

1 A versatile reporter system to monitor virus infected cells and its application to
2 dengue virus and SARS-CoV-2

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26 ABSTRACT

27 Positive-strand RNA viruses have been the etiological agents in several major disease
28 outbreaks over the last few decades. Examples of that are flaviviruses, such as dengue
29 virus and Zika virus that cause millions of yearly infections and spread around the
30 globe, and coronaviruses, such as SARS-CoV-2, which is the cause of the current
31 pandemic. The severity of outbreaks caused by these viruses stresses the importance
32 of virology research in determining mechanisms to limit virus spread and to curb
33 disease severity. Such studies require molecular tools to decipher virus-host
34 interactions and to develop effective interventions. Here, we describe the generation
35 and characterization of a reporter system to visualize dengue virus and SARS-CoV-2
36 replication in live cells. The system is based on viral protease activity causing
37 cleavage and nuclear translocation of an engineered fluorescent protein that is
38 expressed in the infected cells. We show the suitability of the system for live cell
39 imaging and visualization of single infected cells as well as for screening and testing
40 of antiviral compounds. Given the modular building blocks, the system is easy to
41 manipulate and can be adapted to any virus encoding a protease, thus offering a high
42 degree of flexibility.

43 IMPORTANCE

44 Reporter systems are useful tools for fast and quantitative visualization of viral
45 replication and spread within a host cell population. Here we describe a reporter
46 system that takes advantage of virus-encoded proteases that are expressed in infected
47 cells to cleave an ER-anchored fluorescent protein fused to a nuclear localization
48 sequence. Upon cleavage, the fluorescent protein translocates to the nucleus, allowing
49 for rapid detection of the infected cells. Using this system, we demonstrate reliable

50 reporting activity for two major human pathogens from the *Flaviviridae* and the
51 *Coronaviridae* families: dengue virus and SARS-CoV-2. We apply this reporter
52 system to live cell imaging and use it for proof-of-concept to validate antiviral activity
53 of a nucleoside analogue. This reporter system is not only an invaluable tool for the
54 characterization of viral replication, but also for the discovery and development of
55 antivirals that are urgently needed to halt the spread of these viruses.

56 **INTRODUCTION**

57 Positive sense single stranded RNA viruses constitute a major fraction of endemic and
58 emerging human viruses (1). Among the positive-strand RNA viruses, flaviviruses
59 such as dengue virus (DENV) and Zika virus (ZIKV) are some of the most prevalent
60 arboviral pathogens and are considered a major public health problem (2, 3).
61 Currently, there are no universal vaccines or specific antiviral drug approved for the
62 prevention or treatment of infections with these viruses (4). Members of the
63 *Coronaviridae* family also have a positive-strand RNA genome and have caused
64 major outbreaks in the last two decades (5, 6). Currently, the world is facing the
65 pandemic outbreak of SARS-CoV-2, the causative agent of coronavirus disease 2019
66 (COVID-19) (7, 8). As of August 2020, over 19 million confirmed cases and more
67 than 700,000 confirmed deaths have been reported in 216 countries (9). Despite
68 immense efforts by research teams around the world, there is still a dire need for
69 effective and widely available treatment options and a prophylactic vaccine.

70 Once released into the cell, the full genome of flaviviruses and the large open reading
71 frame (ORF1ab) of coronaviruses are translated as polyproteins. Signal peptides and
72 internal transmembrane regions direct polyprotein synthesis to the endoplasmic
73 reticulum (ER) membrane where co-translational cleavage generates the mature viral

74 proteins (10, 11). The flaviviral protease NS2B/3, together with host proteases,
75 cleaves the flavivirus polyprotein into three structural and seven non-structural
76 proteins (12, 13). In the case of coronaviruses, ORF1ab is expressed as two
77 polyproteins, which are cleaved into sixteen non-structural proteins (nsp) by the viral
78 papain-like protease (PL_{pro}) residing in nsp3 and the 3C-like protease ($3CL_{pro}$) of nsp5
79 (14–17). The replication of viral RNA of both virus groups was shown to occur on ER
80 derived membranes, in specialized virus-induced membrane compartments termed
81 replication organelles (10, 11, 18–20).

82 Reporter systems for detection of virus infection are an invaluable tool for the
83 characterization and quantification of virus infection kinetics, for the characterization
84 of virus-host cell interactions and for the identification of antiviral compounds. One
85 approach is the insertion of tags into the viral genome that, upon replication and
86 translation, allow for visualization of the infected cells. However, this approach
87 requires functional molecular clones of a given genome, which are not always
88 available. In addition, insertion of a tag frequently causes attenuation of viral
89 replication competency and therefore, the search for adequate insertion sites is time-
90 consuming or might fail.

91 An alternative approach is the use of engineered fluorescent reporter proteins stably
92 expressed in cells and altering their subcellular distribution upon viral infection (21–
93 23). Building on this idea, here we established a reporter system based on an ER-
94 anchored green fluorescent protein (GFP) that upon cleavage by a viral protease is
95 released from the ER and translocated into the nucleus. Using this system, we
96 demonstrate the reliable reporting activity of DENV and SARS-CoV-2 infected cells.
97 Moreover, we apply this reporter cell system to live cell imaging and assessment of an
98 antiviral compound.

99 **MATERIALS AND METHODS**

100 **Cell lines and virus strains.** HEK-293T, A549 and VeroE6 cells were purchased
101 from ATCC; Huh7 cells (24) were obtained from Heinz Schaller (Center for
102 Molecular Biology, Heidelberg). Generation of the cell lines Huh7-Lunet and the
103 derivative Huh7-Lunet-T7, stably expressing the RNA polymerase of bacteriophage
104 T7, have been previously described (25, 26). All cells were cultured at 37°C and 5%
105 CO₂ in Dulbecco's modified Eagle medium (DMEM, Life Technologies) containing
106 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 1% non-
107 essential amino acids (complete medium). Huh7-Lunet-T7 cells were cultured in
108 complete medium, supplemented with 5 µg/mL zeocin. A549-ACE2 were generated
109 by transduction of A549 with lentiviruses encoding for the human Angiotensin-
110 converting enzyme 2 (ACE2) gene as previously described (41).

111 Wild-type (WT) DENV-2 was produced from an infectious molecular clone based on
112 strain 16681 as described elsewhere (27). The DENV reporter virus genome encoding
113 the Turbo far red fluorescent protein FP635 (DENV-faR) has been previously
114 described (28). SARS-CoV-2 (strain BavPat1) was kindly provided by Prof. Christian
115 Drosten (Charité Berlin, Germany) through the European Virology Archive. Except
116 for DENV-faR that was generated by electroporation of BHK-21 cells as previously
117 described (28), all virus stocks were generated by infection of VeroE6 cells.
118 Supernatants were harvested, filtered, and virus concentration was determined by
119 plaque assay. For infection experiments, cells were inoculated as specified in the
120 results section for 1 h at 37°C. Fresh complete medium was then added, and cells
121 were incubated for the indicated time spans.

122

123

124 **Antibodies.** The antibodies used in this study are listed in Table 1.

TABLE 1 List of antibodies used in this study.

Antibodies	Concentration		Source
	WB	IF	
Mouse IgG1 anti-DENV NS3 ^a	1:1000	1:200	GeneTex
Mouse anti-GAPDH ^a	1:1000	n.a.	Santa Cruz Biotechnology
Rabbit anti-GFP ^a	1:1000	n.a.	Roche
Mouse IgG2a anti-dsRNA ^a	n.a.	1:400	Scicons
Mouse IgG1 anti-SARS-CoV N ^a	n.a.	1:500	Sino biologicals
Goat anti-mouse IgG-HRP ^b	1:10000	n.a.	Sigma-Aldrich
Goat anti-rabbit IgG-HRP ^b	1:10000	n.a.	Sigma-Aldrich
Alexa Fluor 568 donkey anti-mouse IgG ^b	n.a.	1:1000	Thermo Fisher Scientific
Alexa Fluor 568 donkey anti-rabbit IgG ^b	n.a.	1:1000	Thermo Fisher Scientific

a: primary antibody, b: secondary antibody; WB: western blot; IF: immunofluorescence.

