

Supplementary Information

Exploring the dynamic nature of divalent metal ions involved in DNA cleavage by CRISPR-Cas12a

Heyjin Son,^{†a} Jaeil Park,^{†ab} You Hee Choi,^{ac} Youngri Jung,^d Joong-Wook Lee,^b Sangsu Bae^d
and Sanghwa Lee^{*a}

^aAdvanced Photonics Research Institute, Gwangju Institute of Science and Technology, Gwangju 61005, Republic of Korea

^bDepartment of Physics and Optoelectronics Convergence Research Center, Chonnam National University, Gwangju 61186, Republic of Korea.

^cMinistry of Food and Drug Safety (MFDS), Cheongju 28159, Republic of Korea.

^dDepartment of Chemistry, Hanyang University, Seoul 04763, Republic of Korea

[†]These authors contributed equally to this work.

*E-mail: sanglee@gist.ac.kr

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Materials and Methods

Expression and purification of Cas12a

Competent BL21-Pro cells (CP111, Enzynomics) were transformed with recombinant protein plasmids pET28b-AsCas12a and single colonies were streaked on agar plates with kanamycin. A single colony was inoculated into LB containing kanamycin and grown at 37 °C with shaking at 200 rpm to OD₆₀₀~0.4-0.5. The cells induced with 0.8mM IPTG were incubated overnight at 18°C with shaking at 200 rpm. Then, the cells were collected by centrifugation at 6,000 g for 10 min at 4 °C and resuspended in lysis buffer (50 mM NaH₂PO₄, 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 °C. The supernatant was filtered through a 0.45-µm filter and incubated with Ni-NTA agarose for 1 h at 4 °C with rotation. The resin was washed twice with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole) and the proteins were eluted with elution buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole. The eluted protein was concentrated using a protein concentrator (100K MWCO) and diluted with storage buffer (20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 2 % sucrose, and 20 % glycerol).

***In vitro* DNA cleavage assay**

Target plasmid pRG2 containing the cognate target sequence DNMT1 was linearized with restriction enzyme *ScaI* (New England Biolabs) and then purified using a PCR purification kit (GeneAll) before the cleavage assay. Next, 100 ng of the linearised plasmid DNA, 100 ng of

AsCas12a, and 35 ng of DNMT1 crRNA were incubated for 30min in 1x reaction buffer (100mM NaCl, 50 mM Tris-HCl, and 100 µg/ml BSA) with different divalent metal ions Mg²⁺ or Ca²⁺ (10 mM) in 15 µl reaction volume at 37 °C. After incubation, the sample, to which SDS and loading dye was added, was loaded into 1.5% agarose gel.

DNA and RNA preparation for single-molecule FRET

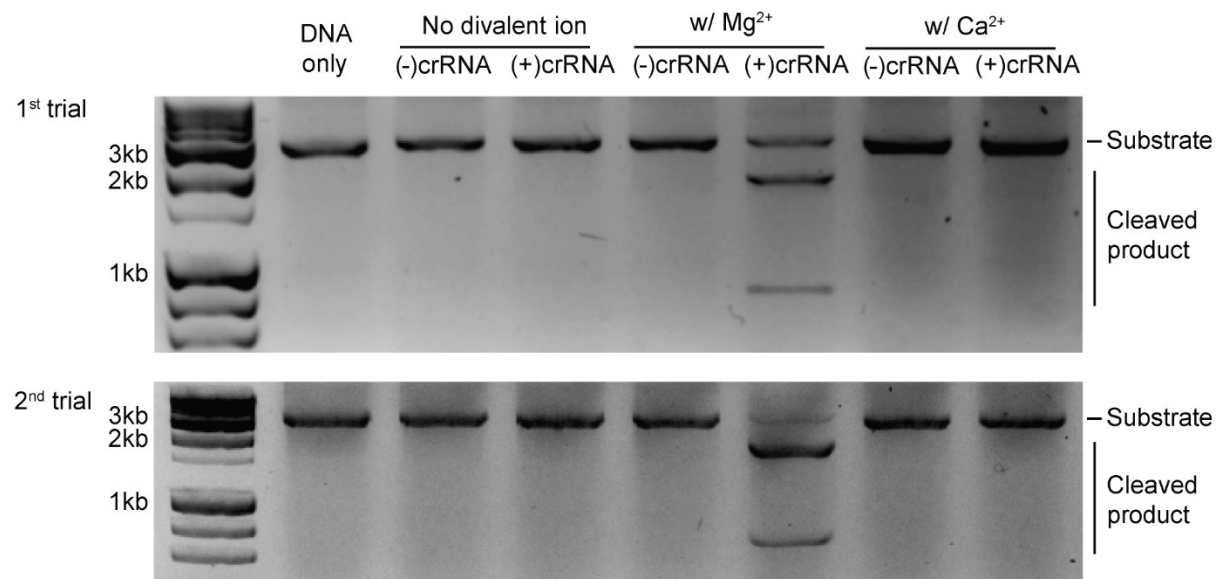
The RNA and DNA strands used in the single-molecule FRET experiment were purchased from Integrated DNA Technologies with HPLC purification and their sequence information is listed in Table S1. The purified RNA strands were labelled with Cy5 at the 3' end of the strand. The target DNA strands comprised a non-target strand labelled with Cy3 at the amine group of an internal amino modifier (dTc6) and the complementary target strand was labelled with biotin at the 5' end. To construct duplexes, non-target and target strands in a 2:1 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 experiments

