STRATEGIES FOR THE SITE-SPECIFIC DECORATION OF DNA ORIGAMI NANOSTRUCTURES WITH FUNCTIONALLY INTACT PROTEINS

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Fig. S1. DNA origami scaffold routing. The DNA origami rectangular tile (65x54 nm) was designed based on the M13mp18 scaffold\textsuperscript{1} using caDNAno.\textsuperscript{2} At the site chosen for ligand attachment, the staple strand was elongated at its 3’-end with 21 bases (green arrow). For attachment to the SLB via complementary cholesterol-oligonucleotides, staple strands were elongated at predefined positions at their 5’-end with 25 bases as indicated by the red arrows.
Fig. S2. Mobility of functionalized DNA origami structures on fluid-phase SLBs. Individual DNA origami structures were tracked on POPC SLBs at a frame rate of 100 Hz. Mean square displacements were determined and plotted as a function of time lags. Assuming free Brownian motion, the diffusion coefficient D was derived by fitting the first two data points with a linear fit. An exemplary plot for H57-dSAv is shown.
Fig. S3. Determining functionalization efficiencies for construct H57-dSAv. Single molecule two-color co-localization imaging was applied to determine the efficiency of each modification step. (A) First, we evaluated the availability of elongated staple strands (handles) for site-specific hybridization. For this purpose, we used fluorophore-conjugated handles (DNA-AS635P) and labeled the DNA origami structure with YOYO, a DNA-intercalating fluorophore. The percentage of co-localized signals in the blue (YOYO) and the red (DNA-AS635P) color channel yielded the handle incorporation efficiency. Representative TIRF images of DNA origami on an SLB are shown. Green open circles indicate signals detected in both color channels; red open circles indicate signals detected only in one channel. (B) Two-color colocalization of fluorescently labeled biotinylated oligo nucleotides (bt-DNA-AS635P) and YOYO yielded the efficiency of hybridization to the handle. (C) Binding of dSAv to the biotinylated handle was then determined via colocalization of fluorescently labeled biotinylated oligos (bt-DNA-AS635P) and dSAv (dSAv-AF555). (D) Finally, two-color colocalization of hybridized bt-DNA-AS635P and site-specifically biotinylated AF555-labeled H57-scFv yielded the overall functionalization efficiency of DNA origami with the TCR-ligand. The functionalization efficiency was then assessed as described in the methods section. Scale bar, 2 µm.
Fig. S4. Determining functionalization efficiencies for construct H57-dSAv-NL. (A) To determine the incorporation efficiency of the biotinylated handle, DNA origami structures featuring a fluorescently labeled, biotinylated handle (AF647-DNA-bt) were produced and unspecifically stained with the DNA-intercalating dye YOYO. Representative TIRF images of DNA origami on an SLB are shown. Green open circles indicate signals detected in both color channels; red open circles designate signals detected only in one channel. The percentage of co-localized signals in the blue (YOYO) and red (AF647-DNA-bt) color channel yielded the incorporation efficiency. (B) The binding efficiency of dSAv to the biotinylated handle was determined via two-color colocalization of fluorescently labeled dSAv (dSAv-AF555) and AF647-DNA-bt. (C) Two-color colocalization of AS635P-labeled H57-scFvS and YOYO-stained DNA origami structures yielded the overall functionalization efficiency of DNA origami with the TCR-ligand. Scale bar, 2 µm.
Fig. S5. Determining functionalization efficiencies for construct H57-DNA. (A) Handle availability was determined via two-color co-localization of DNA-AS635P and YOYO. Representative TIRF images of DNA origami on a SLB are shown. Green open circles indicate signals detected in both color channels; red open circles designate signals detected only in one channel. (B) Colocalization of AF555-labeled DNA-conjugated H57-scFv s (H57-DNA) and DNA-AS635P yielded the overall functionalization efficiency of DNA origami with ligand. Scale bar, 2 µm.
Fig. S6. Determining functionalization efficiencies for the construct H57-PNA. (A) Handle availability was determined via two-color co-localization of DNA-AS635P and YOYO. Exemplary TIRF images of DNA origami on a SLB are shown. Green open circles indicate signals detected in both color channels; red open circles designate signals detected only in one channel. (B) Colocalization of AF555-labeled PNA-conjugated H57-scFvs (H57-PNA) and DNA-AS635P yielded the overall functionalization efficiency of DNA origami with ligand. Scale bar, 2 µm.
Fig. S7. Determining functionalization efficiencies for the construct H57-mSAv. (A) Handle availability was determined by two-color co-localization of DNA-AS635P and YOYO. Exemplary TIRF images of DNA origami on a SLB are shown. Green open circles indicate signals detected in both color channels; red open circles indicate signals detected only in one channel. (B) Colocalization of AS635P-labeled DNA-coupled mSAv (mSAv-DNA-AS635P) and YOYO yielded functionalization efficiency with mSAv. (C) Finally, two-color colocalization of mSAv-DNA-AS635P and AF555-labeled biotinylated H57-scFv yielded the overall functionalization efficiency of DNA origami with ligand. Scale bar, 2 µm.
Fig. S8. Determining functionalization efficiencies for construct H57-tSAv. (A) Handle availability was determined by two-color co-localization of DNA-AS635P and YOYO. Exemplary TIRF images of DNA origami on a SLB are shown. Green open circles indicate signals detected in both color channels; red open circles indicate signals detected only in one channel. (B) Next, two-color colocalization of fluorescently labeled biotinylated oligos (bt-DNA-AS635P) with YOYO yielded the efficiency of hybridization of biotinylated oligo to the handle. (C) Colocalization of fluorescently labeled tSAv (tSAv-AS635P) and YOYO yielded functionalization efficiency with tSAv. (D) Finally, colocalization of hybridized biotin-DNA-AS635P and AF555-labeled H57-scF\textsubscript{v}s yielded the overall functionalization efficiency of DNA origami with ligand. Scale bar, 2 µm.
Fig. S9. Determining functionalization efficiencies for construct H57-tSAv-NL. (A) Incorporation of the biotinylated handle on DNA origami for functionalization was assessed via colocalization of a fluorescently labeled biotinylated oligo (AF647-DNA-bt) and YOYO. Exemplary TIRF images of DNA origami on a SLB are shown. Green open circles indicate signals detected in both color channels; red open circles indicate signals detected only in one channel. (B) The binding efficiency of tSAv to the biotinylated handle was determined via two-color colocalization of fluorescently labeled tSAv (tSAv-AS635P) and YOYO. (C) Last, two-color colocalization of tSAv-AS635P and AF555-labeled H57-scFv yielded the overall functionalization efficiency of DNA origami with ligand. Scale bar, 2 μm.
Fig. S10. Determining functionalization stoichiometries for H57-scFv-functionalized DNA origami constructs. Exemplary TIRF images and corresponding brightness distributions $\rho$ of DNA origami constructs functionalized with AF555-labeled H57-scFv on SLBs. The detected signals were fitted and deconvolved into monomer and multimer contributions$^3$ (see methods section). Scale bar, 2 µm.
Fig. S11. Axial dimensions at the T-cell – SLB interface. Schematic sketches of TCR engagement for the different ligand-functionalized DNA origami structures: H57-dSAv (A), H57-dSAv-NL (B), H57-DNA (C), H57-PNA (D), H57-mSAv (E), H57-tSAv (F), H57-tSAv-NL (G). Distances were estimated from the protein crystal structures\(^5\)–\(^8\) and are given in nm.

