Supporting Information

Efficient Small-Scale Conjugation of DNA to Primary Antibodies for Multiplexed Cellular Targeting

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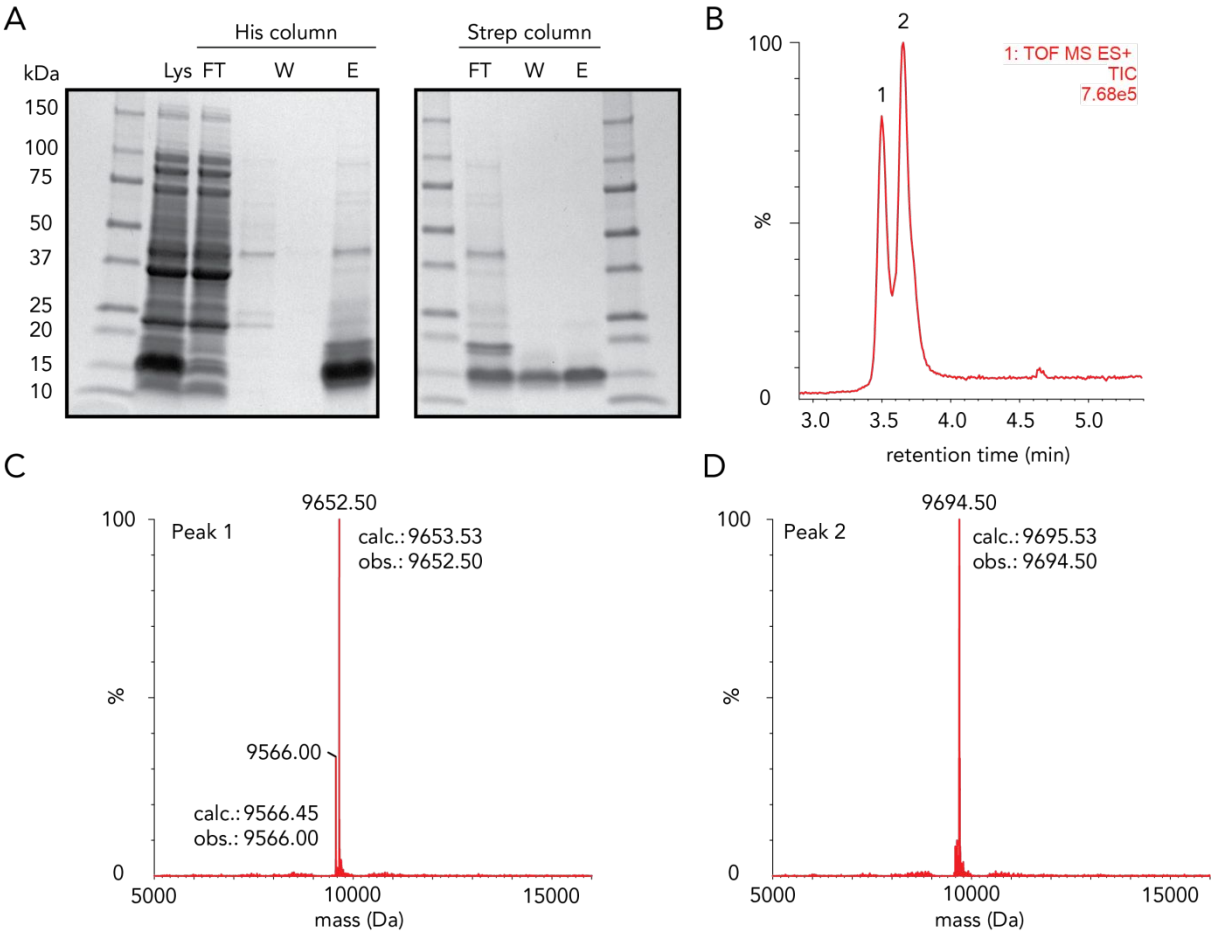

Figure S1. Purification and characterization of pG. (A) SDS-PAGE gel analysis of affinity-tag based purification of pG. (calculated mass: 9784.64 Da). pG runs at an apparent mass of 14 kDa. Labels: Lys, lysate; FT, flow through; W, wash; E, elution. (B) Chromatogram of pG analyzed by liquid chromatography quadrupole time-of-flight mass spectrometry. (C) Deconvoluted mass spectrum of peak 1 shows 2 peaks corresponding to pG without the N-terminal methionine (9652.5 Da) and serine (9566.0 Da). (D) Deconvoluted mass spectrum of peak 2 shows 1 peak corresponding to acetylated pG without the N-terminal methionine (9694.5 Da).

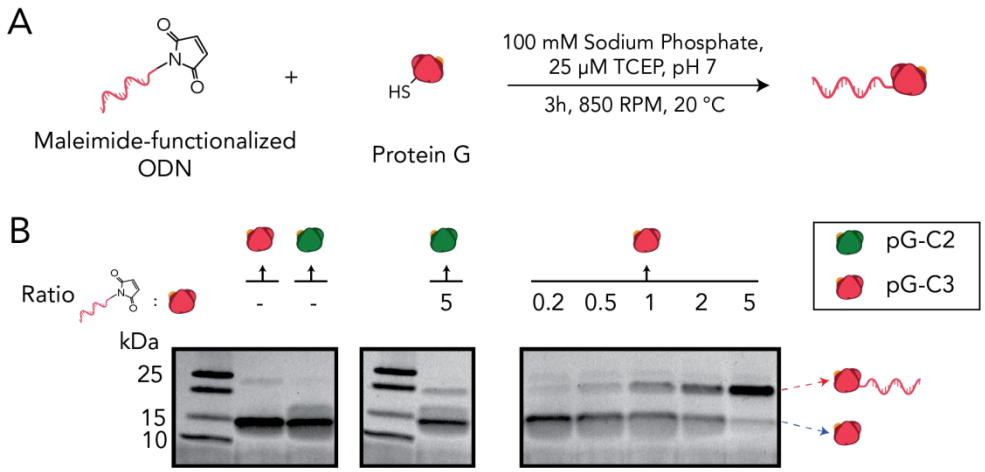

Figure S2. Schematic overview of the protein G-oligonucleotide (pG-ODN) coupling. (A) Reaction conditions for pG-ODN coupling. (B) SDS-PAGE gel analysis under non-reducing conditions of the pG-ODN reaction mixture. Using a 5-fold molar excess of maleimide-functionalized ODN, >90% of pG-C3 is successfully labeled with an ODN compared to ~15% of pG-C2. Labels: pG-C2, pG with the cysteine directly after the N-terminal methionine; pG-C3, pG with a serine placed N-terminally of the cysteine. Amino acid sequence of both pG constructs is shown in table 2.

