Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information

DNA-Free Directed Assembly in Single-Molecule Cut-and-Paste

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1. Protein Expression, Purification, and Labelling

1.a. Preparation of Monovalent Streptavidin

Monovalent Streptavidin (mSA) with N-terminal immobilisation (N-mSA) was previously expressed, purified and assembled by Sedlak *et al*.[1] C-terminally immobilised mSA (C-mSA) was created here using the same protocol. In brief, three different streptavidin subunits were designed: a functional subunit with a polyhistidine tag and a single cysteine at its N-terminus, a functional subunit with a polyhistidine tag and a single cysteine at its C-terminus, and a non-functional subunit (N23A, S27D, S45A).[2]

Functional SA subunit with C-terminal cysteine (orange) and His-tag (green):

MEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYDSAPATDGSGTALGWTVA WKNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTTEANAWKSTLVGHDTFTKVKPSAASCLEH HHHHH

Functional SA subunit with N-terminal cysteine (orange) and His-tag (green):

MGSSHHHHHHMCGSEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYDSAP ATDGSGTALGWTVAWKNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTTEANAWKSTLVGHD TFTKVKPSAAS

Non-functional SA subunit (mutated residues in red):

MEAGITGTWYAQLGDTFIVTAGADGALTGTYEAAVGNAESRYVLTGRYDSAPATDGSGTALGWTVA WKNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTTEANAWKSTLVGHDTFTKVKPSAAS

The three different subunits were cloned into pET vectors and expressed separately in *E.coli* BL21(DE3)-CodonPlus. In the following steps, the different subunits were treated separately: Each harvested cell pellet was dissolved in B-PER reagent. Lysozyme and DNAse I was added. Full cell lysis was achieved by sonication. Inclusion bodies formed and were regained by centrifuging the solution at 20,000 x g for 30 min and discarding the supernatant. The inclusion bodies containing pellet was suspended in washing buffer (phosphate buffered saline, 0.1% Triton X-100, 1 mM DTT). The centrifugation and washing was repeated until the supernatant was clear. The inclusion bodies were then dissolved in denaturation buffer (phosphate buffered saline, 6 M guanidine hydrochloride, pH 7.5). Non-functional and functional subunits (either with N- or C-terminal tags, again treated separately in the next steps) were mixed in a 10:1 ratio as given by the absorption at 280 nm. Refolding into streptavidin tetramers was accomplished by slowly dissolving the mixtures in 500 ml refolding buffer (phosphate buffered saline, 10 mM beta-mercaptoethanol) and stirring it at 4°C overnight. The refolding solution was centrifuged to remove precipitated protein, filtered with a 0.22 µm cellulose filter, and loaded onto a 5 ml HisTrap FF column (GE Healthcare Life Sciences, Little Chalfont, UK). Monovalent streptavidin was eluted from the column using a linear gradient from 10 mM to 250 mM imidazole. Elution fractions were analysed by gel electrophoresis. The eluted monovalent streptavidin was dialysed against phosphate buffered saline, pH 7.4 and stored at 4°C.

1.b. Preparation of N-SdrG

SdrG N2N3 was expressed and purified as previously described by Milles *et al*.[3] In brief, ybbr-SdrG-6xHIS was expressed in a pET28a vector with a 6xHis-tag and an N-terminal ybbR tag for covalent immobilisation to Coenzyme A.

The protein sequence is (ybbR tag in blue, His tag in green):

MATDSLEFIASKLATEQGSNVNHLIKVTDQSITEGYDDSDGIIKAHDAENLIYDVTFEVDDKVKSGDTM TVNIDKNTVPSDLTDSFAIPKIKDNSGEIIATGTYDNTNKQITYTFTDYVDKYENIKAHLKLTSYIDKSKVP NNNTKLDVEYKTALSSVNKTITVEYQKPNENRTANLQSMFTNIDTKNHTVEQTIYINPLRYSAKETNVN ISGNGDEGSTIIDDSTIIKVYKVGDNQNLPDSNRIYDYSEYEDVTNDDYAQLGNNNDVNINFGNIDSPY IIKVISKYDPNKDDYTTIQQTVTMQTTINEYTGEFRTASYDNTIAFSTSSGQGQGDLPPEKTELKLPRSR HHHHHH

A 5 ml preculture of LB medium containing 50 µg/ml Kanamycin grown overnight at 37°C was inoculated in 200 ml ZYM-5052 autoinduction medium[4] containing 100 µg/ml Kanamycin and grown at 37°C for 6 h, then at 18°C overnight. Cells were harvested by centrifugation at 8,000 x g, and pellets were stored frozen at -80°C until purification.

