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Live imaging and quantification of viral infection in K18 hACE2 transgenic mice using reporter-expressing recombinant SARS-CoV-2

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TITLE:

Live Imaging and Quantification of Viral Infection in K18 hACE2 Transgenic Mice using Reporter-Expressing Recombinant SARS-CoV-2

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KEYWORDS:

SARS-CoV-2, coronavirus, fluorescence, bioluminescence, replication-competent viruses, reporter virus, NanoLuc, luciferase, venus, fluorescent protein, viral infection, *in vivo* imaging, IVIS, Ami HT, K18 hACE2 transgenic mice.

SUMMARY:

This protocol describes the dynamics of viral infections using luciferase- and fluorescence-expressing recombinant (r)SARS-CoV-2 and an *in vivo* imaging systems (IVIS) in K18 hACE2 transgenic mice to overcome the need of secondary approaches required to study SARS-CoV-2 infections *in vivo*.

ABSTRACT:

The coronavirus disease 2019 (COVID-19) pandemic has been caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). To date, SARS-CoV-2 has been responsible for over 186 million infections and more than 4 million deaths worldwide. Similar to other viruses, studying SARS-CoV-2 requires the use of experimental methods to detect the presence of virus in infected cells and/or in animal models. To overcome this limitation, we generated replication-competent recombinant (r)SARS-CoV-2 that expresses bioluminescent (nanoluciferase, Nluc) or fluorescent (Venus) proteins. These reporter-expressing rSARS-CoV-2 allow tracking viral infections *in vitro* and *in vivo* based on the expression of Nluc and Venus reporter genes. Here the study describes the use of rSARS-CoV-2/Nluc and rSARS-CoV-2/Venus to detect and track SARS-CoV-2 infection in the previously described K18 human angiotensin-converting enzyme 2 (hACE2) transgenic

mouse model of infection using *in vivo* imaging systems (IVIS). This rSARS-CoV-2/Nluc and rSARS-CoV-2/Venus show rSARS-CoV-2/WT-like pathogenicity and viral replication *in vivo*. Importantly, Nluc and Venus expression allow us to directly track viral infections *in vivo and ex vivo*, in infected mice. These rSARS-CoV-2/Nluc and rSARS-CoV-2/Venus represent an excellent option to study the biology of SARS-CoV-2 *in vivo*, to understand viral infection and associated COVID-19 disease, and to identify effective prophylactic and/or therapeutic treatments to combat SARS-CoV-2 infection.

INTRODUCTION:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped, positive-sense, single-stranded RNA virus that belongs to the Betacoronavirus lineage in the *Coronaviridae* family[1]. This viral family is divided into Alpha-, Beta-, Gamma-, and Delta-coronavirus[1]. Alpha- and Betacoronaviruses mainly infect mammals, whereas Gamma- and Deltacoronavirus infect almost exclusively birds[2]. To date, seven coronaviruses (CoV) have crossed species barriers and emerged as human coronaviruses (HCoV): two alpha-CoVs (HCoV-229E and HCoV-NL63) and five beta-CoVs (HCoV-OC43, HCoV-HKU1, SARS-CoV, Middle East respiratory syndrome coronavirus [MERS-CoV], and SARS-CoV-2)[3–6]. SARS-CoV, MERS-CoV, and SARS-CoV-2 are highly pathogenic, causing severe lower respiratory tract infection[7]. Prior to the emergence of SARS-CoV-2, there were two epidemic outbreaks caused by CoVs: SARS-CoV in Guangdong Providence, China, from 2002–2003, with a case fatality rate (CFR) of about 9.7%; and MERS-CoV in the Middle East from 2012 to present, with a CFR of about 34%[7,8]. SARS-CoV-2 has an overall CFR between 3.4%–49%, with underlying conditions contributing to a higher CFR[8,9]. Since its discovery in December 2019, in Wuhan, China, SARS-CoV-2 has been responsible for over 186 million human infections and more than 4 million human deaths worldwide[7,10–12]. Notably, since late 2020, new SARS-CoV-2 variants of concern (VoC) and variants of interest (VoI) have impacted virus characteristics, including transmission and antigenicity[9,13], and the overall direction of the COVID-19 pandemic. For the treatment of SARS-CoV-2 infections, there is currently only one United States (U.S.) Food and Drug Administration (FDA) therapeutic antiviral (remdesivir) and one Emergency Use Authorization (EUA) drug (baricitinib, to be administered in combination with remdesivir)[14]. There are also 5 approved EUA monoclonal antibodies: REGEN-COV (casirivimab and imdevimab, administered together), sotrovimab, and bamlanivimab and etesevimab administered together[15–19]. There is currently only one FDA-approved prophylactic vaccine, Pfizer-BioNTech, and two other prophylactic vaccines (Moderna and Janssen) have been EUA approved[20–24]. However, with the uncontrolled infection rate and the emergence of VoC and VoI, SARS-CoV-2 still poses a threat to human health. Therefore, new approaches are urgently needed to identify efficient prophylactics and therapeutics to control SARS-CoV-2 infection and the still ongoing COVID-19 pandemic.

Studying SARS-CoV-2 requires laborious techniques and secondary approaches to identify the presence of the virus in infected cells and/or validated animal models of infection. The use of reverse genetics has allowed for the generation of recombinant viruses to answer important questions in the biology of viral infections. For instance, reverse genetics techniques have provided means to uncover and understand the mechanisms of viral infection, pathogenesis, and disease. Likewise, reverse genetics approaches have paved the way to engineer recombinant

viruses lacking viral proteins to understand their contribution in viral pathogenesis. In addition, reverse genetics techniques have been used to generate recombinant viruses expressing reporter genes for *in vitro* and *in vivo* applications, including identifying prophylactic and/or therapeutic approaches for the treatment of viral infections. Fluorescent and bioluminescent proteins are the most commonly used reporter genes due to their sensitivity, stability, and easy detection based on the improvement of new technologies[25,26]. *In vitro*, fluorescent proteins have been shown to serve as a better option for the localization of viruses in infected cells, while luciferases are more convenient for quantification studies[27–29]. *In vivo*, luciferases are preferred over fluorescent proteins for whole animal imaging, while fluorescent proteins are preferred for the identification of infected cells or *ex vivo* imaging[30–32]. The use of reporter-expressing recombinant viruses has served as a powerful tool for the study of viruses in many families, including, among others, flaviviruses, enteroviruses, alphaviruses, lentiviruses, arenaviruses, and influenza viruses[28,33–36].

To overcome the need for secondary approaches to study SARS-CoV-2 and characterize real-time SARS-CoV-2 infection *in vivo*, we have generated replication-competent recombinant (r)SARS-CoV-2 that expresses bioluminescent (nanoluciferase, Nluc) or fluorescent (Venus) proteins using our previously described bacterial artificial chromosomes(BAC)-based reverse genetics, which are maintained as a single copy in *E. coli* in order to minimize toxicity of virus sequences during its propagation in bacteria[37,38]. Notably, rSARS-CoV-2/Nluc and rSARS-CoV-2/Venus showed rSARS-CoV-2/WT-like pathogenicity *in vivo*. The high level of Venus expression from rSARS-CoV-2/Venus allowed detecting viral infection in the lungs of infected K18 hACE2 transgenic mice using an *in vivo* imaging system (IVIS)[39]. The levels of Venus expression correlated well with viral titers detected in the lungs, demonstrating the feasibility of using Venus expression as a valid surrogate of SARS-CoV-2 infection. Using rSARS-CoV-2/Nluc, we were able to track the dynamics of viral infection in real-time and longitudinally assess SARS-CoV-2 infection *in vivo* using the same IVIS approach in K18 hACE2 transgenic mice.

