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Monitoring SARS-CoV-2 infection using a double reporter-expressing virus

Kevin Chiem, Jun-Gyu Park, Desarey Morales Vasquez, Richard Plemper, Jordi Torrelles, James Kobie, Mark Walter, Chengjin Ye, and Luis Martínez-Sobrido

Corresponding Author(s): Luis Martínez-Sobrido, Texas Biomedical Research Institute

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

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July 20, 2022

Dr. Luis Martínez-Sobrido
Texas Biomedical Research Institute
San Antonio, TX

Re: Spectrum02379-22 (Monitoring SARS-CoV-2 infection using a double reporter-expressing virus)

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Daniela Rajao

Editor, Microbiology Spectrum

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American Society for Microbiology
1752 N St., NW
Washington, DC 20036
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Reviewer comments:

Reviewer #1 (Comments for the Author):

This is a game-changing study that reports a carefully constructed and analyzed an advanced double reporter system for SARS-CoV-2. Overall, the authors should be commended for the amount of effort spent on this high quality study. This system could greatly assist preclinical study of COVID-19 infection. A couple of minor comments are presented to consider:
1) The high uptake of the virus in the brain by NLuc warrants discussion. Has that been observed in prior literature? Also this was not detected by viral plaque assay.
2) SARS-CoV-2 vaccines are mentioned in the introduction but not cited in ref 9-18. Some recent literature reviews could be included (e.g. Rudan et al., doi:10.1097/MCP.0000000000000868, 180, 2022; Mabrouk et al., 10.1002/adma.202107781,2107781, 2022)
3) As the NLuc kinetics demonstrate in Fig2, expression is present from 24 to at least 96 hr. Would cells be expected to start dying and losing expression?

4) In the conclusion or discussion, it would be useful for the authors to briefly mention examples of how fluorescence could be used for, what NLuc could be used for, and also how these modalities are expected to track with actual viral load.

Staff Comments:

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July 24th, 2022

Daniela Rajao
Editor, Microbiology Spectrum
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E-mail: spectrum@asmusa.org

Dear Dr. Daniela Rajao,

Thank you very much for your positive reply to our manuscript Spectrum02379-22 **"Monitoring SARS-CoV-2 infection using a double reporter-expressing virus"** by Chiem et al.

We are delighted that the reviewer was very positive and interested in our paper. We truly appreciate the reviewer for her/his time and for the constructive comments and suggestions that helped us to improve the manuscript. We have introduced some changes highlighted in gray in the revised version of the document. The following below is a point-by-point response to the comments made by the reviewer.

Reviewer general comments: This is a game-changing study that reports a carefully constructed and analyzed an advanced double reporter system for SARS-CoV-2. Overall, the authors should be commended for the amount of effort spent on this high quality study. This system could greatly assist preclinical study of COVID-19 infection.

Response: *We appreciate all the positive comments made by this reviewer, indicating that our manuscript is "a game-changing study that reports a carefully constructed and analyzed an advanced double reporter system for SARS-CoV-2". We also thank the reviewer for indicating that "the authors should be commended for the amount of effort spent on this high quality study". We also agree with the reviewer that the virus described in our manuscript could assist with preclinical studies of COVID-19 infection.*

Specific comments:

Comment 1: The high uptake of the virus in the brain by NLuc warrants discussion. Has that been observed in prior literature? Also this was not detected by viral plaque assay.

Response: *We concur with the comment made by the reviewer on the importance of the virus detected in the brain using IVIS of the entire mice by assessing Nluc expression. We have previously observed similar levels of Nluc expression in the brain of infected animals starting, in some cases, at day 4 post-infection with a peak of Nluc expression by day 6 post-infection, just before the infected animals succumb to SARS-CoV-2 infection. We have described these findings in our previous manuscript were we described our single reporter expressing rSARS-CoV-2/Nluc (**Analysis of SARS-CoV-2 infection dynamic in vivo using reporter-expressing viruses.** PMID: 34561300). As shown in Figure 7B of the current manuscript, we were able to detect the presence of the virus in two of the animals infected with rSARS-CoV-2/mCherry-Nluc at day 6 post-infection. Notably, this correlated with the levels of Nluc expression detected in the brain of infected mice by luciferase assay (Figure 7A). More importantly, these results correlate with those we previously described with the single reporter expressing rSARS-CoV-2/Nluc (**Analysis of SARS-CoV-2 infection dynamic in vivo using reporter-expressing viruses.** PMID: 34561300). Furthermore, we and others have also demonstrate the presence of wild-type SARS-CoV-2 in the brain of infected K18 hACE2 mice at similar days post-infection **(Lethality of SARS-CoV-2 infection in K18 human angiotensin-converting enzyme 2 transgenic mice**. PMID: 33257679; **Animal Models of COVID-19: Transgenic Mouse Model.** PMID: 35554912).*

Comment 2: SARS-CoV-2 vaccines are mentioned in the introduction but not cited in ref 9-18. Some recent literature reviews could be included (e.g. Rudan et al., doi:10.1097/MCP.0000000000000868, 180, 2022; Mabrouk et al., 10.1002/adma.202107781,2107781, 2022).

Response: *We agree with the comment made by the reviewer and apologize for not including this important references that have now been included in the revised manuscript.*

Comment 3: As the NLuc kinetics demonstrate in Fig2, expression is present from 24 to at least 96 hr. Would cells be expected to start dying and losing expression?

Response: *We thank the reviewer for bringing this important comment to our consideration. We have observed Nluc expression in cells infected with rSARS-CoV-2/Nluc or rSARS-CoV-2/mCherry-Nluc as early as 12 hours post-infection that increase in a time dependent matter and peak at 72 hours (Figure 2B). The decrease in Nluc expression at 96 hours, as indicated by the reviewer, is because of the cytopathic effect induced by the virus that results in losing expression. Similar findings were observed in the manuscript describing the single reporter expressing rSARS-CoV-2/Nluc (**Analysis of SARS-CoV-2 infection dynamic in vivo using reporter-expressing viruses.** PMID: 34561300).*

Comment 4: In the conclusion or discussion, it would be useful for the authors to briefly mention examples of how fluorescence could be used for, what NLuc could be used for, and also how these modalities are expected to track with actual viral load.

Response: *We agree with the comment made by the reviewer and we thank her/him for this suggestion. Following the comment made by the reviewer, we have included in the revised manuscript a brief description on how the fluorescent (mCherry) and luciferase (Nluc) expression from our rSARS-CoV-2/mCherry-Nluc could be used.*

We hope that by taking into account the comments and suggestions made by the reviewer the manuscript has improved, and we hope that now it could be accepted for publication at Microbiology Spectrum. Finally, we want to thank again the reviewer for her/his helpful and constructive comments that contributed to improve our manuscript.

Sincerely,

Luis Martinez-Sobrido, PhD

Texas Biomedical Research Institute.

August 2, 2022

Dr. Luis Martínez-Sobrido
Texas Biomedical Research Institute
San Antonio, TX

Re: Spectrum02379-22R1 (Monitoring SARS-CoV-2 infection using a double reporter-expressing virus)

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Thank you for submitting your paper to Spectrum.

Sincerely,

Daniela Rajao
Editor, Microbiology Spectrum

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Monitoring SARS-CoV-2 infection using a double reporter-expressing virus

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Running title: A double reporter-expressing recombinant SARS-CoV-2

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the highly contagious agent responsible for the coronavirus disease 2019 (COVID-19) pandemic. An essential requirement for understanding SARS-CoV-2 fundamental biology and the impact of anti-viral therapeutics are robust methods to detect for the presence of the virus in infected cells or animal models. Despite the development and successful generation of recombinant (r)SARS-CoV-2 expressing fluorescent or luciferase reporter genes, knowledge acquired from their use in *in vitro* assays and/or in live animals are limited to the properties of the fluorescent or luciferase reporter genes. Herein, for the first time, we engineered a replication-competent rSARS-CoV-2 that expresses both fluorescent (mCherry) and luciferase (Nluc) reporter genes (rSARS-CoV-2/mCherry-Nluc) to overcome limitations associated with the use of a single reporter gene. In cultured cells, rSARS-CoV-2/mCherry-Nluc displayed similar viral fitness as rSARS-CoV-2 expressing single reporter fluorescent and luciferase genes (rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc, respectively), or wild-type (WT) rSARS-CoV-2, while maintaining comparable expression levels of both reporter genes. *In vivo*, rSARS-CoV-2/mCherry-Nluc has similar pathogenicity in K18 human angiotensin converting enzyme 2 (hACE2) transgenic mice than rSARS-CoV-2 expressing individual reporter genes, or WT rSARS-CoV-2. Importantly, rSARS-CoV-2/mCherry-Nluc facilitates the assessment of viral infection and transmission in golden Syrian hamsters using *in vivo* imaging systems (IVIS). Altogether, this study demonstrates the feasibility of using this novel bireporter-expressing rSARS-CoV-2 for the study SARS-CoV-2 *in vitro* and *in vivo*.