**Note on length estimates**

The two *trans* biotin binding sites in SAv are separated by \(\sim 3.5\) nm;\(^8\) hence the contribution to the total length of the construct was assumed with 4 nm (A, F). For mSAv (E), this contribution was estimated with 3 nm from the crystal structure.\(^7\)

The DNA linker consists of 17 paired bases in constructs A, C, E, F; with the following variations:

**A, F:** (constructs H57-dSAv and H57-tSAv): 4 unpaired Ts on the handle, 17 paired bases, PEG4 linker and biotin. (\(\sim 7\) nm)

**C, E:** (constructs H57-DNA, H57-mSAv)): 4 unpaired Ts on the handle, 17 paired bases and 4 unpaired Ts on the hybridized DNA oligo, PEG4 linker (\(\sim 8\) nm)

**D:** The DNA/PNA linker consists of 4 unpaired Ts on the handle, 17 paired bases, 2 unpaired Ts and an O-linker (\(\sim 6\) nm).

**B, G:** (constructs H57-dSAv-NL and H57-tSAv-NL): The biotin is attached to the handle *via* 2 unpaired Ts and a PEG4 linker.
Fig. S12. Calcium imaging experiments to assess the T-cell activation state. T-cells were loaded with the ratiometric Ca$^{2+}$-sensitive dye Fura-2 AM, seeded onto SLBs and fluorescence emission was recorded at excitation wavelengths 340 nm and 380 nm over 10 min. Activation was tracked via a change of the intensity ratio (340/380nm). Exemplary ratio images recorded at activating (ICAM-1 100 µm$^{-2}$, B7-1 100 µm$^{-2}$, pMHC 150 µm$^{-2}$, (A)) and non-activating (ICAM-1 100 µm$^{-2}$, B7-1 100 µm$^{-2}$, (B)) conditions at 37°C are shown 5 min after cell seeding. Scale bar, 4 µm. (C,D) For each cell, the intensity ratio 340/380nm was determined and plotted over time. Exemplary calcium traces for a T-cell under non-activating (C) and activating (D) conditions are shown. The threshold ratio for counting a cell as “activated” was set to 0.4 for all experiments, indicated by a red dashed line.
Fig. S13. Determination of the TCR labeling efficiency of the different H57-scFv variants. (A) TCRs on T-cells were stained with different AF555-conjugated H57-scFv variants (H57-DNA, H57-PNA, bt-H57, H57) and allowed to adhere to SLBs presenting 100 molecules per µm² ICAM-1 for imaging in TIRF microscopy mode. T-cells were labeled under saturating conditions for AF555-conjugated H57-scFv (20 µg ml⁻¹). The cell outline is indicated by a dashed white contour line. Images were recorded 5-10 min after cell seeding. Scale bar, 2 µm. (B) Surface densities of labeled TCRs were quantified (n ≥ 27 cells). Data are the mean of two independent experiments and two different mice (± s.e.m.).
Table S1. Diffusion coefficients of constructs on SLBs. Single-molecule trajectories of DNA origami structures functionalized with H57-scFv were recorded on SLBs, pooled and diffusion coefficients were determined by mean square displacement analysis. Diffusion coefficients are given as mean ± s.e.m. Data are from at least two independent experiments.

