A fluorescence activatable reporter of flavivirus NS2B-NS3 protease activity enables live imaging of infection in single cells and viral plaques

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Table S1. Reporter variants tested *in vitro* for cleavage and fluorescence activation upon treatment with recombinant DENV-2 NS2B-NS3 protease.

Reporter variant	Linker sequence													State after treatment with recombinant DENV-2 NS2B-NS3 protease
	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'	P7'	
DENVA-GFPv1	*GFP*[*]			G	R	R	D	F	Q	G	P	C	*QP*[+]	Uncleaved, Non-fluorescent
DENVA-GFPv2	*GFP*			G	R	R	G	F	Q	G	P	C	*QP*	Uncleaved, Non-fluorescent
DENVA-GFPv3	*GFP*	D	E	G	R	R	G	G	P	C	*QP*			Uncleaved, Non-fluorescent
DENVA-GFPv4	*GFP*	D	K	K	R	R	G	G	S	G	*QP*			Cleaved, Non-fluorescent
FlaviA-GFP	*GFP*	A	A	Q	R	R	G	R	I	G	*QP*			Cleaved, Fluorescent
ZIKVA-GFP	*GFP*	K	T	G	K	R	S	G	A	L	*QP*			Cleaved, Fluorescent
DENV2A-GFP	*GFP*	V	K	K	Q	R	A	G	V	L	*QP*			Cleaved, Fluorescent

[*]*Position within the green fluorescent protein sequence.*
[+]*Position within the quenching peptide sequence.*