All purification steps were performed at 4°C or on ice when possible. The bacteria pellet was resuspended in a Lysis Buffer and cells were lysed through sonication followed by centrifugation at 40,000 x g for 45 min. The supernatant was applied to a Ni-NTA column for purification by Ni-IMAC and eluted with a buffer containing 200 mM imidazole. Protein-containing fractions were concentrated in centrifugal filters, exchanged into measurement buffer by desalting columns, and frozen in aliquots with 10% (v/v) glycerol in liquid nitrogen

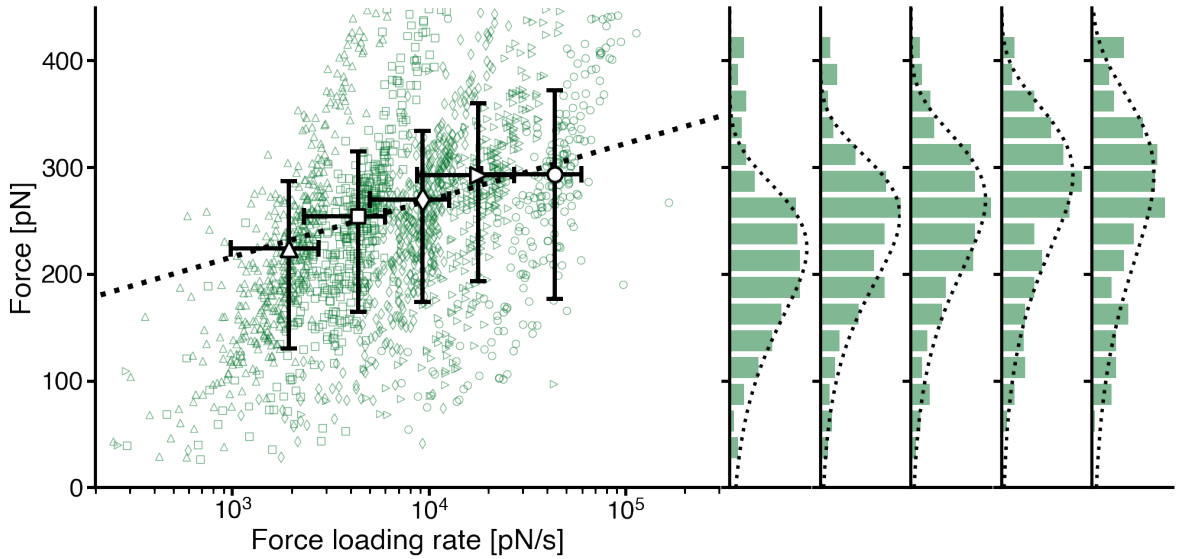

Figure S1. Dynamic force spectrum of the rupture of N-terminally immobilised SdrG and an N-terminal (*i.e.* C-terminally pulled) Fgβ-tag measured with AFM-based SMFS at retraction velocities from left to right: 200 nm/s, 400 nm/s, 800 nm/s, 1600 nm/s, 3200 nm/s. In this geometry, the N-SdrG:Fgβ bond ruptures between approximately 200-300 pN, depending on the loading rate.

to be stored at -80°C until used in experiments. The final protein concentration was 848 M as measured by the absorbance at 280 nm via NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

N-terminally immobilised SdrG bound to its target peptide ligand Fgβ as an N-terminal tag was additionally probed with AFM-based SMFS to acquire a dynamic force spectrum of the rupture force.

1.c. Preparation ddFLN4 Transfer Protein

A transfer construct whose main fold consists of the fourth filamin domain from *Dictyostelium discoideum* (ddFLN4) with a crucial C18S mutation to prevent disulfide bond formation and undesired reaction to the Maleimide-dye used here was designed with several handling and purification tags. The construct harbours an N-terminal Fgβ-tag (NEEGFFSARGHRPLD) to enable direct binding to SdrG. An internal 6xHis tag was included for purification by Ni-IMAC. The construct also harbours an internal ybbR-tag (DSLEFIASKLA) to covalently modify the protein with Biotin. A Sortase tag (LPETGG) was also included,