| Construct       | D (µm²/s)   | trajectories (n) |
|-----------------|-------------|-----------------|
| H57-dSAv        | 0.380 ± 0.013 | 8,203           |
| H57-dSAv-NL     | 0.381 ± 0.015 | 9,857           |
| H57-DNA         | 0.383 ± 0.012 | 7,601           |
| H57-PNA         | 0.421 ± 0.003 | 3,797           |
| H57-mSAv        | 0.385 ± 0.012 | 8,669           |
| H57-tSAv        | 0.355 ± 0.015 | 11,108          |
| H57-tSAv-NL     | 0.399 ± 0.001 | 5,699           |
| Construct          | Fluorophore ID | Handle incorporation | bt-DNA hybridization | SAv attachment | POI attachment |
|-------------------|----------------|----------------------|----------------------|---------------|---------------|
| 1: H57-dSAv       | DYE 1          | DNA origami (YOYO)   | DNA origami (YOYO)   | bt-DNA (AS635P) | bt-DNA (AS635P) |
|                   | DYE 2          | DNA (AS635P)         | bt-DNA (AS635P)      | dSAv (AF555)   | bt-H57 (AF555)  |
| 2: H57-dSAv-NL    | DYE 1          | DNA origami (YOYO)   | ---                  | DNA (AS635P)   | DNA origami (YOYO) |
|                   | DYE 2          | DNA-bt (AF647)       | ---                  | dSAv (AF555)   | bt-H57 (AS635P)  |
| 3: H57-DNA        | DYE 1          | DNA origami (YOYO)   | ---                  | ---           | DNA (AS635P)    |
|                   | DYE 2          | DNA (AS635P)         | ---                  | ---           | H57-DNA (AF555) |
| 4: H57-PNA        | DYE 1          | DNA origami (YOYO)   | ---                  | ---           | DNA (AS635P)    |
|                   | DYE 2          | DNA (AS635P)         | ---                  | ---           | H57-PNA (AF555) |
| 5: H57-mSAv       | DYE 1          | DNA origami (YOYO)   | ---                  | DNA origami (YOYO) | mSAv-DNA (AS635P) |
|                   | DYE 2          | DNA (AS635P)         | ---                  | mSAv-DNA (AS635P) | bt-H57 (AF555)  |
| 6: H57-tSAv       | DYE 1          | DNA origami (YOYO)   | DNA origami (YOYO)   | DNA origami (YOYO) | bt-DNA (AS635P)  |
|                   | DYE 2          | DNA (AS635P)         | bt-DNA (AS635P)      | tSAv (AS635P)  | bt-H57 (AF555)  |
| 7: H57-tSAv-NL    | DYE 1          | DNA origami (YOYO)   | ---                  | DNA origami (YOYO) | tSAv (AS635P)  |
|                   | DYE 2          | DNA-bt (AF647)       | ---                  | tSAv (AS635P)  | bt-H57 (AF555)  |
Table S3. Optimization of functionalization conditions for construct H57-dSAv. For each step, the yield of functionalized DNA origami construct was determined for different conditions (incubation times and molar ratios) via two-color colocalization microscopy as sketched in SI Fig. S3. Optimal conditions for each step are marked in red; these were then used as the basis for subsequent steps. Cumulative yields are shown. Data are the mean (± s.e.m.) of at least two independent experiments.

| Handle incorporation | Molar ratio DNA origami : handle oligo |
|----------------------|----------------------------------------|
|                      | 1 : 1        | 1 : 2        | 1 : 5        | 1 : 10       | 1 : 30       | 1 : 50       | 1 : 100      |
|                      | 19.1 ± 0.9   | 32.8 ± 0.8   | 46.6 ± 0.6   | 84.4 ± 2.1   | 83.4 ± 2.7   | 86.4 ± 0.3   | 86.4 ± 0.0   |

| bt-DNA hybridization | Molar ratio DNA origami : bt-DNA |
|----------------------|----------------------------------|
|                      | 1 : 10 | 1 : 20 | 1 : 50 | 1 : 100 | 1 : 300 | 1 : 1000 |
|                      | 26.9 ± 0.3 | 39.1 ± 2.0 | 86.4 ± 0.5 | 85.4 ± 0.3 | 85.4 ± 0.2 | 85.9 ± 0.0 |

| SAv attachment | Molar ratio DNA origami : dSAv |
|----------------|-------------------------------|
|                | 1 : 1 | 1 : 5 | 1 : 10 | 1 : 100 | Incubation time [min] |
|                | 33.1 ± 2.4 | 34.1 ± 0.6 | 32.6 ± 1.5 | 67.1 ± 0.6 | 10 |
|                | 34.3 ± 1.7 | 61.0 ± 0.8 | 68.7 ± 0.4 | 67.9 ± 1.2 | 30 |
|                | 34.2 ± 1.0 | 61.2 ± 0.7 | 68.1 ± 1.2 | 67.6 ± 1.5 | 60 |
|                | 33.7 ± 0.5 | 65.0 ± 1.4 | 68.4 ± 0.9 | 68.5 ± 0.5 | 300 |