125 **Generation of the reporter construct.** A synthetic DNA construct containing the
126 sequence encoding the reporter protein was generated by Integrated DNA
127 technologies (Coralville, IA, USA). The reporter sequence was inserted into the
128 lentiviral vector pWPI via AscI and SpeI restriction sites (pWPI-RC).
129 Oligonucleotides encoding the protease cleavage sites were designed to allow
130 insertion into the vector via MluI and BamHI restriction sites. The primer pairs (Table
131 2) spanning a given protease cleavage site were heated to 95°C and allowed to anneal
132 by decreasing the temperature in 5°C increments every 2 min. The resulting double-
133 stranded DNA product was inserted via MluI and BamHI into pWPI-RC and ligation
134 products were amplified in *E. coli* (strain DH5 α). Integrity of amplified plasmids was
135 confirmed by restriction pattern analysis and sequence analysis of the insert region,
136 respectively. The complete nucleotide and amino acid sequences of the reporter
137 construct are available on request. The expression of the reporter construct was under
138 the control of the eukaryotic translation elongation factor 1-alpha promoter.

TABLE 2 Sequences of oligonucleotides used in this study.

¹Capital letters indicate sequence non-complementary to the reverse primer. ²fwd: forward, rvs: reverse.

primer. Fwd: forward, Rev: reverse.

140 **Lentiviral transduction and generation of reporter cell lines.** Cells stably
141 expressing the protease reporter constructs were generated by lentiviral transduction.
142 Subconfluent HEK-293T cells were transfected with the pWPI vector encoding the
143 reporter construct together with packaging plasmids pCMV-Gag-Pol and pMD2-
144 VSV-G (kind gifts from D. Trono, EPFL, Lausanne). After 2 days, the supernatant of
145 transfected cells was harvested, filtered, and stored at -80°C. Lentiviruses were
146 titrated by SYBR green I-based real-time PCR-enhanced reverse transcriptase (SG-
147 PERT) assay (29, 30) using the Takyon SYBR green kit (Eurogentec). The titer was
148 determined by comparison with a standard curve of known RNA concentrations.
149 Lentiviral transduction was performed by addition of the filtered supernatant to Huh7,
150 Huh7-Lunet-T7 or A549-ACE2 cells (multiplicity of infection (MOI) = 5) in presence
151 of 4 µg/mL of polybrene. For the generation of stable cell lines expressing the
152 reporter constructs, cells were cultured in medium containing 1 µg/mL puromycin.
153 Cells stably expressing the SARS-CoV-2 optimized reporter construct were FACS-
154 sorted to obtain single cell clones with homogenous expression levels of the
155 fluorescent reporter.

156 **Indirect immunofluorescence (IF).** Cells were seeded on glass cover slips and
157 harvested at the indicated time points. The cells were washed once with PBS and
158 fixed with paraformaldehyde (PFA, 4% in PBS) at room temperature (RT). PFA was
159 removed, cells were washed once with PBS and the cover slips were covered with
160 PBS containing Triton X-100 (0.2%) to permeabilize the cells. Cells on cover slips
161 were blocked with skimmed milk (2%) in PBS containing Tween20 (PBS-T (0.02%))
162 for 1 h. After blocking, the cover slips were placed on 30 µL of primary antibody,
163 diluted in the blocking buffer, with the cell side facing the drop. Cells were incubated
164 for 1.5 h at RT and washed thrice with PBS-T (0.02%). The cover slips were then

165 placed on 30 μ L of secondary antibody with the cell side facing the drop. After
166 45 min of incubation at RT, the cells were washed thrice with PBS-T (0.02%) and
167 cover slips were mounted on microscopy slides using Dapi-Fluoromount-G mounting
168 media (Southern BioTech).

169 **Western blot.** Cells were washed once with PBS and lysed in western blot lysis
170 buffer (1% Triton X-100). After sonification and denaturation at 95°C, protein
171 concentration was measured by Bradford assay. Cell lysates were mixed with
172 Bradford reagent (1:5; Bio-Rad) and absorbance was measured at 595 nm. For each
173 sample, 10 μ g of total lysate was resolved by electrophoresis into a 10% or 15%
174 sodium dodecyl sulfate-polyacrylamide gel for NS3 or GFP, respectively. Proteins
175 were transferred to a polyvinylidene fluoride membrane overnight at 4°C. Membranes
176 were blocked in skimmed milk (5%) in PBS-T (0.2%) for 1 h at RT. After washing
177 thrice with PBS-T (0.2%) for 15 min, membranes were incubated with primary
178 antibody for 1 h at RT. The membranes were washed thrice and HRP-conjugated
179 secondary antibody was added. After incubation for 1 h at RT, the membrane was
180 washed thrice, and the bound antibodies were detected using enhanced
181 chemiluminescence solution (Perkin Elmer, Waltham, MA, USA). Images were
182 acquired using the ChemoCam 6.0 ECL system (INTAS Science Imaging,
183 Goettingen, Germany).

184 **Live cell imaging.** Huh7-Lunet-T7 cells expressing the dengue reporter constructs
185 (Lunet-T7-RC) were seeded onto a glass bottom 35 cm² dish (Mattek) at a density of
186 2 x 10⁴. Transfection of the pIRO-D system (40) was performed 24 h post-seeding
187 using TransIT-LT1 (Mirus Bio) transfection reagent according to the manufacturer's
188 instructions. Four hours post transfection (hpt) the transfection medium was
189 exchanged for complete medium lacking phenol red (imaging medium). Images were

190 collected with a Perkin Elmer spinning disk confocal microscope. For SARS-CoV-2
191 live cell imaging, A549-ACE2 or a selected clone of A549-ACE2 stably expressing
192 the fluorescent reporter (A549-ACE2-RC), were seeded on 35 mm dishes (Ibidi) with
193 gas permeable membrane and sealable lid. Cells were infected for 1 h with
194 SARS-CoV-2 (MOI = 10) and 2 hpi the medium was exchanged for imaging medium.
195 Lid was moved to the locked position and silicon was used to seal the dish in order to
196 prevent evaporation. Images were collected with a 20x lambda air objective on a
197 Nikon Eclipse Ti widefield microscope. Multiple observation fields were imaged for 8
198 h or 18 h at an interval of 10 min for transfection or infection, respectively.

199 **Compound screening assay.** A549-ACE2 cell clones were seeded in duplicates for
200 each condition. On the next day, the cells were treated with a serial dilution of 1:3,
201 starting at 1.1 μ M Remdesivir (Hoelzel-biotech, Germany) or with the solvent
202 dimethyl sulfoxide (DMSO) serving as a control. After 30 min, cells were infected
203 with SARS-CoV-2 (MOI = 5) in the presence of the compound and 16 h later, cells
204 were fixed and stained for N protein by IF. Images were acquired with a Perkin Elmer
205 spinning disk confocal microscope. Signal intensity of N protein staining and nuclear
206 GFP signal was quantified on a single cell level by a semi-automated image analysis
207 workflow (31). Cells were considered as positive for infection in the reporter cell line
208 when the nuclear GFP signal intensity was greater than 7,000 arbitrary fluorescence
209 units. Inhibition was quantified by normalizing the values to those obtained with cells
210 that were treated with DMSO only (no inhibition).