IMPORTANCE

Despite the availability of vaccines and antivirals, the coronavirus disease 2019 (COVID-19) pandemic caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) continues to ravage health care institutions worldwide. Previously, we have generated replication-competent recombinant (r)SARS-CoV-2 expressing fluorescent or luciferase reporter proteins to track viral infection *in vitro* and/or *in vivo*. However, these rSARS-CoV-2 are restricted to express only a single fluorescent or a luciferase reporter gene, limiting or preventing their use to specific *in vitro* assays and/or *in vivo* studies. To overcome this limitation, we have engineered a rSARS-CoV-2 expressing both fluorescent (mCherry) and luciferase (Nluc) genes and demonstrated its feasibility to study the biology of SARS-CoV-2 *in vitro* and/or *in vivo*, including the identification and characterization of neutralizing antibodies and/or antivirals. Using rodent models, we visualize SARS-CoV-2 infection and transmission through *in vivo* imaging systems (IVIS).

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the coronavirus disease 2019 (COVID-19) pandemic (1). Since the first reported case in Wuhan, China, SARS-CoV-2 has spread worldwide and has been associated with more than 500 million confirmed cases and over 6 million deaths (https://coronavirus.jhu.edu/map.html) (2), in part due to its innate high transmissibility (3, 4). In the past two decades, two other human coronaviruses have been responsible for severe disease in humans, including severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002 and the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 (5, 6). Further, four endemic human coronaviruses are responsible for common cold-like respiratory disease: OC43, NL63, 229E, and HKU1 (7, 8). A unique feature of SARS-CoV-2 compared to known betacoronaviruses is the addition of a furin cleavage site in the viral spike (S) glycoprotein which is a major contributor to the virus's increased transmissibility and pathogenicity (9, 10). Several prophylactic (vaccines) and therapeutic (antivirals or monoclonal antibodies) options have been approved by the United States (US) Food and Drug Administration (FDA) to prevent or treat, respectively, SARS-CoV-2 infection. These include three vaccines [Spikevax (former Moderna), COMIRNATY (former BioNTech & Pfizer), and Janssen] (11, 12), several therapeutic antiviral drugs (remdesivir, baricitinib, molnupiravir, and nirmatrelvir), and one monoclonal antibody (MAb, bamlanivimab) (13-15). Unfortunately, SARS-CoV-2 has rapidly accumulated mutations, leading to the emergence of variants of concern

(VoC) and variants of interest (VoI) jeopardizing the effectiveness of existing preventive and/or treatment options (16-20).

Reverse genetics systems have permitted the generation of recombinant RNA viruses entirely from cloned cDNA, facilitating studies to better understand multiple aspects of the biology of viruses, including, among others, mechanisms of viral infection, pathogenesis, transmission, and disease (21-31). Another application of reverse genetics is the generation of recombinant viruses containing gene mutations and/or deletions that result in viral attenuation for their implementation as safe, immunogenic, and protective live-attenuated vaccines (LAV) (22, 32-37). Moreover, reverse genetics have been used to generate recombinant viruses expressing reporter proteins, thereby abolishing the need of secondary approaches for viral detection (38-44). In this regard, genetically modified recombinant viruses expressing reporter genes have been generated to monitor viral infection in cultured cells and/or in animal models using reporter expression as a valid surrogate readout for viral infection (38, 45-49). Notably, these reporter-expressing viruses have the potential to be used in high-throughput screening (HTS) settings to identify antivirals or neutralizing antibodies that can inhibit or neutralize, respectively, viral infection; and to visualize the dynamics of viral infection in validated animal models using *in vivo* imaging systems (IVIS).

Fluorescent and luciferase proteins are used to generate reporter-expressing viruses and represent ideal choices due to their high sensitivity and stability (50-56). Since these reporter genes have dissimilar characteristics, their selection is largely motivated by the type of study or application. Fluorescent proteins are easily detected when excited by absorbing energy at a particular wavelength, which is subsequently emitted

as light at higher wavelength as the molecules drop to a lesser energy state (55). Hence, reporter viruses expressing fluorescent proteins are typically used for *in vitro* studies to observe cellular localization and/or to identify the presence of infected cells (27, 38, 39, 47, 57). Moreover, fluorescence-expressing recombinant viruses are used to identify the presence of the virus in infected cells in validated animal models using *ex vivo* imaging (38, 39, 45-47). However, *in vivo*, fluorescent signals are often obscured by autofluorescence and have insufficient detection due to light scattering. Conversely, luciferases produce bright and localized signals in live organisms to be monitored in real-time using IVIS and represent a viable surrogate of viral replication (38, 39, 58). Moreover, viruses expressing luciferase genes are more sensitive and convenient for quantitative analyses compared to their fluorescent-expressing counterparts (39, 45, 48). Despite the clear advantages of both fluorescence and luciferase reporter genes, only recombinant viruses expressing either fluorescent or luciferase reporter genes have been previously described in the literature (38, 45, 46, 48, 49). In the past, we overcame this issue by generating dual reporter viruses expressing both luciferase and fluorescent reporter genes and demonstrated its advantages with influenza and vaccinia viruses (38, 39, 58).

In this study, we used our previously described bacterial artificial chromosome (BAC)-based reverse genetics (22, 23, 59-61) and the innovative 2A approach (45, 46) to pioneer a rSARS-CoV-2 expressing both fluorescence mCherry and luciferase Nluc reporter genes (rSARS-CoV-2/mCherry-Nluc). Our results demonstrate that rSARS-CoV-2/mCherry-Nluc has similar properties in cultured cells than rSARS-CoV-2 expressing individual mCherry or Nluc reporter genes, or wild-type (WT) rSARS-CoV-2.

Importantly expression of the double reporter gene mCherry-Nluc did not affect viral replication or pathogenesis in K18 human angiotensin converting enzyme 2 (hACE2) transgenic mice or golden Syrian hamsters, validating its use for both *in vitro* and/or *in vivo* studies.

MATERIAL AND METHODS

Biosafety and ethics statement

In vitro and *in vivo* experiments involving infectious rSARS-CoV-2 were conducted in a biosafety level 3 (BSL3) laboratory at Texas Biomedical Research Institute. Experimental procedures involving cell culture and animal studies were approved by the Texas Biomedical Research Institute Biosafety and Recombinant DNA Committees (BSC and RDC, respectively) and the Institutional Animal Care and Use Committee (IACUC).

Cells and viruses

African green monkey kidney epithelial cells (Vero E6; CRL-1586) were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM; Corning) supplemented with 5% fetal bovine serum (FBS; VWR) and 1% PSG (100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine; Corning) at 37°C with 5% CO_2.

Recombinant (r)SARS-CoV-2 were generated based on the whole genomic sequence of the USA-WA1/2020 (WA-1) strain (accession no. MN985325) (45, 60) using a previously described bacterial artificial chromosome (BAC)-based reverse genetics system (22, 23, 59-61). Viral titers (plaque forming units per milliliter, PFU/ml) were determined by plaque assay in Vero E6 cells.

Rescue of recombinant double reporter-expressing SARS-CoV-2

A BAC plasmid was used for the rescue of rSARS-CoV-2 expressing mCherry and Nanoluciferase (Nluc), referred as rSARS-CoV-2/mCherry-Nluc, as previously described (45). Briefly, a fused version of mCherry and Nluc was inserted in front of the viral N protein gene along with a porcine teschovirus 1 (PTV-1) 2A autocleavage site, within the pBeloBAC11 plasmid (NEB) containing the whole genomic sequence of SARS-CoV-2 WA-1 strain. We chose mCherry because red fluorescent proteins are more readily detectable in biological tissues, enabling lower absorbance and scattering of light, as well as less autofluorescence (47, 62-64). We selected Nluc due to its small size, ATP independence, and greater sensitivity and brightness compared with other luciferases (45, 48, 50, 65). Vero E6 cells (1.2 x 10^6 cells/well, 6-well plate format, triplicates) were transfected in suspension with 4.0 µg/well of SARS-CoV-2/mCherry-Nluc BAC plasmid using Lipofectamine 2000 (Thermo Fisher Scientific). Transfection media was changed to post-infection media (DMEM containing 2% FBS and 1% PSG) after 24 h, and cells were split and seeded into T75 flasks 2-days post-transfection. After 3 days, viral rescues were detected by fluorescence microscopy, and cell culture supernatants were collected, labeled as P0 and stored at -80ºC. After viral titration, P1 viral stocks were generated by infecting fresh Vero E6 cells at low multiplicity of infection (MOI) 0.0001 for 3 days and following stored at -80°C.

Reverse transcription (RT)-PCR

Total RNA was extracted from rSARS-CoV-2/mCherry-Nluc-infected (MOI 0.01) Vero E6 cells (1.2 x 10^6 cells/well, 6-well format) using TRIzol reagent (Thermo Fisher Scientific) based on the manufacturer's instructions. The viral genome between 27,895-

29,534 nucleotides based on the SARS-CoV-2 WA-1 strain was RT-PCR amplified using Super Script II Reverse transcriptase (Thermo Fisher Scientific) and Expanded High Fidelity PCR system (Sigma Aldrich). Amplified DNA products were separated on a 0.7% agarose gel, purified using a Wizard Genomic DNA Purification kit (Promega), and sent for Sanger sequencing (ACGT). Primer sequences are available upon request.