| POI attachment | Molar ratio DNA origami : bt-H57-scFv |
|----------------|--------------------------------------|
|                | 1 : 1 | 1 : 5 | 1 : 10 | 1 : 100 | Incubation time [min] |
|                | 34.7 ± 2.2 | 59.8 ± 0.9 | 62.0 ± 0.4 | 66.9 ± 1.5 | 10 |
|                | 32.0 ± 1.2 | 59.1 ± 1.5 | 62.2 ± 1.3 | 67.5 ± 0.5 | 30 |
|                | 31.1 ± 1.4 | 60.1 ± 0.8 | 67.4 ± 1.0 | 68.1 ± 0.7 | 60 |
|                | 34.6 ± 1.0 | 66.6 ± 0.9 | 67.3 ± 1.0 | 67.5 ± 0.5 | 300 |
Table S4. Optimization of functionalization conditions for construct H57-dSAv-NL. For each step, the yield of functionalized DNA origami construct was determined for different conditions (molar ratios, incubation times) via two-color colocalization microscopy as sketched in SI Fig. S4. Cumulative yields are shown. Optimal conditions for each step are marked in red. Data are the mean (± sem) of at least two independent experiments.

| Handle incorporation | Molar ratio DNA origami : biotinylated handle oligo |
|----------------------|-----------------------------------------------------|
|                      | 1 : 1 | 1 : 2 | 1 : 5 | 1 : 10 | 1 : 30 | 1 : 100 |
| 21.2 ± 1.2           | 53.8 ± 7.0 | 59.0 ± 7.0 | 84.0 ± 2.7 | 79.9 ± 6.2 | 79.4 ± 0.5 |

| SAv attachment       | Molar ratio DNA origami : dSAv |
|----------------------|--------------------------------|
|                      | 1 : 1 | 1 : 5 | 1 : 10 | 1 : 100 | Incubation time [min] |
| 43.2 ± 2.7           | 44.6 ± 6.6 | 64.7 ± 2.8 | 66.5 ± 3.8 | 10 |
| 46.8 ± 4.6           | 55.4 ± 8.6 | 71.7 ± 3.3 | 67.8 ± 2.6 | 30 |
| 44.2 ± 1.9           | 67.4 ± 3.6 | 72.0 ± 2.6 | 68.0 ± 2.5 | 60 |
| 46.1 ± 8.2           | 66.3 ± 3.0 | 69.1 ± 4.2 | 70.1 ± 3.1 | 300 |

| POI attachment       | Molar ratio DNA origami : bt-H57-scFv |
|----------------------|--------------------------------------|
|                      | 1 : 1 | 1 : 5 | 1 : 10 | 1 : 100 | Incubation time [min] |
| 58.4 ± 4.8           | 63.5 ± 4.9 | 66.2 ± 4.5 | 68.5 ± 4.5 | 10 |
| 59.5 ± 5.7           | 66.1 ± 3.2 | 67.0 ± 5.0 | 69.6 ± 3.7 | 30 |
| 64.0 ± 2.4           | 66.4 ± 4.0 | 71.2 ± 2.6 | 69.8 ± 3.2 | 60 |
| 64.3 ± 5.3           | 67.4 ± 4.5 | 72.4 ± 0.3 | 73.2 ± 2.5 | 300 |
Table S5. Optimization of functionalization conditions for construct H57-DNA. For each step, the yield of functionalized DNA origami construct was determined for different conditions (molar ratios, incubation times) via two-color colocalization microscopy as sketched in SI Fig. S5. Cumulative yields are shown. Optimal conditions for each step are marked in red. Data are the mean (± sem) of at least two independent experiments.

| Handle incorporation | Molar ratio DNA origami : handle oligo |
|----------------------|----------------------------------------|
|                      | 1 : 1        | 1 : 2        | 1 : 5        | 1 : 10       | 1 : 30       | 1 : 50       | 1 : 100      |
|                      |             |             |             |             |             |             |             |
|                      | 19.1 ± 0.9  | 32.8 ± 0.8  | 46.6 ± 0.6  | 84.4 ± 2.1  | 83.4 ± 2.7  | 86.4 ± 0.3  | 86.4 ± 0.0  |

| POI attachment       | Molar ratio DNA origami : H57-scFv-DNA |
|----------------------|----------------------------------------|
|                      | 1 : 1        | 1 : 5        | 1 : 10       | 1 : 100      | Incubation time [min] |
|                      |             |             |             |             |                     |
|                      | 32.6 ± 2.6  | 62.8 ± 2.9  | 65.7 ± 1.9  | 67.1 ± 3.0  | 10                   |
|                      | 34.9 ± 4.3  | 63.7 ± 2.5  | 66.9 ± 2.4  | 67.6 ± 1.9  | 30                   |
|                      | 36.1 ± 3.3  | 65.4 ± 4.1  | 67.9 ± 2.4  | 67.6 ± 2.1  | 60                   |
|                      | 58.5 ± 1.7  | 66.3 ± 3.6  | 67.7 ± 1.8  | 67.8 ± 2.6  | 300                  |
Table S6. Determination of functionalization yields after each step for construct H57-PNA. For each step, the yield of functionalized DNA origami construct was determined for different conditions (incubation times and molar ratios) via two-color colocalization microscopy as sketched in SI Fig. S6. Cumulative yields are shown. Optimal conditions for each step are marked in red. Data are the mean (± s.e.m.) of at least two independent experiments.