PROTOCOL:

Protocols involving K18 hACE2 transgenic mice were approved by the Texas Biomedical Research Institute (TBRI) Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC). All experiments follow the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council[40]. The appropriate Personal Protection Equipment (PPE) is required when working with mice.

1. Use of K18 hACE2 transgenic mice

1.1. Purchase and maintain 4–6-week-old female B6.Cg-Tg(K18-ACE2)2Prlmn/J mice (K18 hACE2 transgenic mice) in a biosafety level (BSL)-2 animal care facility under specific pathogen-free conditions.

1.1.1. After arrival at the BSL2 facilities, allow the animals to acclimate for 7 days and transfer them to the BSL-3 animal facility for infections and other experimental procedures.

1.1.2. Following IACUC protocols, place 4 mice per cage. To ensure that the animal is deceased, after mice infection with rSARS-CoV-2 and *in vivo* imaging, euthanize the animals with a lethal dose of Fatal-Plus (>100 mg/kg).

2. Biosafety

NOTE: In this manuscript, rSARS-CoV-2 is generated using the BAC-based reverse genetic systems for SARS-CoV-2 USA-WA1/2020 strain, as previously described[37]. All *in vivo* procedures involving rSARS-CoV-2/Nluc or rSARS-CoV-2/Venus infections must be performed in a biological safety cabinet under BSL-3 conditions.

2.1 Clean the biosafety cabinet with 2% Wexicide and 70% Ethanol disinfectant sequentially before and after performing all the experimental procedures described in this article.

2.2 Sterilize all dissection material (scissors, dissecting forceps, etc.) and the homogenizer before and after each use.

2.3 Clean the isolation chamber and disinfect using MB10 tablets before and after each use according to the manufacturer's instruction.

2.4 Discard all biological material produced during the procedures following IBC and IACUC guidelines.

3. *In vivo* characterization of rSARS-CoV-2

3.1 Mouse infections

3.1.1 Place female 4–6-week-old K18 hACE2 transgenic mice in labeled cages, identify the mice in each cage using an ear punch code and place 4 mice per cage. Use a group of mice for body weight and survival and another group of animals for viral titration and imaging.

3.1.2 Before infection, shave mice chest on the ventral side from the pectoral nipple to the inguinal nipple area to facilitate the bioluminescence signal.

3.1.3 Prepare the rSARS-CoV-2 inoculum with 10^5 plaque-forming units (PFU)/mouse under sterile conditions in a total volume of 50 µL using sterile 1x phosphate buffer saline (PBS).

NOTE: Calculate the amount of rSARS-CoV-2 to be used in the viral dilution with the formula: virus needed = (10^5 PFU per mouse / 50 µL) x final volume) / stock viral titer.

3.1.4 Keep the virus inoculum chilled on ice.

3.1.5 Use the mock-infected (1x PBS) and rSARS-CoV-2/WT-infected mice as internal controls. Place the mock-infected mice in a separate cage than rSARS-CoV-2-infected mice.

3.1.6 Anesthetize the mice using 5% isoflurane gaseous sedation in an anesthesia chamber and maintain with 3% isoflurane.

3.1.7 Once postural reaction and righting reflex are confirmed, place the mouse in the dorsal recumbency position.

3.1.8 Scruff the neck of the mouse between the index finger and thumb and hold the tail against the palm of the hand with the pinky finger to hold the animal in a dorsal position.

3.1.9 Place the pipette tip containing 50 µL of the virus inoculum in the nostril and slowly eject the solution. Ensure that the virus inoculum is inhaled and there is no reflux by observing the inoculum drop disappearing.

3.2 Bioluminescence monitoring K18 hACE2 transgenic mice infected with rSARS-CoV-2/Nluc (**Figure 1** and **Figure 2**)

NOTE: This experiment follows the schematic representation and uses the viruses displayed in **Figure 1**. An isoflurane anesthesia attachment is required for the *in vivo* imaging system (IVIS). See **Table of Materials** for details instruments and systems required.

3.2.1 Initiate the imaging software and set up the parameters, click image mode to **Bioluminescence**, open filter, and set the exposure time to auto.

3.2.2 Upon initializing the IVIS machine, place the mice in the isolation chamber while still inside the biosafety cabinet. Induce mice with isoflurane at a 5% concentration. Once loss of the postural reaction and righting reflex is confirmed, maintain with 3% isoflurane.

3.2.3 Once mice are anesthetized, remove them from the isolation chamber and retro-orbitally administer the luciferase substrate diluted 1:10 in 1x PBS (final volume 100 µL/mouse) with a 25 G needle.

3.2.4 After luciferase substrate administration, place the mice back in the isolation chamber with their chests facing up and nasal cavity inside the manifold cone to keep the animal anesthetized during the imaging procedure.

3.2.5 Transfer the isolation chamber to the IVIS machine, and instantaneously after closing the IVIS imager door, select **Acquire** in the software program to initialize (**Figure 2A**).

3.2.6 To analyze bioluminescence images acquired, utilize the **ROI** (region of interest) tool in the software to designate the precise signal and measure the flux (**Figure 2B**).

3.2.7 Click on **Measure** and allow the system to begin assessing the bioluminescence in photons to provide the absolute photon emission comparable to the output measurements provided by the various parameters.

3.3 Fluorescence analysis in K18 hACE2 transgenic mice infected with rSARS-CoV-2/Venus (**Figure 3** and **Figure 4**)

NOTE: This experiment follows the schematic representation and rSARS-CoV-2 shown in **Figure 3**. See **Table of Materials** for details instruments and systems required.

3.3.1 Initiate the imaging software and set up the parameters, set excitation (500 nm) and emission filters (530 nm), click image mode to **Fluorescence** and set the exposure time to auto.

3.3.2 Euthanize the mice using a lethal dose of Fatal-Plus (>100 mg/kg). Following euthanasia, disinfect the incision site with 70% ethanol.

3.3.3 Using tweezers, pull the skin, make an incision from the sternum to the abdomen and cut the incision from the sides with scissors. Cut the hepatic vein to minimize the amount of blood in the lungs and avoid high background signals during imaging.

3.3.4 Using scissors, cut the sternum and open the ribcage. Next, snip the end of the trachea with scissors and remove the lungs with tweezers.

3.3.5 Place the excised lungs in a 6-well plate containing 2 mL of 1x PBS and rinse to remove excess blood. Minimize the possibility of contamination between samples by disinfecting and cleaning surgery tools between samples using 70% ethanol.

3.3.6 After initializing the IVIS machine, place the lungs in a black tray and separate the tissues from each other.

3.3.7 Place the tray inside the isolation chamber inside the biosafety cabinet, and then transfer the isolation chamber to the IVIS. Close the door and click on **Acquire** to initiate the imaging system (**Figure 4A**).

3.3.8 To analyze fluorescence, utilize the **ROI** tool and draw ROIs around each of the individual lungs. Measure each ROI manually and then use the average radiant efficiency values given and subtract from those of the mock-infected mice (**Figure 4B**).

3.3.9 Once imaging is complete, place the tissues on ice for same-day analysis or in a cryotube for dry ice freezing to store at -80 °C for later processing.