Deep sequencing

RNA sequencing library was prepared with a KAPA RNA HyperPrep kit, involving 100 ng of viral RNA and 7 mM of adaptor, and was subjected to 45 min adaptor ligation incubation and 6 cycles of PCR. An Illumina Hiseq X was used to sequence all samples and raw sequencing reads were trimmed and filtered using Trimmomatic V0.32 (66, 67). Bowtie2 V2.4.1 (68) and MosDepth V0.2.6 (69) were used to map sequence reads and quantify genome coverage to reference SARS-CoV-2-WA1/2020 viral genome (MN985325.1), respectively. LoFreq V2.1.3.1 (70) was used to determine low frequency variants and eliminate sites that were less than 100 read depth or less than 1% allele frequencies.

Immunofluorescence assays

Vero E6 cells (1.2 x 10^6 cells/well, 6-well format, triplicates) were mock-infected or infected (MOI 0.01) with rSARS-CoV-2/WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc. At 24 h post-infection (hpi), cells were fixed in 10% neutral buffered formalin at 4ºC overnight and permeabilized using 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 10 min at room temperature (RT). Cells were washed with PBS, blocked with 2.5% bovine albumin serum (BSA) in PBS for 1 h

and then incubated with 1 µg/ml of SARS-CoV anti-N monoclonal antibody (MAb) 1C7C7 in 1% BSA at 4ºC overnight. Cells were washed with PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Dako; 1:200). Cell nuclei were stained with 4', 6'-diamidino-2-phenylindole (DAPI; Research Organics). Representative images (20X) were acquired using an EVOS M5000 imaging system (Thermo Fisher Scientific).

SDS-PAGE and Western blot

Cell lysates were prepared from either mock- or virus-infected (MOI 0.01) Vero E6 cells (1.2 x 10^6 cells/well, 6-well format) after 24 hpi using passive lysis buffer (Promega) based on the manufacturer's instructions. After centrifugation (12,000 x *g*) at 4ºC for 30 min, proteins were separated with 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% dried skim milk in 0.1% Tween 20 PBS (T-PBS) and incubated at 4ºC overnight with the following specific primary MAbs or polyclonal antibodies (PAbs): N (mouse MAb 1C7C7), mCherry (rabbit Pab; Raybiotech), and Nluc (rabbit Pab, Promega). Then, membranes were incubated at 37ºC for 1 h with goat anti-mouse IgG StarBright Blue 520 or anti-rabbit IgG Starbright Blue 700 (Bio-Rad) secondary antibodies. Tubulin was used as a loading control using anti-tubulin hFAB rhodamine antibody (Bio-Rad). Proteins were detected using a ChemiDoc MP imaging system (Bio-Rad).

Plaque assay

Vero E6 cells (2 x 10^5 cells/well, 24-well plate format, triplicates) were infected with 25-50 PFUs of rSARS-CoV-2/WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc for 1 h, overlaid with post-infection media containing 0.6%

agar (Oxoid) and incubated at 37ºC in a 5% CO_2 incubator. At 72 hpi, cells were fixed in 10% neutral buffered formalin at 4ºC overnight and then mCherry-positive plaques were visualized using a ChemiDoc MP imaging system (Bio-Rad). Afterwards, cells were permeabilized in T-PBS for 10 min at RT, blocked in 2.5% BSA in PBS for 1 h, and incubated with specific primary MAb or PAb against the viral N protein (mouse MAb 1C7C7) or Nluc (rabbit PAb). To detect Nluc-positive viral plaques, cells were stained with a FITC-conjugated goat anti-rabbit IgG (Dako; 1:200) and visualized using a ChemiDoc MP imaging system (Bio-Rad). Next, viral plaques were stained with an anti-mouse Vectastain ABC kit and DAB HRP Substrate kit (Vector laboratories) following the manufacturers' recommendations.

Viral growth kinetics

Vero E6 cells (1.2 x 10^6 cells/well, 6-well plate format, triplicates) were infected (MOI 0.01) with rSARS-CoV-2/WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc. After 1 h adsorption, cells were washed with PBS and incubated at 37ºC in post-infection media. Viral titers in cell culture supernatants at each of the indicated time points (12, 24, 48, 72, and 96 hpi) were determined by plaque assay as described above. At each time point, mCherry expression was visualized with an EVOS M5000 imaging system. Nluc activity in the cell culture supernatants at the same times post-infection was quantified using a microplate reader and a Nano-Glo Luciferase Assay system (Promega) following the manufacturers' recommendations. Mean values and standard deviation (SD) were calculated with Microsoft Excel software.

Reporter-based microneutralization and antiviral assays

253 Microneutralization and antiviral assays were performed as previously described (47,
254 71). Human MAb 1212C2 (72) against the Spike protein receptor-binding domain (RBD)
255 of SARS-CoV-2 was serially diluted (3-fold) in post-infection media (starting
256 concentration of 500 ng), combined with 100-200 PFUs/well of rSARS-CoV-2/WT,
257 rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc and
258 incubated at RT for 1 h. Then, Vero E6 cells (4 x 10^4 cells/well, 96-well plate format,
259 quadruplicates) were infected with the antibody-virus mixture and incubated at 37ºC in a
260 5% CO_2 incubator. Cells infected with rSARS-CoV-2/WT were overlaid with 1% Avicel
261 as previously described (71). Nluc activity in cell culture supernatants of cells infected
262 with rSARS-CoV-2/Nluc or rSARS-CoV-2/mCherry-Nluc was quantified at 24 hpi using
263 Nano-Glo luciferase substrate as per manufacturer's instructions, and a Synergy LX
264 microplate reader and analyzed using a Gen5 data analysis software (Bio-Tek). To
265 measure mCherry signal, cells infected with rSARS-CoV-2/mCherry or rSARS-CoV-
266 2/mCherry-Nluc were fixed in 10% neutral buffered formalin overnight and washed with
267 PBS before quantified in a Synergy LX microplate reader. For cells infected with rSARS-
268 CoV-2/WT, plaques were detected using the anti-N MAb 1C7C7 as indicated above and
269 quantified using an ImmunoSpot Analyzer (CTL). Total viral infection (100%) was
270 determined from the number of plaques, fluorescence and luciferase values obtained
271 from virus-infected cells without the 1212C2 hMAb. Viral infection means and SD values
272 were calculated from quadruplicate individual wells of three independent experiments
273 with Microsoft Excel software. Non-linear regression curves and 50% neutralization titer
274 (NT_{50}) values were determined using GraphPad Prism Software (San Diego, CA, USA,
275 V. 8.2.1).

Inhibition of SARS-CoV-2 in antiviral assays was conducted as previously described (47, 71). Briefly, Vero E6 cells (4 x 10^4 cells/well, 96-well plate format, quadruplicates) were infected with 100-200 PFUs/well of rSARS-CoV-2/WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc and incubated at 37ºC for 1 h. Afterwards, the virus inoculum was removed and replaced with post-infection media containing 3-fold serial dilutions of remdesivir (starting concentration of 100 µM) and cells were incubated at 37ºC in a 5% CO_2 incubator. Cells infected with rSARS-CoV-2/WT were overlaid with 1% Avicel as previously described (71). After 24 hpi, Nluc activity from cell culture supernatants infected with rSARS-CoV-2/Nluc or rSARS-CoV-2/mCherry-Nluc was determined using Nano-Glo luciferase substrate and a Synergy LX microplate reader. For cells infected with rSARS-CoV-2/mCherry or rSARS-CoV-2/mCherry-Nluc, mCherry expression was quantified in a Synergy LX microplate reader. Lastly, rSARS-CoV-2/WT was detected using the anti-N MAb 1C7C7 and quantified using an ImmunoSpot Analyzer (CTL). Total viral infection (100%) was calculated from number of plaques, fluorescence, and luciferase values of infected cells in the absence of remdesivir. Means and SD values were calculated from quadruplicates from three independent experiments with Microsoft Excel software. The 50% effective concentration (EC_{50}) was calculated by sigmoidal dose-response curves on GraphPad Prism (San Diego, CA, USA, Version 8.2.1).

Mice experiments

Female 4-6 weeks old K18 hACE2 transgenic mice [B6.Cg-Tg(K18-ACE2)2Prlmn/J, The Jackson laboratory] were maintained in the animal care facility at Texas Biomedical Research Institute under specific pathogen-free conditions. For viral infections, groups

of mice were anesthetized with gaseous isoflurane and inoculated intranasally with the indicated viruses. A separate group of K18 hACE2 transgenic mice were also mock-infected with PBS and served as a negative control.

For body weight and survival studies, K18 hACE2 transgenic mice (n=4) were intranasally infected with 10^5 PFUs/mouse of the indicated viruses and monitored daily for body weight loss and survival to access morbidity and mortality, respectively, over a period of 12 days. Mice that were below 75% of their initial body weight were considered to have reached their experimental endpoint and were humanly euthanized.

In vivo bioluminescence imaging of live mice (n=4) was conducted with an Ami HT *in vivo* imaging system (IVIS; Spectral Instruments) at 1, 2, 4, and 6 days post-infection (DPI). At each time points, mice were anesthetized with isoflurane and retro-orbitally injected with 100 µl of Nano-Glo luciferase substrate diluted by 1:10 in PBS. Mice were immediately placed in an isolation chamber and imaged using the Ami HT IVIS. Radiance within the region of interest (ROI) of each mouse was analyzed using the Aura software (Spectral Instruments) and total flux values (protons/s) were normalized to background signal of mock-infected control.