211 **Bioinformatics analysis.** Images were analyzed using the Fiji software (32, 33).
212 Graph generation and statistical analysis was performed using the GraphPad Prism 8.1
213 software package. The scheme of the assumed reporter topology was designed with

214 the Illustrate software (34) using the RCSB PDB entries 4EVL, 4RXH (chain A),
215 4CG5 (chain C) and 2VBC (chain B).

216

217 **RESULTS**

218 **Design and characterization of DENV reporter constructs.**

219 In order to generate a reporter system that can specifically indicate virus infection, we
220 designed a construct expressing a GFP fusion protein that could selectively be cleaved
221 by viral proteases. The reporter construct was engineered for viruses that produce ER
222 tethered polyproteins that are processed by viral proteases in close proximity to ER
223 membranes. The transmembrane (TM) domain of the ER-resident protein sec61 β was
224 used to target the reporter protein to ER membranes (Figure 1). This ER anchor was
225 connected to a GFP moiety containing the nuclear localization signal (NLS) sequence
226 from simian virus 40 large T-Antigen via a variable linker. The linker region was
227 flanked by restriction enzyme recognition sites allowing the easy insertion and
228 screening of different protease cleavage sequences (Figure 1A). Protease cleavage of
229 the linker would result in GFP translocation from the cytosolic ER to the nucleus,
230 which can be easily detected and quantified by light microscopy.

231 The DENV polyprotein is cleaved into the individual viral proteins by either the host
232 signal peptidase of the viral NS2B/3 serine protease (12, 13). The ER-resident NS2B
233 protein acts as a co-factor of NS3 protease and anchors it to ER membranes (35, 36).
234 To determine an optimal system for reporting DENV infection, several previously
235 described NS2B/3 specific cleavage sequences were inserted into the reporter
236 construct (Table 3).

TABLE 3 List of DENV cleavage site sequences inserted into the reporter construct.

No.	Name	Cleavage site sequence ¹	Source
1	Capsid	RRRR↓SAGM	Shiryaev <i>et. al.</i> 2007 (37)
2	DV _{opt}	GKKRR↓PVK	Shiryaev <i>et. al.</i> 2007 (37)
3	NS2AB	SKKR↓SWPL	Shiryaev <i>et. al.</i> 2007 (37)
4	NS2B3	KKQR↓AGVL	Shiryaev <i>et. al.</i> 2007 (37)
5	NS3hel	AQRR↓RRIG	Shiryaev <i>et. al.</i> 2007 (37)
6	NS3prohel	RKRR↓LTIM	Shiryaev <i>et. al.</i> 2007 (37)
7	panFlav	GLKR↓GGAK	Shiryaev <i>et. al.</i> 2007 (37)
8	ZV _{opt}	KTGKR↓SGAL	Arias-Arias <i>et. al.</i> 2020 (38)

237 ¹ Cleavage site is indicated with ↓.

238 Reporting activity of the cells expressing each of the constructs was first tested by
239 assessing the subcellular GFP localization by IF. Reporter cell lines were generated by
240 lentiviral transduction of Huh7 cells at an MOI of 5 to ensure maximal transduction
241 efficiency. Subsequently, the cell pools expressing an individual construct were
242 infected with DENV for 48 h (MOI = 5) to observe GFP localization in infected
243 versus non-infected cells. To ensure specificity of GFP translocation, cells were fixed
244 and stained for DENV NS3 protein.

245 In DENV-infected cells, NS3 was observed in the perinuclear region as previously
246 described (10, 18, 39). While the GFP signal from the reporter constructs 1, 2, 3 and 7
247 showed an ER-like pattern in mock infected cells, nuclear signal was observed in cells
248 expressing constructs 4, 5, 6 and 8 already in the absence of DENV (Figures 2A and
249 2B). In infected cells expressing the reporter construct 1, a noticeable increase of
250 nuclear GFP signal was found (Figure 2C). Increased nuclear GFP localization, upon
251 DENV infection, was also observed in reporter cell lines expressing constructs 3, 4, 5,
252 7, and 8. No clear differences of GFP localizations were seen for constructs 2 and 6
253 following virus infection. For construct 1, all cells with nuclear GFP signal were also
254 positive for NS3 staining and an additional ~6% of cells were positive for NS3
255 staining alone (Figures 2C and 2D). The other constructs showed a higher percentage

256 of cells only positive for NS3 staining and in cells expressing constructs 2, 6 and 7,
257 nuclear GFP signal was observed in the absence of NS3 staining (Figure 2C).

258 To investigate cleavage of the various reporter proteins in DENV infected cells, we
259 used western blot analysis of cell lysates prepared 48 h post infection (Figure 2E).
260 Based on the construct design, the cleaved GFP-NLS fusion protein was predicted to
261 have a molecular weight of ~34 kDa. In the mock infected cell lines, we observed a
262 GFP protein with the expected molecular weight and additional bands that were also
263 found in cells transfected with empty plasmid. Upon DENV infection, additional ~30
264 kDa GFP-positive bands were detectable in cell lysates containing reporter constructs
265 1, 4, 5, 7 and to a lesser extent in cell lysates with constructs 3 and 8, which is
266 consistent with the predicted cleavage product size. Notably, construct 1 showed
267 higher levels of specific cleaved product compared to the other cell lines.

268 To summarize, DENV infected cells expressing reporter construct 1, containing the
269 NS2B/3 cleavage site from the capsid region, showed the highest level of the correct
270 cleavage product in western blot analysis as well as robust and specific nuclear
271 translocation of GFP signal by fluorescence microscopy. Therefore, reporter construct
272 1 was chosen for generation of stable cell lines for further characterization. These
273 Huh7-derived cell lines were designated reporter construct (RC) cell lines Huh7-RC
274 and Lunet-T7-RC.

275 **Time-course experiments confirm early and reliable identification of DENV**
276 **NS2B/3 positive cells.**

277 Next, we analyzed the kinetics of GFP translocation in the reporter cell line Huh7-RC.
278 Cells were infected with either the WT DENV or with a DENV reporter expressing a
279 far red fluorescent protein (DENV-faR; MOI = 5) (28). Cells were fixed at 24 h, 48 h

280 or 72 h post infection and subsequently analyzed by wide-field fluorescence
281 microscopy (Figure 3A).

282 Mock infected reporter cells exhibited the predicted ER-like localization of the GFP
283 signal. In contrast, reporter cells infected with WT virus showed nuclear GFP
284 localization as early as 24 h after infection and the proportion of nuclear signal
285 increased at later time points (Figure 3A). Reporter cells infected with DENV-faR
286 showed a similar trend, although a lower number of reporting cells was observed at 24
287 h and 48 h post infection in comparison to WT infected reporter cells (Figure 3A),
288 consistent with lower replication capacity of the reporter virus (28). Cells infected
289 with DENV-faR showed an increase of red fluorescence in a time-dependent manner
290 providing evidence for viral replication and spread. Importantly, ~83% to 100% of
291 cells exhibiting red fluorescence also showed nuclear translocation of GFP at 48 h or
292 72 h post infection, respectively (Figure 3A). Additionally, only 2-3% of cells were
293 positive for nuclear GFP in absence of reporter virus signal. These results indicate that
294 the reporter cell line successfully detected DENV infected cells and that the infected
295 cells can be reliably identified as early as 24 h post infection. Furthermore, this
296 experiment demonstrated the suitability of the reporter construct to define the
297 percentage of DENV infected cells without the need for intracellular staining.

