Supplementary Information

Effects of steric hindrance from single-stranded overhangs on target-strand loading into the Cas12a active site

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Materials and Methods

Expression and purification of AsCas12a

Competent BL21-Pro cells (CP111, Enzynomics) were transformed with pET28b-AsCas12a expression vector and single colonies were streaked on kanamycin agar plates. A single colony was inoculated into LB containing kanamycin and grown at 37 ℃ with shaking at 200 rpm up to OD_{600}~0.4-0.5. The 0.8mM IPTG-induced cells were incubated overnight at 18℃ with shaking at 200 rpm. Then, the cells were collected by centrifugation at 6,000 g for 10 min at 4 ℃ and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). The solution, to which a lysozyme and 1mM PMSF were added, was incubated on ice for 1 h. Cells were sonicated repeatedly (5 times) and the lysate was clarified by centrifuging at 18,000 g for 30 min at 4 ℃. The filtered supernatant with a 0.45-μm filter is incubated with Ni-NTA agarose for 1 h at 4 ℃ with rotation. Then, the resin was washed twice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole) and the AsCas12a recombinant proteins were eluted with elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole. The eluted protein was concentrated using a 100K MWCO concentrator and diluted with storage buffer (20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 2 % sucrose, and 20 % glycerol).

RNA and DNA preparation for single-molecule FRET measurements

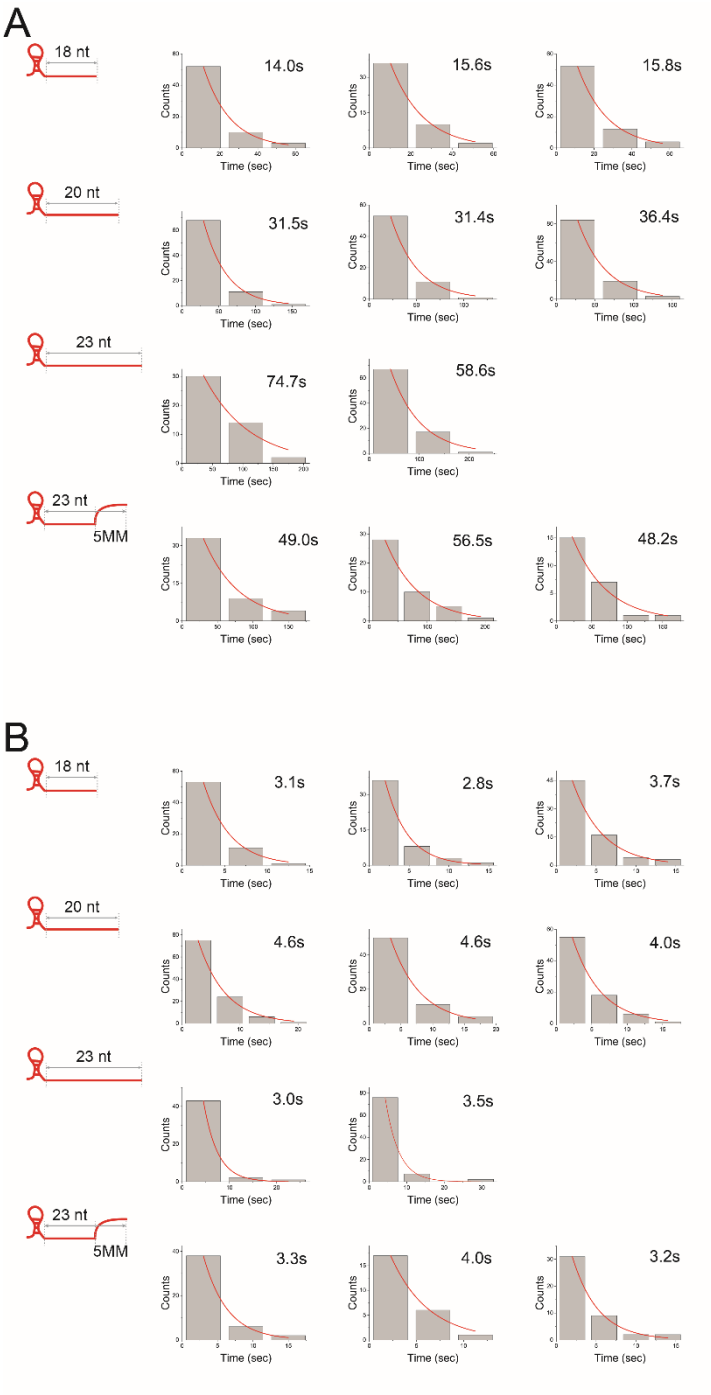
All RNA and DNA strands used in this study were purchased from Integrated DNA Technologies with HPLC purification and their sequence information is listed in Table S1. The RNA strands were labelled with Cy5 at the 3’ end of the strand. The target DNA strands were