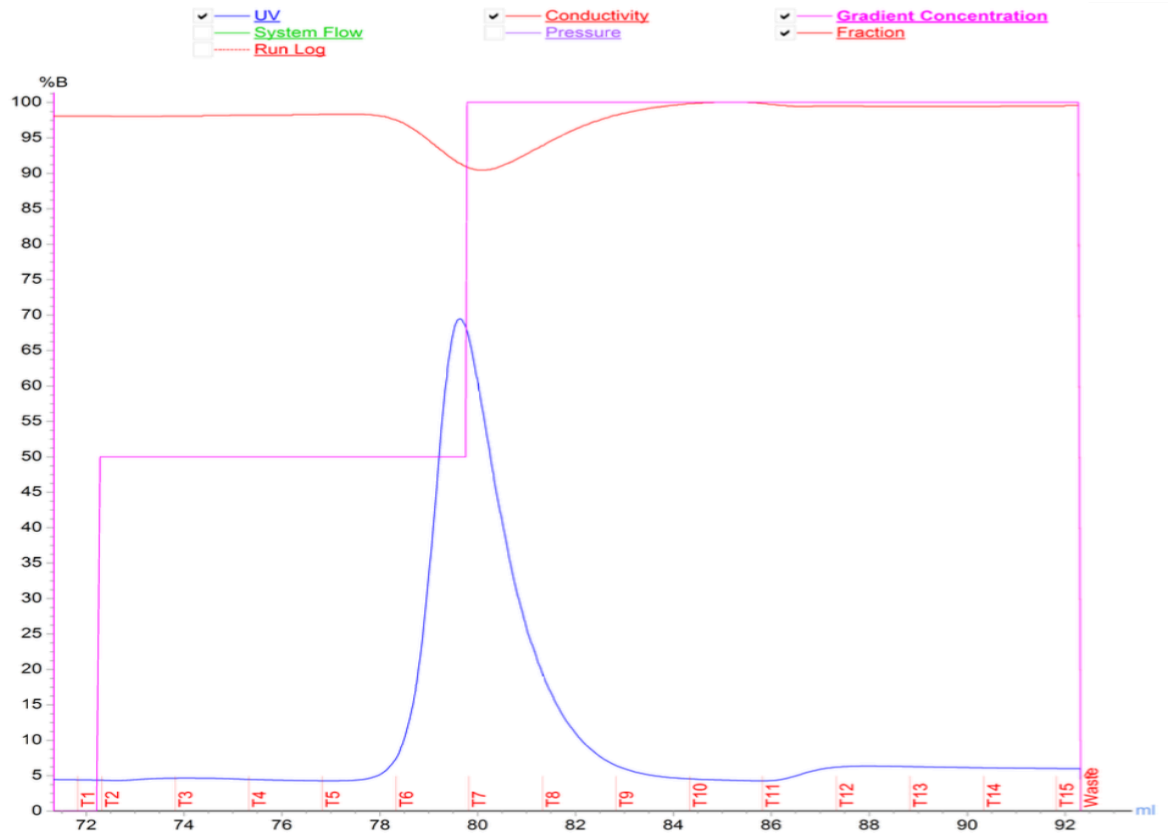

Figure S2. Chromatogram of ddFLN4 construct purification by Ni-IMAC. The 6xHis-tagged protein was purified by step gradient and eluted with high imidazole. Fractions 6-8 span the majority of the major peak, with a smaller peak spanning fractions 11-13. Note that there is a 6 ml delay in reported percentage elution buffer as the program does not take into account tubing length and column dead volume. Therefore, the major peak is observed at 50% elution buffer, or 135 mM imidazole.

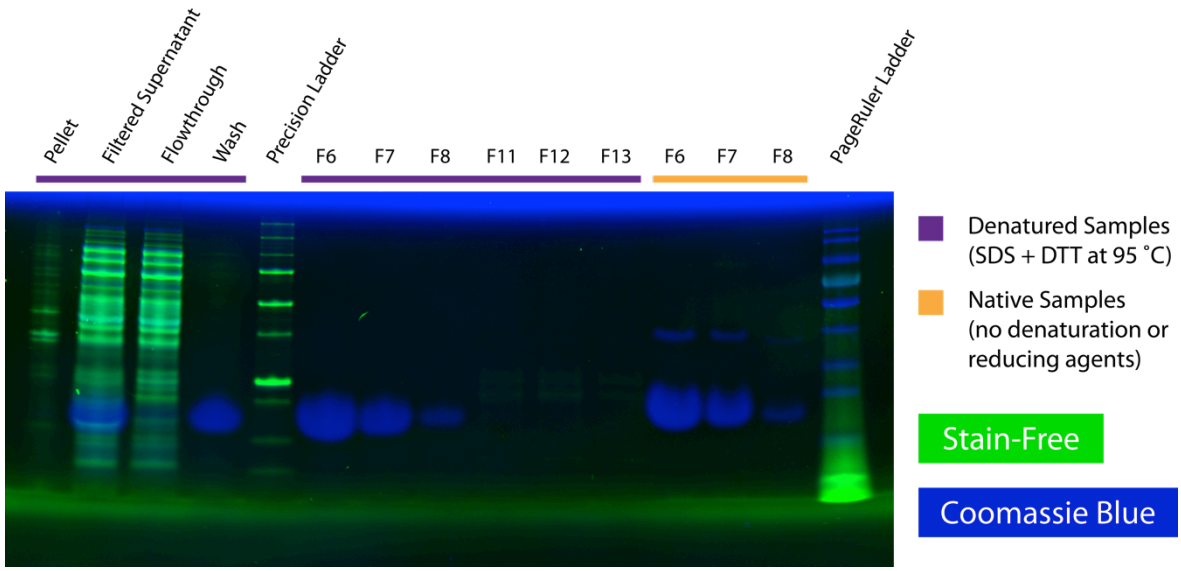

Figure S3. SDS-PAGE and Native PAGE analysis of the ddFLN4 protein construct purification by Ni-IMAC. The ddFLN4 construct was obtained in the soluble fraction and efficiently eluted by step gradient purification. Fractions 6-8 contained the majority of the protein, visible by the overloaded blue bands. These fractions were additionally analysed under native conditions to examine the extent of dimerisation via C-terminal cysteine. Most of the protein is in a reduced state and therefore does not require further reduction before maleimide-Cy5 coupling. Precision Unstained Ladder (Bio Rad) and PageRuler Prestained Ladder (Thermo Fisher Scientific) were used as molecular mass markers

