



Replication of Severe Acute Respiratory Syndrome Coronavirus 2 in Human Respiratory Epithelium

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ABSTRACT Currently, there are four seasonal coronaviruses associated with relatively mild respiratory tract disease in humans. However, there is also a plethora of animal coronaviruses which have the potential to cross the species border. This regularly results in the emergence of new viruses in humans. In 2002, severe acute respiratory syndrome coronavirus (SARS-CoV) emerged and rapidly disappeared in May 2003. In 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) was identified as a possible threat to humans, but its pandemic potential so far is minimal, as human-to-human transmission is ineffective. The end of 2019 brought us information about severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emergence, and the virus rapidly spread in 2020, causing an unprecedented pandemic. At present, studies on the virus are carried out using a surrogate system based on the immortalized simian Vero E6 cell line. This model is convenient for diagnostics, but it has serious limitations and does not allow for understanding of the biology and evolution of the virus. Here, we show that fully differentiated human airway epithelium cultures constitute an excellent model to study infection with the novel human coronavirus SARS-CoV-2. We observed efficient replication of the virus in the tissue, with maximal replication at 2 days postinfection. The virus replicated in ciliated cells and was released apically.

IMPORTANCE Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged by the end of 2019 and rapidly spread in 2020. At present, it is of utmost importance to understand the biology of the virus, rapidly assess the treatment potential of existing drugs, and develop new active compounds. While some animal models for such studies are under development, most of the research is carried out in Vero E6 cells. Here, we propose fully differentiated human airway epithelium cultures as a model for studies on SARS-CoV-2.

KEYWORDS COVID-19, *Coronaviridae*, FISH, HAE, NCoV-2019, SARS, SARS-CoV-2, coronavirus, human airway epithelium, model

Coronaviruses constitute a large family of RNA viruses that mainly infect mammals and birds. In humans, there are four species associated with mild-to-moderate respiratory infections. While these viruses have been infecting humans for a long time, they are believed to have entered the human population by zoonotic events, and one

Citation Milewska A, Kula-Pacurar A, Wadas J, Suder A, Szczepanski A, Dabrowska A, Owczarek K, Marcello A, Ochman M, Stacel T, Rajfur Z, Sanak M, Labaj P, Branicki W, Pyrc K. 2020. Replication of severe acute respiratory syndrome coronavirus 2 in human respiratory epithelium. *J Virol* 94:e00957-20. <https://doi.org/10.1128/JVI.00957-20>.

Editor Rozanne M. Sandri-Goldin, University of California, Irvine

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Received 16 May 2020

Accepted 18 May 2020

Accepted manuscript posted online 20 May 2020

Published 16 July 2020

may speculate that they may have caused epidemics similar to the one observed for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Analysis of the time to the most recent ancestor suggests that human coronavirus NL63 (HCoV-NL63) is the oldest species in humans, followed by its cousin HCoV-229E and two betacoronaviruses, which emerged in humans in the relatively near past (1–4). In the 21st century, we have already faced the emergence of three novel coronaviruses in humans, of which severe acute respiratory syndrome coronavirus (SARS-CoV) disappeared after one season, never to come back, and Middle East respiratory syndrome coronavirus (MERS-CoV) never fully crossed the species border, as its transmission between humans is highly ineffective (5–7). The 2019 zoonotic transmission, however, resulted in the emergence of a novel human coronavirus which seems to carry an optimal set of features allowing for its rapid spread with considerable mortality. Whether the virus will become endemic in humans is an open question (8–10).

At present, studies on the virus are carried out using a surrogate system based on the immortalized simian Vero E6 cell line (11). While this model is convenient for diagnostics and testing of some antiviral drugs, it has serious limitations and does not allow for understanding of virus biology and evolution. For example, the entry routes of human coronaviruses vary between cell lines and differentiated tissues, as do immune responses and virus-host interactions (12–14).

Here, we used fully differentiated epithelium cultures to study infection with the novel human coronavirus SARS-CoV-2. We observed efficient replication of the virus in the tissue, with maximal replication at 2 days postinfection (p.i.). At the time of the study, no antibodies were available. Therefore, we developed immunofluorescent *in situ* hybridization (immunoFISH) to show that the virus primarily infects ciliated cells of the respiratory epithelium.

