Supporting Information

A flow extension tethered particle motion assay for single-molecule proteolysis

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Table S1. Relationship between DNA substrate binding density and trajectory mobility

[substrate], pM	*Total beads per fov, count*[*]	*Trackable trajectories per fov, count*[*]	*Fully mobile trajectories*[*]
0.2	641 ± 93	298 ± 63	60.3 ± 6.5%
1	1,308 ± 218	418 ± 114	27.9 ± 9.3%
5	2,561 ± 202	1,038 ± 239	3.2 ± 1.4%
25	2,475 ± 409	1,206 ± 346	0.3 ± 0.1%

[*]n = 3, reported as mean ± S.E.M.

Table S2. Peptides, oligonucleotides[a], oligonucleotide-peptide conjugates[b], and fluorogenic peptides used in this study.

Biopolymer ID	*Sequence*	*Description*	*Source*
peptide 1	H_2N-GGG[K-(5-FAM)]GC-COOH	Sortase donor peptide with ε-[5-amino-fluorescein (5-FAM)]-Lys (6aa)	Genscript
peptide 2	H_2N-CNIPYKIEAVQSLPATGGH-COOH	Human Notch1 S2 containing peptide (19aa)	Genscript
peptide 3	H_2N-CGENLYFQGLPRTGGH-COOH	TEV consensus containing peptide (16 aa)	Genscript
oligo-peptide conjugate 1 (**1**)	H_2N-GGG{K-(5-FAM)}G{C-[SMCC-HN-(oligo 1)-3']}-COOH	Polyglycine-oligonucleotide Sortase donor peptide (6 aa-17 nt)	This study
oligo-peptide conjugate 2-N (**2**)	H_2N-CNIPYKIEAVQSLPAT-GGG[K-(5-FAM)]G{C-[SMCC-HN-(oligo 1)-3']}-COOH	Notch1 S2 oligo-peptide conjugate, intermediate (20 nt-23 aa-17 nt)	This study
oligo-peptide conjugate 2-T (**2**)	H_2N-CGENLYFQGLPRT-GGG{K-(5-FAM)}G{C-[SMCC-HN-(oligo 1)-3']}-COOH	TEV oligo-peptide conjugate, intermediate (20 nt-19 aa-17 nt)	This study
oligo-peptide-conjugate 3-N (**3**)	H_2N-{[3'-(oligo 2)-NH-SMCC]-C}-NIPYKIEAVQSLPAT-GGG{K-(5-FAM)}G{C-	Notch1 S2 double oligo-peptide conjugate (20 nt-23 aa-17 nt)	This study

	(SMCC-HN-(oligo 1)-3')}-COOH		
oligo-peptide conjugate 3-T (**3**)	H_2N-{[3'-(oligo 2)-NH-SMCC]-Cys}-GENLYFQGLPRTGGG[K-(5-FAM)]G{C-[SMCC-HN-(oligo 1)-3']}-COOH	TEV double oligo-peptide conjugate (20 nt-19 aa-17 nt)	This study
oligonucleotide 1	/5AmMC6/GTAAAACGACGGCCAGT	5'-amino-oligonucleotide, from M13F sequencing primer (17 nt)	IDT
activated oligonucleotide 1	4-(*N*-maleimidomethyl)-cyclohexane-1-amide-HN-5'-(oligo 1)	5'-maleimide-oligonucleotide 1 (17 nt)	This study
oligonucleotide 2	/5AmMC6/G*TCAGAGGTGGCGAAACCC	5'-amino-oligonucleotide, derived from pUC19 vector *ori* (19 nt) * indicates phosphoramidite bond	IDT
activated oligonucleotide 2	4-(*N*-maleimidomethyl)-cyclohexane-1-amide-HN-5'-(oligo 2)	5'-maleimide-oligonucleotide 2 (20 nt)	This study
oligonucleotide 3	CTAGACTGGCCGTCGTTTTAC	5' XbaI overhang-containing bridging oligonucleotide, contains the reverse-complement to M13F primer (oligo 1) (21 nt)	IDT

oligonucleotide 4	/5DigN/CAGGGTCGGAACAGGAGAGC	5'-digoxigenin-oligonicleotide from the pUC19 *ori*, HPLC purified (20 nt)	IDT
oligonucleotide 5	GCTTAATTAACGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTG	Forward strand of the 100 bp duplex from pUC19 *ori*, with 3' overhang complimentary to dig-oligonucleotide 4, contains a unique PacI site (81 nt)	IDT
oligonucleotide 6	GCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGTTAATTAAGCGGTTTCGCCACCTCTGAC	Reverse strand of the 100 bp duplex from pUC19 *ori* with 3' overhang complimentary to oligonucleotides 2 and 8, contains a unique PacI site (80 nt)	IDT
oligonucleotide 7	GGGCGGCGACCT/3BioTEG/	3'-biotin-oligonucleotide from λ phage cohesive site, cosR (12 nt)	IDT
oligonucleotide 8	CTAGGTCAGAGGTGGCGAAACCC	5' XbaI overhang-containing bridging oligonucleotide from pUC19 vector *ori*, for the all-DNA substrate (23 nt)	IDT

[a] Listed 5' to 3'. Purified by desalting except where HPLC purification is indicated.