3.4 Lung bright field imaging and pathology scoring of K18 hACE2 transgenic mice infected with rSARS-CoV-2/Nluc (**Figure 2A–C**) and rSARS-CoV-2/Venus (**Figure 4A–C**)

3.4.1 After imaging bioluminescence of mice infected with rSARS-CoV-2/Nluc, rSARS-CoV-2/WT, and mock-infected, return the mice to their cages. Proceed with euthanasia and the *ex vivo* bright field imaging of mice lungs (**Figure 2A**).

3.4.2 After imaging *ex vivo* fluorescence of lungs of infected mice, take bright-field images of the lungs (**Figure 4A**).

3.4.3 Analyze the gross lesions on the surface of the lung using ImageJ (**Figures 2C** and **4C**).

3.4.4 Open the lung image to be analyzed in ImageJ.

3.4.5 Calculate the ratio of pixel to cm, use the **Straight** tool and measure the actual length of the image.

3.4.6 After selecting, click on **Analyze** > **Measure** to calculate the length of pixels for the length.

3.4.7 Click on **Analysis** > **Set Scale** and input the numbers calculated from the previous step.

3.4.8 Use the **Freehand Selections** tool and select the entire lung surface. Click on **Analyze > Measure** to measure total lung area.

3.4.9 Click on **Edit** > **Selection** > **Make Inverse** to select the rest of the lung area, then press delete or backspace to remove the background.

3.4.10 Remove the selected area, then click on **Image** > **Adjust** > **Color Threshold**.

3.4.11 To select pathologic lesion area, adjust **Brightness** to a minimum (between 1 to 50) and a maximum (between 50 to 200), depending on the levels of congestion and hemorrhages present.

3.4.12 Once pathologic lesions are selected, click on **Select** at the bottom of the threshold color panel, then click on **Analyze** > **Measure** to measure pathologic areas.

3.4.13 Calculate the percentage of gross lesions with the formula: % of pathologic area = (measurement of pathologic area/total lung surface) x 100.

3.5 Viral titrations (**Figure 2D** and **Figure 4D**)

3.5.1 Upon imaging, complete the mice euthanasia and collect lungs, brain, and nasal mucosa.

3.5.2 Place the lungs, brain, and nasal mucosa into separate sterile tissue homogenizers and add 1 mL of cold 1x PBS.

3.5.3 Homogenize the samples by centrifuging at 21,500 x *g* for 10 min to pellet cell debris. Collect and transfer the supernatants into a new sterile tube and discard the pellet.

3.5.4 If viral titrations are performed the same day, store the supernatants at 4 °C. Alternatively, freeze the supernatant of the homogenized samples at -80 °C until being evaluated later.

3.5.5 Utilizing the supernatants obtained from the tissue homogenates make 10-fold serial dilutions and infect confluent monolayers of Vero E6 cells with 1 mL of each dilution of the supernatant (6-well plate format, 1.2×10^6 cells/well, triplicates).

3.5.6 Let the virus adsorb for 1 h at 37 °C in a humidified 5% CO_2 incubator.

3.5.7 After viral adsorption, wash the cells with 1 mL of 1x PBS and incubate in 2 mL of post-infection media containing 1% Agar in the humidified 5% CO_2 incubator at 37 °C for 72 h.

3.5.8 After the incubation, inactivate the plates in 10% neutral buffered formalin for 24 h at 4 °C, ensure the entire plate is submerged.

3.5.9 Take plates out of BSL3 and wash the cells three times with 1 mL of 1x PBS and permeabilize with 1 mL of 0.5% Triton X-100 for 10 min at room temperature (RT).

3.5.10 Block the cells with 1 mL of 2.5% bovine serum albumin (BSA) in 1x PBS for 1 h at 37 °C, followed by incubation in 1 mL of 1 µg/mL of the SARS-CoV nucleocapsid (N) protein cross-reactive monoclonal antibody (1C7C7), diluted in 2.5% BSA for 1 h at 37 °C.

3.5.11 Wash the cells three times with 1 mL of 1x PBS and develop the plaques using the ABC kit and DAB Peroxidase Substrate kit according to the manufacturers' instructions.

3.5.12 Calculate the viral titers as PFU/mL.

NOTE: Calculate with the formula PFU/mL = dilution factor x number of plaques x (1 mL/inoculum volume).

3.6 Nluc activity in tissues of K18 hACE2 transgenic mice infected with rSARS-CoV-2/Nluc (**Figure 2E**)

3.6.1 Quantify the presence of Nluc in the organ homogenates from mock, rSARS-CoV-2/WT, and rSARS-CoV-2/Nluc infected K18 hACE2 transgenic mice using a luciferase assay following the manufacturers' instructions.

3.7 Evaluation of morbidity and mortality (**Figure 2F**,**G** and **Figure 4E**,**F**)

3.7.1 Intranasally infect 4–6-week-old female K18 hACE2 transgenic mice with 10^5 PFU of rSARS-CoV-2/WT, rSARS-CoV-2/Venus, rSARS-CoV-2/Nluc, or mock-infected as described in section 3.1.

3.7.2 Monitor and weigh the mice over 12 days at the same time to minimize the weight variation due to food ingestion. Euthanize the mice that lose 25% of their initial body weight since they have reached a humane endpoint and note these mice as succumbing to viral infection.

3.7.3 After 12 days, euthanize the mice that survive viral infection and calculate the % of body weight change and survival.

REPRESENTATIVE RESULTS:

rSARS-CoV-2/Nluc infection in K18 hACE2 transgenic mice (Figures 1 and 2)

Figure 1A shows a schematic representation of the rSARS-CoV-2/WT (top) and rSARS-CoV-2/Nluc (bottom) used to assess infections *in vivo*. **Figure 1B** shows the schematic flow chart applied to assess rSARS-CoV-2/Nluc infection dynamics in K18 hACE2 transgenic mice. Four-to-six-week-old female K18 hACE2 transgenic mice (N = 4) were either mock-infected with 1x PBS or infected with 10^5 PFU of rSARS-COV-2/WT or rSARS-CoV-2/Nluc intranasally. At 1-, 2-, 4- and 6-days post-infection, mice were sedated using the isolation chamber and then injected with Nluc substrate retro-orbitally. The isolation chamber was immediately placed in the IVIS and Nluc signal was assessed *in vivo* using the imaging software. Nluc expression was readily detected in mice infected with rSARS-CoV-2/Nluc but not those infected with rSARS-CoV-2/WT, or mock-infected (**Figure 2A**). Quantitative analyses showed Nluc intensity at different days post-infection (**Figure 2B**). Gross lesions on the lung surface of mice infected with rSARS-CoV-2/Nluc were comparable to those in the rSARS-CoV-2/WT infected group (**Figures 2C**). Lastly, mice organs (lungs, nasal turbinate, and brain) were homogenized, and viral titers were determined by plaque assay (PFU/mL) and Nluc activity was determined using the luciferase assay following the manufacturer's instructions. Plaques were assessed by immunostaining using the cross-reactive SARS-CoV N monoclonal antibody 1C7C7. Viral titers detected in the rSARS-CoV-2/Nluc infected mice were comparable to those infected with rSARS-CoV-2/WT in all organs at different days post-infection (**Figure 2D**). Nluc activity was only detected in the organs from rSARS-CoV-2/Nluc-infected mice (**Figure 2E**). A separate group of mock-infected and virus-infected mice were monitored for 12 days for changes in body weight (**Figure 2F**) and survival (**Figure 2G**). Mice infected with rSARS-CoV-2/Nluc and rSARS-CoV-2/WT lost up to 25% of their body weight and all succumbed to viral infection between 7–8 days post-infection (**Figure 2F–G**).