RESULTS AND DISCUSSION

Human airway epithelium (HAE) cultures reconstitute the tissue lining the conductive airways of humans. Being fully differentiated, they are among the best tools for studying viral infection in a natural microenvironment (15). These air-liquid interface cultures contain a number of cell types (e.g., basal, ciliated, and goblet). At the same time, they also functionally reflect the natural tissue with extensive cross talk and production of protective mucus and surfactant proteins (16–18). These cultures were previously shown by us and others to be superior to those with standard cell lines in terms of their ability to support replication of human coronavirus HKU1 (HCoV-HKU1) and also as a model to study the biology of infection (19). For example, human coronaviruses were shown some time ago to use a very different entry pathway in immortalized cell lines than in the natural human epithelium. In immortalized cell lines, coronaviruses enter via the pH-dependent endocytic pathway, whereas in the natural human epithelium, they utilize surface serine proteases, such as TMPRSS2 or kallikreins, for activation, and fusion occurs on the cell surface. This difference may affect not only understanding of their basic biology but also antiviral drug development, including that of chloroquine (12–14, 20).

Here, we sought to verify whether HAE cultures may be used to study SARS-CoV-2 infection and identified cellular targets in the tissue. First, HAE cultures or Vero E6 cells were inoculated with SARS-CoV-2 stock and cultured for 4 days. Every day (days 0 to 3), the apical and basolateral release of the virus was evaluated by reverse transcriptase quantitative PCR (RT-qPCR), and the results for the apical release of the virus are presented in Fig. 1A.

Clearly, the increase in virus titer on the apical side is visible at 24 h postinoculation and reaches a plateau at 48 h postinoculation. Also, the virus yield was comparable to that obtained *in vitro* using permissive cells. We did not observe any release of the virus from the basolateral side of the HAE culture, and therefore, we have not shown the relevant data on the graph. The results we observed are consistent with the previously reported polarity of the HAE cultures and apical infection/apical release reported previously for other human coronaviruses. Similarly as for other human coronaviruses,

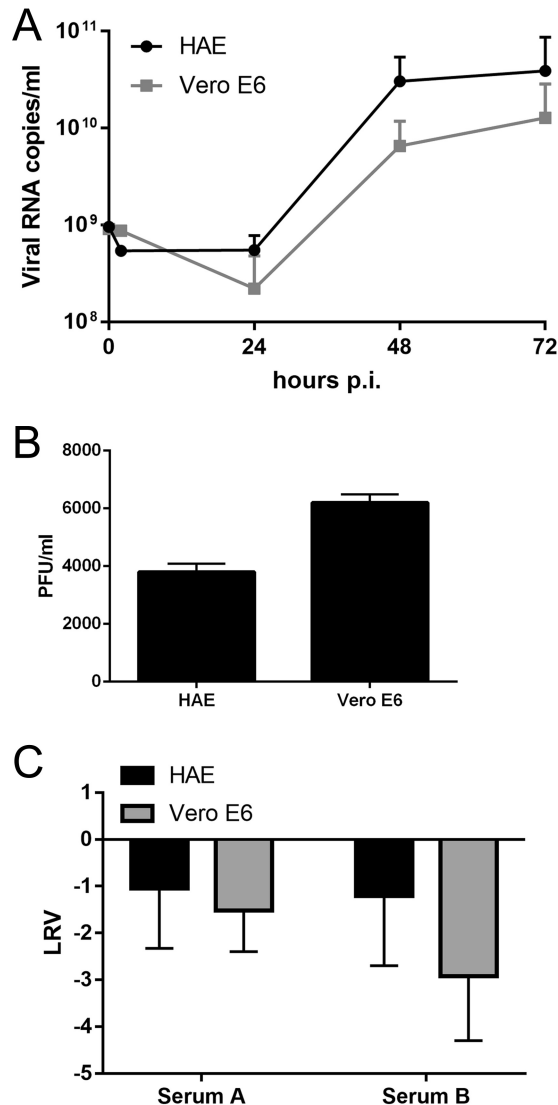


FIG 1 SARS-CoV-2 replicates in HAE cultures. (A) Replication of SARS-CoV-2 in HAE and Vero E6 cultures was evaluated using RT-qPCR, and the data are presented as RNA copy numbers per ml. The experiment was carried out twice, each time in triplicate, and average values with standard deviations are presented. (B) Plaque assay of SARS-CoV-2 stock collected from HAE and Vero E6 cells. The assay was performed using A549 cells overexpressing the ACE2 protein. (C) Inhibition of SARS-CoV-2 replication by patient serum evaluated using RT-qPCR. The data are presented as log reduction values (LRVs) compared to results for the untreated control. The experiment was carried out in triplicate, and average values with standard deviations are presented.

the apical-apical polarity of SARS-CoV-2 infection release restricts the virus to the airway lumen (16). Additionally, plaque assay results showed comparable amounts of infectious virions released from HAE and Vero E6 cells (Fig. 1B). Also, SARS-CoV-2 replication *in vitro* and *ex vivo* was effectively blocked by serum obtained from patients who had recovered from COVID-19 (Fig. 1C).