298 **Live cell imaging of cells expressing the DENV polyprotein.**

299 Recently, a plasmid-based expression system for induction of DENV replication
300 organelles in transfected cells has been described (40). This system, designated
301 "plasmid-induced replication organelle - dengue (pIRO-D), encodes the viral
302 polyprotein that is translated from an RNA generated in the cytoplasm by a stably
303 expressed T7 RNA polymerase. In this way, the pIRO-D system allows the analysis of

304 viral proteins in cells, independent of viral replication. However, since no fluorescent
305 protein coding sequence is incorporated into the construct, expression of the DENV
306 polyprotein cannot be followed by live cell imaging.

307 To overcome this limitation, we determined whether our DENV reporter cell line
308 could be combined with the pIRO-D system to analyze the expression of the viral
309 polyprotein in real-time. Huh7-Lunet cells stably expressing the T7 RNA polymerase
310 and the reporter construct (Lunet-T7-RC) were seeded in dishes with glass bottoms
311 and on the next day transfected with the pIRO-D construct (Figure 3B). The growth
312 medium was changed to imaging medium at 4 h after transfection and the dishes
313 transferred to a live cell imaging microscope. The GFP signal was recorded every 10
314 min for 8 h (final time point at 12 h post transfection). Representative images of mock
315 transfected and pIRO-D-transfected cells at 2 h increments are shown in Figure 3C; a
316 video spanning an 8 h observation period of transfected cells is shown in
317 supplementary movie S1.

318 No nuclear translocation of the GFP reporter was detected in mock transfected Lunet-
319 T7-RC cells (Figure 3C, upper row). In pIRO-D-transfected cells, nuclear localization
320 was already detected in a few cells as early as 4 h post transfection, suggesting a
321 robust expression of the viral polyprotein. The number of cells with nuclear GFP
322 signal as well as the intensity of the signal increased over time.

323 **Development of a reporter system for identification of SARS-CoV-2 infected
324 cells.**

325 The ability to rapidly detect virus infection in cell culture on a large scale is a valuable
326 tool for studies of virus-host interactions, but also for identification and evaluation of
327 antiviral drugs. The recent outbreak of SARS-CoV-2 has created a dire need for such

328 tools to characterize virus infection and develop therapeutics. Therefore, we adapted
329 and optimized the reporter system to sense SARS-CoV-2 infection. The first two open
330 reading frames of coronaviruses are expressed as polyproteins ORF1a/b, which are
331 cleaved into the individual proteins by viral proteases PL_{pro} and 3CL_{pro} (14–16). The
332 sequence of the SARS-CoV-1 Frankfurt isolate was analyzed to determine the
333 protease cleavage sites between individual nsps, and the deduced sequences were
334 inserted into the linker region of our reporter construct (Table 4). The generation of
335 constructs 2, 11 and 13 failed and they were not further pursued.

TABLE 4 List of cleavage site sequences inserted into the SARS-CoV-2 reporter construct.

No.	Name	Cleavage site sequence ¹	Protease ²
1	nsp1/2	ELNGG↓AVTR	PL _{pro}
2	nsp2/3	RLKGG↓APIKG	PL _{pro}
3	nsp3/4	SLKGG↓KIVST	PL _{pro}
4	nsp4/5	SAVLQ↓SGFRK	3CL _{pro}
5	nsp5/6	GVTFQ↓GKFKK	3CL _{pro}
6	nsp6/7	VATVQ↓SKMSD	3CL _{pro}
7	nsp7/8	RATLQ↓AIASE	3CL _{pro}
8	nsp8/9	AVKLQ↓NNELS	3CL _{pro}
9	nsp9/10	TVRLQ↓AGNAT	3CL _{pro}
10	nsp10/RdRp	EPLMQ↓SADAS	3CL _{pro}
11	RdRp/Hel	HTVLQ↓AVGAC	3CL _{pro}
12	Hel/nsp14	VATLQ↓AENVT	3CL _{pro}
13	nsp14/15	FTRLQ↓SLENV	3CL _{pro}
14	nsp15/16	LQASQ↓AWQPG	3CL _{pro}

336 ¹ Cleavage site is indicated with ↓; ²PL_{pro}: Papain-like protease/nsp3, 3CL_{pro}: 3C-like
337 protease/nsp5

338 Cells expressing the GFP reporter containing the individual cleavage site linkers were
339 generated by lentiviral transduction of A549 cells stably expressing the SARS-CoV-2
340 receptor ACE2 (A549-ACE2) (41). Productive infection of the cells with SARS-CoV-
341 2 (strain BavPat1) was determined by detection of double-stranded (ds) RNA 48 h

342 post infection. Cytosolic GFP localization was observed in all mock infected cells
343 expressing the reporter constructs except for construct 3, where nuclear signal was
344 evident (Figure 4A and 4B). No clear differences in GFP localization between mock
345 and virus infected cells were observed for reporter constructs 1, 5 and 7 (Figure 4C),
346 while all the others showed an increase in nuclear GFP signal upon infection
347 (Figure 4C and 4D). Among the different constructs, the reporter construct 14 was the
348 most sensitive, showing the highest number of cells double positive for dsRNA and
349 nuclear GFP together with the lowest number of cells single positive for dsRNA
350 staining (Figure 4D) and therefore was selected for further investigation.

351 **Live cell imaging of SARS-CoV-2 infection.**

352 To determine the kinetics of the reporting activity we investigated SARS-CoV-2
353 infection in reporter cells by live cell imaging. Since transiently transduced cells
354 expressing the SARS-CoV-2 reporter construct showed highly heterogenous GFP
355 signal intensity as well as the formation of large fluorescent aggregates (Figures 4A
356 and 4C), we generated single cell clones by FACS sorting for cells with low
357 expression. Among the twenty cell clones generated, clone C2 was selected based
358 both on SARS-CoV-2 infection susceptibility and reporting activity (data not shown).
359 The A549-ACE2 cells were transiently transduced with lentivirus encoding for the
360 SARS-CoV-2 reporter construct 14 and seeded on glass bottom plates. After 24 h,
361 cells were infected with SARS-CoV-2 (Figure 5A). Previous studies have determined
362 that a complete virus replication cycle can occur within 6 h after infection but that
363 virus replication and assembly continues to increase up to 24 h in A549-ACE2 cells
364 (41). Therefore, live cell imaging was started at 2 h post infection and images were
365 acquired every 10 min for 18 h (final time point 20 h post infection) (supplemental

366 Movie S2). Representative images of mock and SARS-CoV-2 infected cells are
367 shown in Figure 5B.

368 Mock infected cells exhibited an ER-like GFP signal throughout the observed time
369 frame. In infected cells, a time-dependent increase in the numbers of cells showing
370 nuclear GFP signal was observed with the earliest translocation event starting at 5.5 h
371 post infection (supplementary Movie S2). These data demonstrate the suitability of
372 our reporter system for live cell imaging analysis of SARS-CoV-2 infection.

373 **Application of the SARS-CoV-2 reporter for drug screening.**

374 The need for effective treatment for COVID-19 prompted us to investigate the
375 suitability of the reporter cell line for drug screening. A proof-of-concept experiment
376 was performed by using the nucleoside analogue Remdesivir, which is currently the
377 only FDA-approved drug for treatment of SARS-CoV-2 infection. Both the A549-
378 ACE2 C2 reporter clone and the parental A549-ACE2 cells without reporter construct
379 expression were incubated with serial dilutions of Remdesivir for 30 min and infected
380 with SARS-CoV-2 (MOI = 5). The compound remained present throughout the
381 duration of the experiment. Cells were fixed at 16 h post infection and GFP
382 translocation was evaluated using confocal microscopy.