rSARS-CoV-2/Venus infection in K18 hACE2 transgenic mice (Figures 3 and 4)

Figure 3A shows a schematic representation of the rSARS-CoV-2/WT (top) and rSARS-CoV-2/Nluc (bottom) used to assess infections *ex vivo*. **Figure 3B** shows the schematic flow chart applied to assess rSARS-CoV-2/Venus dynamics in K18 hACE2 transgenic mice. Four-to-six-week-old female K18 hACE2 transgenic mice (N= 4/group) were either mock-infected with 1x PBS or infected with 10^5 PFU of rSARS-COV-2/WT or rSARS-CoV-2/Venus intranasally. At 1-, 2-, 4- and 6-days post-infection, mice were euthanized, and their lungs were excised and imaged *ex vivo* using an IVIS. Venus expression was readily detected in all lungs from mice infected with rSARS-CoV-2/Venus but not those infected with rSARS-CoV-2/WT, or mock-infected (**Figure 4A**). Quantitative analyses showed that Venus intensity peaks at 2 days post-infection and decreases over the course of infection in the lungs of infected mice (**Figure 4B**). Images of the lung surface revealed

gross lesions of mice infected with rSARS-CoV-2/Venus was comparable to that of rSARS-CoV-2/WT infected mice (**Figure 4C**). Finally, mice organs (lungs, nasal turbinate, and brain) were homogenized, and viral titers were determined by plaque assay and assessed by immunostaining using the SARS-CoV N protein cross-reactive monoclonal antibody 1C7C7. Infection with rSARS-CoV-2/Venus resulted in comparable viral titers to those observed in mice infected with rSARS-CoV-2/WT in all organs (**Figure 4D**). A separate group of mock-infected and virus-infected mice were monitored for 12 days for changes in body weight (**Figure 4E**) and survival (**Figure 4F**). Mice infected with rSARS-CoV-2/Venus and rSARS-CoV-2/WT lost up to 25% of their body weight and all succumbed to viral infection by day 9 post-infection with no survival (**Figures 4E-4F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Assessment of rSARS-CoV-2/Nluc infection *in vivo* using K18 hACE2 transgenic mice. (**A**) Schematic representation of rSARS-CoV-2/WT (top) and rSARS-CoV-2/Nluc (bottom). (**B**) Schematic flow chart for the assessment of rSARS-CoV-2/Nluc *in vivo*.

Figure 2: rSARS-CoV-2Nluc expression in infected K18 hACE2 transgenic mice. (**A–B**) Four-to-six-week-old female K18 hACE2 transgenic mice were mock-infected (N = 4) or infected with rSARS-CoV-2/WT (N = 4) or rSARS-CoV-2/Nluc (N = 4) using 10^5 PFU per animal. The mice were anesthetized at 1-, 2-, 4- and 6-days post-infection, after being retroorbital injected with the Nluc substrate. (**A**) Nluc expression was determined under an *in vivo* imaging system, and lungs from mock-infected and infected mice were excised and photographed at 1-, 2-, 4- and 6-days post-infection . (**B**) Nluc intensity was quantitively analyzed by the image analysis software and (**C**) gross lesions on the lung surface were quantitively analyzed by ImageJ (**C**) **P < 0.01. (**D**) Viral titers in the nasal turbinate (left), lungs (middle), and brain (right) from mice infected with rSARS-CoV-2/WT and rSARS-CoV-2/Nluc were determined by plaque assay. (**E**) Nluc activity in the nasal turbinate (left), lungs (middle) and brain (right) were measured under a luciferase multi-plate reader. ns, not significant. Mock- and virus-infected mice were monitored for 12 days for changes in (**F**) body weight and (**G**) survival. All data are presented as mean ± SD for each group and analyzed by SPSS13.0 (IBM). A P value of less than 0.05 ($P < 0.05$) was considered statistically significant. This figure has been modified from Ye C. et al.[41].

Figure 3: Assessment of rSARS-CoV-2/Venus infection *in vivo* using K18 hACE2 transgenic mice. (**A**) Schematic representation of rSARS-CoV-2/WT (top) and rSARS-CoV-2/Venus (bottom). (**B**) Schematic flow chart for the assessment of rSARS-CoV-2/Venus *in vivo*.

Figure 4: rSARS-CoV-2/Venus expression in infected K18 hACE2 transgenic mice. (**A–B**) Four-to-six-week-old female K18 hACE2 transgenic mice were mock-infected (N = 4) or infected (10^5 PFU/mouse) with rSARS-CoV-2/WT (N = 4) or rSARS-CoV-2/Venus (N = 4). Lungs were excised at 1-, 2-, 4-, and 6- days post-infection, images of lungs were photographed at 1-, 2-, 4- and 6-days post-infection. (**A**) Venus expression was assessed under an IVIS, (**B**) fluorescence intensity was quantitively analyzed by the image analysis software and (**C**) the gross lesions on the lung surfaces were quantitively analyzed by ImageJ. **P < 0.01. (**D**) Viral titers in the nasal turbinate (left), lungs (middle) and brain (right) from mice infected with rSARS-CoV-2/WT and rSARS-CoV-2/Venus were

determined by plaque assay. ns, not significant. Mock- and SARS-CoV-2-infected mice were monitored for 12 days for (**E**) body weight loss and (**F**) survival. All data are presented as mean ± SD for each group and analyzed by SPSS13.0 (IBM). A P value of less than 0.05 ($P < 0.05$) was considered statistically significant. This figure has been modified from Ye C. et al.[41].

DISCUSSION:

This protocol demonstrates the feasibility of using these rSARS-CoV-2 expressing reporter genes to monitor viral infections *in vivo*. Both reporter-expressing recombinant viruses provide an excellent tool for studying SARS-CoV-2 infections *in vivo*. The described *ex vivo* (rSARS-CoV-2/Venus) and *in vivo* (rSARS-CoV-2/Nluc) imaging systems represent an excellent option to understand the dynamics of SARS-CoV-2 infection, viral pathogenesis and to identify infected cells/organs at different times after viral infection. In order to conduct *ex vivo* (rSARS-CoV-2/Venus) and *in vivo* (rSARS-CoV-2/Nluc) studies, it is eminent to have accurate and reproducible infections as well as adequate inoculations of rSARS-CoV-2/Nluc.

When studying *in vivo* infections using rSARS-CoV-2/Nluc, mice should be shaved to facilitate Nluc visualization under the IVIS. There is also a need to inoculate the Nluc substrate for visualization of Nluc. This may require some experimental testing to determine the concentrations and volume of the Nluc substrate to ensure a high Nluc signal. When studying *ex vivo* infections using rSARS-CoV-2/Venus, and because of limitations of detecting Venus directly from the entire mice using IVIS, only *ex vivo* imaging of the lungs allow detection of Venus expression. This requires having a group of mice to be euthanized at different times points. This is contrary to the situation of mice infected with rSARS-CoV-2/Nluc since the same group of mice can be imaged at different days post-infection, without requiring euthanasia at each of the times post-infection required for rSARS-CoV-2/Venus. An advantage of rSARS-CoV-2/Venus over rSARS-CoV-2/Nluc is that it can be used with flow cytometry to identify what type of cells are infected by SARS-CoV-2 by simply sorting infecting cells from non-infected cells coupled with the use of specific cellular markers as we previously described with influenza (REF). One important aspect of our rSARS-CoV-2/Venus and rSARS-CoV-2/Nluc is that both exhibited WT−like growth properties *in vitro* and *in vivo* without displaying signs of attenuation, allowing us to monitor virus infection *ex vivo* in the lungs of infected mice (rSARS-CoV-2/Venus) and the dynamic of viral replication in the entire mouse (rSARS-CoV-2/Nluc) using noninvasive longitudinal *in vivo* imaging.