Furthermore, we sampled the tissue at 96 h postinfection to verify whether subgenomic mRNAs (sgmRNAs) were present. Analysis was carried out with RT-qPCR, and the results are presented in Fig. 2.

Analysis clearly showed that sgmRNAs were abundant in the infected HAE cultures. As this is generally considered the hallmark of active replication, we believe that it provides sufficient proof that the virus is indeed actively replicating in the cultures.

Next, we made an effort to visualize infection in the tissue. Because at the time of the study no antibody for confocal microscopy was available, we developed an

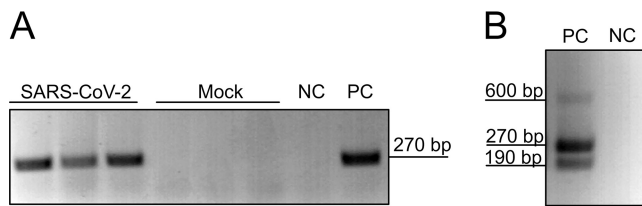


FIG 2 sgmRNAs of SARS-CoV-2 in HAE cultures. The presence of the N sgmRNAs 4 days p.i. in the HAE cultures infected with SARS-CoV-2 was evaluated using RT-PCR. NC, negative control; PC, positive control. (A) N sgmRNA in HAEs obtained after the first PCR. (B) N sgmRNA obtained after the second PCR. An additional band of ~600 bp represents sgmRNA8. The experiment was performed three times, each time using cells obtained from different donors.

immunoFISH assay, where the viral RNA was visualized in the context of the cell using 20 sequence-specific probes and signal amplification. At the same time, β -tubulin was labeled using specific antibodies to visualize ciliated cells. The obtained results are shown in Fig. 3.

Furthermore, we analyzed assay specificity using HAE cultures infected with HCoV-NL63 and HCoV-HKU1. The results are presented in Fig. 4.

In summary, we show that SARS-CoV-2 effectively replicates in HAE cultures and that this *ex vivo* model constitutes a convenient tool for studying this viral infection. We also show that the virus infects ciliated cells. The infection is polarized: infection and release occur at the apical side of the epithelium. It is worth noting that in the absence of immunodetection tools, the new generation of immunoFISH tools offers an interesting alternative.

MATERIALS AND METHODS

Cell culture. Vero E6 (*Cercopithecus aethiops*; kidney epithelial; ATCC CRL-1586) and A549 cells expressing the ACE2 protein (A549_ACE2+) (20) were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Poland) supplemented with 3% heat-inactivated fetal bovine