383 Treatment with 1.1 μ M Remdesivir lead to the loss of viral N protein fluorescence
384 signal in both cell lines confirming the previously described antiviral activity (42, 43)
385 (Figure 5C). Nuclear GFP signal was observed in DMSO treated reporter cells
386 infected with SARS-CoV-2 confirming the reporting activity of the cell line. The
387 percentage of cells displaying nuclear GFP signal and cytosolic N protein staining in
388 the different Remdesivir dilutions was quantified by a semi-automated image analysis
389 workflow (31) (Figure 5D). The IC₅₀ calculated for the N protein staining was 40 nM

390 ($R^2 = 0.987$, 95% CI = 27.05 – 55.89 nM), in line with IC_{50} values found in our in-
391 house assay (H. Kim and R. Bartenschlager, unpublished). Quantification with the
392 reporter signal showed an IC_{50} of 14 nM ($R^2 = 0.997$, 95% CI = 15.21 – 17.97 nM).
393 This decrease in IC_{50} value is likely due to reduced sensitivity of the reporter
394 construct that relies on 3CL_{pro} activity and the higher background in the read-out in
395 comparison to IF staining of the highly abundant N protein. Nevertheless, the two IC_{50}
396 values differ by only a factor of ~3 and therefore are quite comparable. Thus, our
397 reporter system can be reliably used in primary screens to screen e.g. large compound
398 libraries.

399 **DISCUSSION**

400 This study describes the generation and characterization of a fluorescence-based
401 reporter system for detection of DENV and SARS-CoV-2 infection. The reporter
402 construct contains three functional elements: a fluorescent protein fused to an NLS,
403 the TM domain of sec61 β for ER membrane anchoring and an exchangeable protease
404 cleavage site cassette located within the linker region that connects the fluorescent
405 protein and the ER anchor. This design allows easy adaptation of the reporter system
406 to other viruses that encode for specific viral proteases, especially for other positive-
407 stranded RNA viruses replicating in the cytoplasm. The high selectivity and
408 specificity of the selected constructs, as shown by IF, western blot and live cell
409 imaging, render this tool suitable for applications that require single cell analysis,
410 such as live cell imaging and correlative light-electron microscopy (CLEM)
411 approaches. Moreover, combining our reporter cell lines with image analysis pipelines
412 that quantify nuclear translocation events allows for rapid and robust assessment of
413 antiviral efficacy of compounds or other antiviral interventions, including high-
414 throughput screening of large compound libraries.

415 Among the different cleavage sites tested for DENV, the reporter construct 1,
416 composed of the cleavage site between capsid and prM, allowed reliable and selective
417 identification of infected or transfected cells with a construct expressing the viral
418 polyprotein (Figures 2 and 3). Interestingly, for several DENV reporter constructs,
419 nuclear GFP localization was observed in absence of the viral protease (Figure 2A). A
420 possible explanation for this could be that unspecific cleavage of the linker region
421 might be mediated by cellular proteases due to high levels of expression of the
422 reporter construct upon transient transduction.

423 Reporter constructs for detection of flavivirus infection have been described
424 previously were either cytosolic or employed viral non-structural proteins as ER
425 anchors (21–23, 38). Most of these reporter systems rely on the expression of large
426 fragments of viral proteins (21–23) which can alter the physiological stoichiometry of
427 the viral proteins and induce undesired pleiotropic effects. Indeed, even expression of
428 single NS proteins can affect cellular functions, such as alteration of mitochondrial
429 morphodynamics by NS4B (44). In contrast, since our reporter construct does not
430 contain viral sequences, except for the cleavage site, it is less prone to affect cellular
431 pathways and processes.

432 The flexibility granted by the modular nature of our constructs allows for simple
433 adaptation of the reporter system to different viruses that encode proteases acting in
434 close proximity of the ER membranes. This allowed us to rapidly adapt the system to
435 the detection of SARS-CoV-2 infection in cell culture. Transient transduction of cells
436 with lentiviruses coding for the different constructs allowed fast screening and
437 identification of the most suitable cleavage site (Figure 4). Notably, while it was
438 sufficient to use cell pools under antibiotic selection for DENV reporter cell lines
439 Huh7-RC and Lunet-T7-RC, we had to establish single cell clones for the

440 SARS-CoV-2 reporter proteins because in most cells large fluorescent aggregates
441 were observed (Figure 4A). This is likely due to differences in the ability of cell lines
442 to respond to high levels of GFP fusion proteins. In addition to sorting for cells with
443 lower reporter expression as done here, this problem might be overcome by
444 employing less active promoter or by using an alternative fluorescent protein.

445 Live cell imaging demonstrated that SARS-CoV-2 infected cells can be identified as
446 early as 5.5 h post infection (Figure 5B). Real-time identification of SARS-CoV-2-
447 infected cells is currently mainly performed by employing recombinant viruses
448 expressing reporter genes (45–47). While these studies report robust and reliable
449 identification of infected cells, our reporter cell line has advantages in certain settings.
450 The use of reporter viruses requires molecular clones and the adaptation of the
451 genomic sequence for each different isolate, which for viruses with large RNA
452 genomes like SARS-CoV-2 involves substantial cloning efforts. Additionally, the
453 relatively high mutation and recombination rate of RNA viruses during genome
454 replication makes reporter viruses inherently unstable. Moreover, integration of a
455 reporter gene into the recombinant virus may attenuate the replication efficiency, as
456 observed when comparing WT to DENV-faR virus infection (Figure 3B). In contrast,
457 our cell line allows the detection of wildtype virus isolates, although in this system
458 conserved cleavage site sequences are required.

459 We tested the suitability of the SARS-CoV-2 optimized reporter cell line to assess the
460 antiviral activity of Remdesivir and determined an IC₅₀ of 16 nM in our reporter
461 system. By using N staining as an alternative read-out, we obtained a somewhat lower
462 efficacy of Remdesivir (IC₅₀ = 40 nM), which is closer to the data reported in the
463 literature (42, 43, 48). The reduced sensitivity of the reporter construct, which relies
464 on 3CL_{pro} activity and GFP translocation, might stem from failure to detect cells with

465 low levels of infection whereas the highly expressed N protein is already detectable
466 by IF. Additionally, the selected cell clone 2 might differ in Remdesivir metabolism
467 which can impact the antiviral efficacy of the nucleoside analogue. It is important to
468 note that the IC_{50} value varies depending on which cell lines and assays are employed
469 and the IC_{50} values determined in this study are in a similar range with only a 3-fold
470 difference. Nevertheless, compounds found to exhibit antiviral activity in our reporter
471 construct should be further validated, e.g. by plaque assay and/or viral RNA level
472 quantification. Therefore, the reporter cell line can be employed in a primary screen to
473 lower the number of candidates for validation in more sophisticated and time-
474 consuming assays, thus reducing costs and increasing speed.

475 Two additional applications of our system shall be mentioned. The first is the use for
476 CLEM, a powerful imaging method that can be used to mark and identify cells of
477 interest amongst a large number of non-infected or un-transfected cells and
478 subsequent analysis of this traced cell by electron microscopy methods (49).
479 Secondly, the reporter cell line as described here can also be employed for protease
480 inhibitor screens in areas that do not have access to biosafety level 3 laboratories. This
481 can be done by transient or stable expression of the protease individually and
482 monitoring of the reduction of nuclear GFP as a result of protease inhibition, similar
483 to a recent study (50).

484 In conclusion, we describe a reporter system suitable for the detection of DENV and
485 SARS-CoV-2 infected cells. The system is easy to handle and flexible and should be
486 applicable to any virus encoding a cytoplasmic protease. It is suitable for a large
487 number of methods and assays, including high content screening. In all these respects,
488 we expect this tool to facilitate investigations of virus-host interactions, but also the

489 development of antiviral drugs that are urgently needed to curb pandemic viruses such
490 as SARS-CoV-2.