Single-molecule FRET experiments were conducted using a detection chamber assembled using a quartz slide and coverslip. To prevent the non-specific adsorption of proteins, the quartz slides and coverslips were cleaned and coated with polyethylene glycol (PEG) and biotinylated polyethylene glycol in a 40:1 ratio. The biotinylated DNA duplexes were immobilised on the PEG-coated surface using the streptavidin-biotin interaction. All 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 MgCl₂/CaCl₂, and an oxygen scavenging system (0.4 % (w/v) glucose, 1 %

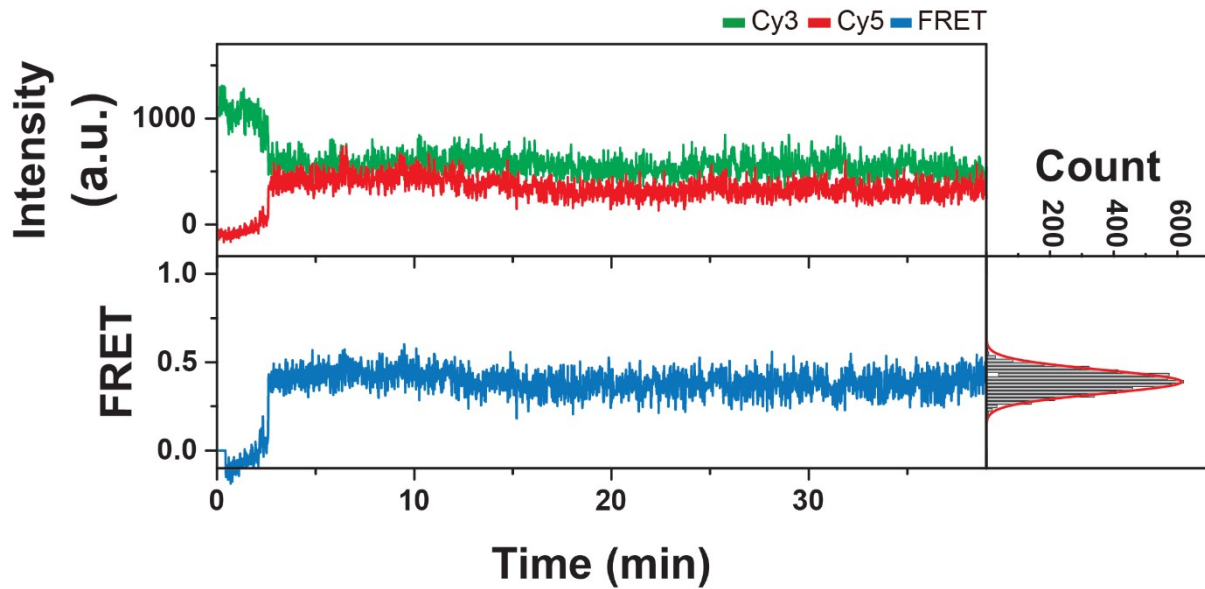
(v/v) Trolox, 1 mg/mL glucose oxidase, and 0.04 mg/mL catalase). Single-molecule fluorescence images were acquired by a home-built prism type total internal reflection (TIRF) microscope with 1 s time resolution. For real-time buffer exchange, the substituting buffer containing 20 nM of Cas12 RNP or 10 mM of MgCl₂ was introduced into the chamber using a syringe pump while fluorescence signals were being taken under excitation of Cy3 by a 532-nm laser. Cy3 and Cy5 signals were collected using a 60x water immersion objective lens filtered through a 532-nm notch filter, separated with a 635-nm dichroic mirror, and imaged onto an electron-multiplying charged device camera.

Figure S1. Effects of Mg²⁺ and Ca²⁺ ions on DNA cleavage by Cas12a RNP



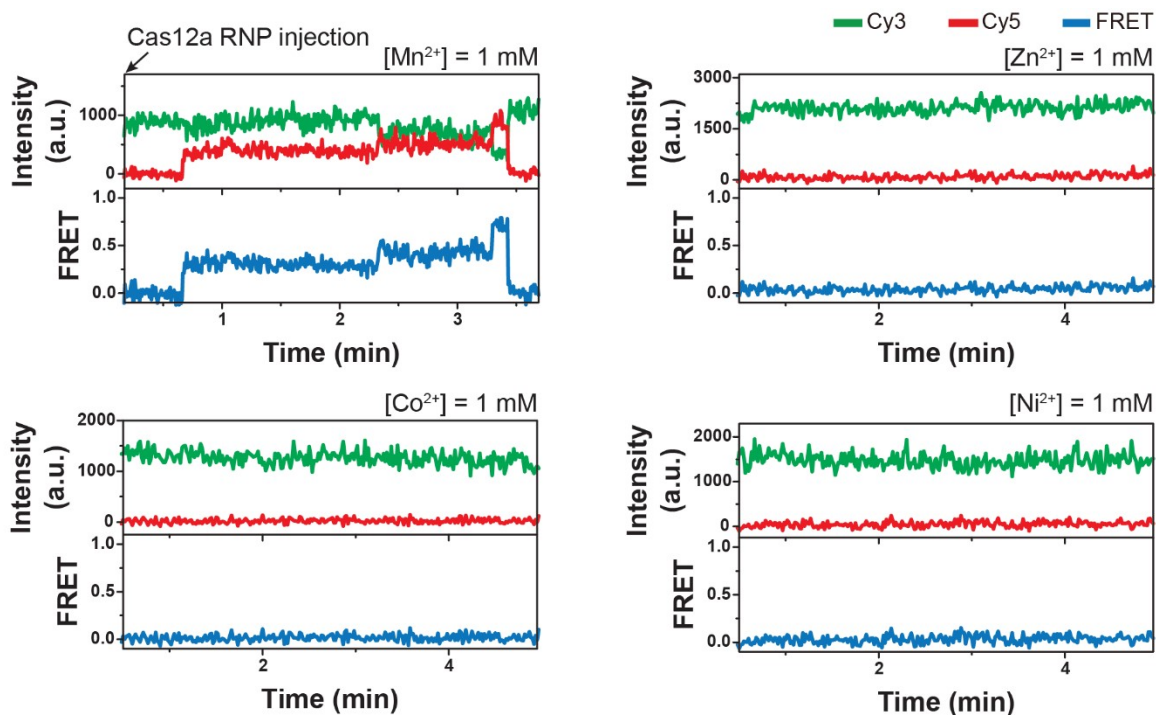
The influence of the divalent metal ions Mg²⁺ and Ca²⁺ on the DNA cleavage activity of Cas12a RNP was evaluated using agarose gel electrophoresis (1.5%) as described in the Materials and Methods section. The concentration of both divalent ions was 10 mM. The experiments were repeated twice independently.