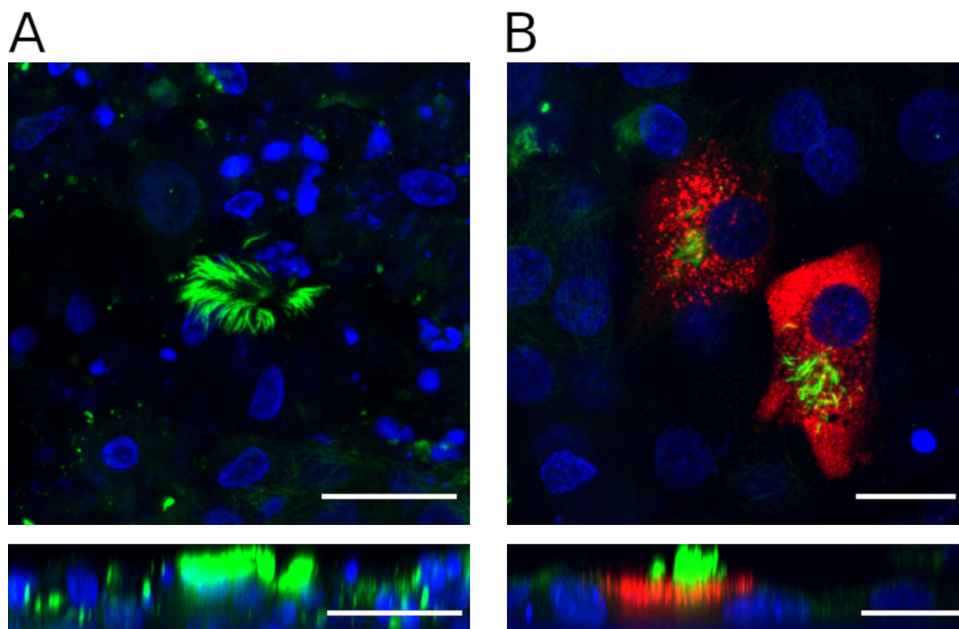


FIG 3 SARS-CoV-2 infects ciliated cells of the human airway epithelium. Three-dimensional immuno-RNA FISH demonstrating localization of SARS-CoV-2 subgenomic RNA in ciliated HAE cultures. Three-dimensionally reconstructed confocal image stacks of mock-inoculated control cells (A) or cells infected with SARS-CoV-2 (B). The bottom lanes of panels A and B show the xz plane in orthogonal views. SARS-CoV-2 RNA was visualized by FISH using a set of probes against viral nucleocapsid RNA and is shown in red. Cilia were visualized by an anti- β 5 tubulin antibody and are shown in green. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) and are shown in blue. Bar = 20 μ M.