although not directly utilised in this work. Lastly, a C-terminal cysteine (Cys) enabled covalent modification with Cy5. The ddFLN4 gene was PCR amplified from a synthetic template with primers containing the respective tag coding sequences. The construct was cloned into a modified pET28a vector (GE Healthcare Life Sciences, Little Chalfont, UK). The resulting fusion protein (Fgβ-ddFLN4-6xHis-ybbR-LPETGG-Cys) was expressed in *E.coli* Nico(DE3)-RIPL cells. A preculture of 5 ml LB containing 50 µg/ml Kanamycin was grown overnight at 37°C for 16 h. The preculture was then inoculated in 500 ml of ZYM-5052 autoinduction medium[4] containing 100 µg/ml Kanamycin and grown at 37°C for 20 h.

All purification steps were performed at 4°C or on ice when possible. Following expression, cells were separated from the medium by centrifugation at 500 x g for 20 min. Cells were then resuspended in His Lysis Buffer (30 mM Tris-HCl pH 7.8, 150 mM NaCl, 20 mM imidazole) and lysed by pulse sonication. The soluble fraction and insoluble fractions were separated by centrifugation at 20,000 x g for 45 min. The transfer construct was obtained in the soluble fraction and filtered with a 0.22 µm syringe filter. The filtered supernatant was purified by Ni-IMAC on a 5 ml HisTrap HP Ni-NTA column (GE Healthcare Life Sciences, Little Chalfont, UK) via step gradient elution from 20 mM to 250 mM imidazole (His Elution Buffer: 30 mM Tris-HCl pH 7.8, 150 mM NaCl, 250 mM imidazole) using an Äkta Start HPLC (GE LifeSciences, Little Chalfont, UK), producing a chromatogram with a single major peak (Figure S2).

Selected fractions from the major peak in the chromatogram were analysed by SDS-PAGE and Native PAGE. Samples were loaded to a Mini-PROTEAN TGX Stain-Free Precast Gel (Bio-

Rad Laboratories, CA, USA), which contains within its matrix a proprietary imaging molecule that is activated by exposure to UV light and then specifically labels tryptophan residues. While this imaging method is much faster than traditional coomassie staining, proteins that have no tryptophan residues (such as the ddFLN4 construct) do not produce a signal. Therefore, after first imaging with the stain-free method, the gel was additionally stained with coomassie blue. This has the advantage of enabling direct discrimination between the ddFLN4 construct and other co-eluting proteins. Gels were imaged with a ChemiDoc MP (Bio-Rad Laboratories) using stain-free imaging as well as and coomassie blue imaging. The images were overlaid using Image Lab software (Bio-Rad Laboratories).

Glycerol (10% v/v final concentration) was directly added to fractions 6-8 of purified protein. Reducing agents were omitted, as their presence would presumably interfere with cysteine-maleimide coupling later. The protein was finally stored at -80°C at a final concentration of about 700 µM as measured by the absorbance at 280 nm via NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

1.d. Cy5- and Biotin-Labelling of ddFLN4

The ddFLN4 transfer construct was modified first with Cy5 in a cysteine-maleimide reaction, followed by Biotin in an Sfp-catalysed transferase reaction. Cy5 Maleimide Mono-Reactive Dye (Mal-Cy5, Sigma Aldrich) was dissolved in DMSO to a stock concentration of 5 mM and stored at -20°C. The cysteine-maleimide reaction consisted of 7 nmol of purified ddFLN4 transfer construct protein and 50 nmol Mal-Cy5 in 1x Cysteine-Maleimide Reaction