composed of a non-target strand labelled with Cy3 at the amine group of an internal amino modifier (dTC6) and the complementary target strand was dual-labelled with biotin at the 5' end and Cy5 internally. To construct nicked duplexes, non-labelled non-target, labelled non-target and target strands in a 2:1:1 molar ratio were annealed in a buffer solution containing 10 mM Tris-HCl (pH 8.0) and 50 mM NaCl by cooling down slowly after incubation at 95 °C for 5 min.

Single-molecule FRET with alternating-laser excitation

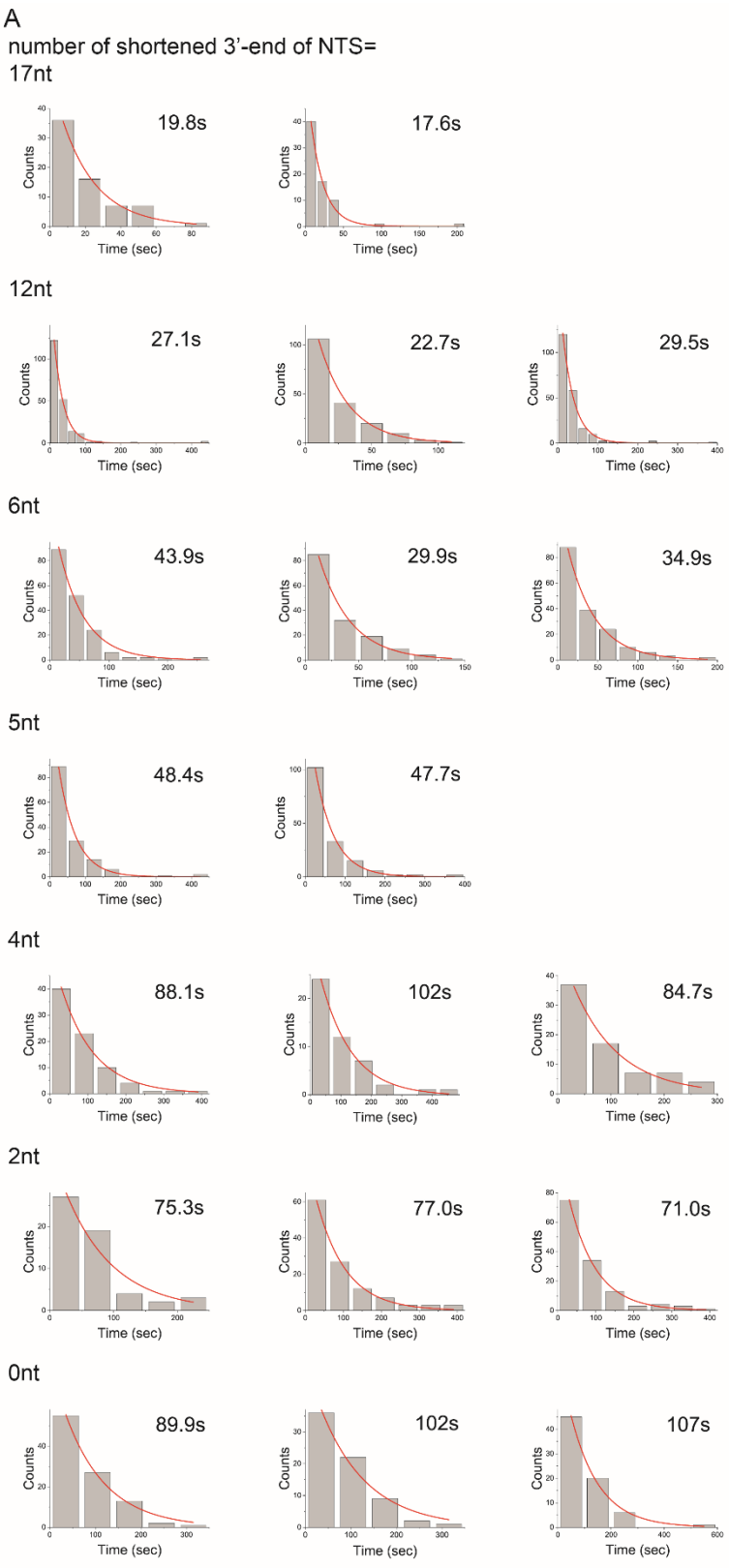
To prevent undesired adsorption of proteins to the surface of detection chamber, a quartz slide and coverslip were coated with PEG and biotin-PEG in a 40:1 ratio. A detection chamber was assembled with the quartz slide and coverslip using a double-sided adhesive tape. Through the streptavidin-biotin interaction, the biotinlayted DNA duplexes were immobilised on the PEG-coated surface. Experiments were performed at 37 °C with the buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.9 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$/$CaCl_2$, and an oxygen scavenging system (0.4 % (w/v) glucose, 1 % (v/v) Trolox, 1 mg/mL glucose oxidase, and 0.04 mg/mL catalase). 20 nM of pre-assembled Cas12a RNP was introduced into the chamber using a syringe pump while the fluorescence images were taken. Cy3 and Cy5 was alternately excited under the green (532-nm, Sapphire; Coherent) and red (637-nm, Obis; Coherent) lasers using mechanical shutters. Fluorescence signals collected through a 60x water immersion objective lens filtered through 532-nm and 633-nm notch filters, separated with a 635-nm dichroic mirror, and imaged onto an electron-multiplying charged device camera (Ixon Ultra DU897U, Andor).