Figure S2. Ca^{2+} ions establish a structurally stable but catalytically inactive state of the Cas12a complex in NTS pre-nicked DNA



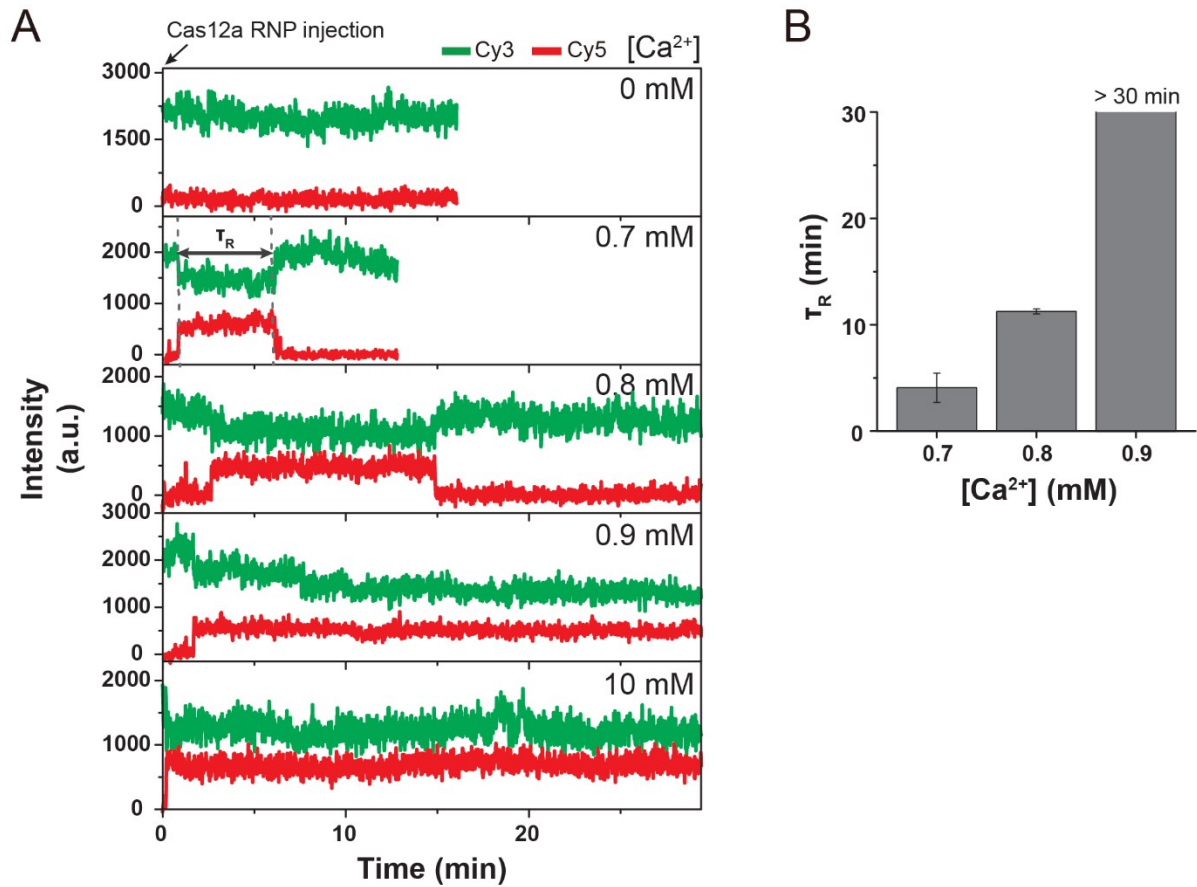
Representative time trace of Cy3 (green), Cy5 (red) and FRET efficiency (blue) showing stable binding of Cas12a and NTS pre-nicked DNA. No additional transition was observed during the measurement (> 30 min). The corresponding FRET histogram is depicted to the right of the panel, and was constructed from at least 50 time traces.