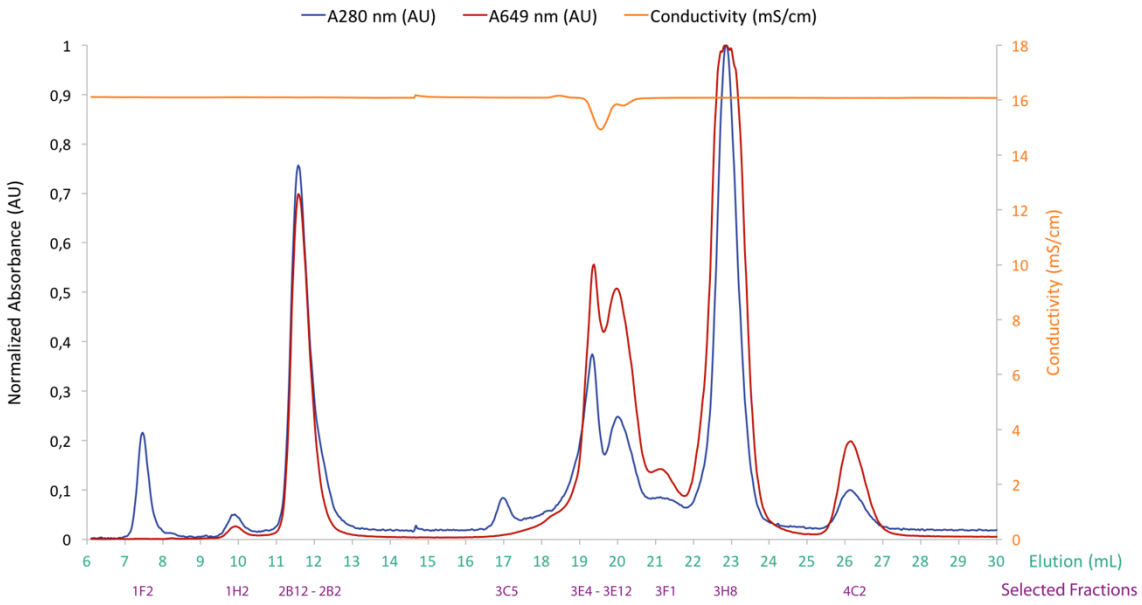

Figure S4. Chromatogram of Cy5- and biotin-labelled ddFLN4 transfer construct purification by size-exclusion chromatography. The absorbance at 280 nm and 649 nm is used to estimate the concentrations of protein and Cy5, respectively. Selected fractions (bottom purple labels) were further analysed by SDS-PAGE

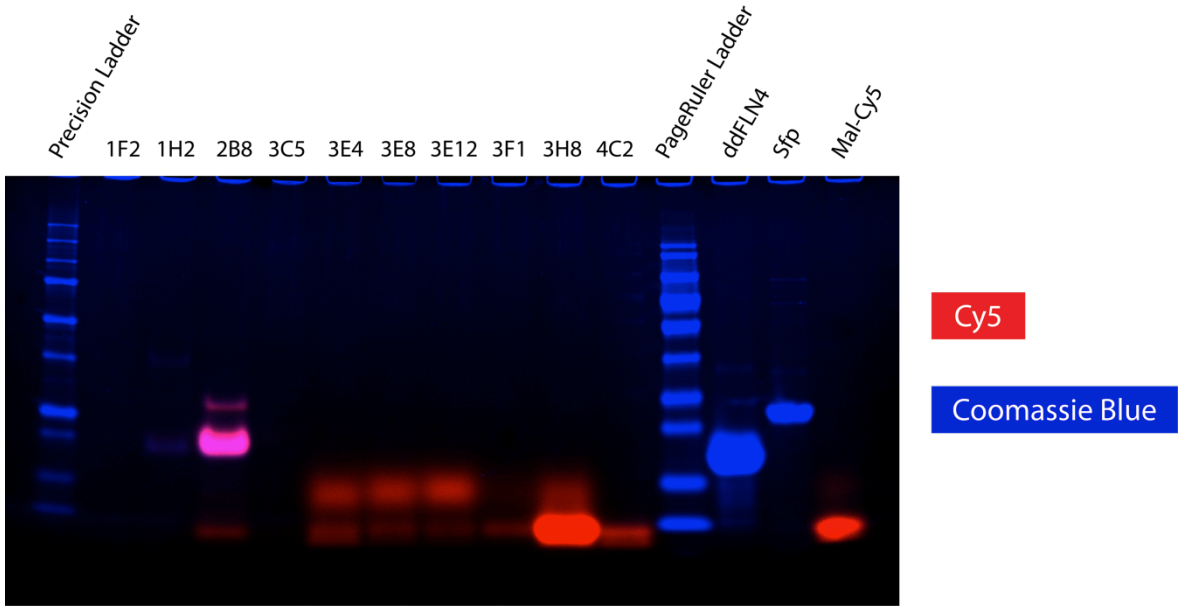

Figure S5. SDS-PAGE analysis of selected fractions of labelled ddFLN4 transfer construct purification. In addition to fractions from size-exclusion chromatography, samples of unreacted ddFLN4, Sfp and Mal-Cy5 were included as controls. The major peak spanning fractions 2B12-2B2 contained a high concentration of Cy5-labelled ddFLN4 as well as a small amount of co-eluted Cy5-labelled Sfp by-product.

Buffer (30 mM Tris-HCl pH 7.2, 150 mM NaCl) in a total volume of 40 µl at room temperature. The reaction was incubated at room temperature for 1 h followed by overnight incubation at 4°C. Subsequently, 9 nmol CoA-Biotin (Sigma Aldrich) and 1 nmol Sfp transferase were added to the reaction volume. Sfp Buffer Reaction Buffer was added to a 1x concentration (120 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 2% Glycerol, 2 mM DTT) to give a final total volume of 100 µl. The reaction was incubated at 37°C for 1 h and then overnight at 4°C.