To access fluorescence expression in the lungs and to determine viral titers, a separate cohort of mice (n=4) were similarly infected with the indicated recombinant viruses and were humanely euthanized at 2 and 4 DPI after *in vivo* imaging. Lungs were surgically excised, washed in PBS, and fluorescent and brightfield images were obtained using an Ami HT IVIS and an iPhone 6s (Apple), respectively. Fluorescence signal (radiance efficiency) around the ROI of the lungs were quantified using the Aura software and mean values were normalized to the autofluorescence of mock-infected

lungs. The macroscopic pathology score was determined in a blinded manner by a certified pathologist from brightfield images of the lungs, in which the percent of total surface area of lungs affected by consolidation, lesions, congestion, and/or atelectasis was quantified with NIH ImageJ software as previously described (60, 73). Nasal turbinate and brains were also collected, and tissues were individually homogenized in 1 ml of PBS using a Precellys tissue homogenizer (Bertin Instruments). Tissue homogenates were centrifuged at 12,000 x *g* at 4ºC for 5 min to pellet cell debris, and supernatants were collected. Viral titers were determined by plaque assay and immunostaining as described above. Nluc activity in the tissue homogenates were determined using Nano-Glo luciferase substrate kit and a Synergy LX microplate reader.

Hamster experiments

Female 4-6 weeks old golden Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratories and maintained in the animal care facility at Texas Biomedical Research Institute under specific pathogen-free conditions. For viral infections, hamsters were anesthetized with isoflurane and intranasally infected with rSARS-CoV-2/mCherry-Nluc. One day later, infected hamsters were transferred to cages containing contact naïve hamsters. A separate group of hamsters were also mock-infected with PBS and served as a negative control.

In vivo bioluminescence imaging of live hamsters (n=4) was conducted with an Ami HT IVIS on 2, 4 and 6 DPI. Hamsters were anesthetized with gaseous isoflurane in an isolation chamber, and Nano-Glo luciferase substrate was diluted 1:10 in PBS and retro-orbitally injected into each animal (200 µl). Immediately after, hamsters were

secured in the isolation chamber and imaged with an Ami HT IVIS and bioluminescence analyses were performed. The total flux values were obtained around the ROI of each hamster and normalized to mock-infected hamsters. Next, hamsters were euthanized, and mCherry expression in excised lungs were imaged in an Ami HT IVIS. The Aura software was used to determine the radiant efficiency of the ROI. Fluorescence signal obtained from infected or contact lungs were normalized to mock-infected lungs. Brightfield images of lungs were taken using an iPhone 6s and were used to assess the pathology score in a blinded manner. A trained pathologist determined the percent of lung surface that was affected by lesions, congestion, consolidation, and/or atelectasis using NIH ImageJ (60, 73). Along with the lungs, nasal turbinate were excised and homogenized in PBS using a Precellys tissue homogenizer at 12,000 x *g* for 5 min. Supernatants were collected and used to determine viral titers and Nluc activity as described above.

Statistical analysis

All data are presented as mean values and SD for each group and were analyzed using Microsoft Excel software. A one-way ANOVA or student t-test was used for statistical analysis on GraphPad Prism or Microsoft Word software, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; and ns, no significance.

RESULTS

Generation of rSARS-CoV-2/mCherry-Nluc

Recently, we have generated rSARS-CoV-2 expressing single reporter genes upstream of the viral N gene using a PTV-1 2A autoproteolytic peptide approach (25). These new rSARS-CoV-2 displayed higher levels of reporter gene expression than those previously described in which the reporter gene substitutes the viral ORF7a protein (45-47). To generate a rSARS-CoV-2 expressing two reporter genes, mCherry and Nluc, we implemented a similar method and inserted a fusion sequence of mCherry-Nluc, and the PTV-1 2A autoproteolytic peptide, upstream of the SARS-CoV-2 N gene in the BAC containing a full length copy of the SARS-CoV-2 genome (**Figure 1A**), and rescued rSARS-CoV-2/mCherry-Nluc using our previously described protocol (46, 60). To assess whether mCherry expression could be directly visualized by fluorescence microscopy, Vero E6 cells were mock-infected or infected (MOI 0.01) with

391 rSARS-CoV-2/WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-
392 2/mCherry-Nluc (**Figure 1B**). At 24 hpi, cells were fixed and mCherry expression was
393 directly assessed under a fluorescence microscope, which showed high mCherry
394 fluorescence expression in cells infected with rSARS-CoV-2/mCherry or rSARS-CoV-
395 2/mCherry-Nluc, but not in cells infected with rSARS-CoV-2/WT or rSARS-CoV-2/Nluc
396 (**Figure 1B**). Further, viral infection was detected by indirect immunofluorescence
397 microscopy using an anti-N protein 1C7C7 MAb (**Figure 1B**). As expected, all Vero E6
398 cells infected with the different rSARS-CoV-2 mutants, but not mock-infected cells, were
399 positive for the presence of the virus. Expression of mCherry and Nluc reporter genes
400 were also confirmed by Western blot (**Figure 1C**). As expected, mCherry was readily
401 detected in whole cell lysates from Vero E6 cells infected with rSARS-CoV-2/mCherry
402 or rSARS-Cov-2/mCherry-Nluc but not in those infected with rSARS-CoV-2/WT or
403 rSARS-CoV-2/Nluc; or mock-infected (**Figure 1C**). Likewise, Nluc was detected only in
404 cell extracts from rSARS-CoV-2/Nluc and rSARS-CoV-2/mCherry-Nluc infected cells
405 and not in those infected with rSARS-CoV-2/WT or rSARs-CoV-2/mCherry; or mock-
406 infected (**Figure 1C**). A specific band for the viral N protein appeared in all the virus-
407 infected cell extracts, but not in mock-infected Vero E6 cell extracts, all of which showed
408 comparable protein levels of N protein expression (**Figure 1C**). The identity of the
409 double reporter-expressing rSARS-CoV-2/mCherry-Nluc was further validated by next
410 generation sequencing (**Figure 1D).** The rSARS-CoV-2/mCherry and rSARS-CoV-
411 2/Nluc were also sequenced as reference controls. We found two non-reference alleles
412 with a frequency greater than 10% in rSARS-CoV-2/mCherry in the viral N (C752T) and
413 envelope, E (V5A) proteins (**Figure 1D,** top). Likewise, we identified two amino acid

changes in the rSARS-CoV-2/Nluc S (H655Y) and E (S6L) proteins (**Figure 1D,** middle). No amino acid changes were found in rSARS-CoV-2/mCherry-Nluc compared to the reference viral genome (**Figure 1D,** bottom), indicating that rSARS-CoV-2/mCherry-Nluc resembles the sequence of rSARS-CoV-2/WT apart from the insertion of the mCherry-Nluc reporter gene fusion and the PTV-1 2A autoproteolytic site.

***In vitro* characterization of rSARS-CoV-2/mCherry-Nluc**

Since the cloning of two reporter genes as a fusion protein could affect viral fitness and/or reporter gene expression, we examined the viral fitness of rSARS-CoV-2/mCherry-Nluc in cultured cells by assessing growth kinetics and compared them to those of rSARS-CoV-2 expressing single reporter gene (e.g. rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc) or rSARS-CoV-2/WT (**Figure 2A**). Vero E6 cells were infected at an MOI of 0.01 and viral titers in cell culture supernatants were quantified at different time points. No significant difference in replication kinetics were found between all the indicated viruses, except for rSARS-CoV-2/Nluc, which replicated slightly slower (**Figure 2A**). Conversely, rSARS-CoV-2/mCherry-Nluc reached a high titer of 10^7 PFU/ml by 24-48 hpi like rSARS-Co-2/WT and rSARS-CoV-2/mCherry, suggesting that the expression of the double reporter fused mCherry-Nluc gene did not affect viral fitness in Vero E6 cells (**Figure 2A**). In parallel, Nluc and mCherry expression were evaluated over a period of 96 h by either assessing Nluc activity in cell culture supernatants (**Figure 2B**) or by fluorescence microscopy (**Figure 2C**). Vero E6 cells were similarly infected (MOI 0.01) and Nluc activity in cell culture supernatants were quantified at different time points. We found Nluc activity steadily increased beginning at 12 hpi and peaked at 72 hpi, then decreased at 96 hpi (**Figure 2B**). No Nluc activity was

detected in cell culture supernatants from mock-infected or Vero E6 cells infected with rSARS-CoV-2/WT or rSARS-CoV-2/mCherry (**Figure 2B**). Similarly, mCherry expression was detected as early as 12 hpi and increased in a time dependent matter until 72 hpi (**Figure 2C**). At 96 hpi, mCherry expression was lightly reduced, which coincided with the decrease in Nluc activity and viral titers at the same time point most likely due to the cytopathic effect (CPE) caused by viral infection. As expected, no mCherry expression was detected in Vero E6 cells infected with rSARS-CoV-2/WT or rSARS-CoV-2/Nluc, or mock-infected (not shown). These results suggest that *in vitro* detection and replication of rSARS-CoV-2/mCherry-Nluc could be monitored and quantified based on the expression of either Nluc (**Figure 2B**) or mCherry (**Figure 2C**) reporter genes.