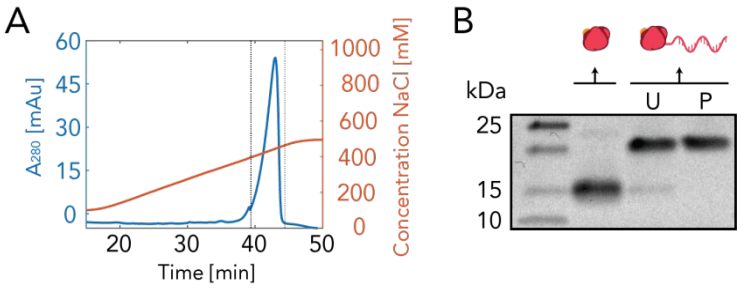

Figure S3. Overview of the purification of pG-ODN constructs. (A) Elution trace of pG-ODN using fast protein liquid chromatography (FPLC) monitored by on-line absorption at 280 nm. The large peak, indicated by the dashed lines, was collected. (B) SDS-PAGE gel analysis under non-reducing conditions of the collected fraction, showing successful removal of uncoupled pG. Labels: U, unpurified pG-ODN reaction mixture; P, purified pG-ODN

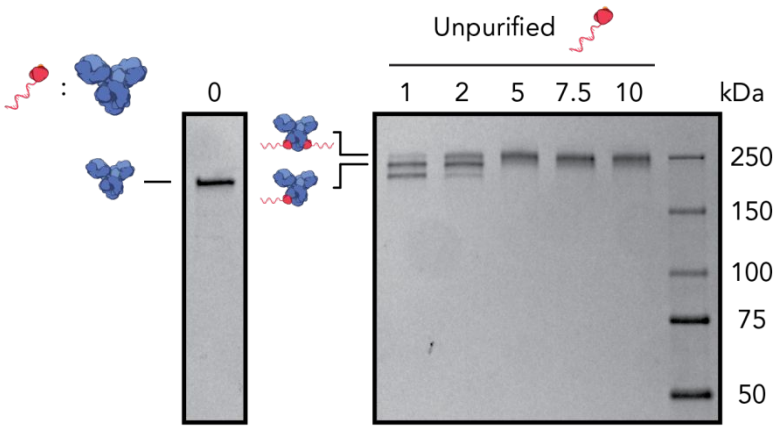

Figure S4. SDS-PAGE gel analysis under non-reducing conditions of Human IgG1 incubated with varying molar equivalents of unpurified pG-ODN. The use of 5 molar equivalents of the pG-ODN construct results in covalent labeling of all the heavy chains of the human IgG1 antibody.

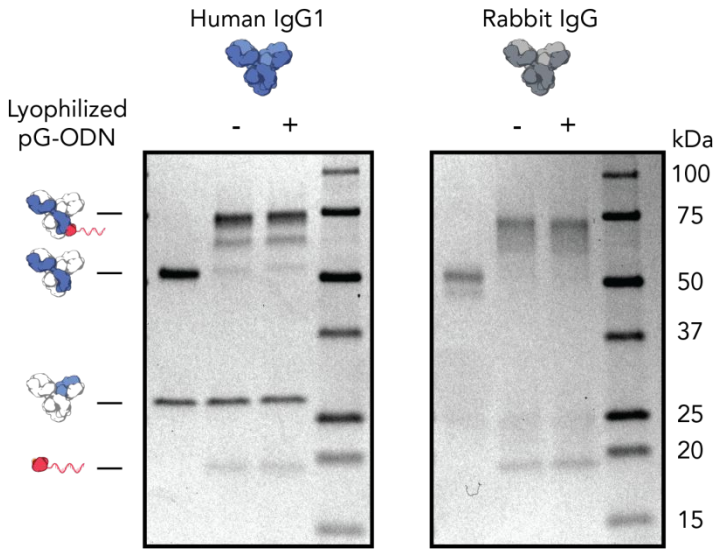

Figure S5. SDS-PAGE gel analysis under reducing conditions of pG-ODN antibody coupling before (-) and after (+) lyophilization of pG-ODN. For both Cetuximab (hIgG1) and Rabbit IgG, no decrease in coupling efficiency is observed when pG-ODN was lyophilized prior to antibody coupling.