In order to isolate the dual-labelled ddFLN4 transfer construct, the reaction volume was purified by size-exclusion chromatography using an Äkta Explorer HPLC (GE LifeSciences, Little Chalfont, UK). A Superdex 75 Increase 10/300 GL column (GE Healthcare Life Sciences, Little Chalfont, UK) was first equilibrated with Size Exclusion Buffer (50 mM HEPES pH 7.5, 200 mM NaCl, 10% v/v glycerol). The unpurified protein was loaded to the column and eluted in Size Exclusion Buffer. Chromatograms of the absorbance at 280 nm and 649 nm were collected during purification (Figure S4), and fractions were collected in 100 µl increments.

Fractions from the most prominent peaks were analysed via SDS-PAGE to determine which peak contained Cy5-labelled ddFLN4 transfer construct (Figure S4). Gels were imaged with a ChemiDoc MP (Bio-Rad Laboratories) using Epi-red LED excitation and 695/55 nm emission filter to detect Cy5 and coomassie blue imaging for protein detection. The images were overlaid using Image Lab software (Bio-Rad Laboratories). The major peak was further analysed to identify the optimal fractions (Figure S5). Fractions from the major peak as well as secondary peaks were also assessed for Biotin labelling via Native PAGE (Figure S6).

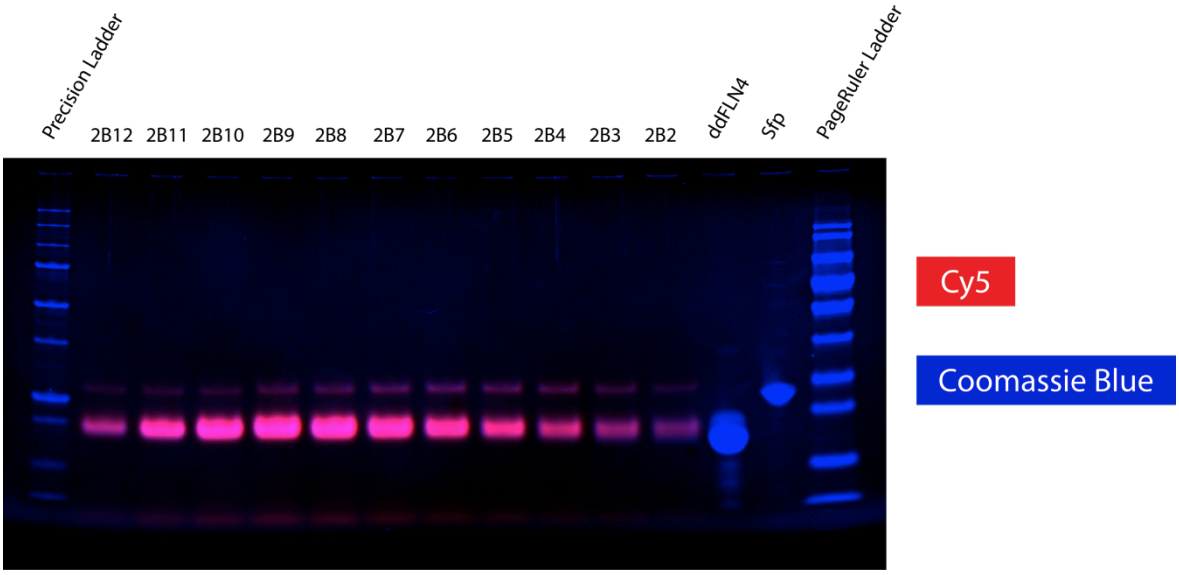

Figure S6. SDS-PAGE analysis of selected fractions from the major peak of labelled ddFLN4 transfer construct purification. In addition to fractions from size-exclusion chromatography, samples of unreacted ddFLN4 and Sfp were included as controls. The fractions contain a high concentration of labelled ddFLN4 as well as a lesser amount of co-eluted Cy5-labelled Sfp.

Selected fractions that demonstrated efficient labelling with both Cy5 and Biotin were pooled (2B12-2B10; 2B9-2B7; 2B6-2B3) and stored at -80°C at a final concentration of about 1 µM as measured by the absorbance at 649 nm via NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