Next, plaque assays were conducted to further corroborate that all rSARS-CoV-2/mCherry-Nluc viral particles express both mCherry and Nluc reporter genes, and to evaluate plaque phenotype and compared them to that of rSARS-CoV-2 expressing individual reporter genes (rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc), and rSARS-CoV-2/WT (**Figure 2D**). When plaques were examined by fluorescence microscopy, mCherry-positive plaques were detected in cells infected with rSARS-CoV-2/mCherry and rSARS-CoV-2/mCherry-Nluc (**Figure 2D**). Then, Nluc-positive plaques were detected using an anti-Nluc specific Ab and FITC-conjugated secondary Ab, which only appeared in cells infected with rSARS-CoV-2/Nluc or rSARS-CoV-2/mCherry-Nluc (**Figure 2D**). Importantly, when viral plaques were immunostained with an anti-N protein Ab, they colocalized with mCherry- and/or Nluc-positive plaques (white arrows) in both Vero E6 cells infected with individual reporter-expressing rSARS-CoV-2/mCherry and

460 rSARS-CoV-2/Nluc, as well as in double reporter-expressing rSARS-CoV-2/mCherry-
461 Nluc (**Figure 2D**), demonstrating that all rSARS-CoV-2 plaques contained the reporter
462 gene(s). Although the overall plaque size phenotype did not vary between the different
463 viruses, we did observe smaller plaques produced (amongst the normal sized plaques)
464 in rSARS-CoV-2/mCherry-Nluc experiments (**Figure 2D**).

465 **A double reporter-based neutralization assay for the identification of SARS-CoV-2**
466 **neutralizing antibodies and antivirals**

467 To demonstrate the feasibility of implementing our rSARS-CoV-2/mCherry-Nluc to
468 identify and characterize neutralizing Abs (NAbs) and antivirals, we developed a double
469 reporter-based microneutralization assay using 1212C2 hMAb (**Figure 3A**) and
470 remdesivir (**Figure 3B**), which are described and shown to neutralize or inhibit,
471 respectively, SARS-CoV-2 (74, 75). The neutralization activity of 1212C2 was assessed
472 by incubating the hMAb with the indicated rSARS-CoV-2 prior to infection of Vero E6
473 cells, and quantifying Nluc activity in cell culture supernatants (**Figure 3A**, right panel)
474 and mCherry expression (**Figure 3A**, middle panel) using a microplate reader at 24 hpi.
475 As internal control, we conducted the microneutralization assay using immunostaining
476 of rSARS-CoV-2/WT, as previously described (**Figure 3A**, left panel) (71). We
477 determined the 50% neutralization concentration (NT_{50}) of 1212C2 hMAb using
478 sigmoidal dose-response curves. The NT_{50} of 1212C2 hMAb against rSARS-CoV-
479 2/mCherry (2.4 ng) and rSARS-CoV-2/mCherry-Nluc (2.7 ng) as determined by
480 fluorescent mCherry expression were similar to that of rSARS-CoV-2/WT (3 ng) using a
481 classical immunostaining assay, and those reported with the SARS-CoV-2 WA-1 natural
482 isolate (46, 74). Moreover, NT_{50} values of 1212C2 hMAb against rSARS-CoV-2/Nluc or

483 rSARS-CoV-2/mCherry-Nluc determined by Nluc expression (3.0 and 2.0 ng,
484 respectively) were also comparable to those of rSARS-CoV-2/WT (3 ng). To determine
485 whether rSARS-CoV-2/mCherry-Nluc could also be used to assess the effectiveness of
486 antivirals, we quantified the Nluc activity (**Figure 3B**, right panel) and mCherry
487 expression (**Figure 3B**, middle panel) in Vero E6 cells infected with the single and
488 double reporter-expressing rSARS-CoV-2 in the presence of serial 3-fold dilutions of
489 remdesivir. As before, we also included rSARS-CoV-2/WT infected cells stained with
490 the MAb against the viral N protein as internal control (**Figure 3B**, left panel). Sigmoidal
491 dose-response curves were developed from reporter expression values and used to
492 calculate the 50% effective concentration (EC_{50}). The EC_{50} values of remdesivir against
493 the indicated viruses were similar to each other, regardless of whether the
494 microneutralization assay used immunostaining (rSARS-CoV-2/WT, 2 μM; left panel),
495 fluorescence (rSARS-CoV-2/mCherry, 1.7 μM; rSARS-CoV-2/mCherry-Nluc, 1.5 μM;
496 middle panel), or luciferase (rSARS-CoV-2/Nluc, 1.4 μM; rSARS-CoV-2/mCherry-Nluc,
497 1.5 μM; right panel) (**Figure 3B**). Overall, these results demonstrate the feasibility of
498 using the rSARS-CoV-2 expressing both mCherry and Nluc reporter genes to reliably
499 and quickly evaluate the neutralizing and inhibitory properties of NAbs and/or antivirals,
500 respectively, against SARS-CoV-2 based on mCherry and/or Nluc expression,
501 respectively.

502 **Characterization of rSARS-CoV-2/mCherry-Nluc in K18 hACE2 transgenic mice**

503 Next, we characterized the pathogenicity and ability of rSARS-CoV-2/mCherry-Nluc
504 to replicate in K18 hACE2 transgenic mice using rSARS-CoV-2 expressing individual
505 fluorescent and bioluminescent reporter genes (rSARS-CoV-2/mCherry and rSARS-

CoV-2/Nluc, respectively), and rSARS-CoV-2/WT as internal control. One group of mice was infected with a mixture of rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc. To assess pathogenicity, groups of K18 hACE2 transgenic mice (n=4/group) were mock-infected or infected with 10^5 PFUs of the indicated viruses and changes in body weight (**Figure 4A**) and survival (**Figure 4B**) were monitored for 12 DPI. All mice infected with rSARS-CoV-2 rapidly lost body weight and succumbed to viral infection (**Figures 4A and 4B,** respectively). Most importantly, the virulence of rSARS-CoV-2/mCherry-Nluc was shown to be identical to that of our previously reporter viruses expressing individual mCherry or Nluc (46), or rSARS-CoV-2/WT (71, 76). These data indicate that expression of the fusion of mCherry and Nluc from rSARS-CoV-2/mCherry-Nluc does not result in viral attenuation in K18 hACE2 transgenic mouse model as compared to rSARS-CoV-2/WT.

Tracking viral dynamics of rSARS-CoV-2/mCherry-Nluc infection and pathogenesis in K18 hACE2 transgenic mice

Since our rSARS-CoV-2/mCherry-Nluc expresses both fluorescent (mCherry) and luciferase (Nluc) reporter genes, we sought to demonstrate the advantage of using this newly double reporter-expressing rSARS-CoV-2/mCherry-Nluc to track viral replication in live animals. Thus, K18 hACE2 transgenic mice were mock-infected or infected with 10^5 PFU of the indicated rSARS-CoV-2 reporter viruses intranasally and Nluc was monitored at 1, 2, 4, and 6 DPI (**Figure 5A**). In mice infected with rSARS-CoV-2/Nluc or rSARS-CoV-2/mCherry-Nluc, or co-infected at the same time with rSARS-CoV-2/Nluc and rSARS-CoV-2/mCherry, we detected Nluc signal as early as 1 DPI, which increased over time (**Figure 5A**). Since IVIS was conducted in the same mouse, viral

529 replication and distribution was followed over time (**Figure 5A**) and bioluminescence
530 intensity around the chest area of the mice was measured in flux (**Figure 5B**). As
531 expected, Nluc expression increased over time until mice succumbed to SARS-CoV-2
532 infection, consistent with previous literature, including ours (45). Notably, and as
533 expected based on the IVIS (**Figure 5A**), Nluc expression was only readily detected in
534 K18 hACE2 transgenic mice infected with rSARS-CoV-2/Nluc, rSARS-CoV-2/mCherry-
535 Nluc, or co-infected with both, rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc (**Figure**
536 **5B**). No significant differences in flux were observed between the groups of mice
537 infected with the Nluc-expressing rSARS-CoV-2 mutants (**Figure 5B**).