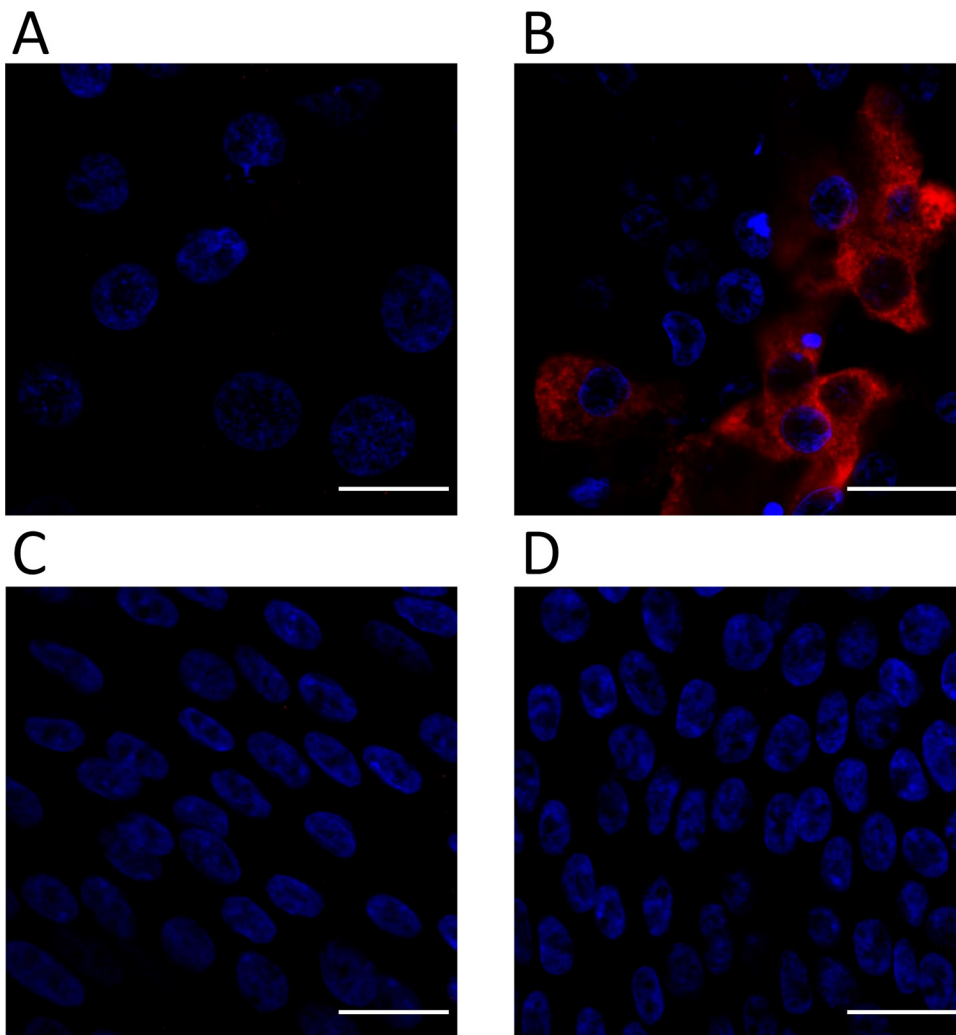


FIG 4 Specificity of the SARS-CoV-2 RNA FISH assay in HAE cultures. Three-dimensionally reconstructed confocal image stacks of mock-infected control cells (A) and cells infected with SARS-CoV-2 (B), NL63 (C), and HKU1 (D) subjected to RNA FISH using a set of probes against SARS-CoV-2 nucleocapsid RNA. SARS-CoV-2 RNA is shown in red. Nuclei were stained with DAPI and are shown in blue. Bar = 20 μ M.

serum (FBS; Thermo Fisher Scientific, Poland) and streptomycin (100 μ g/ml), penicillin (100 U/ml), and ciprofloxacin (5 μ g/ml). A549_ACE2+ cell cultures were supplemented with G418 (5 mg/ml; BioShop, Canada). Cells were cultured at 37°C in an atmosphere containing 5% CO₂.

HAE cultures. Human epithelial cells were isolated from conductive airways resected from transplant patients. The study was approved by the bioethical committee of the Medical University of Silesia in Katowice, Poland (approval no KNW/0022/KB1/17/10, dated 16 February 2010). Written consent was obtained from all patients. Cells were mechanically detached from the tissue after protease treatment and cultured on plastic in bronchial epithelial growth medium (BEGM). Subsequently, cells were transferred onto permeable Transwell insert supports (ϕ , 6.5 mm) and cultured in BEGM. After the cells reached full confluence, the apical medium was removed and the basolateral medium was changed to an air-liquid interface (ALI). Cells were cultured for 4 to 6 weeks to form differentiated, pseudostratified mucociliary epithelia. All experiments were performed in accordance with relevant guidelines and regulations.

Virus. SARS-CoV-2 (isolate 026V-03883; kindly granted by Christian Drosten, Charité–Universitätsmedizin Berlin, Germany, and provided by the European Virus Archive–Global [EVAg]). The virus stock was prepared by infecting fully confluent Vero E6 cells at a 50% tissue culture infective dose (TCID₅₀) of 400 per ml. Three days after inoculation, the supernatant from the cultures was aliquoted and stored at –80°C. Control Vero E6 cell supernatant from mock-infected cells was prepared in the same manner. Virus yield was assessed by titration on fully confluent Vero E6 cells in 96-well plates according to the method of Reed and Muench (21). Plates were incubated at 37°C for 2 days, and the cytopathic effect (CPE) was scored by observation under an inverted microscope.

Virus infection. Fully differentiated human airway epithelium (HAE) cultures were inoculated with SARS-CoV-2 at a TCID₅₀ of 1,000 per ml (as determined on Vero E6 cells). Following 2 h of incubation at

37°C, unbound virions were removed by washing with 200 μ l of 1 \times phosphate-buffered saline (PBS), and HAE cultures were maintained at an air-liquid interface for the rest of the experiment. To analyze virus replication kinetics, each day postinfection (p.i.), 100 μ l of 1 \times PBS was applied at the apical surface of HAE and collected following a 10-min incubation at 32°C. For the neutralization study, patient sera (1:200 dilution in PBS [HAE] or culture medium [Vero E6]) were used. All samples were stored at -80°C and analyzed using RT-qPCR.

Additionally, 48 h postinfection, selected HAE cultures were collected, and the presence of sgRNAs was determined as a hallmark of an active infection.

Isolation of nucleic acids and reverse transcription. A viral DNA/RNA kit (A&A Biotechnology, Poland) was used for nucleic acid isolation from cell culture supernatants, and Fenozol (A&A Biotechnology) was used for total RNA isolation from cells. RNA was isolated according to the manufacturer's instructions. cDNA samples were prepared with a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Poland), according to the manufacturer's instructions.