[b] SMCC: the portion of crosslinker remaining after Sulfo-SMCC reaction with an amino oligo (R_1) and cysteine (C) containing peptide (R_2).

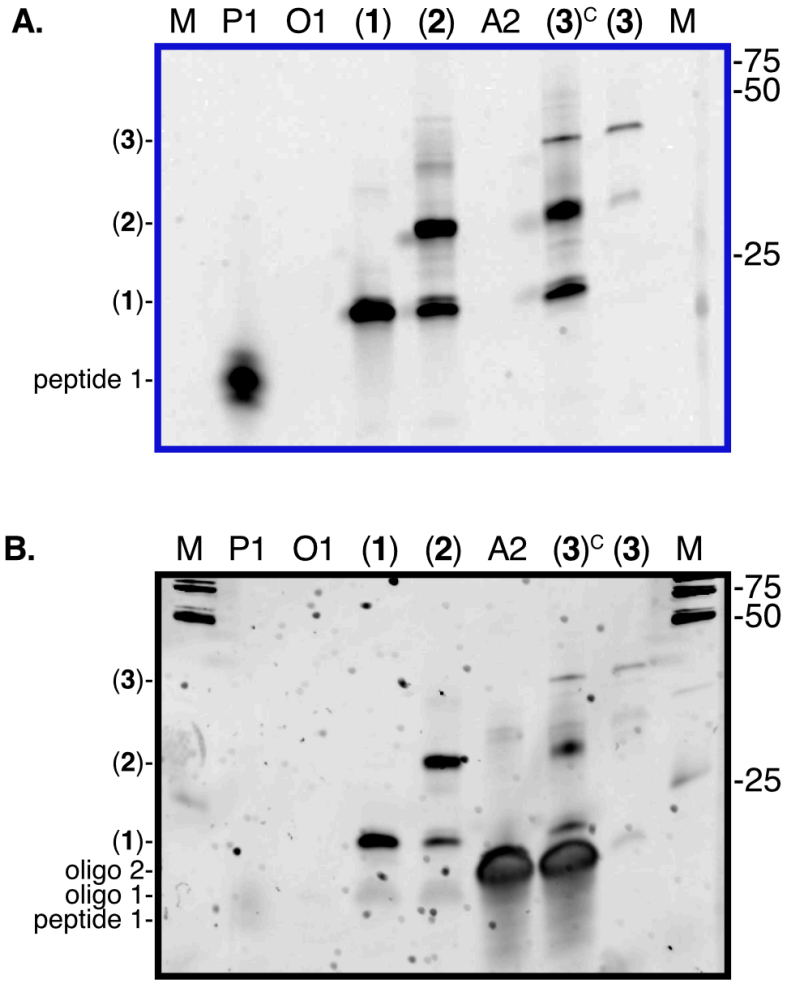

Figure S1. SDS-PAGE analysis of the double oligo-peptide conjugate intermediate for TEV cleavage. 15% TBE-Urea PAGE analysis showing: (A) in gel fluorescence of an unstained gel to detect the fluorescein label, and (B) in gel UV excitation fluorescence, of a SybrSafe stained gel to enable DNA detection. Abreviations above lanes: M, low MW DNA marker (NEB); P1, peptide 1; O1, oligonucleotide 1; (**1**) oligo-peptide conjguate 1; (**2**) partially purified oligo-peptide conjugate 2-T; A2, activated oligonucleotide 2; (**3**)^{C} crude double oligo-peptide conjugate 3-T; (**3**) double oligo-peptide conjugate 3-T.

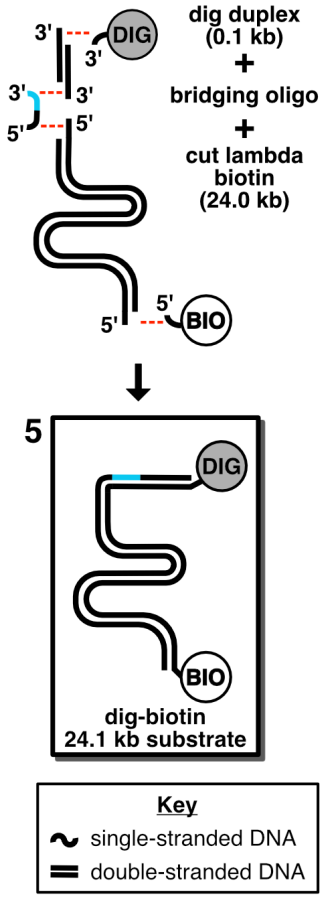

Figure S2. Scheme for assembly of DNA-only control substrate. Product (5) (boxed) is produced by annealing and ligation of a 5' digoxigenin-containing oligonucleotide, a short (100 bp) DNA duplex, a bridging oligonucleotide (oligonucleotide 8, cyan and black), the XbaI-cleaved 24.5 kbp fragment of phage λ, and a terminal biotinylated oligonucleotide.

Supporting Movie V1. Flow extension of all-DNA substrate (5) captured at 0.2 pM. The frame rate is 45 fps. Scale bar is 10 microns. Flow is from left to right. Flow starts at ~8 sec.

Supporting Movie V2. Single molecule proteolysis of TEV consensus DNA-peptide conjugate (4) with TEV protease. The frame rate is 45 fps. Scale bar is 10 microns. Flow is from left to right. Flow starts at ~8 sec. Cleavage starts at ~16 sec.