Figure S3. Effect of various divalent metal ions on the DNA cleavage reaction by Cas12a



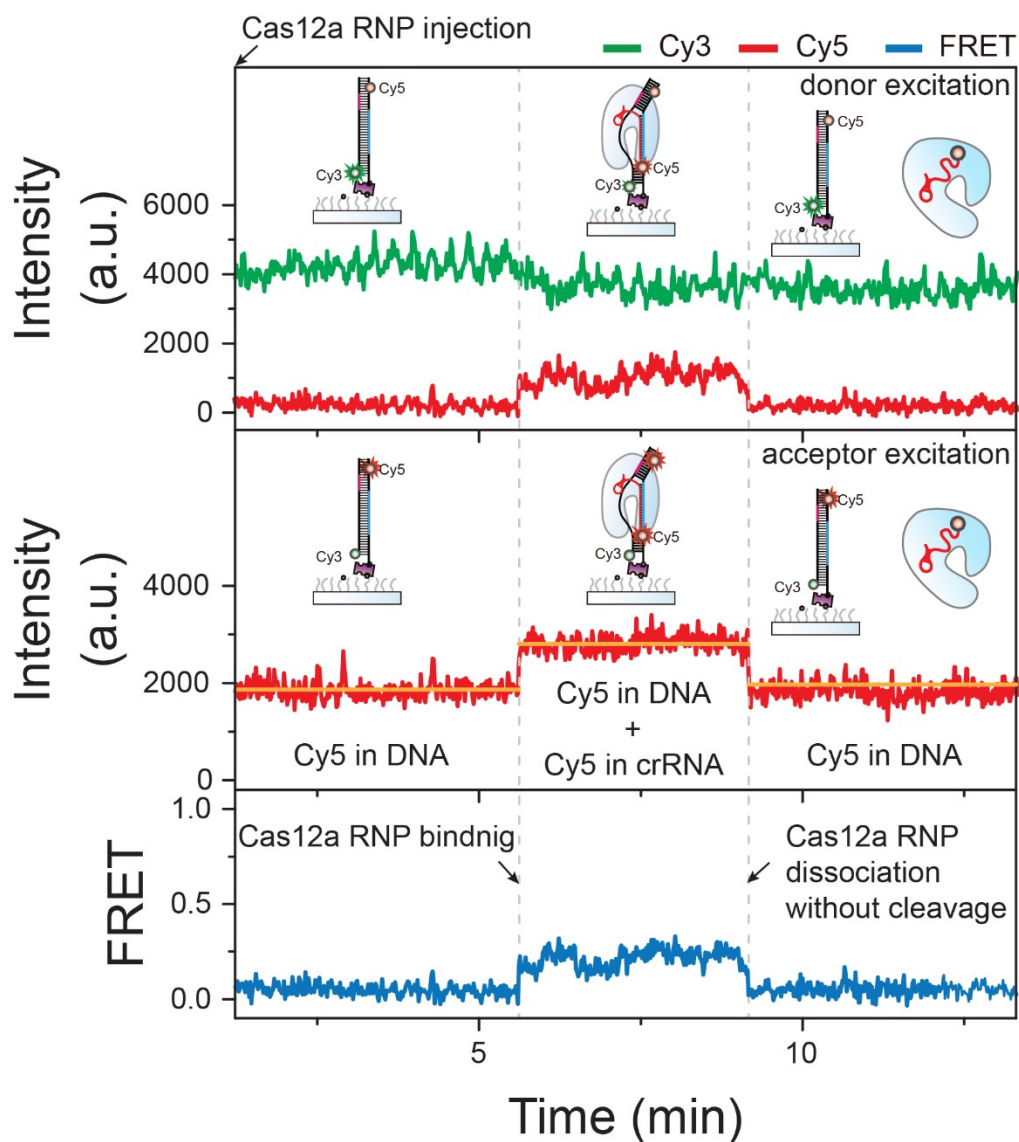
Representative time traces of Cy3 (green) and Cy5 (red) fluorescence, and corresponding FRET efficiencies (blue) in the presence of 20 nM Cas12a RNP and 1 mM Mn^{2+} , Zn^{2+} , Co^{2+} , or Ni^{2+} ions. In these measurements, we found that, similar to the case of the Mg^{2+} ion condition, the DNA cleavage reaction proceeded via three distinct reaction steps in the presence of Mn^{2+} ions. However, in the remaining divalent metal ion conditions, Cas12a RNP not only failed to generate DNA breaks, but also did not induce even the Cas12a R-loop complex.

Figure S4. Stability of the Cas12a R-loop complex under various Ca^{2+} concentrations



(A) Representative time traces of Cy3 (green) and Cy5 (red) fluorescence in the presence of 20 nM Cas12a RNP and Ca^{2+} ions with varying concentrations. In the absence of divalent metal ions (0 mM), no Cas12a R-loop complex was formed. (B) Average dwell times of the Cas12a R-loop complex at 0.7, 0.8, and 0.9 mM Ca^{2+} concentrations. Each experiment was repeated at least more than twice.