Importantly, these reporter-expressing rSARS-CoV-2/Venus and rSARS-CoV-2/Nluc represent an excellent option for the identification of lead prophylactic and/or therapeutics for the treatment of SARS-CoV-2 infection[41]. The use of reporter-expressing rSARS-CoV-2 expressing different fluorescent proteins used (e.g., Venus and mCherry) allow us to combine them in bifluorescent assays to identify if therapeutics can efficiently inhibit infection of two viruses at the same time, in vitro and or in vivo, and to track viral infection, and pathogenesis[39].

ACKNOWLEDGMENTS:

We would like to thank members at our institute (Texas Biomedical Research Institute) for their efforts in keeping our facilities fully operational and safe during the COVID-19 pandemic. We

would also like to thank our Institutional Biosafety Committee (IBC) and spell (IACUC) for reviewing our protocols in a time-efficient manner. We thank Dr. Thomas Moran at the Icahn School of Medicine at Mount Sinai for providing the SARS-CoV cross-reactive 1C7C7 nucleocapsid (N) protein monoclonal antibody. SARS-CoV-2 research in the Martinez-Sobrido's laboratory is currently supported by the NIAID/NIH grants RO1AI161363-01, RO1AI161175-01A1, and R43AI165089-01; the Department of Defense (DoD) grants W81XWH2110095 and W81XWH2110103; the San Antonio Partnership for Precision Therapeutic; the Texas Biomedical Research Institute Forum; the University of Texas Health Science Center at San Antonio; the San Antonio Medical Foundation; and by the Center for Research on Influenza Pathogenesis and Transmission (CRIPT), a NIAID-funded Center of Excellence for Influenza Research and Response (CEIRR, contract # 75N93021C00014).

DISCLOSURES:

The authors declare that the research was conducted in the absence of any commercial, financial and non-financial, or personal conflict of interest in relation to the work described.

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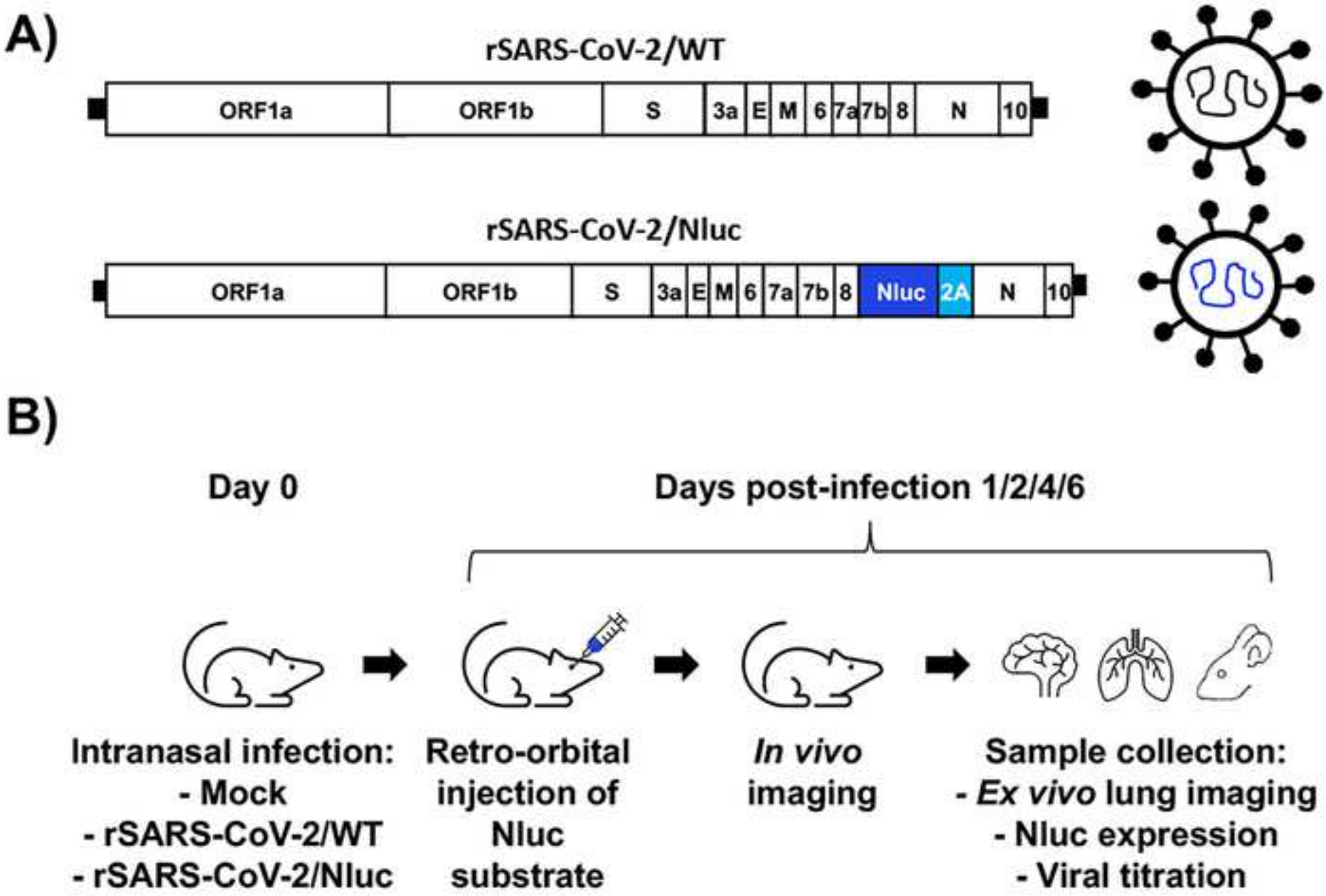

