	AGGCAGGACCAGAAAGAGCGGTCGGCCA
33[130]62[129]	CATGCTGAATGGCTTAATTGAGTTACGCAAGACATTAT
45[109]72[108]	AGGGTAATGATTAGGAGCTCCAGC
38[118]64[108]	AATGACCGGAAGCCGTCAAATAGAGTCA
54[118]80[107]	AATCACCCGGTCATGGGAAACATCGGCC
52[55]78[44]	AGCACCGCATTTGGGTCTGAAACACGAC
48[97]74[87]	ATAAGTTCGCAATAGGTGAGGAGTTGGC
52[139]78[128]	TTTGCTAACGTTGATATCCGCACAGGGC
44[181]70[171]	GCTGACCAGGACGTTTAAATGTTCCTGT
60[144]86[127]	AAACCCCCTGCCGTATTAAGGAACAAATAGGGT
36[97]62[87]	AGGCATTATTCTTAACCTCCGAATAAAG
38[160]64[150]	ATTGAATTCAAAGCTGTGTAGTATTTTA
44[55]70[44]	GATTTTTACAAAATTTTGAGTCAGAAGG
54[76]80[65]	AGCCGCCTGCCTTTCCACCGAATTAGTA
48[139]74[128]	TAAAGGTACTCCTTGTGGTTGTGCAAGG
46[160]72[149]	TTGTGTCCCAACTTCTATTACGGCAAAG
58[118]84[107]	GCTCAGTTATAAGTGCGGTCACCCAGCA
48[55]74[44]	TACCAGCAGCAGATCTGAGAGTTGCTGA
42[76]68[65]	TGCACCCTACCGCGAGATGAAAAAATCG
37[55]64[65]	AAAACAAAAGGTAAAGTAATGTCTTCTGTATCCTTG
44[139]70[129]	CATTACCATTACCTACAAACGTCTGAAT
40[139]66[129]	GATAAAATTTTGCCAACAAGATCTAGCT
56[139]82[128]	TCAGAACGGGATAGAGAGTTGAGGGTGG
40[97]66[87]	TAGAAACGTCCTGAAATCATACTTTTTT
50[76]76[65]	AAGGCCGGACAGCAGACCTGAAAACATC
42[118]68[107]	GCGGGAGCCGGTATAACAGAAGCCCCAA
48[181]74[171]	AGAATACATACCAAGCCAAGCACGACGT

17[30]71[46]	AAAGACAACTCGTCATTTTCTAATTTACGCTAA
13[30]75[46]	AAATGGCTATTACAAATGATTTTAAGGCAATAG
9[77]79[87]	ATAACATGGAAATACCAGTAGGGAGTTA
15[140]73[150]	CGATTAAGGCGAAAGGGAACCGGATATT
13[119]75[130]	TGCTGAAATGCGCACTGGCATAGCCGGA
12[44]79[46]	AATACCAGTCATGGATTATTTACATCACCGAATTCATT
48[196]77[193]	AAAGAAAGAGGCAAA
54[175]83[172]	AAAGAGTTTCGTCAC
12[128]79[130]	GAATGAGTAATCTCCAGAACAATAATAATAACGCCCAC
7[98]81[109]	ACGCGCGGCCAGCTTTCATCGAGAATAG
22[149]69[150]	GTCTAAATTGCAAGAACCTACCATGTTGAGAAGACGAC
3[98]85[109]	GGGAAGACGTAACCGTCGAGAGAGGTTG
3[140]85[151]	TGAGTGTTCAAAAGACCGTACGCCACCC
15[98]73[109]	AAATCAAAACTAATAACAAAGAGTGAAT
15[56]73[66]	ACCTCAATTTAGAAACAGGGAAAGAAAC
44[196]73[193]	AAAGCGCATAGGCTG
13[77]75[87]	GCCATTATATTAACGGAAACCATTGAGT
26[107]65[109]	TACGCTGAGACAACATAAATCAATCAAAAAGTAGGCAG
23[140]65[151]	GATAAATTTCAAAATTTTAATGAGAGTA
6[80]83[87]	GGAGCCCTCAGCCCTCAG
19[56]69[66]	AGCGGAATAACAGTATCGTAGCATTCCA
13[161]75[171]	CCCGCTTGGAGAAGGACCCCCTGATAAA
9[161]79[171]	ATAAAGTGTCATAGCAAAAGGCAGCTTG
21[119]67[130]	AAACAGGTGAACGGTTTTGCAAAACGAG
24[86]67[87]	AATGAATTACTGTTATTCATTTCATCAACAAACTATTA
21[161]67[171]	TAATATTTCAGGTCTTTAGACAATATTC
11[56]77[67]	CAGTAATAGAATACCCTCAGCTATGGTT
7[51]81[68]	AAAGGAACGGTAAGTCTGTGACAGAACGATAGC
7[140]81[150]	TTTTTCTCGTTGCGATTCCACATGGGAT
19[98]69[109]	TTCATCAAATTGCGTATAGAAGAGCATG
17[77]71[87] + Cy5	GGGACGAGTAACCGAGAACGAGCTATTT/3Cy5Sp/
11[140]77[150] + Cy5	TTAAGCTCAACTCGTTCCATTACATACA/3Cy5Sp/
11[98]77[109] + Cy5	CCGAACTGACGCATCTACAGACCACGGA/3Cy5Sp/
5[119]83[130] + Cy5	GGCGAAACGGTCCACACCCTCTAATCAA/3Cy5Sp/
17[161]71[171] + Cy5	CGCCATTGTAACAATCATTATAACAACA/3Cy5Sp/
17[119]71[130] + Cy5	CAGCTTTGACCGTATTTAATCCCGACTT/3Cy5Sp/

Table S3. CpG-functionalized and extra folding staples. Sequence names correspond to the position of the staple in the caDNAno file. * indicates a phosphorothioate bond.