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499

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681

682

683 **FIGURE LEGENDS**

684 **Figure 1: Schematics of the reporter construct (A) and the predicted membrane**
685 **topology (B).** A) Arrows indicate restriction sites for MluI and BamHI that flank the
686 linker region and allow the insertion of the protease cleavage site. B) Proteins and
687 peptides are colored as indicated on the bottom right of the panel.

688

689 **Figure 2: Evaluation of DENV reporter constructs.** A) Huh7 cells were transduced
690 with lentiviruses encoding for the different DENV GFP-based reporter constructs 1-8
691 (Table 3) at an MOI of 5. Cells were fixed 72 hours post-transduction and subcellular
692 localization of GFP was analyzed by confocal microscopy. Scale bar: 100 μ m. B)
693 Quantification of images as in A). The percent of cells showing nuclear or cytosolic
694 GFP localization is shown. C) Huh7 cells were transduced as above for 24 h before
695 being infected with DENV at an MOI of 5. Cells were fixed 48 hpi and NS3 was
696 stained by immunofluorescence. Subcellular localization of NS3 and GFP were
697 analyzed by confocal microscopy. Red: DENV NS3 protein; green: reporter GFP
698 signal. Scale bar: 100 μ m. D) Quantification of images as in C). Percent of cells
699 positive for NS3 (red) and cells positive for both nuclear GFP and NS3 signals was
700 quantified. E) Cells expressing the reporter constructs 1-8 or an empty plasmid
701 (empty) were infected with DENV (MOI = 5). 48 hpi cells were lysed and 10 μ g of
702 total protein for each sample was resolved by SDS-PAGE. NS3 and GFP were
703 detected with a specific antibody. Glyceraldehyde-3-phosphate dehydrogenase
704 (GADPH) served as a loading control. Size of the pre-stained protein ladder bands is
705 indicated in kDa on the side of each panel.

706

707 **Figure 3: Time-course experiments of DENV reporter in infection and**
708 **transfection systems.** A) Huh7 cells stable expressing the reporter construct 1 were
709 mock infected, infected with DENV WT or the reporter virus DENV-faR (MOI = 5).
710 Left panel: Cells were fixed at the indicated hpi and signals of the reporter virus (red)
711 and the GFP-based reporter construct (green) were detected with a wide-field
712 fluorescence microscope. Scale bar: 100 μ m. Right panel: quantification of images in
713 the left panel. Percentage of cells positive for nuclear GFP signal (Nuclear-GFP),
714 DENV-faR reporter virus (Nuclear-faR) and positive for both nuclear GFP and faR
715 reporter signals (orange) was quantified. Values were normalized by setting the total
716 number of cells counted using DAPI staining as 100%. B) Experimental set-up to
717 monitor GFP-reporter activation in pIRO-D transfected cells. C) Lunet-T7-RC cells
718 stably expressing the T7 RNA polymerase and the reporter construct 1 were mock or
719 pIRO-D transfected. 4 hpt the medium was changed, and live cell imaging performed
720 for 10 h with a confocal spinning disc microscope. Images of representative fields of
721 view and the indicated time points are shown. Scale bar: 20 μ m.

722

723 **Figure 4: Screening of SARS-CoV-2 reporter constructs.** A) A549-ACE2 cells
724 were transduced with lentiviruses encoding for the reporter constructs specified on the
725 top left of each panel. Cells were fixed 32 hours post-transduction and GFP
726 localization was analyzed using a wide-field fluorescence microscope. B)
727 Quantification of images acquired as in A). The percentage of nuclear or cytosolic
728 GFP is shown (light green and gray, respectively). C) Cells transduced as in A) were
729 infected after 16 hour post-transduction with SARS-CoV-2 (MOI = 5). Cells were
730 fixed 16 hpi and viral double-stranded RNA (red), a replication intermediate

731 indicative of active viral replication, and the GFP-based reporter construct (green)
732 were detected by immunofluorescence using a wide-field fluorescence microscope.
733 D) Quantification of images acquired as in c). Percentage of infected cells positive for
734 dsRNA only (red) or double positive for nuclear GFP signal and dsRNA (orange) is
735 shown. Scale bars: 50 μ m.

736

737 **Figure 5: Application of the SARS-CoV-2 reporter cell line for live cell imaging**
738 **of viral infection and assessment of antiviral activity of Remdesivir.**

739 A) Experimental set-up to monitor GFP-reporter activation in SARS-CoV-2 infected
740 cells. B) A549-ACE2-RC (clone C2) cells stably expressing the reporter construct 14
741 were infected with SARS-CoV-2 (MOI = 10). 2 hpi live cell imaging was performed
742 for 18 h using a confocal spinning disc microscope. Images of representative fields of
743 view and time points are displayed. Scale bar: 50 μ m. C) A549-ACE2 and A549-
744 ACE2-RC (clone C2) expressing the SARS-CoV-2 reporter construct 14 were
745 incubated with Remdesivir (1.1 μ M) or DMSO control and infected with SARS-CoV-
746 2 (MOI = 5). After 16 h, cells were fixed and stained for N protein before imaging
747 with a confocal spinning disc microscope. Scale bar: 50 μ m. D) IC50 calculation of
748 Remdesivir in reporter cell clone 2 infected with SARS-CoV-2 (MOI = 5). Percentage
749 of inhibition was calculated by quantification of the number of N-positive cells and
750 cells with nuclear GFP signal in duplicate wells for each compound concentration.
751 Values were normalized by setting the average number of infected cells in the DMSO
752 treated sample as 0 % inhibition.

753

754 **Supplemental Movie S1.** Live cell imaging of Lunet-T7-RC cells transfected
755 transfected with the pIRO-D system.

756 **Supplemental Movie S2.** Live cell imaging of A549-ACE2-RC (clone C2) cells