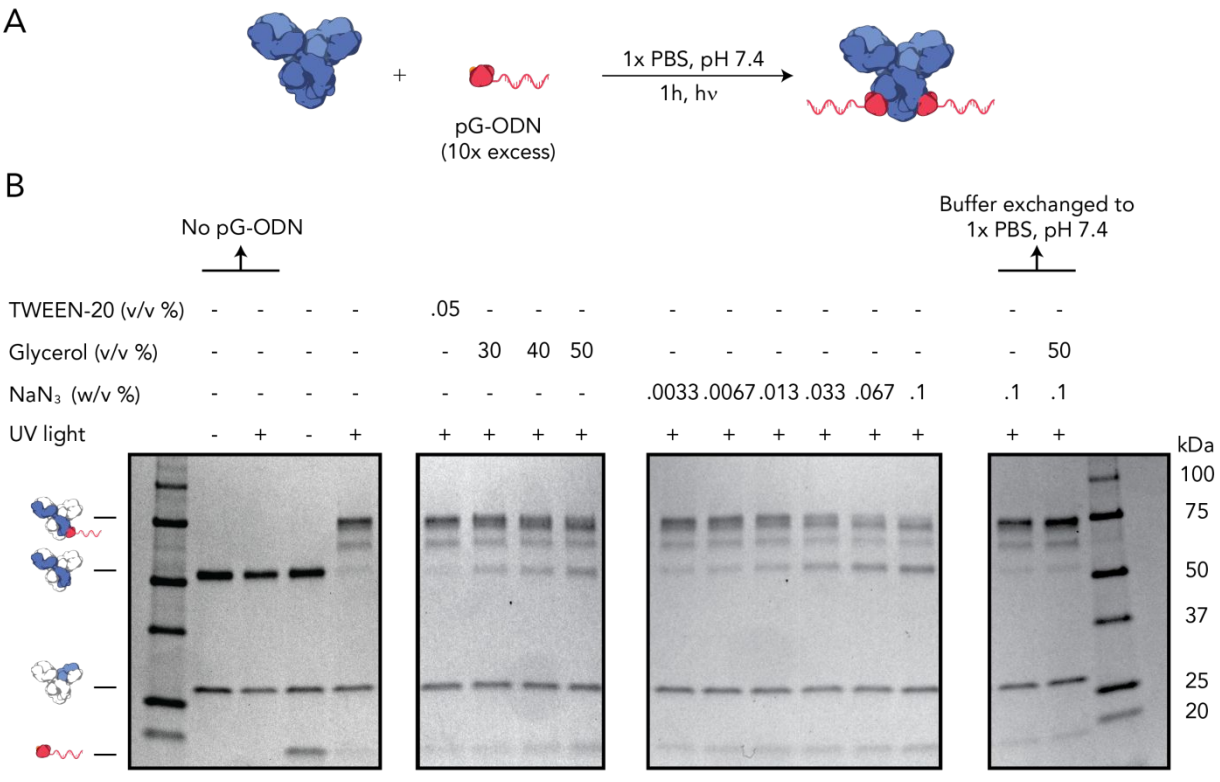

Figure S6. Coupling efficiency of pG-ODN to Cetuximab in the presence of multiple additives. (A) Schematic overview of the reaction conditions. (B) SDS-PAGE gel analysis under reducing conditions of Cetuximab coupled to pG in the presence of TWEEN-20, glycerol and sodium azide (NaN_3). Both glycerol and sodium azide inhibit pG-ODN-antibody formation. Buffer exchanging an antibody solution that contains 0.1% sodium azide in the presence and absence of 50% glycerol to 1x PBS, pH 7.4 using ultrafiltration fully restores the coupling efficiency.

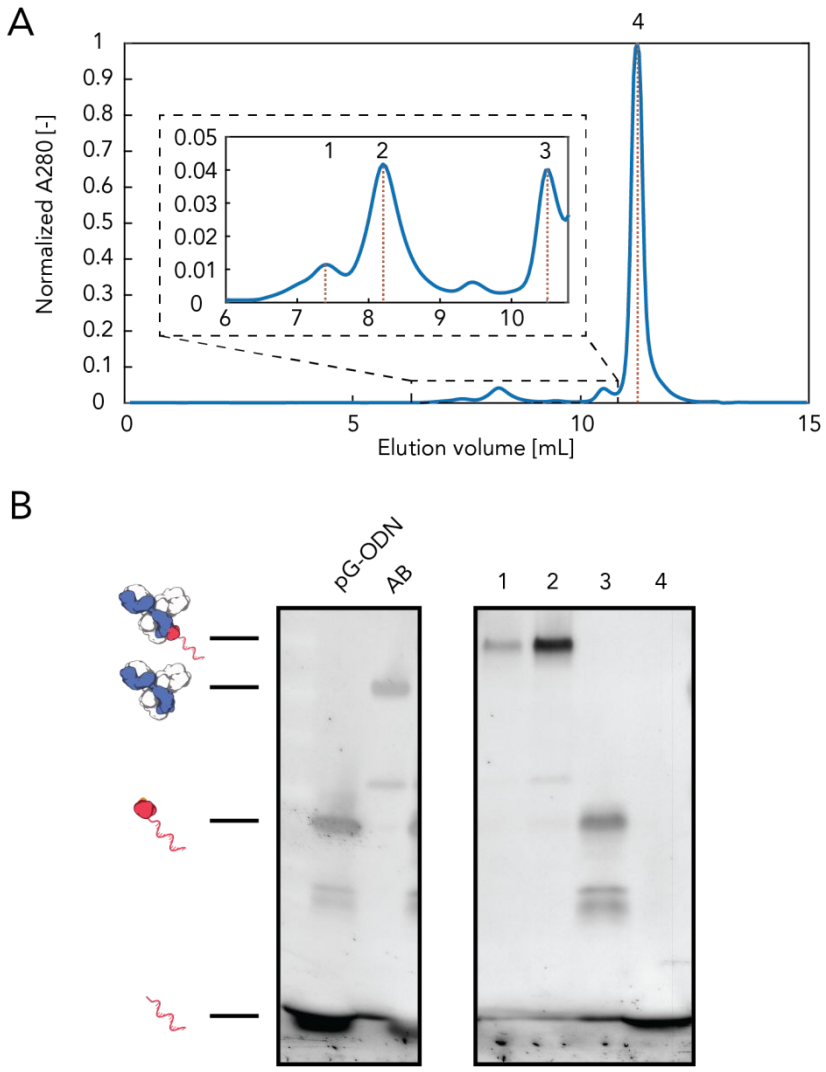

Figure S7. Purification of pG-ODN-antibody constructs using size exclusion chromatography (SEC). (A) Chromatogram showing the separation of pG-ODN-antibody (1, 2), pG-ODN (3) and the uncoupled ODN (4). (B) SDS-PAGE gel analysis of the 4 peaks shown in the chromatogram stained with SYBR Gold shows the successful separation of pG-ODN-antibody and pG-ODN constructs. Label: AB, Antibody (Cetuximab)

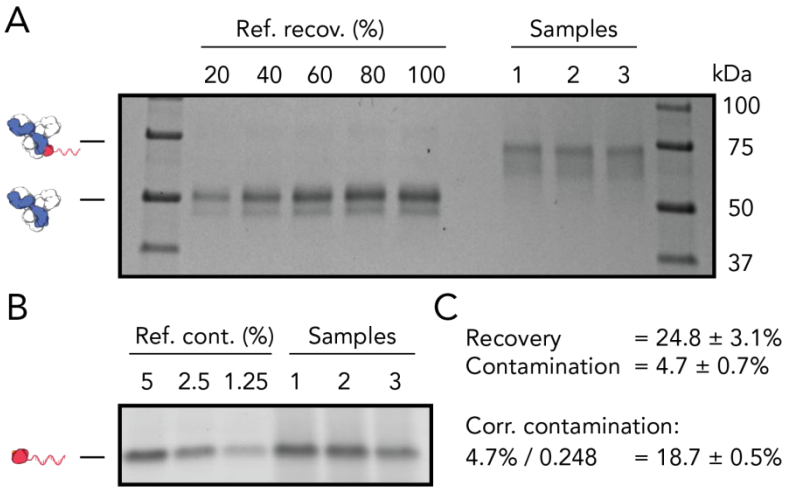

Figure S8. Recovery and purity of pG-ODN-antibody constructs purified by ultrafiltration. (A) SDS-PAGE gel analysis under reducing conditions stained with Coomassie Blue. The gel band intensity corresponding to the recovered heavy chain of the pG-ODN-antibody was compared to a reference. (B) SDS-PAGE gel analysis under non-reducing conditions stained with SYBR Gold. The intensity of the band corresponding to pG-ODN after purification was compared to a reference. (C) Calculation of the recovery and contamination. The contamination was corrected based on the recovery of the pG-ODN-antibody construct.