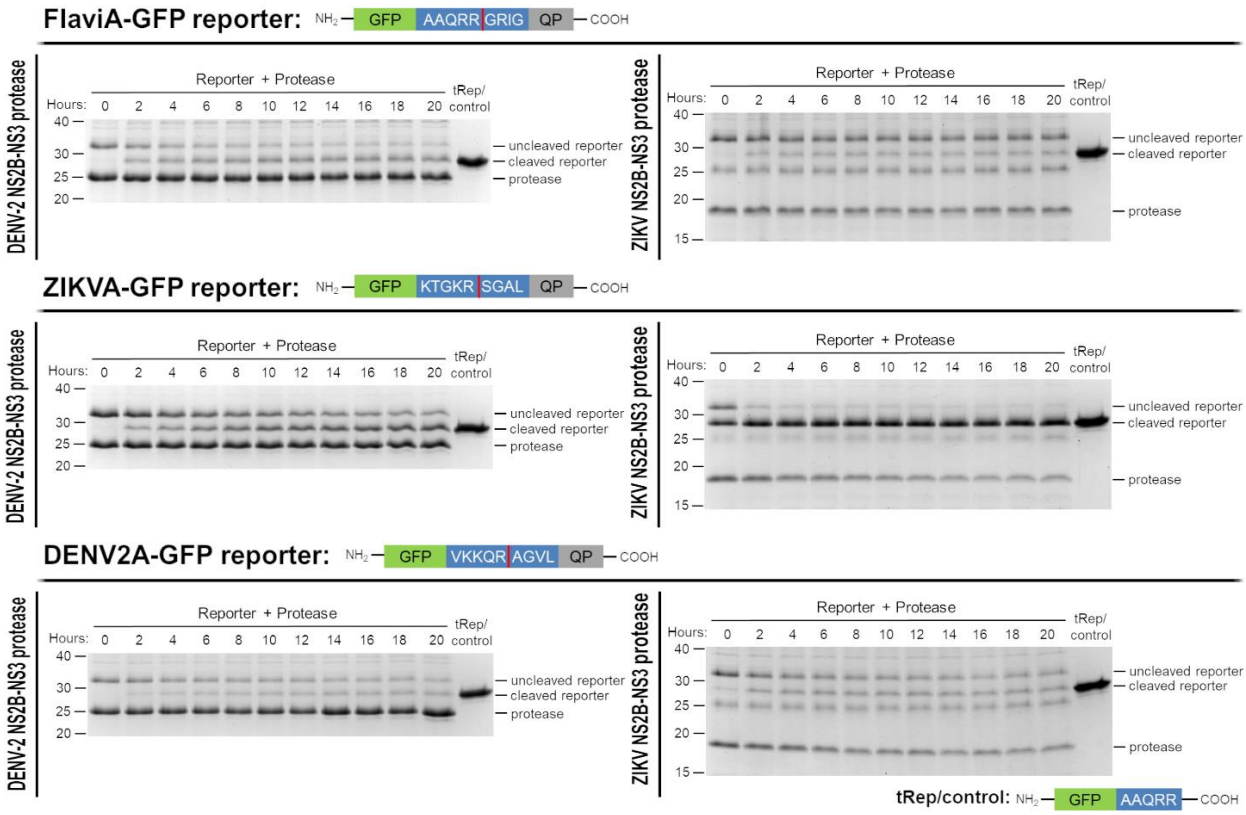

Figure S1. Cleavage kinetics of flavivirus-activatable GFP reporter variants by DENV-2/ZIKV NS2B-NS3 proteases *in vitro*. Three variants of the flavivirus-activatable GFP reporter were developed by changing the linker sequence: ZIKVA-GFP (ZIKV polyprotein NS2B/NS3 cleavage site linker), DENV2A-GFP (DENV-2 polyprotein NS2B/NS3 cleavage site linker), and FlaviA-GFP with the internal NS3 cleavage site linker which is present in many members of the *Flavivirus* genus. For the *in vitro* cleavage kinetics, purified reporter proteins were mixed with purified DENV-2 NS2B-NS3 protease (left panel) or ZIKV NS2B-NS3 protease (right panel) at a molar ratio of 1:1 and incubated for given times. Reactions were quenched by thermal treatment in SDS loading buffer and samples were analyzed by SDS-PAGE and staining of the gels with Coomassie blue. tRep/control is an engineered cleaved version of the FlaviA-GFP protein and was used as size marker of cleaved reporters.