538 As luciferase and fluorescence proteins have different properties and could
539 potentially reveal different readouts as surrogate indicators of viral infection, we next
540 determined and compared Nluc and mCherry expression during infection *in vivo*. Thus,
541 K18 hACE2 transgenic mice (n=4) were mock-infected or infected with rSARS-CoV-
542 2/WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, rSARS-CoV-2/mCherry-Nluc, or co-
543 infected with rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc, then on 2 and 4 DPI, Nluc
544 activity in the entire mouse (**Figures 6A and 6B**) and mCherry expression of whole
545 lungs (**Figures 6C and 6D**) were determined, including the gross pathology score
546 (**Figure 6E**). Like our previous results (**Figure 5**), an increase in Nluc expression from 2
547 to 4 DPI was observed in K18 hACE2 transgenic mice infected with rSARS-CoV-2/Nluc,
548 rSARS-CoV-2/mCherry-Nluc, or co-infected with rSARS-CoV-2/mCherry and rSARS-
549 CoV-2/Nluc (**Figures 6A**). These results were further confirmed when we determined
550 the flux in the *in vivo* imaged mice (**Figures 6B**). After quantifying Nluc expression, the
551 lungs from mock- and rSARS-CoV-2-infected K18 hACE2 transgenic mice were excised

552 and imaged in the IVIS to determine and quantify mCherry expression (**Figures 6C and**
553 **6D,** respectively). We only observed detectable levels of mCherry expression in the
554 lungs of K18 hACE2 transgenic mice infected with rSARS-CoV-2/mCherry, rSARS-coV-
555 2/mCherry-Nluc, or co-infected with both rSARS-CoV-2/mCherry and rSARS-coV-
556 2/Nluc (**Figures 6C and 6D**). Notably, levels of mCherry expression, like those of Nluc
557 were comparable in the lungs of K18 hACE2 transgenic mice infected with the double
558 reporter-expressing rSARS-CoV-2/mCherry-Nluc than those infected with the single
559 rSARS-CoV-2/Nluc, or co-infected with rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc
560 (**Figures 6C and 6D**). Correlating with *in vivo* and *ex vivo* imaging of the lungs, gross
561 lung pathology scores were comparable in all rSARS-CoV-2-infected K18 hACE2
562 transgenic mice and more significant at 4 DPI (**Figure 6E**).
563 Both Nluc activity and viral titers peaked at 2 DPI in the nasal turbinate of mice
564 infected with rSARS-CoV-2/Nluc, rSARS-CoV-2/mCherry-Nluc, or co-infected with
565 rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc (**Figures 7A and 7B**, left panels)
566 However, in the lungs, Nluc activity remained the same at 2 and 4 DPI, while viral titers
567 decreased at 4 DPI as compared to 2 DPI (**Figures 7A and 7B**, middle panels). Nluc
568 activity in brain homogenates was only evident in the samples from mice infected with
569 rSARS-CoV-2/Nluc, rSARS-CoV-2/mCherry-Nluc, or both rSARS-CoV-2/mCherry and
570 rSARS-CoV-2/Nluc and signals increased in a time dependent matter (**Figure 7A**, right
571 panel). Consistent with previous studies, we were only able to detect SARS-CoV-2 in
572 the brain of two of the four mice infected with rSARS-CoV-2/mCherry-Nluc at 4 DPI
573 (**Figure 7B**, right panel) (45)**.** Altogether, these findings demonstrate the feasibility to
574 asses viral infection *in vivo* in the entire mice by bioluminescence (Nluc), and *ex vivo* in

575 the lungs of infected mice by fluorescence (mCherry) with our double reporter-
576 expressing rSARS-CoV-2/mCherry-Nluc and that the mCherry-Nluc fusion does not
577 have a significant impact in the pathogenesis and replication of the virus in K18 hACE2
578 transgenic mice, showing similar levels of Nluc or mCherry reporter gene expression
579 than those of rSARS-CoV-2 expressing individual bioluminescence or fluorescence
580 proteins. Notably, viral titers of rSARS-CoV-2 mCherry-Nluc in the nasal turbinate and
581 lungs were comparable to those of a rSARS-CoV-2/WT.

582 **Assessment of SARS-CoV-2 infection and transmission in golden Syrian**
583 **hamsters**

584 To demonstrate the feasibility of using our double reporter rSARS-CoV-2/mCherry-
585 Nluc to assess viral replication and transmission, golden Syrian hamsters (n=4) were
586 mock-infected or infected with 10^5 PFU/hamster of rSARS-CoV-2/mCherry-Nluc. The
587 day after infection, non-infected naïve contact hamsters were placed in the same cage
588 with infected hamsters. On 2, 4, and 6 DPI Nluc expression in the entire hamsters were
589 evaluated by IVIS, like in our previous studies using K18 hACE2 transgenic mice.
590 Infected hamsters presented detectable levels of Nluc expression in both the nasal
591 turbinate and lungs at 2 and 4 DPI that decreased at 6 DPI (**Figure 8A**). In contrast,
592 contact hamsters had little to no Nluc signal on 2 DPI that drastically increased on 4 and
593 6 DPI (**Figure 8A**). The temporal and spatial differences in Nluc signal between
594 originally infected and contact hamsters is most likely due to the route of
595 transmission/infection and the time frame in which the contact hamsters were exposed
596 to the originally infected hamsters. These initial IVIS results were further confirmed by
597 quantification of bioluminescence in hamsters (**Figure 8B**) that showed a decrease in

598 flux in infected hamsters from 2 to 6 DPI and an increase from 2 to 4 DPI and then
599 decreased on 6 DPI in contact hamsters (**Figure 8B**). Subsequently, lungs were
600 excised and imaged in the IVIS for Nluc and mCherry expression (**Figure 8C**). Nluc and
601 mCherry levels of expression correlate between them and with that observed by IVIS in
602 the whole hamster, revealing a time-dependent effect in reporter expression in both
603 infected and contact hamsters that correlate with the levels observed by IVIS (**Figure**
604 **8D**). Gross pathology scores in the lungs of infected and contact hamsters were
605 determined from brightfield images with increased scores over time in both infected and
606 contact animals (**Figure 8E**).

607 Finally, nasal turbinate and lungs from mock and originally infected or contact
608 hamsters were processed to determine Nluc activity (**Figure 9A**) and viral titers (**Figure**
609 **9B**). In the nasal turbinate of originally infected hamsters, both Nluc activity and viral
610 titers decreased from 2 to 6 DPI, while in contact hamsters, a time-dependent increase
611 was observed (**Figures 9A and 9B,** respectively). A similar trend between Nluc signal
612 and viral titers were observed in the lungs of originally infected and contact hamsters.
613 Notably, Nluc signal and viral titers from both groups of infected and contact hamsters
614 correlated with bioluminescence intensity from whole animals and excised lungs
615 (**Figure 8**). Based on these results with rSARS-CoV-2/mCherry-Nluc, viral infection can
616 be monitored in hamsters solely using reporter expression that correlates well with
617 levels of viral replication. Moreover, rSARS-CoV-2 transmission from originally infected
618 to contact hamsters can be easily tracked *in vivo* in the whole animal or *ex vivo* in the
619 lungs (**Figure 8**), and results correlate with those of viral replication (**Figure 9**).
620

DISCUSSION

Replication-competent, reporter-expressing, recombinant viruses have been previously shown to represent an excellent approach to study, among others, viral infection, replication, pathogenesis, and transmission (21-31). We and others have described the feasibility of generating rSARS-CoV-2 expressing reporter genes encoding either fluorescent or luciferase proteins (38, 45, 46, 48, 49). These reporter-expressing replication-competent rSARS-CoV-2 can be used to assess the prophylactic activity of vaccines and/or the therapeutic potential of NAbs or antivirals (47, 71). Moreover, these rSARS-CoV-2 expressing fluorescent or luciferase proteins represent an excellent option to study the biology of SARS-CoV-2 in cultured cells and/or in

644 validated small animals of infection (46, 47, 49). Moreover, we have described a new
645 approach to express reporter genes from the locus of the viral N protein using a 2A
646 autoproteolytic system where levels of fluorescent or luciferase expression are higher
647 than those where the reporter gene substitutes the viral ORF7a (45, 46). This new 2A
648 strategy to express the reporter gene from the viral N protein locus does not require
649 deleting the viral ORF7a (45, 46). However, these previously described reporter-
650 expressing replication-competent rSARS-CoV-2 only express a single fluorescent or a
651 luciferase protein and, therefore, their experimental applications are limited to the
652 properties of one specific reporter gene and available equipment (45, 46).

653 Using our previously described BAC-based reverse genetics (21, 59), we generated
654 a rSARS-CoV-2 expressing a fusion of the fluorescent mCherry protein to the
655 bioluminescence Nluc protein (rSARS-CoV-2/mCherry-Nluc) upstream of the viral N
656 protein separated by the PTV-1 2A autoproteolytic cleavage site, thereby allowing
657 separate expression of the mCherry-Nluc fusion and the viral N protein (45, 46).
658 Expression of both reporter genes was validated by fluorescence microscopy (mCherry)
659 or by luciferase activity with a microplate reader (Nluc). We further confirmed reporter
660 expression by Western blot, where a specific band was detected for the mCherry-Nluc
661 fusion polyprotein. In cell culture, rSARS-CoV-2/mCherry-Nluc displayed growth kinetics
662 similar to those of rSARS-CoV-2 expressing individual mCherry (rSARS-CoV-
663 2/mCherry) or Nluc (rSARS-CoV-2/Nluc), or a rSARS-CoV-2 lacking reporter genes
664 (rSARS-CoV-2/WT). Likewise, the plaque phenotype of the novel rSARS-CoV-
665 2/mCherry-Nluc were similar in size to those of rSARS-CoV-2/mCherry, rSARS-CoV-
666 2/Nluc, or rSARS-CoV-2/WT but only rSARS-CoV-2/mCherry-Nluc had detectable

levels of expression of both reporter genes. Importantly, levels of mCherry or Nluc reporter expression correlated with the levels of viral replication, further supporting the concept of reporter genes being a valid surrogate to study viral infection.