Figure 2

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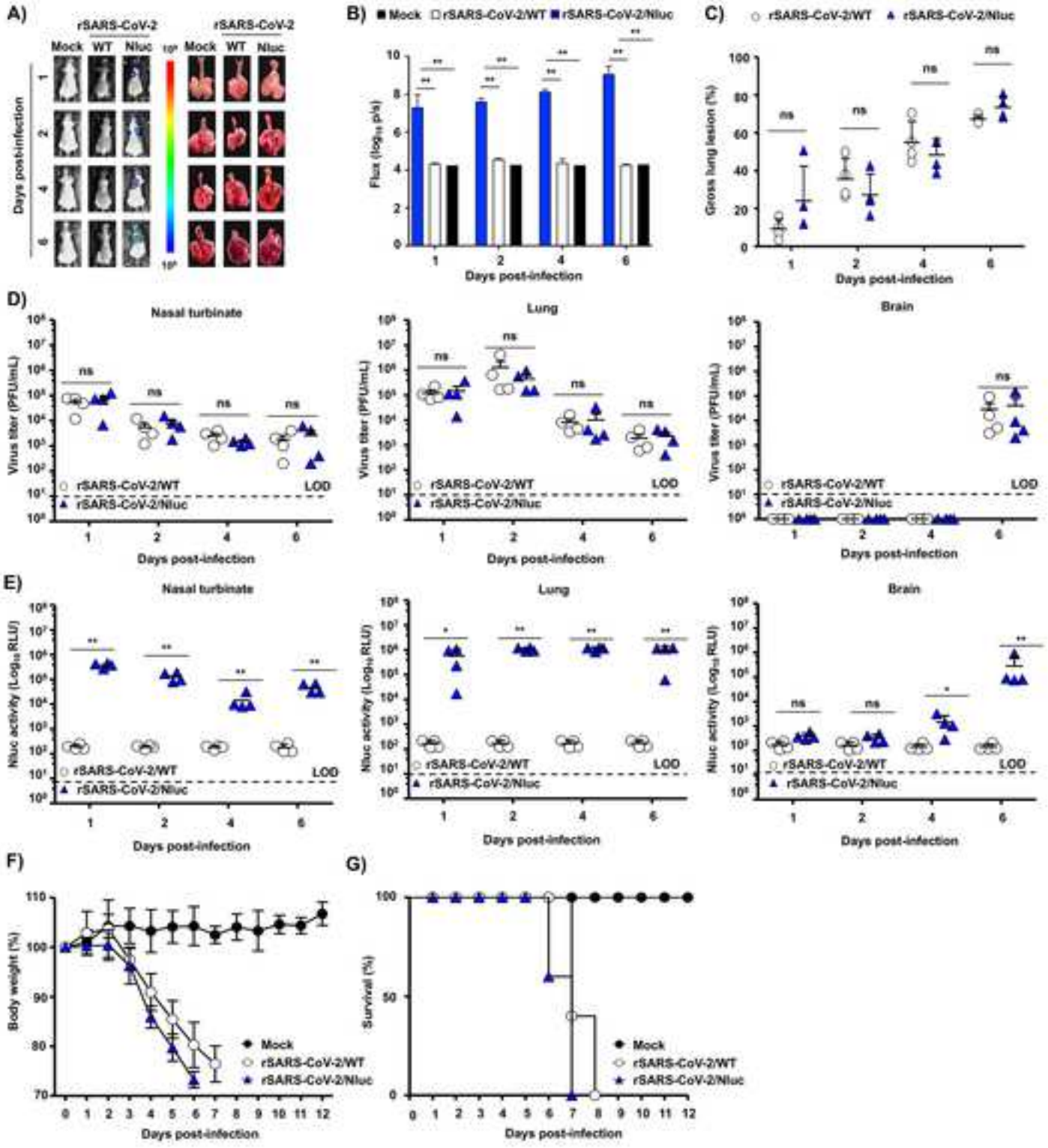