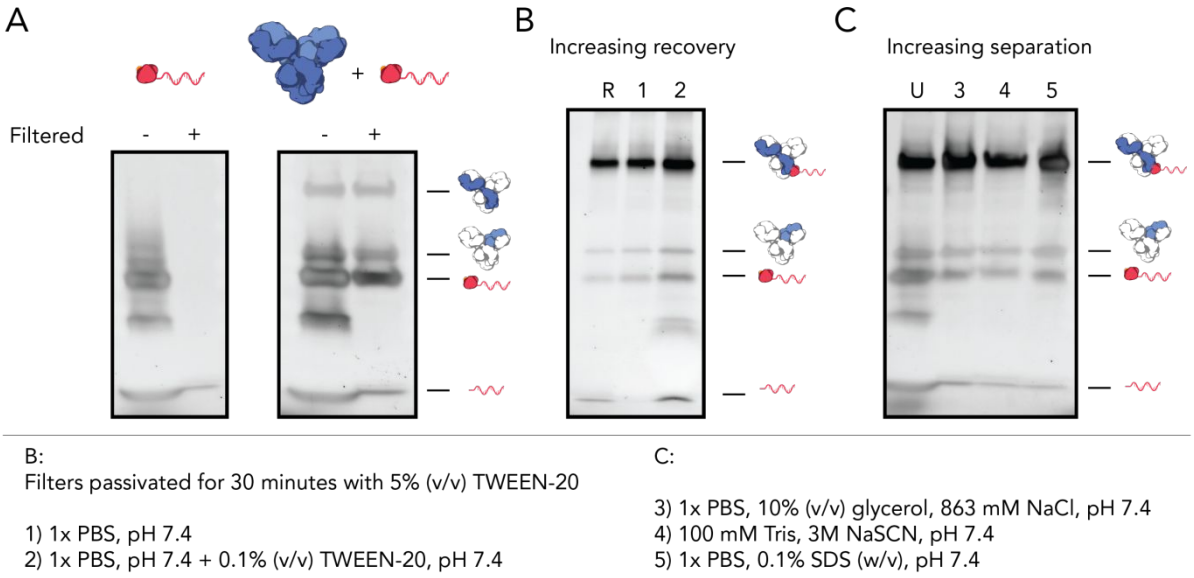

Figure S9. Optimization of pG-ODN-antibody purification using ultrafiltration. All SDS-PAGE gels were performed under reducing conditions and stained with SYBR Gold (A) pG-ODN removal using ultrafiltration in the absence (left) and presence of Cetuximab (right). Only in the absence of Cetuximab, pG-ODN was able to pass the 100 kDa molecular weight cut-off (MWCO) membrane. (B) pG-ODN removal using filters that were passivated with 5% TWEEN-20 for 30 minutes. When 0.1% (v/v) was included in the washing buffer, the recovery was increased to 68% compared to a reference filter that was not passivated (R). (C) pG-ODN removal using alternative (denaturing) washing buffers. In all cases most of the maleimide-ODN was removed successfully, however the separation between pG-ODN and pG-ODN-antibody constructs remained limited. Labels: U, unpurified sample.

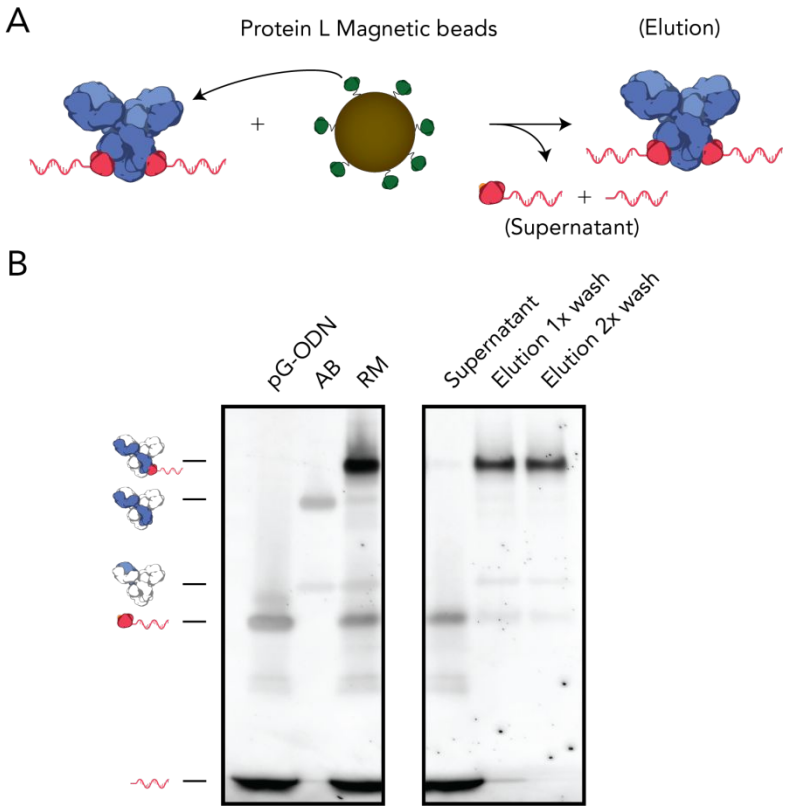

Figure S10. Purification of pG-ODN-Cetuximab using protein L magnetic beads. (A) Schematic overview of the purification process. Protein L binds to the light chain of Cetuximab and beads are recovered using a magnetic stand. (B) SDS-PAGE gel analysis under reducing conditions of different fractions of the purification. Both maleimide-ODN and pG-ODN remain in the supernatant while pG-ODN-antibody constructs are captured by the magnetic beads and recovered in the elution fraction. Labels: AB, Antibody (Cetuximab); RM, unpurified reaction mixture.