Based on the advantages of using a rSARS-CoV-2 expressing both a fluorescent and a bioluminescence protein over those expressing either fluorescence or luciferase reporter genes, we developed a bireporter-based microneutralization assay to identify and characterize NAbs as well as antivirals. Importantly, NT_{50} (NAbs) and EC_{50} (antivirals) values obtained in bireporter-based microneutralization assays using either fluorescence or luciferase signal were comparable to those obtained with rSARS-CoV-2 expressing individual mCherry (rSARS-CoV-2/mCherry) or Nluc (rSARS-CoV-2/Nluc) reporter genes, or rSARS-CoV-2/WT, and those reported previously in the literature (24, 45, 46, 48). Overall, the bireporter rSARS-CoV-2 represents an excellent option in circumstances were fluorescence or luciferase is negated by the properties of an antiviral drug (such as fluorescing chemical entities in certain small molecule compounds) or the host cell being studied (46, 71). Moreover, although in this report the bireporter-based microneutralization assay was performed in 96-well plates, it can be easily adapted to a 384-well format for high-throughput screenings (HTS) to identify NAbs or antivirals using a double reporter screening approach based on expression of both fluorescent mCherry and luciferase Nluc. In this instance, having two reporters allows HTS to have the option to use either reporters, or both, to further validate neutralization results.

One of the major limitations of recombinant viruses expressing reporter genes is their potential attenuation *in vivo* (39). To assess whether expression of mCherry fused

690 to Nluc affected SARS-CoV-2 replication *in vivo,* we infected K18 hACE2 transgenic
691 mice and golden Syrian hamsters with rSARS-CoV-2/mCherry-Nluc. Despite encoding a
692 fusion of two reporter genes from the locus of the viral N protein, rSARS-CoV-
693 2/mCherry-Nluc displayed similar virulence as determined by changes in body weight
694 and survival in K18 hACE2 transgenic mice as rSARS-CoV-2 expressing individual
695 fluorescent (rSARS-CoV-2/mCherry) or luciferase genes (rSARS-CoV-2/Nluc), or
696 rSARS-CoV-2/WT. Importantly we traced viral infection in the same animal over a
697 period of 6 days based on Nluc expression. We were able to detect both luciferase and
698 fluorescent expression in the lungs of mice infected with rSARS-CoV-2/mCherry-Nluc;
699 and mCherry or Nluc expression levels in the lungs of infected mice were comparable to
700 those observed in mice infected with rSARS-CoV-2/mCherry or rSARS-CoV-2/Nluc, or
701 co-infected with both rSARS-CoV-2/mCherry and rSARS-CoV-2/Nluc, or rSARS-CoV-
702 2/WT. Notably, rSARS-CoV-2/mCherry-Nluc replicated in the nasal turbinate, lungs, and
703 brain of infected K18 hACE2 transgenic mice to levels comparable to recombinant
704 viruses expressing individual reporter genes (rSARS-CoV-2/mCherry or rSARS-CoV-
705 2/Nluc) and those of rSARS-CoV-2/WT. Similar results were also observed in the
706 golden Syrian hamster model of SARS-CoV-2 infection and transmission (21, 72).
707 Importantly, in the case of hamsters, we were able to track viral infection and
708 transmission in infected and contact hamsters, respectively, demonstrating the
709 feasibility of using our double reporter-expressing rSARS-CoV-2/mCherry-Nluc in
710 transmission studies in hamsters.

711 In summary, we have generated a rSARS-CoV-2 expressing simultaneously two
712 reporter genes that is suitable for multiple experimental applications currently not

713 available with the use of rSARS-CoV-2 expressing a single fluorescent or luciferase
714 reporter gene. This rSARS-CoV-2/mCherry-Nluc virus is, to our knowledge, the first
715 replication-competent rSARS-CoV-2 stably expressing two reporter genes. With rSARS-
716 CoV-2/mCherry-Nluc, mCherry could be used to identify infected cells *in vitro* while Nluc
717 represents a better option to provide quantify levels of infection. In animal studies,
718 mCherry is a superior option for *ex vivo* imaging and for the identification of infected
719 cells (45). Moreover, by combining two recombinant viruses expressing two different
720 fluorescent proteins, one could evaluate the antiviral or neutralizing activity of antivirals
721 or antibodies, respectively, against multiple viruses by looking at different fluorescent
722 expression (47). In contrast, Nluc is the only viable option for *in vivo* imaging using
723 entire animals (45). Importantly, both reporter genes can be used as valid surrogates of
724 viral infection since their levels of expression correlate with those of viral replication,
725 demonstrating the feasibility of using this novel bireporter-expressing rSARS-CoV-2 to
726 study the biology of SARS-CoV-2 *in vitro* and/or *in vivo*. The feasibility of generating
727 rSARS-CoV-2 expressing a fusion of two reporter genes demonstrates the plasticity of
728 the viral genome to express large ORFs from the locus of the viral N protein. Moreover,
729 the robust levels of reporter gene expression obtained using this 2A autoproteolytic
730 approach and the feasibility of expressing foreign genes without the need of removing a
731 viral protein (e.g. ORF7a) represent an ideal option for the use of rSARS-CoV-
732 2/mCherry-Nluc to study viral infection, pathogenesis and transmission, including newly
733 identified VoC.
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774 **FIGURE LEGENDS**
775 **Figure 1. Generation of a bireporter rSARS-CoV-2 expressing mCherry and Nluc**
776 **(rSARS-CoV-2/mCherry-Nluc). A) Schematic representation of the rSARS-CoV-**
777 **2/mCherry-Nluc viral genome:** SARS-CoV-2 structural, non-structural, and accessory
778 open reading frame (ORF) proteins are indicated in white boxes. mCherry (red), Nluc
779 (blue) and the PTV-1 2A autoproteolytic sequence (black) were inserted in front of the
780 viral N protein. **B) mCherry expression and immunofluorescence assays:** Vero E6
781 cells (1.2 x 10^6 cells/well, 6-well format, triplicates) were mock-infected or infected (MOI

782 0.01) with rSARS-CoV-2 WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-
783 CoV-2/mCherry-Nluc. Cells were fixed in 10% neutral buffered formalin 24 hpi before
784 directly visualizing mCherry expression under a fluorescence microscope or the viral N
785 protein using a specific 1C7C7 MAb. Cell nuclei were strained with DAPI.
786 Representative images are shown. Scale bars = 100 µm. Magnification = X20. **C)**
787 **Western blots:** Vero E6 cells (1.2 x 10^6 cells/well, 6-well format, triplicates) were mock-
788 infected or infected (MOI 0.01) with rSARS-CoV-2 WT, rSARS-CoV-2/mCherry, rSARS-
789 CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc. At 24 hpi, cells were collected and protein
790 expression in cell lysates were evaluated by Western blot using specific antibodies
791 against SARS-CoV-2 N protein, or the mCherry and Nluc reporter proteins. Tubulin was
792 included as a loading control. The molecular mass of proteins is indicated in kilodaltons
793 (kDa) on the left. **D) Deep sequencing analysis of reporter-expressing rSARS-CoV-**
794 **2:** The non-reference allele frequency of rSARS-CoV-2/mCherry (top), rSARS-CoV-
795 2/Nluc (middle), and rSARS-CoV-2/mCherry-Nluc (bottom) was calculated by
796 comparing the short reads to the respective reference SARS-CoV-2 WA-1 viral genome
797 (MN985325.1). Non-reference alleles present in less than 10% of reads are not shown
798 (dotted line) and the non-reference allele frequency that is greater than 10% is
799 indicated.

800 **Figure 2. *In vitro* characterization of the bireporter rSARS-CoV-2/mCherry-Nluc**
801 **virus. A) Viral growth kinetics:** Viral titers (PFU/ml) in the cell culture supernatants of
802 Vero E6 cells (1.2 x 10^6 cells/well, 6-well format, triplicates) infected (MOI 0.01) with
803 rSARS-CoV-2 WT (WT), rSARS-CoV-2/mCherry (mCherry), rSARS-CoV-2/Nluc (Nluc),
804 or rSARS-CoV-2/mCherry-Nluc (mCherry-Nluc) at the indicated time points post-

infection were determined by plaque assay. Data represents the mean values and SD of triplicates. LOD, limit of detection. **B) Nluc activity:** Nluc activity in the cell culture supernatants obtained from the experiment in panel A is represented in relative light units (RLU). **C) mCherry expression kinetics:** Vero E6 cells (1.2 x 10^6 cells/well, 6-well format, triplicates) were infected (MOI 0.01) with rSARS-CoV-2 WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc and mCherry expression was directly visualized under a fluorescence microscope at the indicated times post-infection. Representative images are shown. Scale bars = 300 μm. Magnification = X10. **D) Plaque phenotype:** Viral plaques from Vero E6 cells (2 x 10^5 cells/well, 24-well plate format, triplicates) infected with rSARS-CoV-2 WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc at 3 DPI were observed under a fluorescence imaging system (first column, red filter), fluorescently stained with an antibody against Nluc (second column, FITC), or immunostaining with an antibody against the viral N protein (third column, N). White arrowheads depict the overlapping signal of mCherry fluorescence (left), Nluc bioluminescence (middle), and immunostaining of the viral N protein (right) in Vero E6 cells infected with rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, or rSARS-CoV-2/mCherry-Nluc. ns, not significant. *, $P < 0.05$; **, $P < 0.01$.