Figure 3

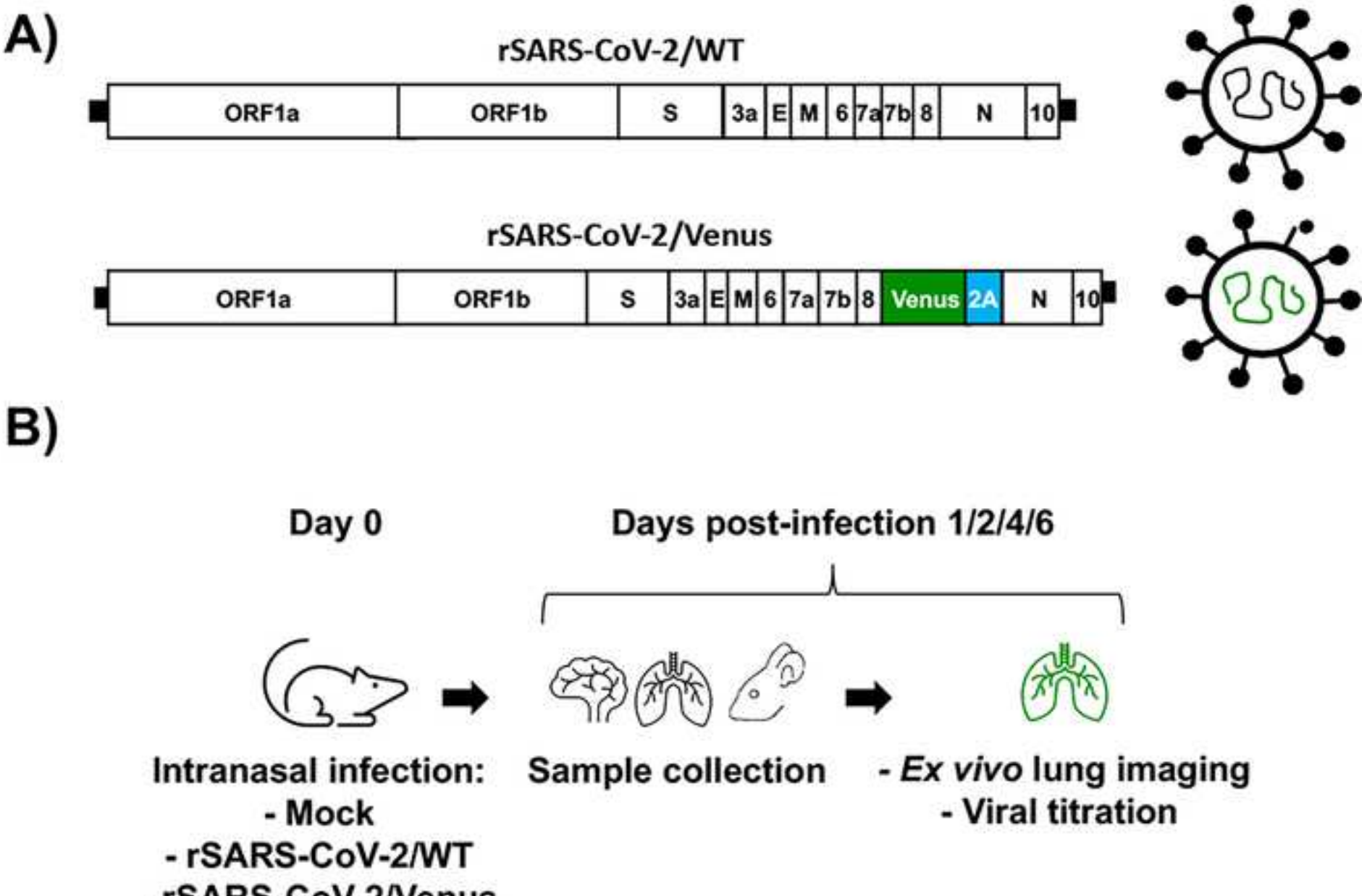

Figure 4

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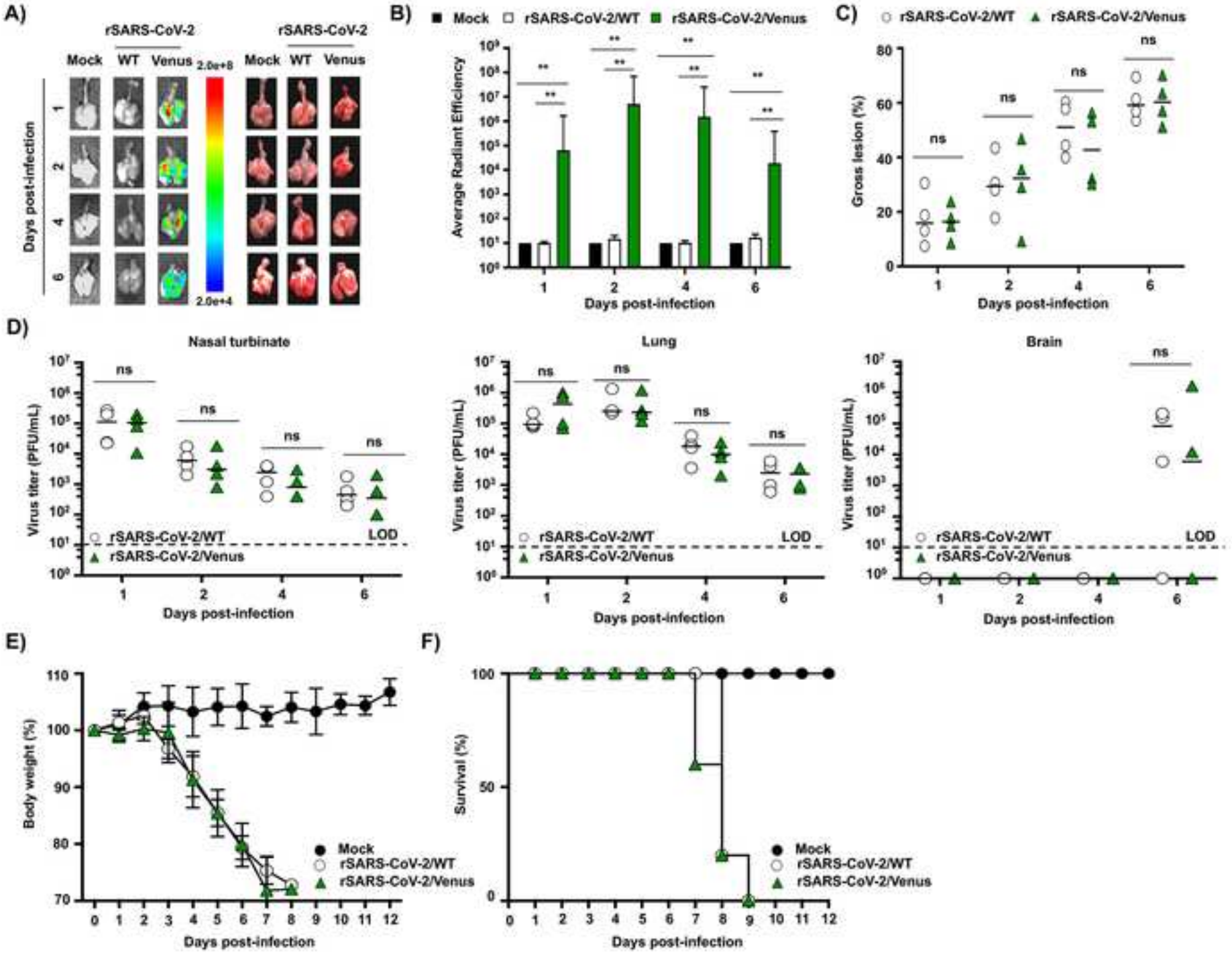

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Table of Materials

JoVE_ rSARS-CoV-2 in vivo Table_of_Materials-63127R3.xlsx

October 12th, 2021

Dear Dr. Amit Krishnan,

Thank you very much for your positive reply to our manuscript JoVE63127R1 **"Live imaging and quantification of viral infection in K18 hACE2 transgenic mice using reporter-expressing recombinant SARS-CoV-2"** by Morales Vasquez et al.

We have carefully considered all the reviewers' and editorial's comments and suggestions, which have helped us to improve the quality of our manuscript. To address the comments made by the reviewers and the editors, as well as to accommodate their suggestions, we have introduced several changes in a revised version of the document. The following is a point-by-point response to the comments raised by each one of the reviewers and the editorial office.

Reviewers comments

Reviewer #1

Manuscript Summary: The authors developed fluorescent and luminescent expressing rSARS-CoV-2 virus for live-imaging use in infected mice, which is an important tool and protocol to study the viral infection in vivo and ex vivo. Even though, similar study had been published elsewhere (Live Imaging of SARS-CoV-2 Infection in Mice Reveals that Neutralizing Antibodies Require Fc Function for Optimal Efficacy, Immunity (2021)). A comparable study and detail protocol is still helpful information.

Response: *We appreciate the overall positive and constructive comments made by this reviewer regarding the importance and relevance of our protocol.*

Major comment: Fig 2 A and C should not be separated as they are the same sample. should combines as Fig.2A panel, just bright and fluorescent view, the same for Fig.4.

Response: *We agree with the comment made by the reviewer. Following his/her recommendation, we have combined the panels A and C in Figures 2 and 4, and included the changes in our revised version of the manuscript.*

Major comment: Fig. 2E and 4E, the data should that virus titer in nasal and lung was decreasing as day passed, which is against all the data published on K18 mice, because the lung titer should increase and retain at high level during infection, see Immunity (2021). The authors should explain why they see decreased viral load in lung, but their Nluc activity maintained(Fig.2F).

Response: *We thank the reviewer for this comment and apologize for any potential confusion. Previous published manuscripts, including ours (**Analysis of SARS-CoV-2 infection dynamic in vivo using reporter-expressing viruses.** Ye C. et al. 2021, **A bifluorescent-based assay for the identification of neutralizing antibodies against SARS-CoV-2 variants of concern in vitro and in vivo.** Chiem K. et al. 2021, **Contribution of SARS-CoV-2 Accessory Proteins to Viral Pathogenicity in K18 Human ACE2 Transgenic Mice.** Silvas J. et al. 2021, **Lethality of SARS-CoV-2 infection in K18 human angiotensin-converting enzyme 2 transgenic mice.** Oladunni F. et al. 2020) have shown that SARS-CoV-2 replication (e.g. viral titers) peak at 2 days post-infection in the nasal turbinate and lungs of infected K18 hACE2 mice; and by 4 days post-infection, an approximately 1-2 log decrease in viral titers is observed. This data has remained consistent throughout each independent experiment in our laboratory, and also work published by others. Regarding the levels of Nluc observed in the lungs of K18 hACE2 mice infected with the rSARS-CoV-2/Nluc, we have observed that the signal of Nluc accumulates throughout the course of infection, even when viral titers decrease (**Analysis of SARS-CoV-2 infection dynamic in vivo using reporter-expressing viruses.** Ye C. et al. 2021). This is most likely because Nluc is very stable and accumulate in the lungs of infected mice, even when viral titers decrease.*

Major comment: survival and weight curve should be shown. aren't the mice lethal to 10^5 pfu of SARS-COV-2？According to literature, K18 mice should be a lethal model.

Response: *We thank the reviewer for this suggestion. Following his/her recommendation, we have included the survival and body weight in our revised version of the manuscript to show the lethality of the different reporter-expressing rSARS-CoV-2 in our experiments, which show WT-like lethality in the K18 hACE2 mouse model.*

Minor Comment: Check spelling throughout. Line 150.

Response: *We apologize for this editorial mistake. This spelling mistake as well as others has been fixed in the revised version of the manuscript.*

Reviewer #2

Manuscript Summary: In the associated manuscript, Vasquez et al generate bioluminescent and fluorescent recombinant SARS-CoV-2 reporter viruses and describe protocols to visualize and quantify infection in vivo and ex vivo in a K18 hACE2 mouse model. This is achieved by the expression of nanoluciferase (Nluc) or Venus proteins via a P2A fusion N-terminal to SARS-CoV-2 nucleoprotein (CoV-2 N) generated in a bacterial artificial chromosome. The authors describe carefully controlled experiments using the reporter viruses, quantifying SARS-CoV-2 infection across a range of samples including live mice, nasal turbinate and lung and brain tissue while showing specificity of reporter signal to their recombinant reporter virus in comparison to mock infection or infection with a WT virus.