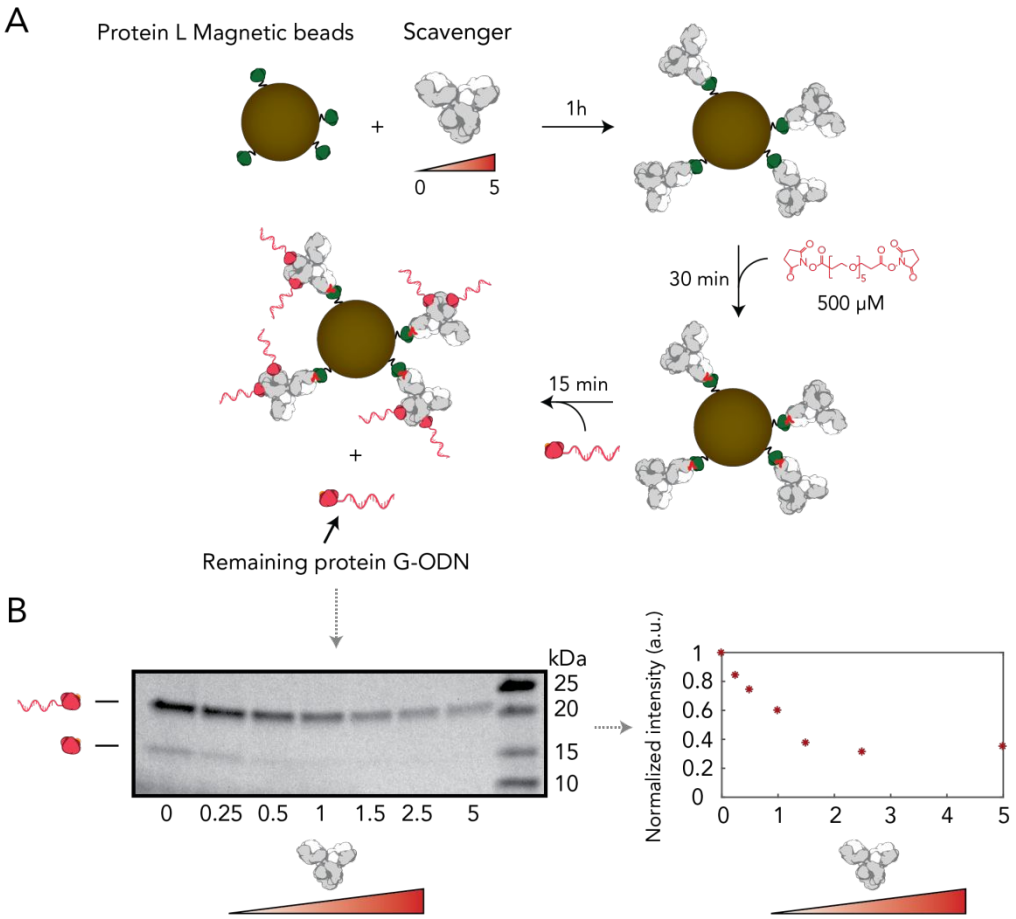

Figure S11. Optimization of the pG-ODN capturing capacity of the antibody-functionalized protein L magnetic beads as a function of the scavenger antibody. (A) Schematic overview of the synthesis of scavenging beads. Protein L magnetic beads were incubated with different equivalents of a scavenging antibody. In this case 1 equivalent was defined as the maximum capacity of a protein L bead (110 µg antibody/mg of bead). The antibody was covalently attached to the beads and the beads were incubated with pG-ODN. The beads were removed using a magnetic stand and the amount of remaining pG-ODN was quantified. (B) SDS-PAGE gel analysis under reducing conditions of the remaining amount of pG-ODN as a function of the amount of scavenging antibody. Gel band intensity shows that ≥ 2 equivalents of scavenger antibody are required to achieve maximum pG-ODN binding capacity.

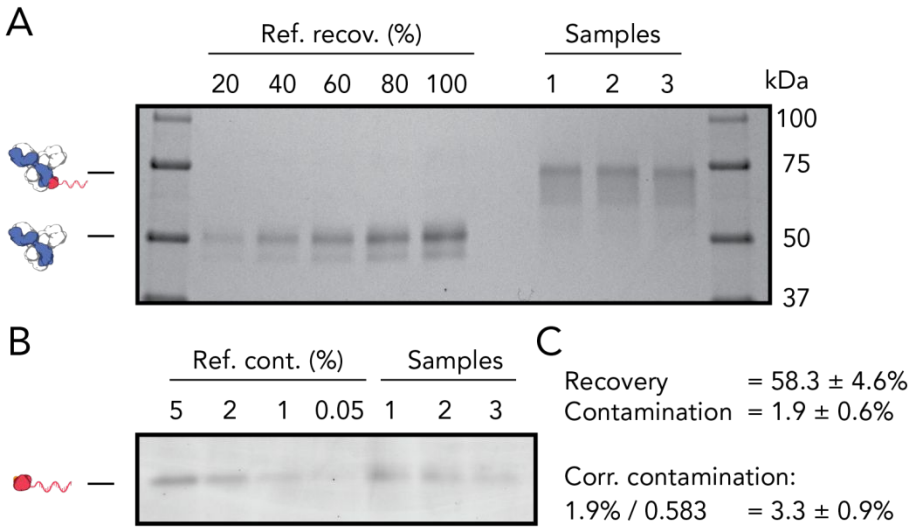

Figure S12. Recovery and purity of pG-ODN-antibody constructs purified using scavenging beads. (A) SDS-PAGE gel analysis under reducing conditions stained with Coomassie Blue. The gel band intensity corresponding to the recovered heavy chain of the pG-ODN-antibody was compared to a reference. (B) SDS-PAGE gel analysis under non-reducing conditions stained with SYBR Gold. The intensity of the band corresponding to pG-ODN after purification was compared to a reference. (C) Calculation of the recovery and contamination. The contamination was corrected based on the recovery of the pG-ODN-antibody construct.

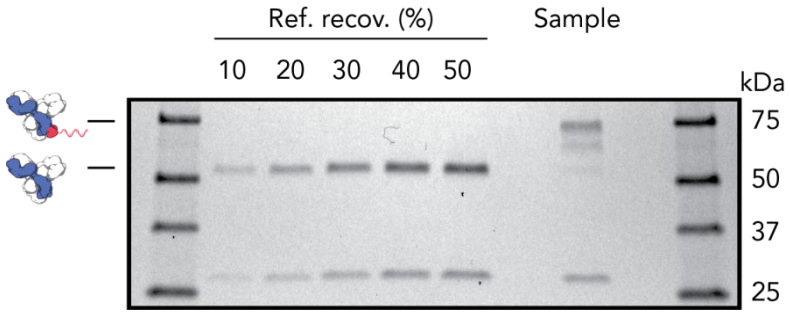

Figure S13. Recovery analysis of pG-ODN-Cetuximab using scavenging beads for purification. SDS-PAGE gel analysis under reducing conditions stained with Coomassie Blue. The gel band intensity corresponding to the recovered light chain of the pG-ODN-antibody was compared to a reference which showed a recovery of 30%

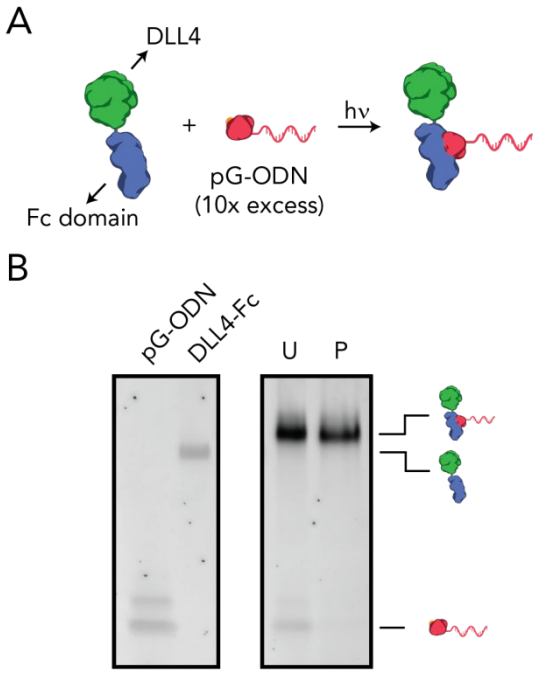

Figure S14. Labeling and purification of the Fc-fusion protein DLL4-Fc. (A) Schematic overview of the protein and labeling of DLL4-Fc using 10-fold molar excess of pG-ODN. (B) SDS-PAGE gel analysis under reducing conditions of unpurified (U) and purified (P) pG-ODN-DLL4-Fc using scavenging beads. The gel was stained with SYBR gold.