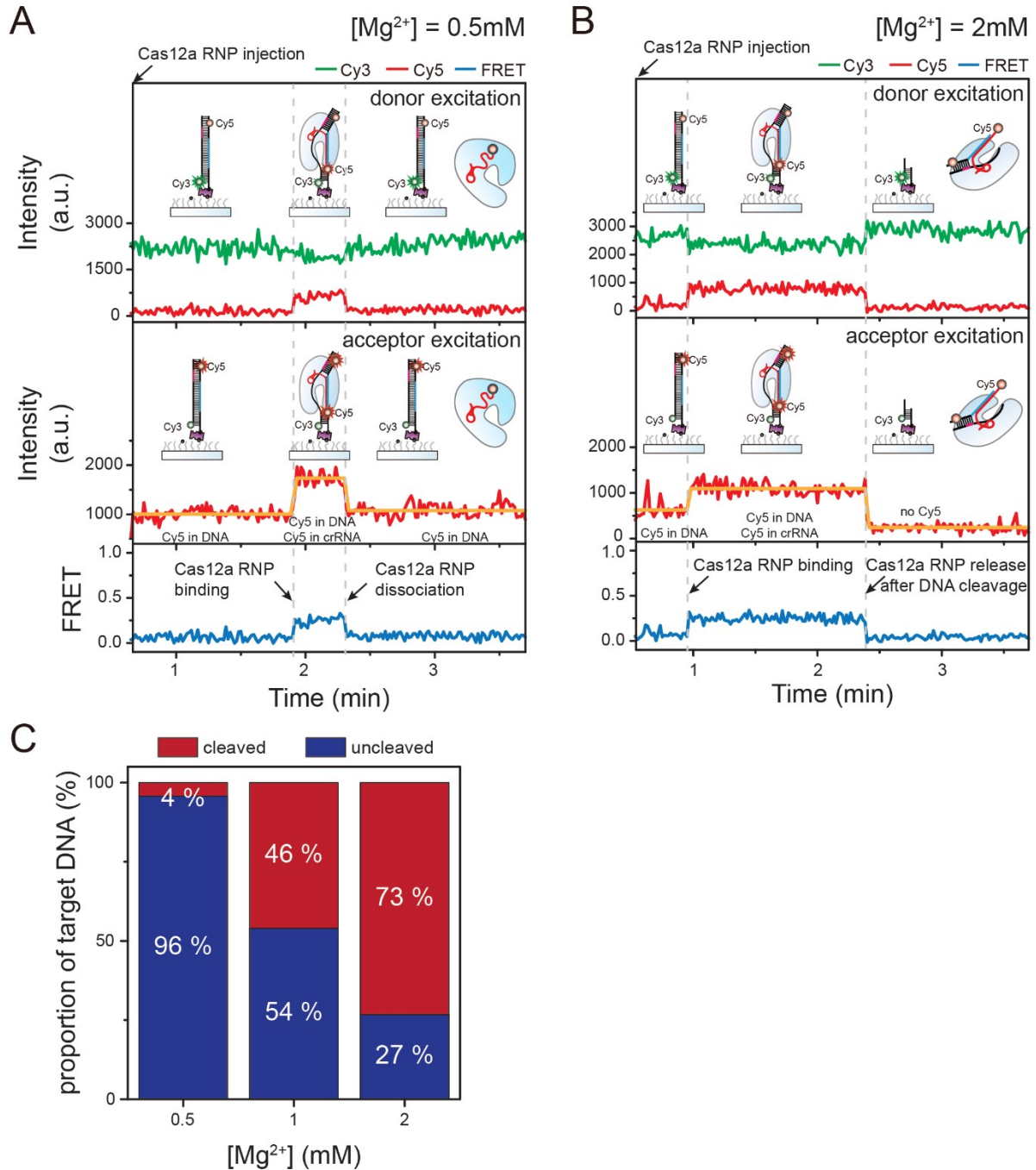
Figure S5. Single-molecule FRET measurement using a doubly labelled target DNA



Representative time traces of Cy3 (green, top) and Cy5 (red, top) intensities and FRET efficiency (blue, bottom) under donor excitation and Cy5 intensity (red, middle) under acceptor excitation showing the dissociation of Cas12a RNP from target DNA without the cleavage reaction at 0.7 mM Ca^{2+} concentration. We performed a single-molecule alternating-laser excitation (ALEX) FRET experiment with Cy3-Cy5 doubly labelled DNA and Cy5-labelled crRNA to confirm that the disappearance of Cy5 signal under donor excitation indicates the

dissociation of Cas12a RNP from target DNA without the cleavage reaction. In this measurement, we observed that even after the Cy5 signal disappeared under donor excitation by the dissociation of Cas12a RNP, the Cy5 signal still remains under acceptor excitation. This observation revealed that, in this condition, Cas12a RNPs are dissociated from target DNA without the cleavage reaction. Orange line is added to a time trace of Cy5 intensity under acceptor excitation as an eye guide. Experimental schemes were illustrated above each time trace to clearly reveal our interpretation.