This work provides a useful resource to the community, instructing other researchers on how to implement experiments with the described reporter viruses which will further understanding of the fundamental biology of SARS-CoV-2, disease mechanisms and provide a platform for development of treatments to prevent or ameliorate disease in a complex animal model.

Response: *We also appreciate the positive comments on our manuscript made by this reviewer indicating that our work "has the potential to have a positive impact on the field", "provides useful resources to the community" to "understand fundamental biology of SARS-CoV-2, disease mechanisms" and "a platform for the development of treatments against SARS-CoV-2 in the K18 hACE2 mouse model of infection".*

Major comments: No major concerns.

Reviewer #3

Manuscript Summary: In this manuscript by Vasquez et al from the Luis Martinez-Sobrido lab, the authors describe the methodology to carry out longitudinal imaging of SARS-CoV-2 infection using luminescence as well as fluorescent reporter viruses. The methods and techniques are described in sufficient detail for others to follow and reproduce.

Response: *We also appreciate the overall positive and constructive comments made by this reviewer regarding the quality of the data, the methodologies and techniques described, that will be useful for others to follow and reproduce.*

Major comment: A comparison of luminescence and fluorescence-based imaging is lacking. Based on the data shown, one can assume that non-invasive longitudinal imaging that can be carried out using luminescence-based approach may not be possible using a fluorescence-based readout as the authors have shown images of only organs (lungs) after necropsy. If true, this needs to be mentioned at least in the discussion so that the readers can decide on optimal method to use for their settings.

Response: *We concur with the comment made by the reviewer and thank him/her for bringing this important comment. Following his/her recommendation, we have included changes in our revised version of the manuscript addressing this reviewer's comment.*

Minor comment: There is a typo in Figure 4A lower panel. It should be rSARS-CoV-2/Venus and not Nluc.

Response: *Results shown in Figure 4 relate to rSARS-CoV-2/Venus. We have carefully review Figure 4A and all the legends refer to rSARS-CoV-2/Venus.*

Editorial comments

Editorial Comment #1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: *We have carefully revised the manuscript for spelling and grammar issues. Any grammar mistakes have been fixed in the revised version of the manuscript.*

Editorial Comment #2: Please provide a 10-50-word summary to clearly describe the protocol and its applications in complete sentences.

Response: *We have included a short summary in the revised version of the manuscript.*

Editorial Comment #3: Please revise the following lines to avoid previously published work: 30-31, 72-75, 137-138, 165-166, 168-169, 176-177,180-181,226-228. Please refer to the iThenticate report attached.

Response: *Similarly worded lines to previous publications have been fixed in the revised version of the manuscript.*

Editorial Comment #4: For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) before punctuation.

Response: *We have applied the JoVE endnote style in the revised version of the manuscript.*

Editorial Comment #5: Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Response: *We have corrected the numbering format in the revised version of the manuscript.*

Editorial Comment #6: JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: The Jackson Laboratory (Bar Harbor, ME, USA), Precelleys homogenizer, QUIP Laboratories, Wilmington, DE, USA, iPhone 6s (Apple, CA, USA), Sigma, Nano-Glo® Luciferase Assay System (Promega), Aura Imaging software, Wexicide, Ami HT, Vectastain ABC kit, Vector Laboratory, etc.

Response: *We have removed all commercial language in the revised version of the manuscript.*

Editorial Comment #7: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

Response: *We have corrected the text to be written in imperative tense in the revised version of the manuscript.*

Editorial Comment #8: Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Response: *We have revised the manuscript and included more detailed information in the protocol steps in the revised version of the manuscript.*

Editorial Comment #9: Please provide the volume of all solutions used (BSA, neutral buffered formalin, etc.)

Response: *We have revised the manuscript and included this information in the revised version of the manuscript.*

Editorial Comment #10: Line 145: Are the mice disinfected before shaving? If yes, please mention how? Also, describe which part of the chest was shave and how?

Response: *We apologize for not providing this information in our initial submission of the manuscript. This information has been now included in the revised document.*

Editorial Comment #11: Line 146: How were PFUs counted?

Response: *We have provide information on how the PFUs are counted in our revised manuscript.*

Editorial Comment #12: Line 150: What is the final volume?

Response: *We have included this information in the revised manuscript.*

Editorial Comment #13: Line 153: What was the percentage of isoflurane used? Please mention how proper anesthetization is confirmed. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

Response: *We have included this information in the revised manuscript.*

Editorial Comment #14: Line 165,186: For steps that involve software or analyzing tools, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed. Please provide all parameter and setting details.

Response: *We have revised our manuscript and included the steps suggested in the revised version of the manuscript.*

Editorial Comment #15: Line 216: How was the ImageJ analysis done? Please provide all details.

Response: *We have included details on ImageJ analysis in the revised version of the manuscript.*

Editorial Comment #16: Line 242: How was the PFU calculated?

Response: *We have provide information on how the PFUs are calculated in our revised manuscript.*

Editorial Comment #17: Please include a single line space between all the steps. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: *We have included a single line spacing in between steps and highlighted sections in the Protocol that identifies the essential steps of the protocol for the video.*

Editorial Comment #18: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol, b) Any modifications and troubleshooting of the technique, c) Any limitations of the technique, d) The significance with respect to existing methods, e) Any future applications of the technique.

Response: *We have revised the Discussion section to follow editors' suggestion. This has been included in the revised version of the manuscript.*

Editorial Comment #19: Figure legends 2/4: Please provide the number of samples used in each group in the Figure legends. Please define what the error bars represent and what the ** stands for in the figure legend. Please also mention what the color intensity bar denotes.

Response: *We have included the sample number and statistics information in the revised version of the manuscript.*

Editorial Comment #20: Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors

have no competing financial interests, then a statement indicating no competing financial interests must be included.

Response: *We have included a Disclosure section in the revised manuscript.*

Editorial Comment #21: Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Response: *We have applied the JoVE endnote style in the revised manuscript.*

Editorial Comment #22: Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Response: *Trademark and registered symbols have been removed in the revised version of the manuscript.*

Editorial Comment #23: Figure 2/4: Please revise “PFU/ml” to “PFU/mL” in the panel E and F.

Response: *We have changes PFU/ml to PFU/mL in the revised manuscript.*

Editorial Comment #24: Please cite the biorxiv article (https://www.biorxiv.org/content/10.1101/2021.06.03.446942v1.full.pdf) in the Figure legends of the Figures common in both articles (JoVE submission and biorxiv).

Response: *We have included the citation of the biorxiv article in the figure legends in the revised version of the manuscript.*

We hope that by considering all the comments and suggestions made by the editors the manuscript could be now accepted for publication at JoVE. Finally, we want to thank again the editors for their very helpful and constructive criticisms that have contributed to improve our manuscript.

Sincerely,

Luis Martinez-Sobrido, Ph.D.

Texas Biomedical Research Institute

We hope that by considering all the comments and suggestions made by the reviewers the manuscript has been improved, and it could be now accepted for publication at JoVE . Finally, we want to thank again the reviewers for their very helpful and constructive criticisms that have contributed to improve our manuscript.

Sincerely,

Luis Martinez-Sobrido, Ph.D.

Texas Biomedical Research Institute

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Best regards,
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