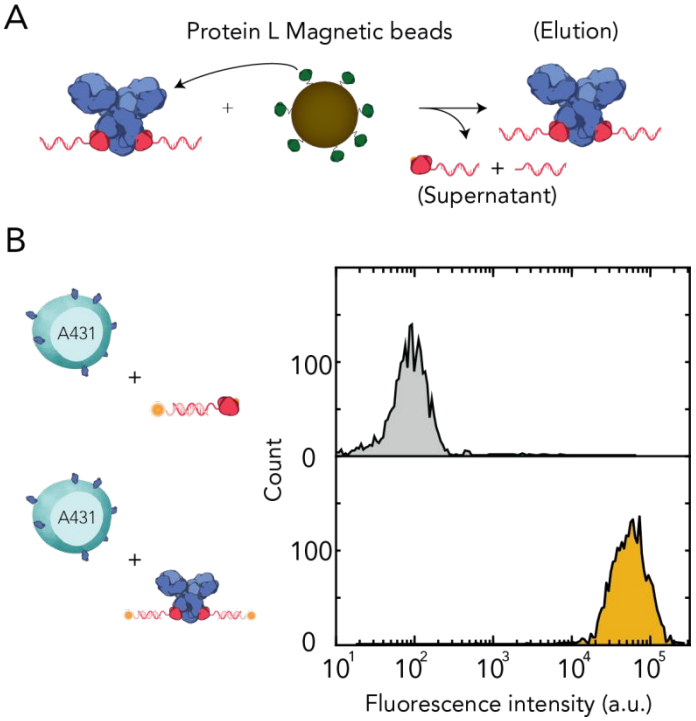

Figure S15. Antibody activity after direct purification of the pG-ODN-antibody construct using protein L functionalized magnetic beads. (A) Schematic overview of the purification process. Protein L binds to the light chain of Cetuximab and beads are recovered using a magnetic stand. (B) Flow cytometric analysis of EGFR-expressing A431 cells using 10 nM pG-ODN-functionalized Cetuximab hybridized to a CY5-labeled imager strand. Fluorescent intensity of pG-ODN-Cetuximab labeled A431 cells was compared to A431 cells incubated with only pG-ODN.

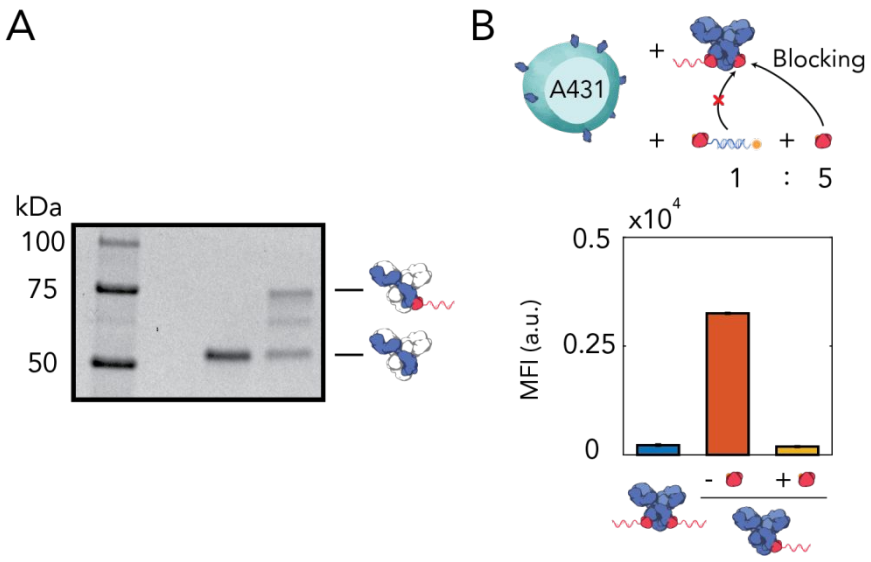

Figure S16. Blocking of free Fc sites using free pG during cellular labeling. (A) SDS-PAGE gel analysis under reducing conditions shows that ~50% of the Fc chains are covalently labeled with pG-ODN. (B) Partly labeled pG-ODN-Cetuximab was incubated with 20-fold molar excess of a competing pG-ODN sequence and 100-fold molar excess of pG to block free Fc sites. Flow cytometric analysis of EGFR-expressing A431 cells using 10 nM pG-ODN-functionalized Cetuximab shows that cross-contamination is observed when free Fc sites are not blocked with pG. Introduction of a 100-fold molar excess of pG shows the same median fluorescence intensity compared to quantitatively labeled pG-ODN-Cetuximab, indicating that successful blocking of Fc sites achieved. MFI represent the median fluorescence intensity and error bars represent SD (n=3).

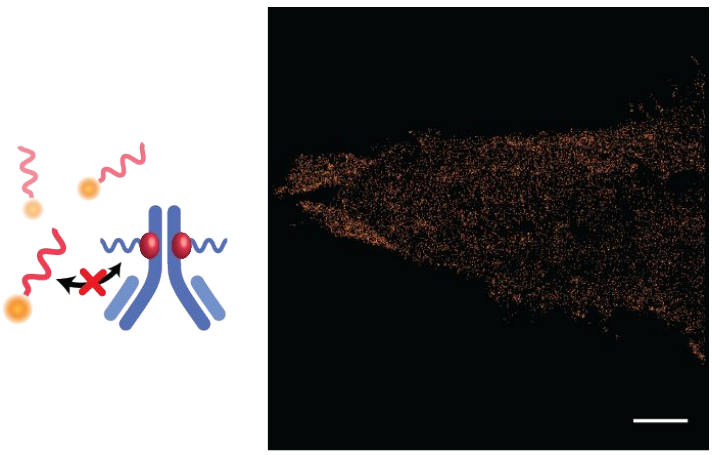

Figure S17. Aspecific interaction of imager strands in DNA-PAINT with cellular components. A431 carcinoma cells are labeled with a pG-ODN-Cetuximab construct containing a short (11 nt) docking strand (docking 4) and fixated to a glass slide. DNA-PAINT super-resolution image obtained using ATTO647N-functionalized imager strands which are not complementary to the docking strand (imager 5) (20,000 frames, 20-Hz frame rate). Scale bar, 5 µm.