757 infected with SARS-CoV-2

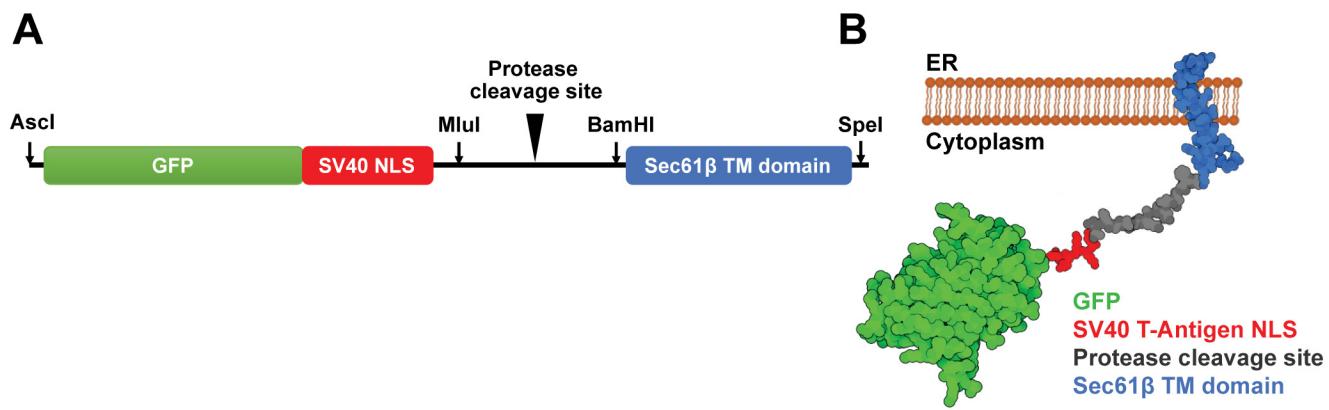


Figure 1

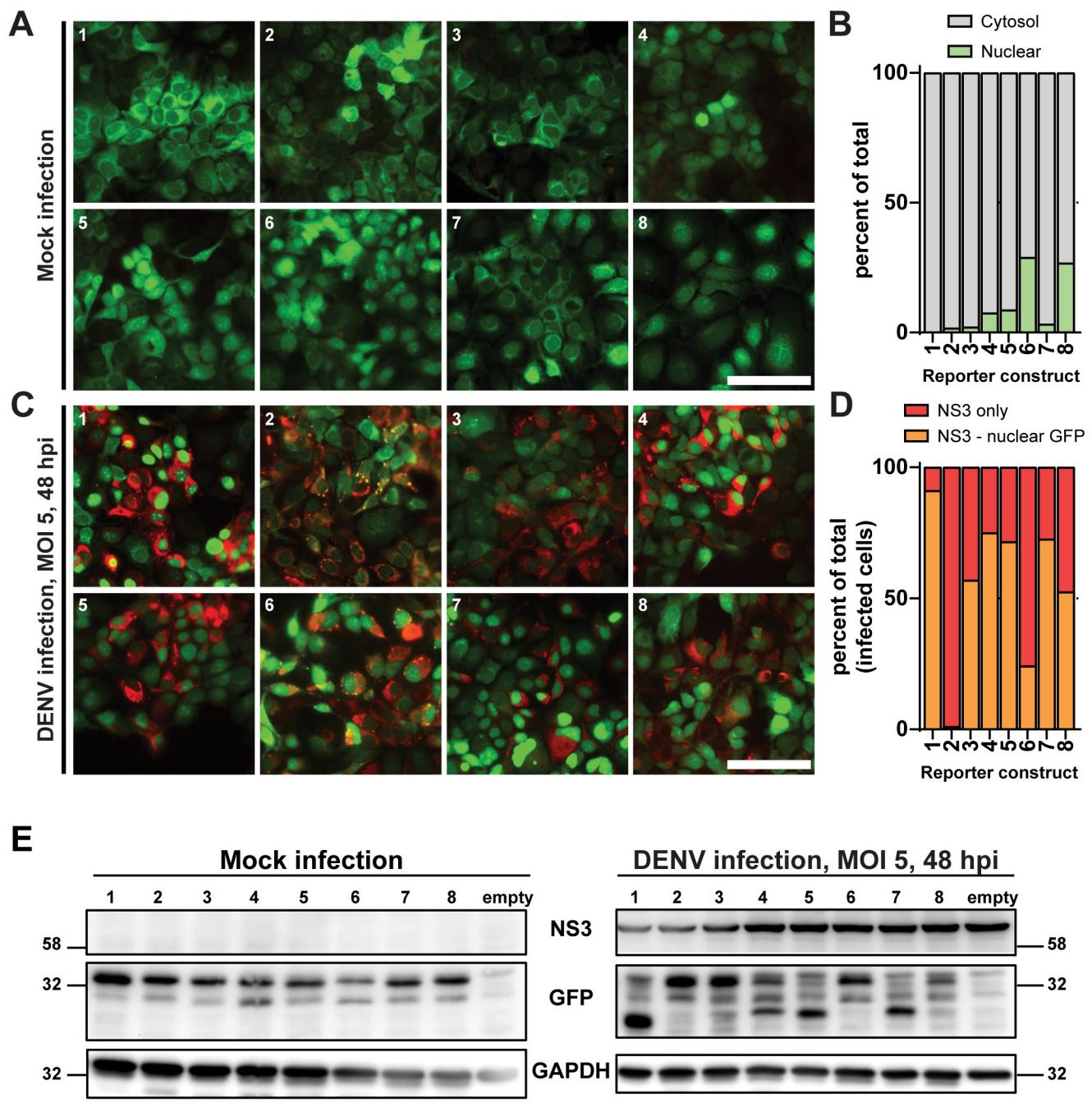


Figure 2