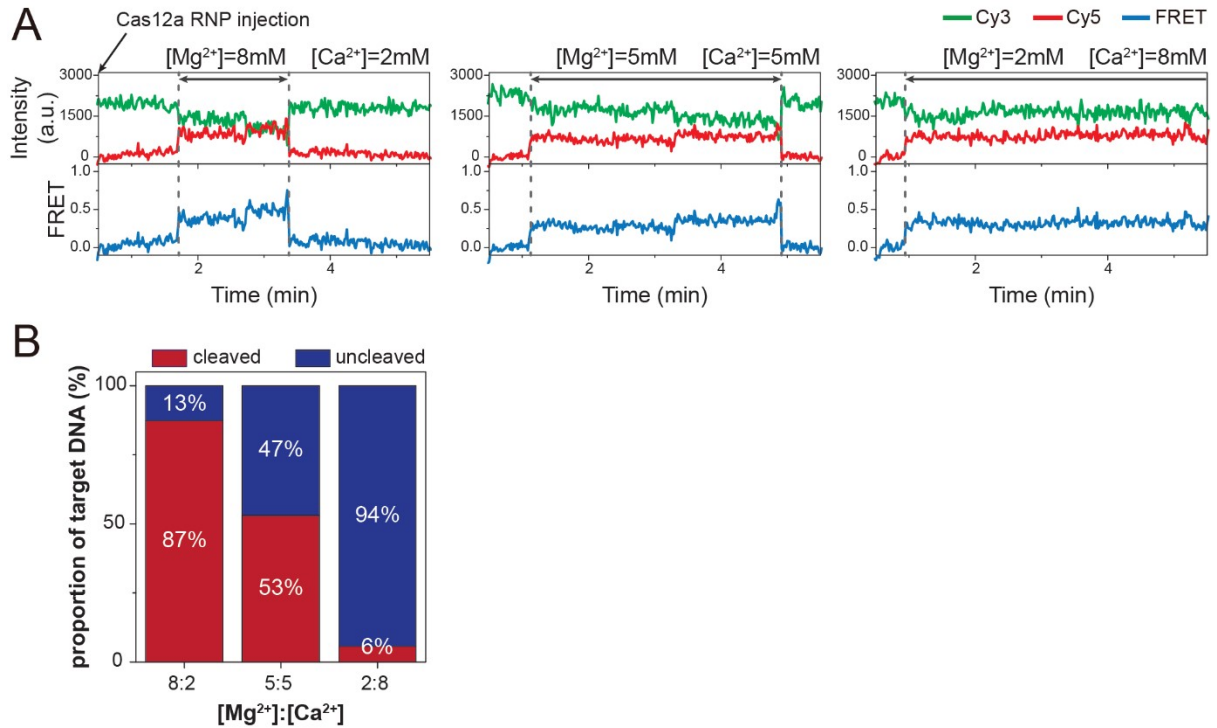
Figure S6. Effect of the Mg^{2+} concentration on stabilisation of the Cas12a R-loop complex and promotion of DNA cleavage



Representative time traces of Cy3 (green) and Cy5 (red) intensities and FRET efficiencies (blue) in the presence of 10 nM Cas12a RNP at (A) 0.5 mM Mg^{2+} and (B) 2 mM Mg^{2+} concentrations for TS pre-nicked DNA. To investigate an effect of the Mg^{2+} concentration on

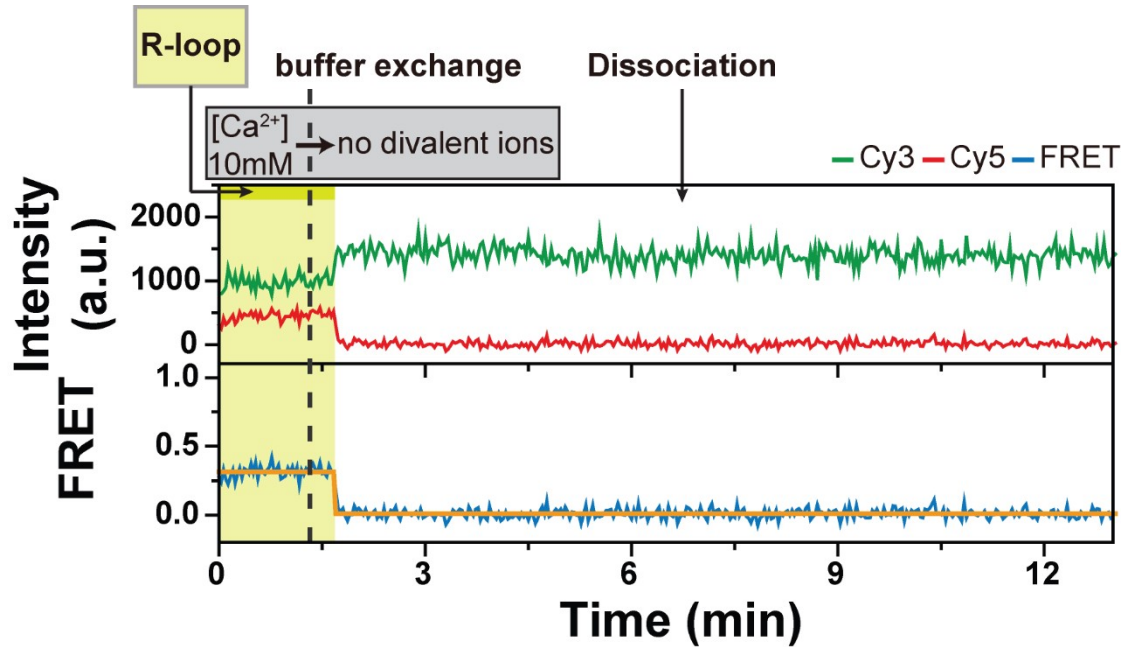
stability of the R-loop complex, we performed Mg^{2+} titration experiments with Cy3-Cy5 doubly labelled DNA and Cy5-labelled crRNA using single-molecule alternating laser excitation (ALEX) FRET assay, which allows us to distinguish between the release event of cleaved DNA products after DNA cleavage and the dissociation event of Cas12a RNP without DNA cleavage. In (A), the disappearance of Cy5 signal under donor excitation indicates the dissociation of Cas12a RNP from target DNA without DNA cleavage. This is because Cy5 signal still remains under acceptor excitation even after the dissociation of Cas12a RNP. In (B), the disappearance of Cy5 signal under donor excitation indicates the release of cleaved DNA product after DNA cleavage because no Cy5 signal is observed under acceptor excitation after the dissociation of Cas12a RNP. Orange lines were added to time traces of Cy5 intensity under acceptor excitation as eye guides. Experimental schemes were illustrated above each time trace to clearly reveal our interpretation. (C) Proportions of cleaved and uncleaved DNAs observed in the experiments at different Mg^{2+} concentrations. The proportions were calculated by counting the number of cleaved and uncleaved DNA molecules and dividing them into the total number of DNA molecules (70 molecules at least).