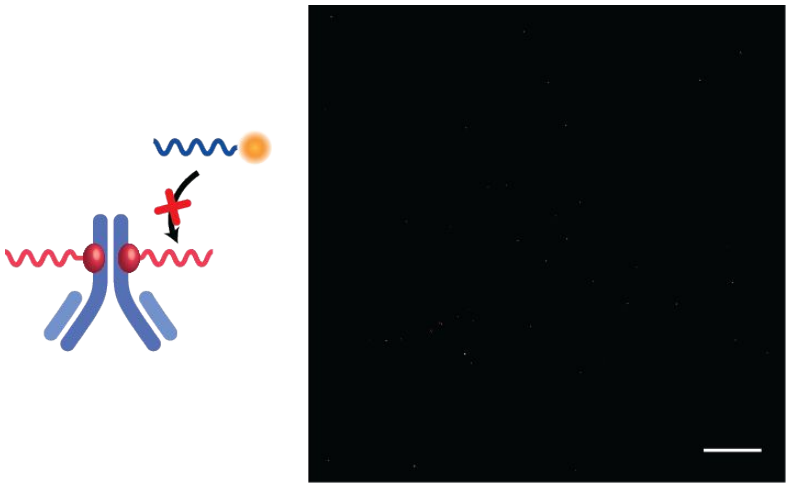

Figure S18. Aspecific interaction of imager strands in dSTORM with cellular components. A431 carcinoma cells are labeled with a pG-ODN-Cetuximab construct containing a long (20 nt) docking strand, not complementary to the imager (docking 5), and fixated to a glass slide. dSTORM super-resolution image obtained using CY5-functionalized imager strands (imager 1) (20,000 frames, 65.5-Hz frame rate). Scale bar, 5 μm.

Supplementary materials and methods:

Antibodies/Fc-fusion proteins

The following antibodies/Fc-fusion proteins were used to characterize the coupling of pG-ODN to antibodies from different host species: Cetuximab (anti-EGFR red.) (Erbitux, Merck), monoclonal anti-GFP mouse IgG2a (JL-8; catalog number: 632380, Clontech), Rabbit IgG Isotype Control (catalog number: 02-6102, Invitrogen) and DLL4-hIgG1-Fc (catalog number: 10171-H02H, Sino Biological). For the cellular labeling the following antibodies were used: monoclonal anti-CD45 mouse IgG2a (F10-89-4; catalog number: MA5-16669, Invitrogen) and monocolonal anti-CD31 mouse IgG2a (HEC7; catalog number: MA3100, Invitrogen)

Cells and complementary culture medium

A431 and Jurkat T cells were cultured in Roswell Park Memorial Institute (RPMI, Gibco) 1640 Medium fortified with 10% FBS and 1% penicillin and streptomycin. Human Umbilical Vein Endothelial Cells (HUVECs) were a kind gift from Dr. Cecilia Sahlgren. HUVECs were cultured in Endothelial Base Medium (EBM-2, Lonza) with additives of 2% FBS, 0.04% Hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, and 0.1% heparin (Lonza) supplemented with 1% penicillin/streptomycin. All cells were incubated at 37 °C and 5% CO_2.

SDS-PAGE

For SDS-PAGE analysis 4-20% SDS-PAGE Mini-PROTEAN® TGX Precast gels (Bio-rad) were used (Novex® by Life Technologies). The running buffer consists of 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Samples were heated at 95 °C for 5 min in 1x SDS Sample Buffer (62.5 mM Tris, 10% Glycerol, 2.5% SDS (w/v), 0.01% Bromophenolblue, pH 6.8) before loading. When SDS-PAGE was performed under reducing conditions DTT to a final concentration of 50 mM was added. The gels were run at 150 V for either 40 minutes (analyzing pG-ODN coupling) or 60 minutes (analyzing pG-ODN-antibody labeling). To visualize DNA bands, the gel was stained in 50 mL 1x SYBR Gold (Thermo scientific) and to visualize protein bands Coomassie Brilliant Blue G-250 (Bio-Rad) was used.

Buffer exchange/ODN removal using Amicon spin filters

A spin-filter was pre-wetted with 500 µL of the buffer of interest and spun for 5 minutes at 14,000 xg at 4 °C. The remaining concentrate was removed after centrifugation. Before loading, the reaction mixture was diluted to a final volume of 500 µL in the buffer of interest. The sample was added to the filter and centrifuged for 5 minutes at 14,000 xg at 4 °C. This step was repeated for a total of three washing steps. Eventually, the desalted concentrate was recovered by inverting the filter and spinning for 6 minutes at 1,000 xg at 4 °C.

Fast protein liquid chromatography (FPLC) with an anion-exchange column

For FPLC an anion-exchange a HiTrap Q HP column (1 mL, GE Healthcare) was used. The column was equilibrated with equilibration buffer (50 mM Tris-HCl, pH 7.5). The conjugation reaction mixture was diluted five-fold in equilibration buffer and applied manually to the column. The column was washed with equilibration buffer supplemented with 100 mM NaCl and subsequently a salt gradient was applied with a start and end concentration of 100 and 500 mM NaCl, respectively, and a flow rate of 1 mL/min. Elution fractions of 0.5 mL were collected and analyzed by measuring on-line absorption at 280 nm.

Quadrupole time-of-flight mass spectrometry (Q-Tof)

For Q-Tof an aliquot of pG was buffer exchanged using Amicon 3 kDa MWCO centrifugal filters (Merck Millipore) to ultrapure H_2O to a final concentration of 1 mg/mL. A sample of 0.1 µL pG was injected to an Agilent Polaris C18A RP column with a flow of 0.3 mL/min and a 15-60% acetonitrile gradient containing 0.1% formic acid. Mass spectra were measured on a Xevo G2 QTof mass spectrometer (Waters) in positive mode and deconvoluted with MaxEnt Deconvolution software.