Quantitative PCR. Viral RNA was quantified using quantitative PCR (qPCR; CFX96 Touch real-time PCR detection system; Bio-Rad, Poland). cDNA was amplified using 1 \times qPCR master mix (A&A Biotechnology, Poland), in the presence of probe (100 nM; FAM/BHQ1, ACT TCC TCA AGG AAC AAC ATT GCC A) and primers (450 nM each; CAC ATT GGC ACC CGC AAT C and GAG GAA CGA GAA GAG GCT TG). The heating scheme was as follows: 2 min at 50°C and 10 min at 92°C, followed by 30 cycles of 15 s at 92°C and 1 min at 60°C. In order to assess the copy number for the N gene, standards were prepared and serially diluted.

Detection of SARS-CoV-2 N sgRNA. Total nucleic acids were isolated from virus or mock-infected cells at 4 days p.i. using Fenozol reagent (A&A Biotechnology, Poland) according to the manufacturer's instructions. Reverse transcription was performed using a high-capacity cDNA reverse transcription kit (Life Technologies, Poland) according to the manufacturer's instructions. Viral cDNA was amplified in a 20- μ l reaction mixture containing 1 \times Dream Taq green PCR master mix (Thermo Fisher Scientific, Poland) and primers (500 nM each). The following primers were used to amplify SARS-CoV-2 subgenomic mRNA (sgmRNA): common sense primer (leader sequence, 5'-TAT ACC TTC CCA GGT AAC AAA CCA-3') and nucleocapsid antisense primer (5'-GTA GCT CTT CGG TAG TAG CCA AT-3'). The conditions were as follows: 3 min at 95°C; 35 cycles (30 cycles for second PCR) of 30 s at 95°C, 30 s at 55°C, and 20 s at 72°C; followed by 5 min at 72°C and 10 min at 4°C. The PCR products were run on 1% agarose gels (Tris-acetate-EDTA [TAE] buffer) and analyzed using molecular imaging software (Thermo Fisher Scientific).

Plaque assay. A549_{ACE2+} cells were seeded in 24-well plates and cultured for 24 h at 37°C with 5% CO₂. Virus samples were serially diluted 10-fold in culture medium and layered on the cells in triplicate. Following 1 h of incubation at 37°C with gentle rocking, cells were overlaid with agarose medium (2% FBS with 0.05% agarose). Cells were cultured for 72 h at 37°C with 5% CO₂ and fixed with 4% paraformaldehyde (PFA) for 1 h. The supernatant was discarded, and cells were stained with crystal violet to visualize the plaques.

RNA fluorescent *in situ* hybridization and immunofluorescence. HAE cultures were infected with SARS-CoV-2 (TCID₅₀ of 1,000, as assessed for the Vero E6 cells) and fixed at 4 days postinfection with 3.7% paraformaldehyde (PFA) overnight. As controls, HAE cultures were infected with HCoV-NL63 (isolate Amsterdam 1) or HCoV-HKU1 (strain Caen 1) and fixed at 4 days postinfection with 3.7% PFA for 2 h.

The next day, cells were subjected to an RNA-FISH protocol using hybridization chain reaction (HCR) technology from Molecular Instruments, Inc. Briefly, cells were permeabilized with 100% methanol overnight and then subjected to graded rehydration with methanol-PBS and 0.1% Tween 20. The set of DNA HCR v3.0 probes complementary to SARS-CoV-2 nucleocapsid RNA was incubated for 12 h at 37°C, extensively washed, and hybridized with HCR amplifiers for 12 h at room temperature in the dark. Next, cells were subjected to immunostaining with antibodies against mouse β 5-tubulin from Santa Cruz Biotechnology (sc-134234; 1:100) and rinsed three times with PBS and 0.1% Tween 20, followed by 1 h of incubation with Alexa Fluor 488 secondary antibodies (1:400; Invitrogen). The cells were finally washed three times with PBS and 0.1% Tween 20, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (D1306; Thermo Fisher Scientific), and cells were mounted on slides with Prolong diamond antifade mounting medium (P36970; Invitrogen). Fluorescent images were acquired using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy GmbH) with ZEN 2012 SP1 black edition software and processed in ImageJ Fiji (National Institutes of Health, Bethesda, MD).

ACKNOWLEDGMENTS

This work was supported by funds provided by the Ministry of Science and Higher Education for research on SARS-CoV-2 and grants from the National Science Center (grants UMO-2017/27/B/NZ6/02488 to K.P. and UMO-2018/30/E/NZ1/00874 to A.K.-P.).

We declare no conflict of interest.

The funders had no role in the design of the study, in the collection, analysis, or interpretation of data, in writing of the manuscript, or in the decision to publish the results.

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