| Handle incorporation | Molar ratio DNA origami : handle oligo |
|----------------------|----------------------------------------|
|                      | 1 : 1        | 1 : 2        | 1 : 5        | 1 : 10       | 1 : 30       | 1 : 50       | 1 : 100      |
|                      | 19.1 ± 0.9   | 32.8 ± 0.8   | 46.6 ± 0.6   | **84.4 ± 2.1** | 83.4 ± 2.7   | 86.4 ± 0.3   | 86.4 ± 0.0   |

| POI attachment       | Molar ratio DNA origami : H57-scFv-PNA |
|----------------------|----------------------------------------|
|                      | 1 : 1        | 1 : 3        | 1 : 10       | 1 : 100      | Incubation time [min] |
|                      | 24.7 ± 1.9   | 28.0 ± 1.5   | 47.9 ± 3.2   | 48.9 ± 3.2   | 10             |
|                      | 50.2 ± 6.0   | 71.5 ± 2.5   | 71.7 ± 2.3   | 73.1 ± 2.4   | 30             |
|                      | 52.0 ± 4.5   | **74.2 ± 2.2** | 72.9 ± 2.5   | 73.0 ± 3.0   | 60             |
|                      | 51.4 ± 5.0   | 73.6 ± 2.0   | 72.9 ± 2.5   | 72.9 ± 2.7   | 300            |
Table S7. Determination of functionalization yields after each step for construct H57-mSAv. For each step, the yield of functionalized DNA origami construct was determined for different conditions (incubation times and molar ratios) via two-color colocalization microscopy as sketched in SI Fig. S7. Cumulative yields are shown. Optimal conditions for each step are marked in red. Data are the mean (± s.e.m.) of at least two independent experiments.

| Handle incorporation | Molar ratio DNA origami : handle oligo |
|----------------------|----------------------------------------|
|                      | 1 : 1        | 1 : 2        | 1 : 5        | 1 : 10       | 1 : 30       | 1 : 50       | 1 : 100      |
|                      | 19.1 ± 0.9   | 32.8 ± 0.8   | 46.6 ± 0.6   | 84.4 ± 2.1   | 83.4 ± 2.7   | 86.4 ± 0.3   | 86.4 ± 0.0   |

| SAv attachment       | Molar ratio DNA origami : mSAv-DNA |
|----------------------|-------------------------------------|
|                      | 1 : 3     | 1 : 10    | 1 : 100   | Incubation time [min] |
|                      | 68.8 ± 2.7| 74.0 ± 2.5| 76.4 ± 3.1| 10               |
|                      | 75.4 ± 0.4| 75.7 ± 1.5| 78.1 ± 0.6| 30               |
|                      | 81.0 ± 2.4| 78.5 ± 2.7| 78.2 ± 5.8| 60               |
|                      | 78.0 ± 0.6| 80.0 ± 1.9| 81.1 ± 3.0| 300              |

| POI attachment       | Molar ratio DNA origami : bt-H57-scFv |
|----------------------|---------------------------------------|
|                      | 1 : 1      | 1 : 5      | 1 : 10     | 1 : 100     | Incubation time [min] |
|                      | 47.4 ± 3.0 | 50.0 ± 4.8 | 56.4 ± 6.3 | 62.1 ± 2.6 | 10               |
|                      | 49.5 ± 1.5 | 58.3 ± 5.0 | 63.2 ± 2.4 | 65.5 ± 3.0 | 30               |
|                      | 50.7 ± 1.6 | 63.1 ± 1.9 | 66.6 ± 3.2 | 66.3 ± 2.9 | 60               |
|                      | 53.7 ± 1.7 | 65.5 ± 3.2 | 66.5 ± 3.1 | 67.6 ± 3.8 | 300              |
Table S8. Determination of functionalization yields after each step for construct H57-tSAv. For each step, the yield of functionalized DNA origami construct was determined for different conditions (incubation times and molar ratios) via two-color colocalization microscopy as sketched in SI Fig. S8. Cumulative yields are shown. Optimal conditions for each step are marked in red. Data are the mean (± s.e.m.) of at least two independent experiments.

| Handle incorporation | Molar ratio DNA origami : handle oligo |
|----------------------|-----------------------------------------|
|                      | 1 : 1         | 1 : 2         | 1 : 5         | 1 : 10        | 1 : 30        | 1 : 50        | 1 : 100        |
|                      | 19.1 ± 0.9    | 32.8 ± 0.8    | 46.6 ± 0.6    | **84.4 ± 2.1**| 83.4 ± 2.7    | 86.4 ± 0.3    | 86.4 ± 0.0    |

| bt-DNA hybridization | Molar ratio DNA origami : bt-DNA |
|----------------------|---------------------------------|
|                      | 1 : 10    | 1 : 20     | 1 : 50    | 1 : 100    | 1 : 300    | 1 : 1000    |
|                      | 26.9 ± 0.3| 39.1 ± 2.0| **86.4 ± 0.5**| 85.4 ± 0.3| 85.4 ± 0.2| 85.9 ± 0.0  |