Size exclusion chromatography (SEC)

Size exclusion chromatography was performed on an Agilent 1260 Infinity II Bio-inert LC System using a Bio-SEC-5 column (5 µm, 300 Å, 7.8*300 mm, Agilent). The column was equilibrated with 5 column volumes of 1x PBS, pH 7.4 using a flow rate of 1 mL/min. The column was coupled to a 280 nm UV spectrophotometer and a fraction collector. 95 µL sample was injected and elution fractions of 0.2 mL were collected and analyzed on SDS-PAGE under reducing conditions

Direct purification using protein L beads

This procedure is optimized to purify 50 µL of 4 µM antibody-pG-ODN from 40 µM uncoupled pG-ODN using 25 µL of protein L beads. In a typical reaction 25 µL of Pierce™ protein L magnetic beads was added to 75 µL washing buffer (1x PBS, 0.05% TWEEN-20, pH 7.4). The beads were washed twice in 200 µL washing buffer after which the magnetic beads were incubated with 50 µL of the reaction mixture diluted to a final volume of 200 µL in washing buffer. Reaction was rotated at for 1h at 4 °C. Subsequently, the magnetic beads were collected with a magnetic stand and the supernatant was discarded. The protein L beads were washed twice using 200 µL washing buffer. To collect the antibody-pG-ODN, beads were incubated for 5 minutes in elution buffer (100 mM Tris-HCl, 1 M NaSCN, pH 7.5). The elution fraction was desalted using a Zeba™ spin desalting column, 7000 MWCO, 0.5 mL (Thermo scientific).

Blocking of free Fc sites

Cetuximab was incubated for 1h under UV-illumination using 3-fold molar excess of pG-ODN to achieve 50% labeling of Fc domains. 20-fold molar excess of a competing pG-ODN was added to the partly labeled pG-ODN-Cetuximab in the presence and absence of 100-fold molar excess pG. The reaction mixture was shaken at 600 RPM for 1h at 20 °C. Subsequently, 12.5 µL A431 cells ($3.5*10^6$ cells/mL) were incubated with the reaction mixture in a final reaction volume of 250 µL and a final concentration of 10 nM pG-ODN-Cetuximab. Incubation was performed at 400 RPM for 30 minutes at 20 °C. After incubation the cells were pelleted and redissolved in labeling buffer (1x PBS, 0.1% (w/v) BSA, pH 7.4) containing 1 µM pG and 100 nM of a CY5-labeled ODN complementary to the competing pG-ODN sequence. The cells were incubated and centrifuged as described above and analyzed using flow cytometry.

Flow cytometry

Flow cytometry was performed on a FACS Aria III (BD Biosciences) equipped with a 70 µm nozzle. Single cell events were gates based on the linear relation between forward scatter height (FSC-H) and forward scatter area (FSC-A). For each sample 2,000 gated events were collected and analyzed using custom written Matlab scripts.

Table S1: DNA sequences

All DNA oligonucleotides were purchased from Integrated DNA Technologies. Amino-functionalized ODNs were obtained desalted and dissolved in DNas/Rnase-free water at a concentration of 1 mM and fluorescently labeled ODNs were HPLC purified and dissolved at a concentration of 250 µM

SDS-PAGE analysis pG-ODN/antibody coupling

Name	**Sequence (5' to 3')**	**Length (nt)**
Docking 1 (Cetuximab), ***a***	CCC TAG AGT GAG TCG TAT GA/3AmMO/	20

Flow cytometry

Name	**Sequence (5' to 3')**	**Length (nt)**
Imager 1 (Cetuximab), ***a'***	TCA TAC GAC TCA CTC TAG GGT T/3Cy5Sp/	22
Docking 2 (aCD45), ***b***	/5AmMC6/AC TGA CTG ACT GAC TGA CTG	20
Imager 2 (aCD45), ***b'***	/5Cy5/CA GTC AGT CAG TCA GTC AGT	20
Docking 3 (aCD31), ***c***	GTC CAT GCT CAG GAT TGC GA /3AmMO/	20
Imager 3 (aCD31), ***c'***	TCG CAA TCC TGA GCA TGG ACT T /3Cy5Sp/	22

DNA-PAINT & dSTORM

Name	**Sequence (5' to 3')**	**Length (nt)**
Docking 4 (DNA-PAINT)	/5AmMC6/TTA TAC ATC TA	11
Imager 4 (DNA-PAINT, c.[1])	CTA GAT GTA T/3ATTO647NN/	10
Imager 5 (DNA-PAINT, n.c. [2])	TAT GTA GAT C/3ATTO647NN/	10
Docking 5 (dSTORM, n.c.[2])	TTA TAC ATC TAG TCG TGT GA/3AmMO/	20

[1] complementary
[2] non-complementary

For dSTORM imaging docking 1 and imager 1 were used to generate figure 5.

Table S2: Protein G sequences

The single-letter amino acid code is shown in uppercase above the corresponding DNA sequence. The cysteine is shown in purple, the *strep*-tag in orange, pG in blue, the hexahistidine tag in green and the amber stop codon, coding for the non-natural amino acid *p*-Bpa, is indicated in red.

pG-C2:

```
      M  C  W  S  H  P  Q  F  E  K  G  T  M  T  F  K  L  I  I  N
1     ATGTGCTGGTCCCATCCGCAGTTCGAGAAAGGTACCATGACATTTAAACTGATAATCAAC

      G  K  T  L  K  G  E  I  T  I  E  A  V  D  A  *  E  A  E  K
61    GGCAAAACCTTAAAAGGGGAGATCACAATTGAGGCAGTCGATGCCTAGGAAGCCGAGAAA

      I  F  K  Q  Y  A  N  D  Y  G  I  D  G  E  W  T  Y  D  D  A
121   ATCTTTAAACAATATGCTAATGATTATGGTATTGACGGAGAATGGACGTATGACGATGCG

      T  K  T  F  T  V  T  E  E  F  T  S  G  G  S  G  D  D  H  H
181   ACAAAAACTTTCACCGTAACTGAGGAATTCACTAGTGGTGGAAGTGGGGACGATCATCAT

      H  H  H  H  *
241   CATCATCATCATTAA
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pG-C3:

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      M  S  C  W  S  H  P  Q  F  E  K  G  T  M  T  F  K  L  I  I
1     ATGAGTTGCTGGTCCCATCCGCAGTTCGAGAAAGGTACCATGACATTTAAACTGATAATC

      N  G  K  T  L  K  G  E  I  T  I  E  A  V  D  A  *  E  A  E
61    AACGGCAAAACCTTAAAAGGGGAGATCACAATTGAGGCAGTCGATGCCTAGGAAGCCGAG

      K  I  F  K  Q  Y  A  N  D  Y  G  I  D  G  E  W  T  Y  D  D
121   AAAATCTTTAAACAATATGCTAATGATTATGGTATTGACGGAGAATGGACGTATGACGAT

      A  T  K  T  F  T  V  T  E  E  F  T  S  G  G  S  G  D  D  H
181   GCGACAAAAACTTTCACCGTAACTGAGGAATTCACTAGTGGTGGAAGTGGGGACGATCAT

      H  H  H  H  H  *
241   CATCATCATCATCATTAA
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