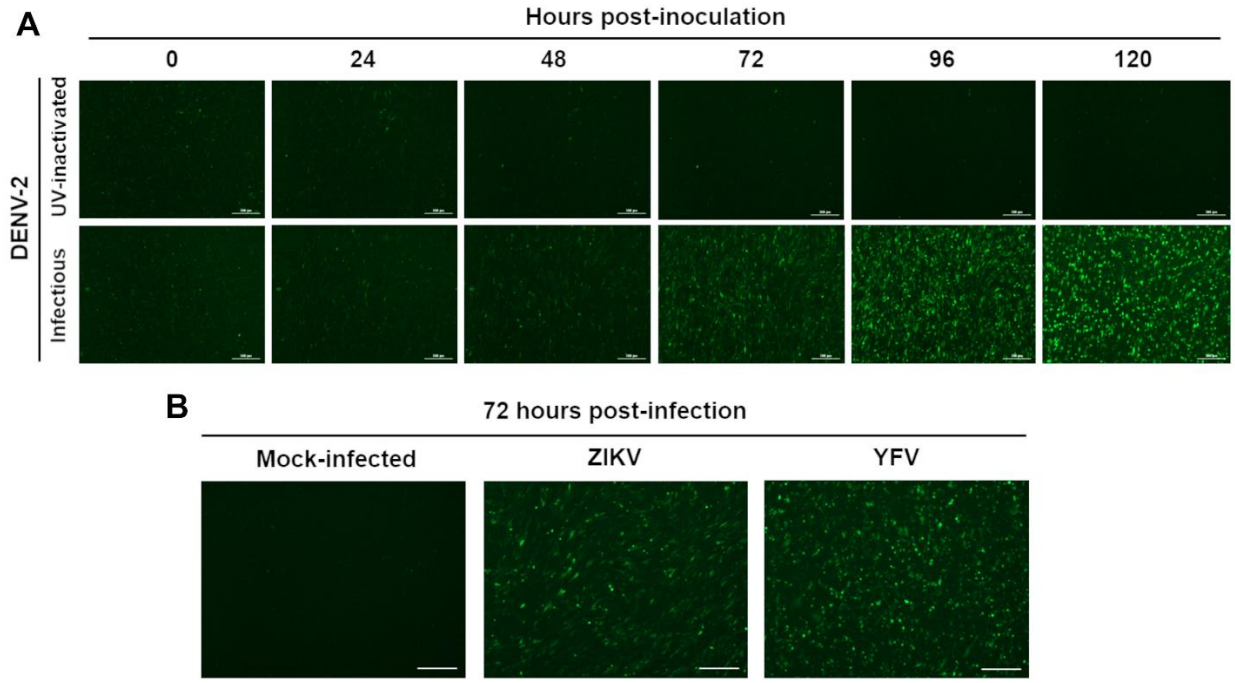

Figure S2. The FlaviA-GFP reporter becomes fluorescent in stably-transduced BHK-21 cells upon DENV-2, ZIKV, and YFV infection. Stable BHK-21 cells expressing the FlaviA-GFP reporter were inoculated with DENV-2 13538, ZIKV CIET-01, and YFV 17D at a low MOI of 0.1, for the specified time periods. **(A)** Fluorescence kinetics of the FlaviA-GFP reporter in stable BHK-21 cells after inoculation with infectious and UV-inactivated DENV-2. **(B)** Fluorescence of the FlaviA-GFP reporter in stable BHK-21 cells after 72 hour post-infection with ZIKV and YFV. Magnification of 40X, scale bar = 100 µm.

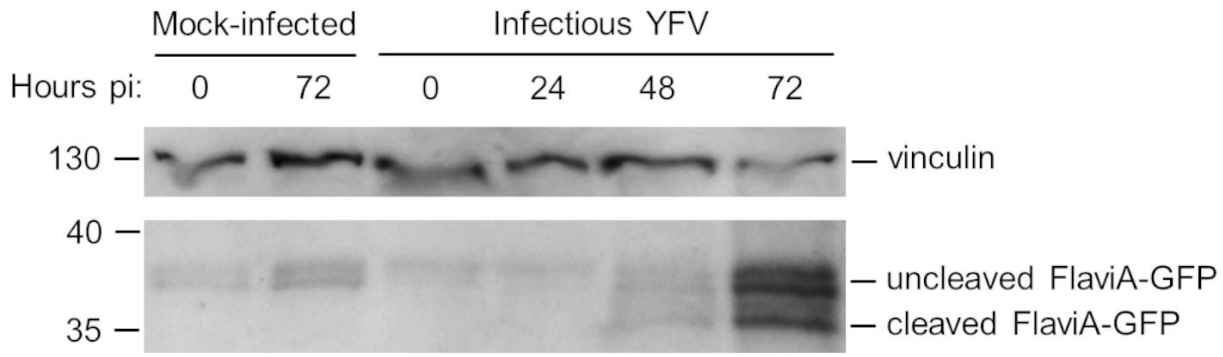

Figure S3. The FlaviA-GFP reporter becomes cleaved in stably-transduced BHK-21 cells upon YFV infection. The cleavage kinetics of the FlaviA-GFP reporter in stable BHK-21 cells upon mock or YFV 17D infection at a low MOI of 0.1 was made by western blot for the depicted time periods post-inoculation (pi) and following the protocol described in the experimental procedures.