| SAv attachment | Molar ratio DNA origami : tSAv |
|----------------|---------------------------------|
|                | 1 : 1    | 1 : 5    | 1 : 10   | 1 : 100   | Incubation time [min] |
|                | 76.6 ± 4.8| 77.9 ± 5.2| 77.5 ± 5.7| 79.0 ± 5.6| 10            |
|                | 77.5 ± 3.6| 79.7 ± 4.8| **79.4 ± 3.2**| 79.4 ± 3.6| 30            |
|                | 76.8 ± 4.3| 78.3 ± 6.0| 79.6 ± 4.0| 79.3 ± 4.0| 60            |
|                | 77.7 ± 4.9| 78.8 ± 6.1| 79.6 ± 4.0| 82.1 ± 2.6| 300           |

| POI attachment | Molar ratio DNA origami : bt-H57-scFv |
|----------------|----------------------------------------|
|                | 1 : 10 | Incubation time [min] |
|                | 69.1 ± 2.3| 10               |
|                | 70.5 ± 1.1| 30               |
|                | **70.1 ± 1.3**| 60               |
|                | 68.1 ± 3.0| 300              |
Table S9. Determination of functionalization yields after each step for construct H57-tSAv-NL. For each step, the yield of functionalized DNA origami construct was determined for different conditions (incubation times and molar ratios) via two-color colocalization microscopy as sketched in SI Fig. S9. Cumulative yields are shown. Optimal conditions for each step are marked in red. Data are the mean (± s.e.m.) of at least two independent experiments.

| Handle incorporation | Molar ratio DNA origami : biotinylated handle oligo |
|----------------------|-----------------------------------------------------|
|                      | 1 : 1  | 1 : 2  | 1 : 5  | 1 : 10 | 1 : 30 | 1 : 100 |
|                      | 21.2 ± 1.2 | 53.8 ± 7.0 | 59.0 ± 7.0 | **84.0 ± 2.7** | 79.9 ± 6.2 | 79.4 ± 0.5 |

| SAv attachment | Molar ratio DNA origami : tSAv | Incubation time [min] |
|----------------|-------------------------------|-----------------------|
| 1 : 1          | 65.2 ± 6.5                    | 10                    |
| 1 : 5          | 78.8 ± 0.8                    |                       |
| 1 : 10         | 79.5 ± 1.4                    |                       |
| 1 : 100        | 81.2 ± 1.7                    |                       |
|                | **83.8 ± 1.3**                | 30                    |
|                | 81.3 ± 1.7                    |                       |
|                | 78.0 ± 2.4                    | 60                    |
|                | 80.6 ± 2.3                    |                       |
|                | 82.7 ± 0.3                    |                       |
|                | 81.5 ± 1.8                    |                       |
|                | **83.9 ± 0.2**                | 300                   |
|                | 81.6 ± 1.1                    |                       |
|                | 82.1 ± 2.4                    |                       |
|                | 83.9 ± 0.2                    |                       |

| POI attachment | Molar ratio DNA origami : bt-H57-scFv | Incubation time [min] |
|----------------|----------------------------------------|-----------------------|
| 1 : 10         |                                        | 10                    |
| 71.0 ± 1.3     |                                        |                       |
| 71.2 ± 1.2     |                                        |                       |
| 72.1 ± 1.9     |                                        | 60                    |
| 71.6 ± 1.8     |                                        | 300                   |
Table S10. Functionalization efficiencies for the different constructs. The degree of functionalization (%, ± s.e.m.) (with one or more ligands) was determined via two-color colocalization microscopy (see Figures SI 3-9). Cumulative yields are shown in (A). To allow for direct comparison of the efficiencies of individual functionalization steps we provide this information in (B). Note that for (B) some step efficiencies could not be determined directly but had to be calculated (indicated with #).

|   | H57-dSAv | H57-dSAv-NL | H57-DNA | H57-PNA | H57-mSAv | H57-tSAv | H57-tSAv-NL |
|---|---------|-------------|---------|---------|----------|----------|-------------|
| A | handle incorporation | 84.4 ± 2.1 | 84.0 ± 2.7 | 84.4 ± 2.1 | 84.4 ± 2.1 | 84.4 ± 2.1 | 84.4 ± 2.1 | 84.0 ± 2.7 |
|   | bt-DNA hybridization | 86.4 ± 0.5 | - | - | - | - | 86.4 ± 0.5 | - |
|   | SAv attachment | 68.7 ± 0.1 | 72.0 ± 0.2 | - | - | 81.0 ± 2.4* | 79.4 ± 3.2 | 83.8 ± 1.3 |
| B | H57-scFv attachment | 67.4 ± 0.7 | 71.2 ± 2.6 | 67.9 ± 0.7* | 74.2 ± 0.3* | 66.6 ± 1.2 | 70.1 ± 0.9 | 72.1 ± 0.7 |

|   | H57-dSAv | H57-dSAv-NL | H57-DNA | H57-PNA | H57-mSAv | H57-tSAv | H57-tSAv-NL |
|---|---------|-------------|---------|---------|----------|----------|-------------|
| B | handle incorporation | 84.0 ± 2.7 | 84.0 ± 2.7 | 84.4 ± 2.1 | 84.4 ± 2.1 | 84.4 ± 2.1 | 84.4 ± 2.1 | 84.0 ± 2.7 |
|   | bt-DNA hybridization | 102.4 ± 3.1# | - | - | - | - | 102.4 ± 3.1# | - |
|   | SAv attachment | 85.7 ± 3.0 | 85.7 ± 3.0 | - | - | 96.0 ± 5.2*# | 91.9 ± 6.5# | 99.8 ± 4.8# |
|   | H57-scFv attachment | 98.9 ± 3.9# | 98.9 ± 3.9# | 80.5 ± 2.8* | 87.9 ± 2.5* | 82.2 ± 3.9 | 88.3 ± 7.4# | 86.0 ± 2.2 |