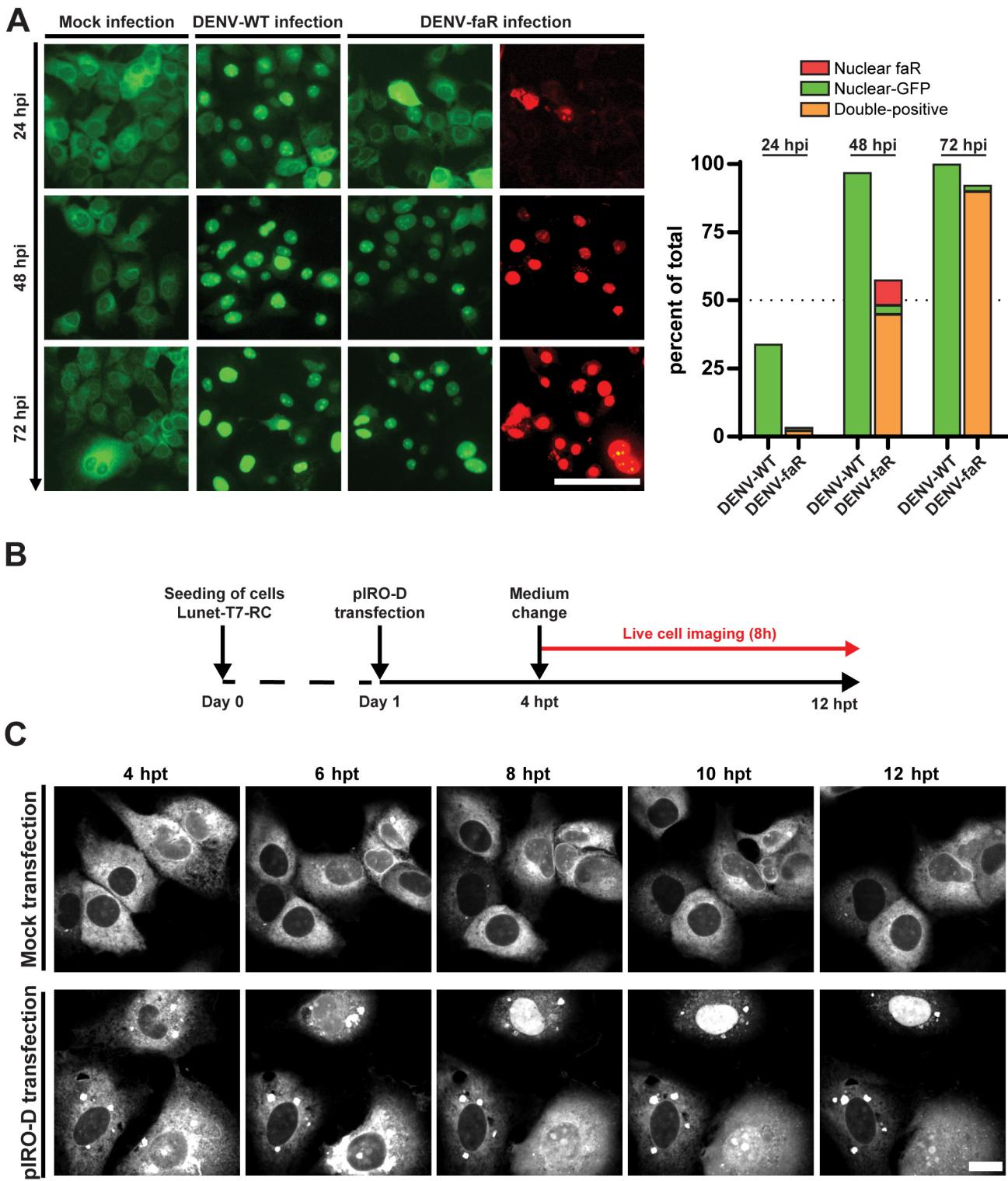


Figure 3

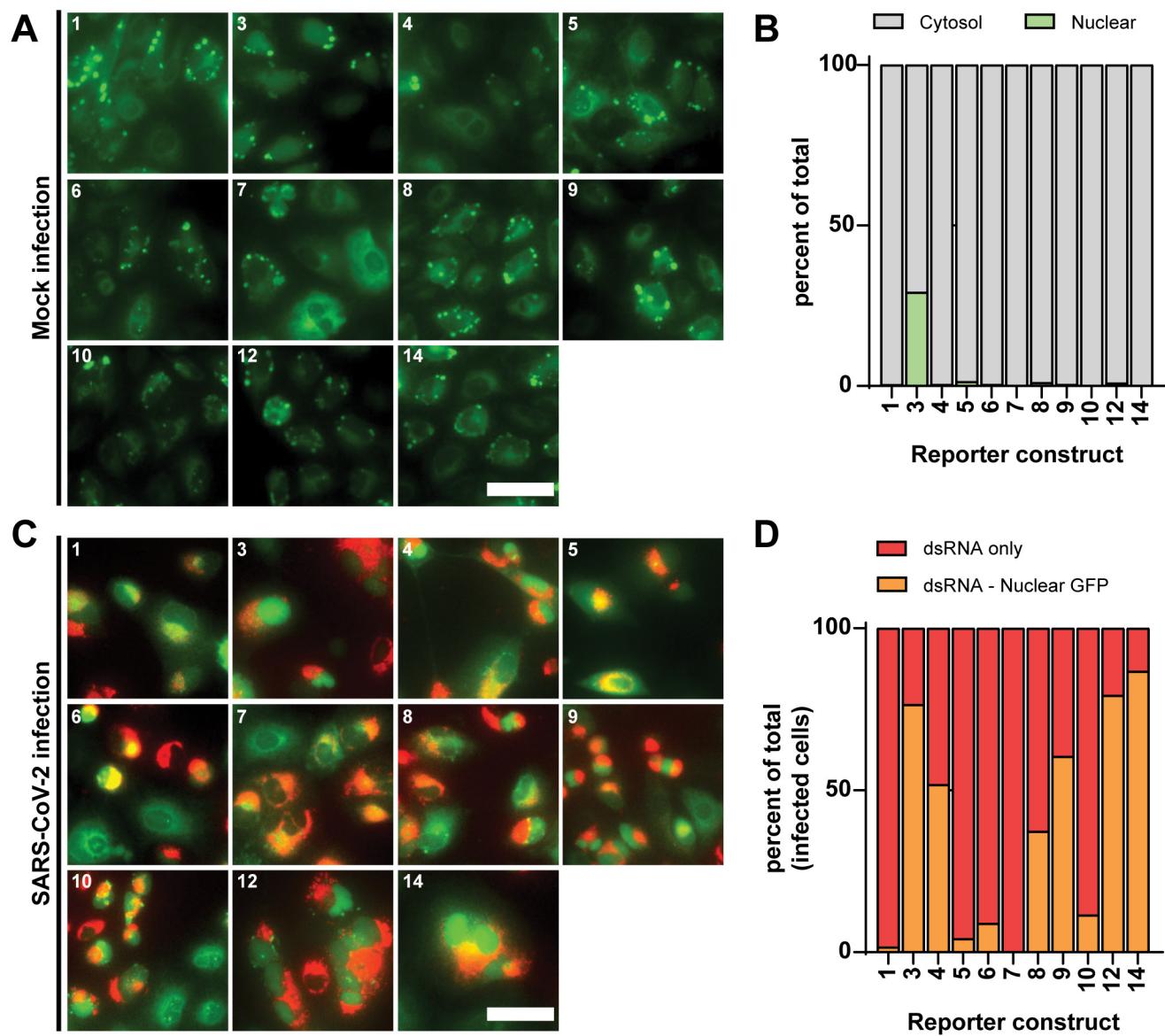


Figure 4

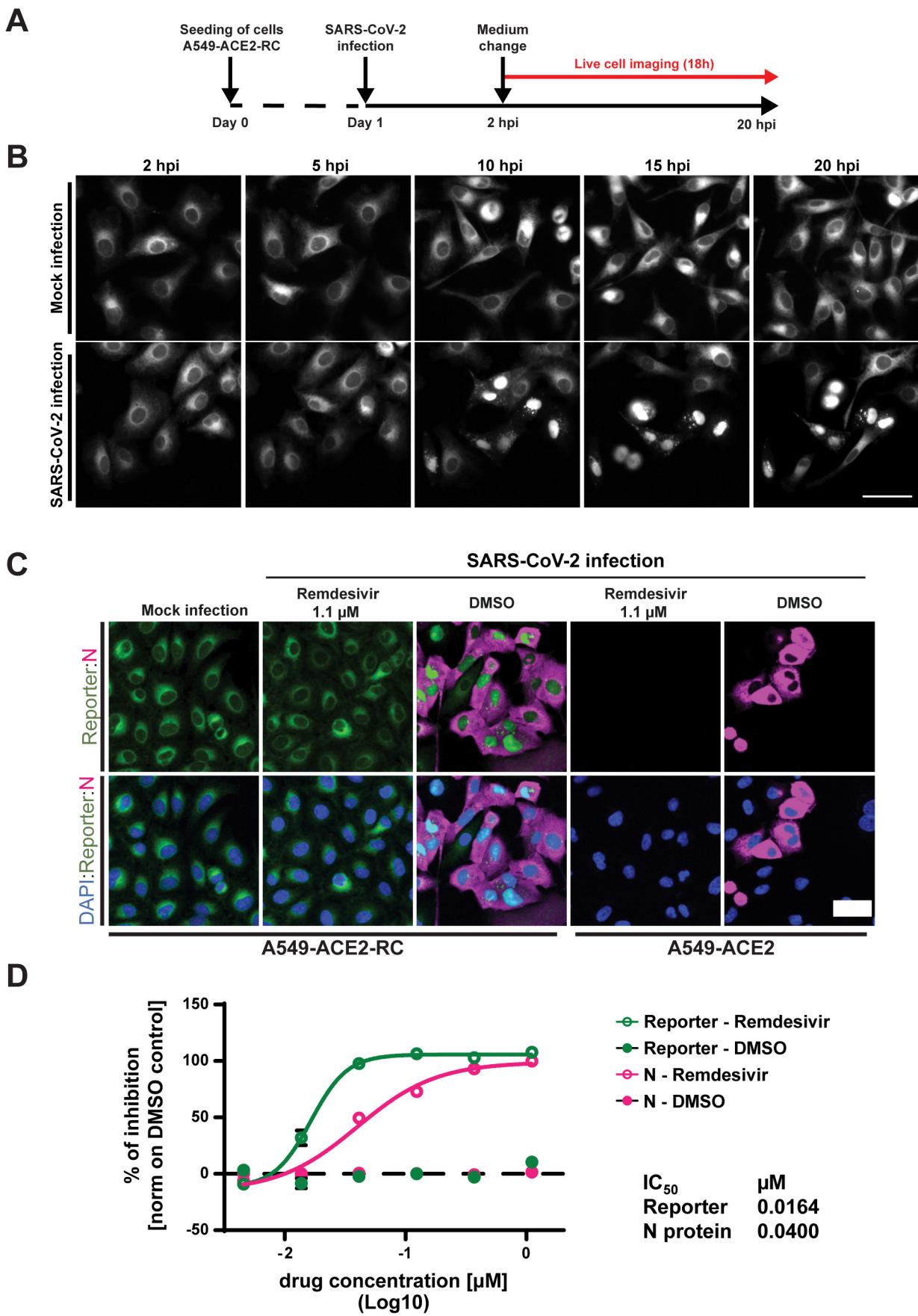


Figure 5