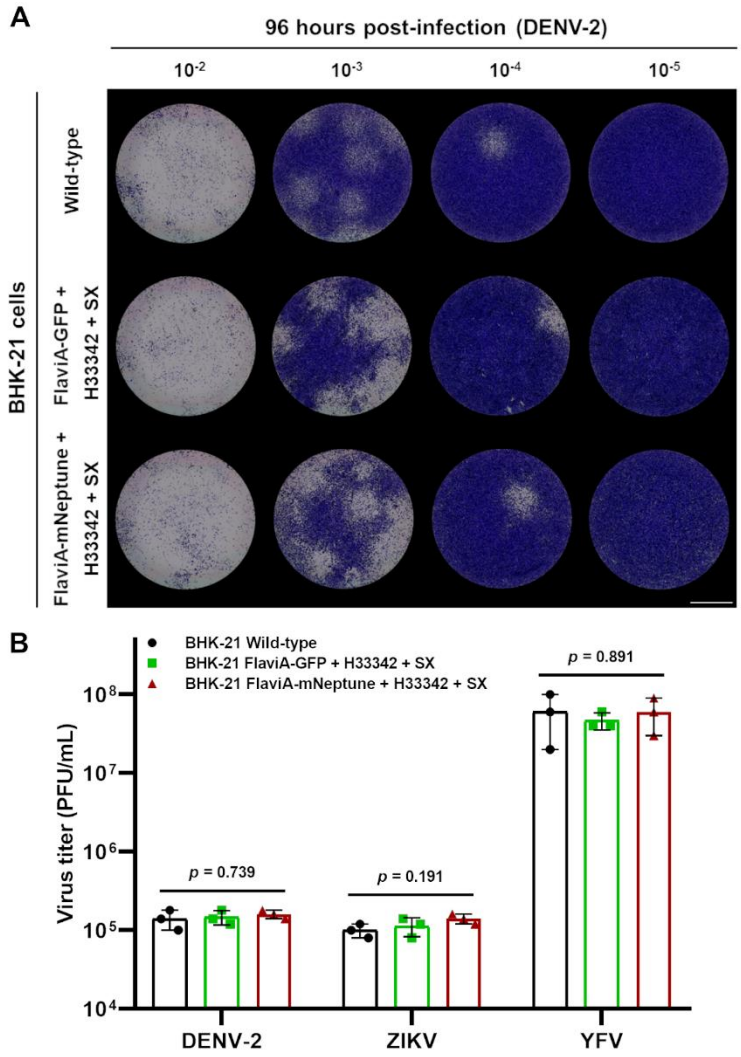

Figure S4. Stable expression of the FlaviA-GFP and FlaviA-mNeptune reporters in combination with dyes of chromatin and cell death has no effect on flaviviruses replication in mammalian cells. Wild-type and stable BHK-21 cells expressing either the FlaviA-GFP or the FlaviA-mNeptune reporter together with dyes of chromatin and cell death were used to perform a plaque assay with viral seeds of DENV-2 13538, ZIKV CIET-01, and YFV 17D. **(A)** Comparison of DENV-2 plaque assay in wild-type and stable BHK-21 cells expressing either the FlaviA-GFP or the FlaviA-mNeptune reporter in combination with Hoechst 33342 (H33342) and SYTOX green (SX) at 96 hours post-infection. Images from a representative experiment are shown (n = three independent experiments, magnification of 40X, scale bar = 1000 µm). **(B)** Virus titers for DENV-2, ZIKV, and YFV in wild-type and stable BHK-21 cells expressing either the FlaviA-GFP or the FlaviA-mNeptune reporter together with Hoechst 33342 (H33342) and SYTOX green (SX) at 96 hours post-infection. Data are expressed as mean ± SD of three independent experiments.

Figure S5. Multiple sequence alignment of the internal NS3 cleavage site from ten medically important flaviviruses. Protein sequences of the internal NS3 cleavage site from DENV-1 to 4, ZIKV, YFV, WNV, SLEV, JEV, and TBEV were obtained from the NCBI reference proteins data base (accession numbers NP_059433.1, NP_056776.2, YP_001621843.1, NP_073286.1, YP_009428568.1, NP_041726.1, YP_001527877.1, YP_001008348.1, NP_059434.1, and NP_043135.1, respectively), aligned by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and visualized with WebLogo (https://weblogo.berkeley.edu/logo.cgi).