Note: in all cases, CpG-ODNs (with and without linkers) are directly conjugated to staples forming the DNA origami disk structures. They are therefore "integrated" in the structure core to avoid loss of active molecules. A double strand configuration for the linkers is then introduced by base-pairing with a complementary sequence (anti-linker) to a single strand linker among staple and CpG-ODN sequences.

Disk type	*Name*	*Sequence*
Empty	3[119]1[140]	GGCGCTAGGGCGAGGAGTCCAGCGAAAAACCGTAAA
Empty	25[119]22[129]	ATAGTGAATTTATAAAAGGCCCAACCGTGAAT
Empty	17[98]42[98]	TCAGGAAGTTGGTGTTGAGATAGCCTTA
Empty	13[98]8[108]	ACCACCAGCAGATGATCGGCTCTGACCTCAGCCATTCAAACTCTGT
7 nm CpG	3[119]1[140]	GGCGCTAGGGCGAGGAGTCCAGCGAAAAACCGTAAA
7 nm CpG	25[119]22[129]	ATAGTGAATTTATAAAAGGCCCAACCGTGAAT
7 nm CpG	CpG1826 + 13[98]8[108]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TACCACCAGCAGATGATCGGCTCTGACCTCAGCCATTCAAACTCTGT
7 nm CpG	CpG1826 + 17[98]42[98]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TTCAGGAAGTTGGTGTTGAGATAGCCTTA
38 nm CpG	17[98]42[98]	TCAGGAAGTTGGTGTTGAGATAGCCTTA
38 nm CpG	13[98]8[108]	ACCACCAGCAGATGATCGGCTCTGACCTCAGCCATTCAAACTCTGT
38 nm CpG	CpG1826 + 25[119]22[129]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TATAGTGAATTTATAAAAGGCCCAACCGTGAAT
38 nm CpG	CpG1826 + 3[119]1[140]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TGGCGCTAGGGCGAGGAGTCCAGCGAAAAACCGTAAA
7 nm CpG +10bp linker	3[119]1[140]	GGCGCTAGGGCGAGGAGTCCAGCGAAAAACCGTAAA
7 nm CpG +10bp linker	25[119]22[129]	ATAGTGAATTTATAAAAGGCCCAACCGTGAAT

7 nm CpG +10bp linker	CpG1826 + 10 LINKER + 13[98]8[108]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TCCATAGACTAACCACCAG CAGATGATCGGCTCTGACCTCAGCCATTCAAACTCTGT
7 nm CpG +10bp linker	CpG1826 + 10 LINKER + 17[98]42[98]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TCCATAGACTATCAGGAAG TTGGTGTTGAGATAGCCTTA
7 nm CpG +10bp linker	anti-linker10	TAGTCTATGG
38 nm CpG +10bp linker	17[98]42[98]	TCAGGAAGTTGGTGTTGAGATAGCCTTA
38 nm CpG +10bp linker	13[98]8[108]	ACCACCAGCAGATGATCGGCTCTGACCTCAGCCATTCAAACTCTGT
38 nm CpG +10bp linker	CpG1826 + 10 LINKER + 25[119]22[129]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TCCATAGACTAATAGTGAA TTTATAAAAGGCCCAACCGTGAAT
38 nm CpG +10bp linker	CpG1826 + 10 LINKER + 3[119]1[140]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TCCATAGACTAGGCGCTAG GGCGAGGAGTCCAGCGAAAAACCGTAAA
38 nm CpG +10bp linker	anti-linker10	TAGTCTATGG
7 nm CpG +20bp linker	3[119]1[140]	GGCGCTAGGGCGAGGAGTCCAGCGAAAAACCGTAAA
7 nm CpG +20bp linker	25[119]22[129]	ATAGTGAATTTATAAAAGGCCCAACCGTGAAT
7 nm CpG +20bp linker	CpG1826 + 20 LINKER + 13[98]8[108]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TATAGACTAGCAACTTTCA CCACCACCAGCAGATGATCGGCTCTGACCTCAGCCATTCAAACTCTGT
7 nm CpG +20bp linker	CpG1826 + 20 LINKER + 17[98]42[98]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TATAGACTAGCAACTTTCA CCTCAGGAAGTTGGTGTTGAGATAGCCTTA
7 nm CpG +20bp linker	anti-linker20	GGTGAAAGTTGCTAGTCTAT
38 nm CpG +20bp linker	17[98]42[98]	TCAGGAAGTTGGTGTTGAGATAGCCTTA
38 nm CpG +20bp linker	13[98]8[108]	ACCACCAGCAGATGATCGGCTCTGACCTCAGCCATTCAAACTCTGT
38 nm CpG +20bp linker	CpG1826 + 20 LINKER + 25[119]22[129]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TATAGACTAGCAACTTTCA CCATAGTGAATTTATAAAAGGCCCAACCGTGAAT
38 nm CpG +20bp linker	CpG1826 + 20 LINKER + 3[119]1[140]	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*TATAGACTAGCAACTTTCA CCGGCGCTAGGGCGAGGAGTCCAGCGAAAAACCGTAAA
38 nm CpG +20bp linker	anti-linker20	GGTGAAAGTTGCTAGTCTAT
free CpG	CpG 1826	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T

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