Figure S7. Effect of the mixtures with different molar ratios of Mg^{2+} and Ca^{2+} ions on DNA cleavage by Cas12a RNP



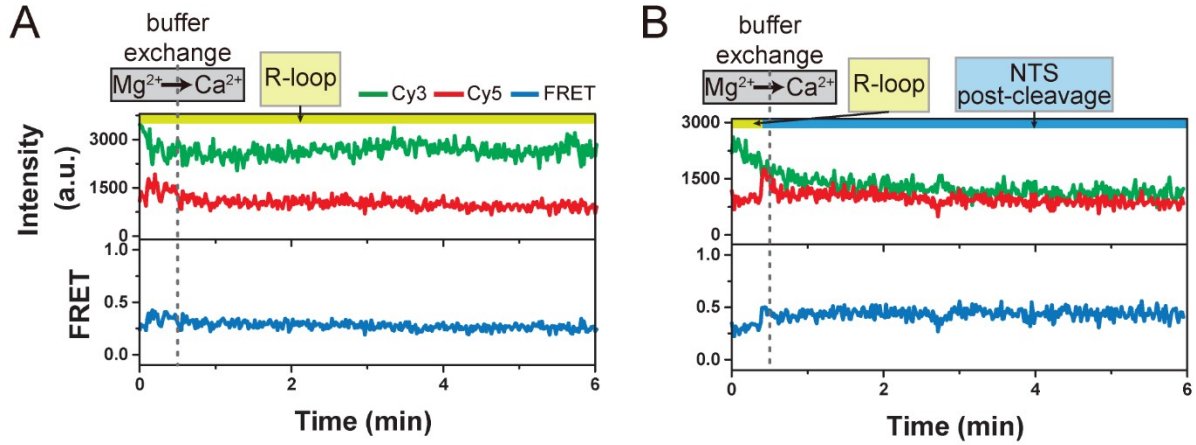
(A) Representative time traces of Cy3 (green) and Cy5 (red) fluorescence and corresponding FRET efficiencies (blue) in the presence of 20 nM Cas12a RNP and mixtures with different molar ratios of Mg^{2+} and Ca^{2+} ions (4:1, 1:1, and 1:4). (B) Proportions of cleaved and uncleaved DNAs observed in the experiments under the mixtures with different molar ratios of Mg^{2+} and Ca^{2+} ions. The proportions were calculated by counting the number of cleaved and uncleaved DNA molecules and dividing them into total number of DNA molecules (264 molecules at least).

Figure S8. Dissociation of Cas12a RNP from the R-loop complex upon introduction of buffer that contains no divalent cations



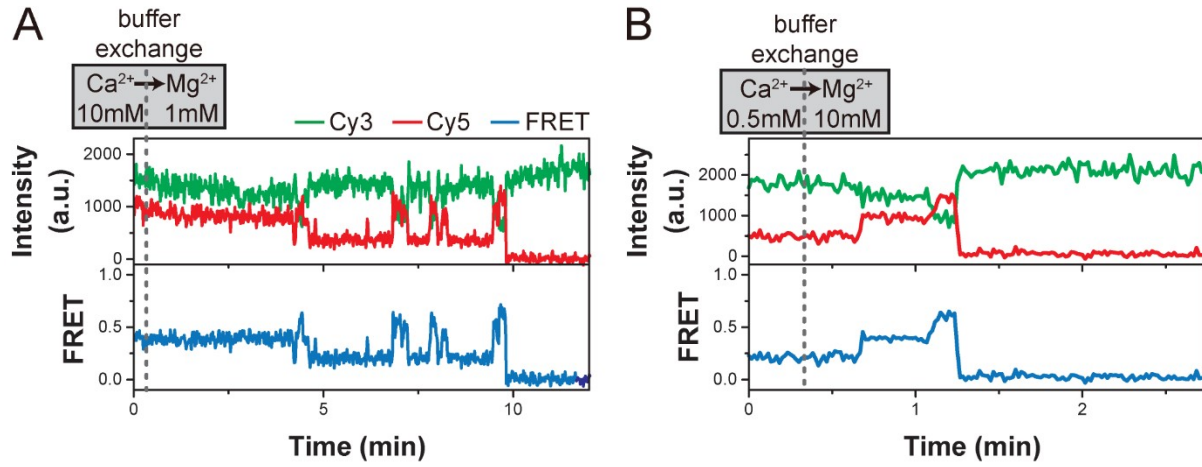
Representative time traces of Cy3 (green) and Cy5 (red) and FRET efficiency (blue) for the real-time buffer exchange experiment. The time at which the subsequent buffer was injected is indicated with a dashed line. A buffer solution without divalent cations was introduced into the detection chamber containing the surface-immobilised Cas12a ternary complex arrested in the pre-cleavage R-loop state in the presence of 10 mM Ca²⁺ ions.