Figure 3. Bireporter-based microneutralization assay to identify NAbs and antivirals against SARS-CoV-2. A) A bireporter microneutralization assay to identify NAbs: Three-fold serial dilutions of the SARS-CoV-2 1212C2 hMAb (starting concentration of 500 ng) were prepared in post-infection media and incubated with 100-200 PFUs/well of rSARS-CoV-2 WT (WT), rSARS-CoV-2/mCherry (mCherry), rSARS-

CoV-2/Nluc (Nluc), or rSARS-CoV-2/mCherry-Nluc (mCherry-Nluc) for 1 h at RT. Vero E6 cells (96-well plate format, 4 x 10^4 cells/well, quadruplicates) were infected and incubated with the virus-antibody mixture at 37^o C for 24 h. Viral neutralization was determined by immunostaining using an anti-N protein MAb (1C7C7) for rSARS-CoV-2/WT (left) or by fluorescence expression for rSARS-CoV-2/mCherry and rSARS-CoV-2/mCherry-Nluc (middle), or bioluminescence for rSARS-CoV-2/Nluc and rSARS-CoV-2/mCherry-Nluc (right) using a microplate reader. The 50% neutralization titer (NT_{50}) was calculated using sigmoidal dose-response curves on GraphPad Prism. Viral neutralization was normalized to wells containing infected cells without the 1212C2 hMAb. The dotted line indicates 50% virus inhibition. Data are represented by the mean values and SD of quadruplicates. **B) A bireporter microneutralization assay to assess antivirals:** Vero E6 cells (96-well plate format, 4 x 10^4 cells/well, quadruplicates) were infected with 100-200 PFUs of rSARS-CoV-2/WT, or reporter viruses expressing mCherry, Nluc, or mCherry-Nluc. After 1 h viral absorption, cells were incubated in post-infection media containing 3-fold serial dilutions of remdesivir (starting concentration of 100 μM). Viral inhibition was determined by immunostaining using an anti-N protein MAb (1C7C7) for rSARS-CoV-2/WT (left) or by fluorescence expression for rSARS-CoV-2/mCherry and rSARS-CoV-2/mCherry-Nluc (middle), or bioluminescence for rSARS-CoV-2/Nluc and rSARS-CoV-2/mCherry-Nluc (right) using a microplate reader. The 50% effective concentration (EC_{50}) was calculated using sigmoidal dose-response curves on GraphPad Prism. Viral inhibition was normalized to wells containing infected cells without remdesivir. The dotted line indicates the 50% virus inhibition. The data is represented by the mean values and SD of quadruplicates.

851 **Figure 4. Virulence of rSARS-CoV-2/mCherry-Nluc in K18 hACE2 transgenic mice:**
852 Four-to-six-weeks-old female K18 hACE2 transgenic mice (n=4) were mock-infected or
853 intranasally inoculated with 10^5 PFU/mouse of rSARS-CoV-2 WT (WT), rSARS-CoV-
854 2/mCherry (mCherry), rSARS-CoV-2/Nluc (Nluc), or the bireporter rSARS-CoV-
855 2/mCherry-Nluc (mCherry-Nluc). A group of four-to-six-weeks-old female K18 hACE2
856 transgenic mice (n=4) were also co-infected with rSARS-CoV-2/mCherry and rSARS-
857 CoV-2/Nluc (mCherry + Nluc). Body weight loss (**A**) and survival (**B**) of mice were
858 monitored for 12 days after viral infection.
859 **Figure 5. *In vivo* kinetics of rSARS-CoV-2/mCherry-Nluc in K18 hACE2 transgenic**
860 **mice:** Four-to-six-weeks-old female K18 hACE2 transgenic mice (n=4) were mock-
861 infected or infected intranasally with 10^5 PFU/mouse of rSARS-CoV-2 WT, rSARS-CoV-
862 2/mCherry, rSARS-CoV-2/Nluc, rSARS-CoV-2/mCherry + rSARS-CoV-2/Nluc, or with
863 rSARS-CoV-2/mCherry-Nluc (mCherry-Nluc). Nluc activity in the whole mouse at the
864 indicated DPI was evaluated with an Ami HT *in vivo* imaging system. Representative
865 images of the same mouse at 1, 2, 4, and 6 DPI are shown (**A**). Means and SD of the
866 radiance (number of photons per second per square centimeter per steradian
867 [p/second/cm2/sr]) and bioluminescence (total flux [$\log_{10}$ photons per second (p/s)] over
868 each mouse are shown) (**B**). ns, not significant. ***, $P < 0.001$.
869 **Figure 6. *In vivo* bioluminescence and *ex vivo* fluorescence in K18 hACE2**
870 **transgenic mice infected with rSARS-CoV-2/mCherry-Nluc. A) *In vivo* Nluc**
871 **expression:** Nluc activity in live mice (n=4) mock-infected or infected (10^5 PFU/mouse)
872 with rSARS-CoV-2 WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, rSARS-CoV-
873 2/mCherry + rSARS-CoV-2/Nluc, or the bireporter rSARS-CoV-2/mCherry-Nluc

(mCherry-Nluc) were determined on 2 and 4 DPI using the Ami HT IVIS. A representative image of a mouse per time point is shown. **B) Quantification of Nluc signal:** Means and SD of the radiance (number of photons per second per square centimeter per steradian [p/second/cm2/sr]) and bioluminescence (total flux [log_{10} photons per second (p/s)] of mock and infected mice is shown. **C) *Ex vivo* mCherry expression:** Excised lungs from mock-infected or infected mice from panel A were monitored for mCherry fluorescent expression (FL, top) and bright field (BF, bottom) at 2 and 4 DPI. Representative lung images from the same mouse used in panel A are shown. **D) Quantification of mCherry expression:** The mean values of mCherry signal around the regions of interest were normalized to the autofluorescence of mock-infected lungs at each time point and the fold changes in fluorescence were calculated. **E) Gross pathology score:** Pathology lesions, consolidation, congestion, and atelectasis, of excised lungs were measured using NIH ImageJ and represented as percentages of total lung surface area affected. ns, not significant. *, $P < 0.05$; ***, $P < 0.001$.

Figure 7. Nluc activity and viral titers in tissue homogenates from infected K18 hACE2 transgenic mice: The nasal turbinate (left), lungs (middle), and brain (right) of four-to-six-weeks-old female K18 hACE2 transgenic mice (n=4) mock-infected or infected intranasally with 10^5 PFU/mouse of rSARS-CoV-2 WT, rSARS-CoV-2/mCherry, rSARS-CoV-2/Nluc, rSARS-CoV-2/mCherry + rSARS-CoV-2/Nluc, or the bireporter rSARS-CoV-2/mCherry-Nluc were collected after imaging on an Ami HT IVIS on 2 and 4 DPI. After homogenization, Nluc activity (**A**) and viral titers (**B**) in tissue homogenates were determined on a microplate reader or by plaque assay, respectively. The results

are the mean values and SD. LOD, limit of detection. ns, not significant. nd, not detected. **, $P < 0.01$; ***, $P < 0.001$.

Figure 8. *In vivo* bioluminescence and *ex vivo* fluorescence in golden Syrian hamsters infected with rSARS-CoV-2/mCherry-Nluc. A) *In vivo* Nluc expression: Nluc activity in four-to-six-weeks-old female golden Syrian hamsters (n=4) mock-infected or infected with 10^5 PFU/hamster of rSARS-CoV-2/mCherry-Nluc were determined on 2, 4 and 6 DPI using the Ami HT IVIS. Contact animals were exposed to infected animals 1 DPI. A representative image of a hamster per time points and experimental condition is shown. **B) Quantification of Nluc signal:** Means and SD of the radiance (number of photons per second per square centimeter per steradian [p/second/cm2/sr]) and bioluminescence (total flux [$\log_{10}$ photons per second (p/s)] were quantified from whole hamsters after IVIS imaging. **C) *Ex vivo* mCherry and Nluc expression:** Excised lungs from mock-infected or infected golden Syrian hamsters from panel A were monitored for mCherry fluorescence (FL, top), Nluc signal (Nluc, middle), and bright field (BF, bottom) at 2, 4, and 6 DPI. Representative lung images from the same hamster used in panel A are shown. **D) Quantification of mCherry expression:** the mean values of mCherry signal around the regions of interest were normalized to the autofluorescence of mock-infected lungs at each time point and the fold changes in fluorescence were calculated. **E) Gross pathology score:** Pathological lesions, consolidation, congestion, and atelectasis, of excised lungs were measured using NIH ImageJ and represented as percentages of total lung surface area affected. ns, not significant. *, $P < 0.05$; ***, $P < 0.001$.

Figure 9. Nluc activity and viral titers in golden Syrian hamster tissue homogenates infected with rSARS-CoV-2/mCherry-Nluc: The nasal turbinate (left) and lungs (right) of four-to-six-weeks-old female golden Syrian hamsters (n=4) mock-infected or infected intranasally with 10^5 PFU/hamster of rSARS-CoV-2/mCherry-Nluc were collected after imaging on an Ami HT IVIS at 2 and 4 DPI. In addition, after 24 hpi, contact golden Syrian hamsters (n=4, contact) were added to the cages of infected animals. After homogenization, Nluc activity (**A**) and viral titers (**B**) in tissue homogenates were determined on a microplate reader or by plaque assay, respectively. Results are the means and SD. LOD, limit of detection. ns, not significant. nd, not detected. ***, $P < 0.001$.

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