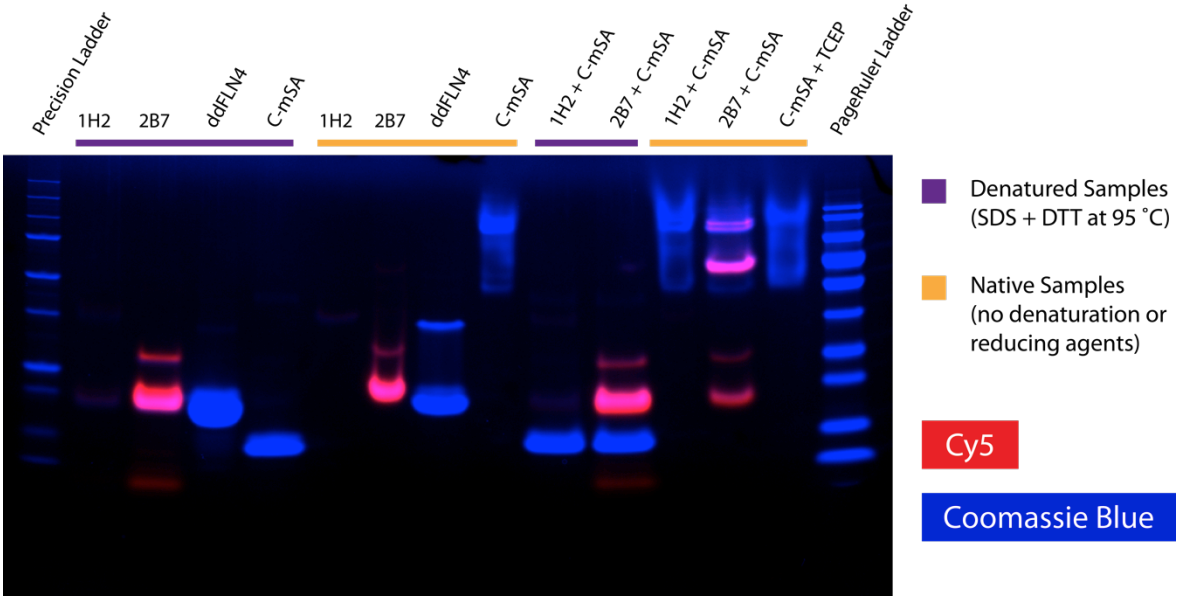

Figure S7. SDS-PAGE and Native PAGE analysis of selected fractions from labelled ddFLN4 transfer construct purification and binding to C-mSA. Fraction 2B7 from the major peak of Cy5-labelled ddFLN4 binds C-mSA under native conditions, visible by a band-shift in both Cy5-labelled ddFLN4 and C-mSA. This strongly suggests that at least the majority of Cy5-labelled ddFLN4 is also biotin-labelled.

2. Surface preparation

2.a. Preparation of Cantilevers

Cantilevers (BL-AC40TS, BioLever mini, Olympus, Japan) were oxidised in a UVOH 150 LAB UV-ozone cleaner (FHR Anlagenbau GmbH, Ottendorf-Okrilla, Germany). Silanisation was accomplished by incubating the cantilevers in (3-Aminopropyl)dimethylethoxysilane (ABCR, Karlsruhe, Germany, 50% v/v in Ethanol) for 2 min. Cantilevers were washed in toluene, then in isopropanol, and finally in ultrapure water and finally baked at 80°C for 45 min. For 30 minutes, the silanised cantilevers were placed in 25 mM heterobifunctional polyethylene glycol crosslinkers of 5,000 Da molecular weight (Rapp Polymere, Tübingen, Germany) dissolved in 50 mM HEPES at pH 7.5. The amines on the cantilevers reacted with the N-hydroxy succinimide on the one end of the crosslinkers. Using ultrapure water unreacted crosslinkers were washed off, before the cantilevers were placed in 1 mM Coenzyme A dissolved in coupling buffer (50 mM sodium phosphate, 50 mM sodium chloride, 10 mM EDTA, pH 7.2) for one hour. The maleimide on the other end of the PEG crosslinker and the thiol of the Coenzyme A formed a stable thioester bond. Unreacted Coenzyme A was washed off by ultrapure water. For several hours, the Coenzyme A-coated cantilevers were incubated with an Sfp-reaction mix containing 85 µM ybbR-SdrG, 3 µM Sfp Synthase, 10 mM magnesium chloride and 50 mM HEPES at pH 7.5. Sfp Synthase covalently joins Coenzyme A on the surface and the ybbR-tagged proteins.[5] The functionalised cantilevers were washed and stored in phosphate buffered saline.

2.b. Preparation of Glass Surfaces

Glass cover slips were sonicated in 50% (v/v) 2-propanol in filtered H_2O for 15 min and oxidised in a solution of 50% (v/v) hydrogen peroxide (30%) and sulfuric acid for 30 min. They were then washed in ddH_2O, dried in a nitrogen stream and then silanised by incubating for 1 h in (3-Aminopropyl)dimethylethoxysilane (ABCR, Karlsruhe, Germany, 1.8% v/v in Ethanol). The silanised surfaces were incubated in sodium borate buffer (150 mM, pH 8.5) for 30 min in order to deprotonate primary amine groups.