*indicates attachment via hybridization.
Table S11. Number of H57-scFv molecules per DNA origami construct. The number of H57-scFv molecules per functionalized DNA origami construct was determined by comparing the signal brightness of the construct to the brightness of a single AF555-labeled H57-scFv as described in the methods section. Data are the mean of at least two independent experiments (± s.e.m.).

| Construct   | 1 H57-scFv | 2 H57-scFv | 3 H57-scFv | signals (n) |
|-------------|------------|------------|------------|-------------|
| H57-dSAv    | 98.6 ± 0.8 | 0.5 ± 0.3  | 0.6 ± 0.5  | 1,352 ± 147 |
| H57-dSAv-NL | 99.9 ± 0.3 | 0.1 ± 0.1  | 0.6 ± 0.2  | 1,369 ± 107 |
| H57-DNA     | 97.5 ± 1.1 | 2.2 ± 1.1  | 0.3 ± 0.2  | 1,273 ± 69  |
| H57-PNA     | 99.5 ± 0.2 | 0.0 ± 0.0  | 0.5 ± 0.2  | 2,276 ± 484 |
| H57-mSAv    | 98.6 ± 0.8 | 1.1 ± 0.7  | 0.3 ± 0.1  | 1,359 ± 63  |
| H57-tSAv    | 30.6 ± 1.8 | 52.8 ± 3.2 | 16.7 ± 5.1 | 5,984 ± 1,627 |
| H57-tSAv-NL | 48.1 ± 3.2 | 47.1 ± 0.7 | 4.8 ± 3.9  | 3,096 ± 565 |
Table S12. Fitting parameters of fits to dose-response curves. Dose-response curves of T-cell activation by via the different ligand-decorated DNA origami constructs were fitted with Eq. 19 to extract the activation threshold $T_A$, the maximum response $A_{\text{max}}$ and the Hill coefficient $n$. The 95% confidence intervals are indicated. The mean number of cells per region (± s.e.m.) and the number of animals used to generate dose-response curves are shown.

| Construct      | n  | n   | n   | $A_{\text{max}}$ (%) | $A_{\text{max}}$, low | $A_{\text{max}}$, high | $T_A$, low | $T_A$, high | # cells | # animals |
|----------------|----|-----|-----|-----------------------|------------------------|------------------------|------------|------------|---------|-----------|
| H57-dSAv       | 2.44 | 1.66 | 3.23 | 91.81 | 84.50 | 99.13 | 3.91 | 3.29 | 4.64 | 163 ± 35 | 3         |
| H57-dSAv-NL    | 3.06 | 1.83 | 4.29 | 100.14 | 91.55 | 108.73 | 3.27 | 2.78 | 3.85 | 199 ± 55 | 2         |
| H57-DNA        | 4.38 | 2.49 | 6.26 | 91.01 | 81.41 | 100.62 | 9.68 | 8.66 | 10.83 | 176 ± 68 | 2         |
| H57-PNA        | 1.73 | 0.94 | 2.52 | 103.83 | 94.52 | 113.15 | 2.75 | 2.20 | 3.43 | 206 ± 65 | 2         |
| H57-mSAv       | 3.35 | 0.99 | 5.71 | 92.55 | 84.01 | 101.09 | 3.15 | 2.58 | 3.86 | 203 ± 53 | 2         |
| H57-tSAv       | 1.23 | 0.33 | 2.12 | 105.74 | 89.16 | 122.31 | 0.81 | 0.45 | 1.49 | 289 ± 81 | 2         |
| H57-tSAv-NL    | 1.42 | 0.72 | 2.12 | 101.65 | 90.28 | 113.02 | 0.83 | 0.45 | 1.54 | 214 ± 62 | 2         |
### Table S13. List of staple strands

