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 |
|------------------|-----------------|------------------------------------------------------------------|
| DENV A-GFPv1     | GFP *            | Uncleaved, Non-fluorescent                                       |
| DENV A-GFPv2     | GFP             | Uncleaved, Non-fluorescent                                       |
| DENV A-GFPv3     | GFP D E G R R G G P C | Uncleaved, Non-fluorescent                                       |
| DENV A-GFPv4     | GFP D K K R R G G S G | Cleaved, Non-fluorescent                                       |
| FlaviA-GFP       | GFP A A Q R R G R I G | Cleaved, Fluorescent                                           |
| ZIKVA-GFP        | GFP K T G K R S G A L | Cleaved, Fluorescent                                           |
| DENV2A-GFP       | GFP V K K Q R A G V L | 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).