A PDMS microfluidic system – based on the system described by Kufer *et al.* [6] – was fixed on the aminosilanised glass and bonded briefly at 60°C for 10 min. The depot and target channels were incubated with a solution of a heterobifunctional PEG crosslinker [7, 8] with N-hydroxy succinimide and maleimide groups (molecular weight 5,000 Da, Rapp Polymere, Tübingen, Germany) dissolved to 30 mM in 100 mM HEPES pH 8.0 for 20 min. Unbound PEG was flushed from the channels with filtered H_2O.

Concurrently with assembling and functionalizing the microfluidics channels, Monovalent Streptavidin was reduced for covalent attachment to maleimide. Streptavidin with a reactive cysteine at the N-terminus (N-mSA) and at the C-terminus (C-mSA) was incubated in 5 mM TCEP at room temperature for 1 h, followed by buffer-exchange to PBS via Zeba

Spin Deslating Columns, 7K MWCO (Thermo Fisher Scientific, Waltham, MA, USA). Freshly reduced mSA was immediately applied to the PEG-functionalised microfluidic system, with N-mSA in the Depot channel and C-mSA in the Target channel.

N- and C-mSA were incubated in the channels for 1 h. Both channels were then flushed with filtered PBS to remove unbound mSA. The channels were then flushed with 0.1 mg/ml filtered BSA and 0.05% TWEEN20 in PBS to passivate the surface and discourage nonspecific adsorption. The labelled ddFLN4 transfer construct was diluted to an approximate concentration of 1 nM in PBS with 0.05 mg/ml BSA and 0.01% TWEEN20 and incubated in the depot channel for 1 h. The depot channel was then extensively flushed with PBS to clear the solution and remove unbound or non-specifically bound ddFLN4. The microfluidic system was then removed and the surface submerged in PBS.

3. Experimental Procedures

The experiments described in the manuscript were performed on an AFM/TIRFM hybrid, the details of which may be found in Gumpp *et al.*[9] This supporting information specifies methods, materials and additional data that are relevant for the conduction of the measurements discussed in the main text.

3.a. AFM Measurements

Measurements employed a custom-built AFM head and an Asylum Research MFP3D controller (Asylum Research, Santa Barbara, USA), which provides ADC and DAC channels as well as a DSP board for setting up feedback loops. Software for the automated control of the AFM head and xy-piezos during the force spectroscopy measurements was programmed in Igor Pro (Wave Metrics, Lake Oswego, USA). BioLever Mini (Olympus, Tokyo, Japan) cantilevers were chemically modified (see Preparation of Cantilevers) and calibrated in solution using the equipartition theorem.[10, 11] Pulling velocities were set to 3200 nm/s in the depot and 200 nm/s in the target area. The positioning feedback accuracy is ±3 nm. However, long-term deviations may arise due to thermal drift. Typical times for one Cut & Paste cycle amount to approximately 3 s in these experiments.

3.b. TIRF Microscopy

The fluorescence microscope of the hybrid instrument excites the sample through the objective in total internal reflection mode. A Nikon Apochromat 100x NA1.49 oil immersion objective (CFI Apochromat TIRF, Nikon, Japan) was employed. Laser excitation was achieved with a fiber-coupled Toptica iChrome MLE-LFA four-colour laser (Toptica Photonics, Gräfelfing, Germany), which is capable of emitting light at 405 nm, 488 nm, 561 nm and 640 nm through one single fiber mode. Specifically, red excitation at 640 nm with an

estimated intensity of approximately 10 W/cm^2 was utilised to monitor the Cy5 fluorescence. Emitted light from the sample was separated from the laser light with a Chroma quad line zt405/488/561/640rpc TIRF dichroic mirror (Chroma, Bellows Falls, VT, USA) and focused with a 20 cm tube lens. Separation of different emission wavelengths for simultaneous multicolour imaging was achieved by a Cairn Research Optosplit III (Cairn Research, Faversham, UK). Images were recorded with a back-illuminated Andor iXon DV860 DCS-BV EMCCD camera (Andor, Belfast, Ireland) in frame transfer mode with 1 MHz readout rate at a frame rate of 10 Hz. The camera was cooled and operated at -80°C. Fluorescent images were evaluated and processed with the analysis software ImageJ.

3.c. SMC&P Experiment

The rocket pattern was written in 442 transfer cycles with 200 nm spacing between each deposition point. The retraction veolcity in the depot was set to 3,200 nm/s and in the target to 200 nm/s. This corresponds to approximate surface contact times[12] (dependent on approach/retraction velocity, indentation force and substrate stiffness) of 5 ms and 80 ms, respectively, sufficient for ligand binding. Considering a single N-SdrG molecule being bound to the cantilever tip and estimating its localisation in a half sphere with r = 30 nm (approximate length of PEG5000 linker), the local concentration of SdrG would be in the μM range. This is several orders of magnitude higher than the measured K_d for the SdrG:Fgβ interaction (about 400 nM)[13] and the mSA:biotin interaction (<1 nM).[1] Taking further into account that bond formation is not diffusion-limited for the SMC&P experiment, successful attachment is very likely even at the given, short contact times.

4. Supplementary References

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