Figure S9. Interruption of the DNA cleavage by Cas12a RNP via real-time buffer exchange from Mg^{2+} buffer with Ca^{2+} buffer



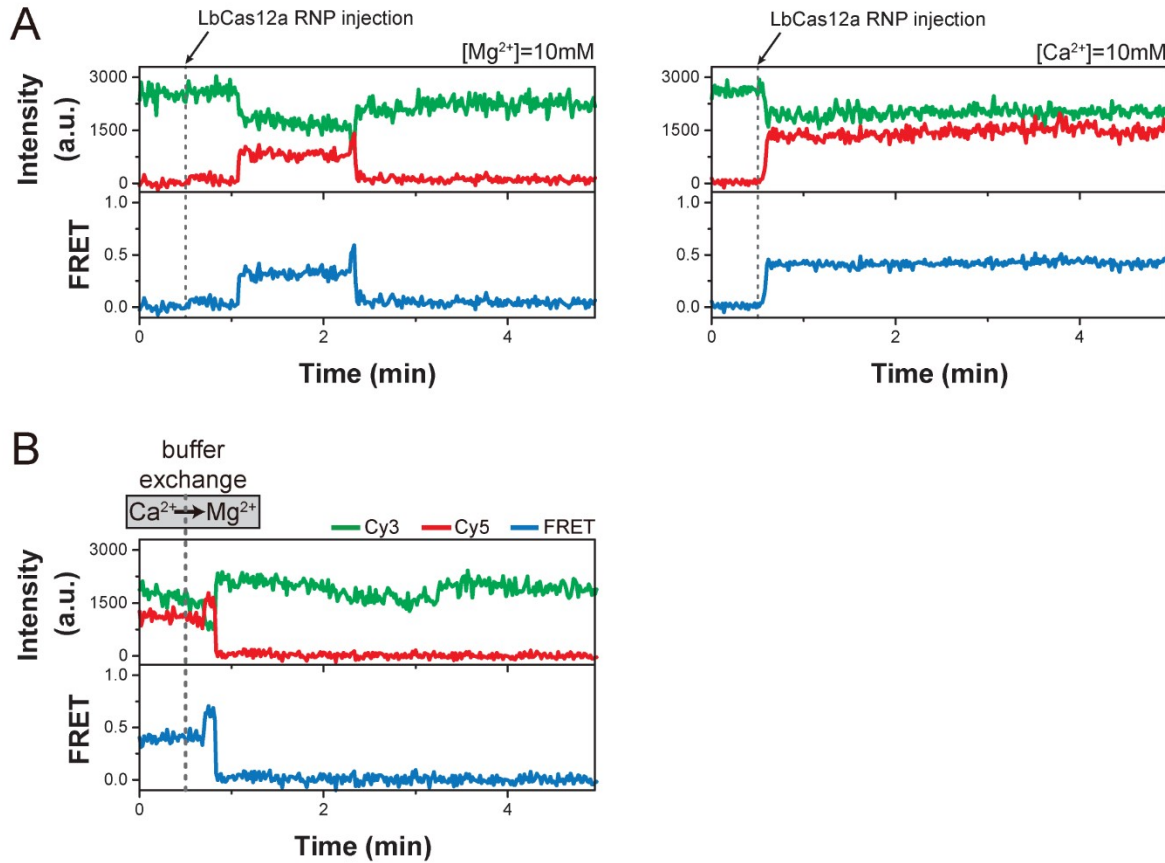
Representative time traces of Cy3 (green) and Cy5 (red) intensities and FRET efficiencies (blue) observed in buffer exchange experiments for WT DNA. A buffer containing 10 mM Ca^{2+} was introduced to the detection chamber in which Cas12a RNP-DNA ternary complexes were formed in the presence of 10 mM Mg^{2+} ions. In these measurements, Cas12a RNP-DNA ternary complexes were mostly arrested in (A) R-loop state or (B) NTS-post cleavage state after introducing the Ca^{2+} buffer.

Figure S10. Real-time buffer exchange experiments with physiological ion concentrations



Representative time traces of Cy3 (green) and Cy5 (red) fluorescence and FRET efficiencies (blue) observed in real-time buffer exchange experiments. A reaction buffer containing (A) 1 mM or (B) 10 mM Mg^{2+} ions was introduced to the detection chambers in which Cas12a ternary complexes were arrested at pre-cleavage state under the reaction condition including 10 mM or 0.5 mM Ca^{2+} ions, respectively.

Figure S11. Exchangeability of divalent metal ions in the DNA cleavage reaction by LbCas12a RNP



(A) Representative time traces of Cy3 (green) and Cy5 (red) intensities and corresponding FRET efficiencies (blue) in the presence of 20 nM LbCas12a RNP at 10 mM Mg^{2+} (left) and 10 mM Ca^{2+} (right) concentrations for NTS pre-nicked DNA. (B) Representative time traces observed in a real-time buffer exchange experiment for NTS pre-nicked DNA. A reaction buffer containing 10 mM Ca^{2+} ions in the detection chamber was replaced with the 10 mM Mg^{2+} reaction buffer.

Table S1. DNA and RNA oligonucleotides used in this study

Name	Sequence
crRNA	5'-AAUUUCUACUCUUGUAGAU CUGAUGGUCCAUGUCUGUUACUC -Cy5-3'
WT DNA	5'-biotin-TTTTTCACTTGACAGGCGAGTAACAGACATGGACCATCAGGAAACATTAACGTACT-3' 3'-GTGA ACTG TCCGCTCATTGTCTGTACCTGGTAGTCCTTTGTAATTGCATGA-5' Cy3
TS pre-nicked DNA	5'-biotin-TTTTTCACTTGACAGGCG AGTAACAGACATGGACCATCAGGAAACATTAACGTACT-3' 3'-GTGA ACTG TCCGCTCATTGTCTGTACCTGGTAGTCCTTTGTAATTGCATGA-5' Cy3
NTS pre-nicked DNA	5'-biotin-TTTTTCACTTGACAGGCGAGTAACAGACATGGACCATCAGGAAACATTAACGTACT-3' 3'-GTGA ACTG TCCGCTCATT GTCTGTACCTGGTAGTCCTTTGTAATTGCATGA-5' Cy3