| Designation | Sequence |
|-------------|----------|
| 10[63]-BLK  | CAGCTTTCCGGCCGATCGTAAACGCAGTCCGCCT |
| 11[112]-BLK | AGCCGGACACAATCATAAGGAACCGGGCGGTAC |
| 12[95]-BLK  | ACACTCATAGGGGACGACAGCTGGTACCTG |
| 13[80]-BLK  | GGGCCAGCTAAGGAGGAACTCCCCGTAAAAC |
| 14[63]-BLK  | GTATCGGGTCGTTTCTGTTGACGTAATCA |
| 15[112]-BLK | TACGAAAGGAGCTACCTAAAAATGGCTATAG |
| 16[95]-BLK  | TGATTCCTAAAGGGGTGGCATCAATAATCATAC |
| 17[32]-BLK  | GGGATAGGTTTCCGGCACCGCTTCCATTCAGG |
| 18[143]-BLK | TAGCCGGGACCTTCTCATCAAGTAGAATCAAGCTA |
| 19[95]-BLK  | GGAGCTAGGTAGCACCCTCCATTCAGG |
| 20[112]-BLK | CTTGCGCAAGCAAGCGGTGACGTAAATCAG |
| 21[95]-BLK  | GGATTCCTAAAGGGGTGGCATCAATAATCATAC |
| 22[32]-BLK  | GGCAGAGTACGGGACGCACTTCAGGGGAAA |
| 23[112]-BLK | CTTTGGCATAGGGGGAACGCACTTCAGGGGAAA |
| 24[95]-BLK  | CCAACCTCAGGTGTAGGGAACGCACTTCAGGG |
| 25[32]-BLK  | TACGGAAGATTTGTATCATCGCCTATGTTACT |
| 26[47]-BLK  | CAGCCGACGCTCAGGTTTGGTAGATATCAACAT |
| 27[63]-BLK  | CAGGAAAGCAGGCGGCCCTCCTTCTGCAAG |
| 28[112]-BLK | CTTTGGCATAGGGGGAACGCACTTCAGGGGAAA |
| 29[32]-BLK  | CTGCGCAAGTTCCTCAGGTCAGGAAGAAGAC |
| 30[143]-BLK | CTGAGGCTACTAAAGACTTTTACATAGCC |
| 31[95]-BLK  | CAGCAGCGGGCGAAAGGGGGATGTCGCTATTA |
| 32[32]-BLK  | AGTGAGCACAGGACGGGCAAGCATAACTCA |
| 33[80]-BLK  | TTGGCTATATATTCATGCCGTATTTTCTTTTAAT |
| 34[143]-BLK | TCCGCTACACGGGTATGCGGACAAGGAAAA |
| 35[95]-BLK  | GGGATGCTAGGTGAGTGCAATAGTGTCGCG |
| 36[32]-BLK  | CAGCAGCGGGCGAAAGGGGGATGTCGCTATTA |
| 37[80]-BLK  | CGTGCCAGATGAATGAATTTTCTCGTCTTTC |
| Designation | Sequence                                                   | Docking Sequence                      |
|-------------|------------------------------------------------------------|---------------------------------------|
| 9[80]-4T-V  | CAAAAATAAAAGAGACAGATGAACTGACCTTTTGAGTGTGTCATGT            | GTGGAGTAGTGTCATGT                      |
| Z'-4T-10[47]| AGAGTCTAGCATATTTAGCCTTATTTAATTGTTGTTAATCTCAGCTCAAGCCCAAA | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-11[128]| AGAGTCCTAGCATATTTAGCCTTTTCAACGGAGGCAACCAACCTAAACGTACAGGG | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-14[47]| AGAGTCCTAGCATATTTAGCCTTTTACCCAGGCTGTTGGGAGAGGCGATCGCCTC | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-15[128]| AGAGTCCTAGCATATTTAGCCTTTTACCCATGTAGTACCGCCACCCTTCAGTACCAG | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-18[47]| AGAGTCCTAGCATATTTAGCCTTTTATACCCATGTAGTACCGCCACCCTTCAGTACCAG | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-19[128]| AGAGTCCTAGCATATTTAGCCTTTTTATACCCATGTAGTACCGCCACCCTTCAGTACCAG | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-2[47] | AGAGTCCTAGCATATTTAGCCTTTTATACCCATGTAGTACCGCCACCCTTCAGTACCAG | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-3[128] | AGAGTCCTAGCATATTTAGCCTTTTATACCCATGTAGTACCGCCACCCTTCAGTACCAG | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-6[47] | AGAGTCCTAGCATATTTAGCCTTTTATACCCATGTAGTACCGCCACCCTTCAGTACCAG | AGAGTCTAGCATATTTAGCC                   |
| Z'-4T-7[128] | AGAGTCCTAGCATATTTAGCCTTTTATACCCATGTAGTACCGCCACCCTTCAGTACCAG | AGAGTCTAGCATATTTAGCC                   |
Table S15. List of modified oligonucleotides

| Designation | Composition                  | Sequence                      | Modification            |
|-------------|------------------------------|-------------------------------|-------------------------|
| bt-DNA      | Biotin-PEG4-V                 | ACATGACACTACTCCAC             | 5'-Biotin-PEG4          |
| bt-DNA-635P | Biotin-PEG4-V-4T-AS635P       | ACATGACACTACTCCACTTTT         | 5'-Biotin-PEG4; 3'-AS635P |
| DNA-635P    | 9[80]-4T-V'-AS635P            | CAAAAATAAAAGAGGACAGATGAAGGACGTTTGTGAGATGTCATTG | 3'-AS635P               |
| AF647-DNA-bt| AF647-2T-9[80]-2T-PEG4-Biotin| TTTTTATTTTCTACTCCAC           | 5'-AF647; 3'-Biotin-PEG4 |
| tetrazine-PEG5-DNA | tetrazine-PEG5-4T-V        | TTACATGACACTACTCCAC           | 5'-tetrazine-PEG5        |
| azido-PEG4-DNA | azido-PEG4-4T-V             | TTTACATGACACTACTCCAC           | 5'-N3-PEG4              |
| PNA-cysteine| O-2T-V (PNA)                 | O- TTACATGACACTACTCCAC         | 5'-O-linker             |
|             | Z-PEG4-Cholesterol           | GGCTAAATATGCATTAGACTCTCT      | 3'-Cholesterol-PEG4      |
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