Figure S1. Dwell-time histograms for U and B states of the target strand cleavage reaction by AsCas12a with crRNA variants



Histograms of dwell times for (A) U and (B) B states of the cleavage reaction by WT AsCas12a with crRNA variants. To obtain dwell time histograms, each event was collected from more than 48 time trajectories. Each histogram was fit to a single-exponential function to obtain corresponding dwell times. Experiments were performed with 20 nM AsCas12a RNP with 10 mM of $MgCl_2$.

Figure S2. Dwell-time histograms for U state of the target strand cleavage reaction by AsCas12a RNP with various lengths of cleaved 3' NTS overhang



Histograms of dwell times for U state of the cleavage reaction by WT AsCas12a RNP on target

DNAs with various lengths of cleaved 3’ NTS overhangs. To obtain dwell time histograms, each event was collected from more than 42 time trajectories. Each histogram was fit to a single-exponential function to obtain corresponding dwell times. Experiments were performed with 10 nM AsCas12a RNP with 10 mM of $MgCl_2$.

Table S1. RNA and DNA oligonucleotides used in this study

Name	Sequence
crRNA_18nt	AAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGU-Cy5
crRNA_20nt	AAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUA-Cy5
crRNA_23nt	AAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-Cy5
crRNA_23nt + 5MM	AAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUAUGAG-Cy5
TS w/ Cy5	Biotin- TTTTTTCACTTGACAGGCGAGTAACAGACATGGACCATCAGGAAACATTAACGT(Cy5)AC T
TS	Biotin- TTTTTTCACTTGACAGGCGAGTAACAGACATGGACCATCAGGAAACATTAACGTACT
NTS_PAM_distal	TTACTCGCCTGTCAAGT(Cy3)G
NTS_PAM_proximal_0 w/o Cy5	AGTACGTTAATGTTTCCTGATGGTCCATGTCTG
NTS_PAM_proximal_0	AG T(Cy5)ACGTTAATGTTTCCTGATGGTCCATGTCTG
NTS_PAM_proximal_2	AG T(Cy5)ACGTTAATGTTTCCTGATGGTCCATGTC
NTS_PAM_proximal_4	AG T(Cy5)ACGTTAATGTTTCCTGATGGTCCATG
NTS_PAM_proximal_5	AG T(Cy5)ACGTTAATGTTTCCTGATGGTCCAT
NTS_PAM_proximal_6	AG T(Cy5)ACGTTAATGTTTCCTGATGGTCCA
NTS_PAM_proximal_1 2	AG T(Cy5)ACGTTAATGTTTCCTGAT
NTS_PAM_proximal_1 7	AGT(Cy5)ACGTTAATGTTTC

* The annealed regions of the target DNA are colored blue.