Report

A method for the generation of pseudotyped virus particles bearing SARS coronavirus spike protein in high yields

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1 **SUMMARY**

2 **The ongoing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic**

3 **has threatened human health and the global economy. Development of additional**

4 **vaccines and therapeutics is urgently required, but such development with live virus**

5 **must be conducted with biosafety level 3 confinement. Pseudotyped viruses have been**

6 **widely adopted for studies of virus entry and pharmaceutical development to overcome**

7 **this restriction. Here we describe a modified protocol to generate vesicular stomatitis**

8 **virus (VSV) pseudotyped with SARS-CoV or SARS-CoV-2 Spike protein in high yield.**

9 **We found that pseudovirions produced with the conventional transient expression**

10 **system lacked coronavirus Spike protein at their surface as a result of inhibition of**

11 **parental VSV infection by overexpression of this protein. Establishment of stable cell**

12 **lines with an optimal expression level of coronavirus Spike protein allowed the efficient**

13 **production of progeny pseudoviruses decorated with Spike protein. This improved VSV**

14 **pseudovirus production method should facilitate studies of coronavirus entry and**

15 **development of antiviral agents.**

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1 **INTRODUCTION**

2 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped, positive-

3 strand RNA virus that belongs to the *Coronaviridae* family and is responsible for the recent

4 pandemic of coronavirus disease 2019 (COVID-19). As of 15 June 2021, there had been >174

5 million confirmed cases of and ~3.7 million deaths from COVID-19 reported in over 220

6 countries, areas, or territories (https://www.who.int/emergencies/diseases/novel-coronavirus-

7 2019).

8 The envelope of SARS-CoV-2, like that of SARS-CoV, contains three structural

9 proteins: the spike glycoprotein (hereafter referred to simply as S protein), membrane protein,

10 and small envelope protein (Schoeman and Fielding, 2019; Wrapp et al., 2020). Together with

11 retrovirus envelope proteins and influenza virus hemagglutinin proteins, S protein is

12 categorized as a class I viral fusion protein (Millet and Whittaker, 2018; White 2008), and it is

13 targeted to the rough endoplasmic reticulum (rER) of host cells by an NH_2-terminal signal

14 sequence (Breitling and Aebi, 2013; Duan et al., 2020; Lontok et al., 2004; Sadasivan et al.,

15 2017). Cleavage of the signal sequence is followed by resumption of peptide chain elongation

16 and insertion of the synthesized S protein into the ER membrane as a homotrimer (Binns et al.,

17 1985; Braakman and Hebert, 2013; Delmas and Laude, 1990; Duan et al., 2020). S protein

18 plays a key role in both specific interaction of the virus with its host cell receptor and

19 subsequent virus internalization via endocytosis or membrane fusion. Binding of the

20 coronavirus to its host cell receptor, angiotensin-converting enzyme 2 (ACE2), has been

21 attributed to the S1 receptor-binding subunit region of S protein, which adopts an active

22 "standing-up" conformation for receptor binding and an inactive "lying-down" conformation

23 for immune evasion (Guan et al., 2020; Wrapp et al., 2020). After the S1 subunit region binds

24 to ACE2, S protein undergoes proteolytic cleavage into S1 and S2 fusion subunits at the

25 S1/S2 site in the presence of transmembrane protease serine 2 (TMPRSS2) and furin (Bestle

26 et al., 2020; Jaimes et al., 2020). Additional cleavage sites that are located within the S2

27 subunit and are cleaved by host cell proteases including cathepsins, furin-like proprotein

28 convertases, and trypsin-like serine protease (Burkard et al., 2014; Coutard et al., 2020;

29 Hoffmann et al., 2020). The exposed S2 subunit mediates membrane fusion either at the

30 plasma membrane or with the endosomal membrane, depending on protease availability in the

31 host cell. Characterization of S protein is thus crucial for a better understanding of SARS-

32 CoV-2 entry, but such studies are often limited because experiments with the live virus must

33 be conducted in a biosafety level 3 (BSL-3) containment facility.

1   Pseudotyped virus systems are highly useful for characterization of viral envelope

2   proteins and investigation of their role in virus entry (Hoffmann et al., 2020; Miyauchi et al.,

3   2009; Nanbo et al., 2010; Rentsch and Zimmer, 2011; Schelhaas et al., 2012; de Vries et al.,

4   2011). Virus particles produced with such a system consist of a surrogate viral core and

5   heterologous viral envelope proteins. The genome of the parental virus is modified to remove

6   essential genes for viral reproduction, so as to prevent the generation of infectious progeny

7   viruses. This approach allows viruses pseudotyped with envelope proteins of highly

8   pathogenic viruses to be handled safely in a BSL-2 containment facility instead of a BSL-3 or

9   BSL-4 facility. Vesicular stomatitis virus (VSV), a negative-strand RNA virus of the

10   *Rhabdoviridae* family, has been widely adopted in both pseudotyped and recombinant virus

11   systems (Hoffmann et al., 2020; Miyauchi et al., 2009; Nanbo et al., 2010; Rentsch and

12   Zimmer, 2011). It possesses five structural proteins—the glycoprotein (G), large polymerase

13   protein (L), matrix protein (M), nucleocapsid (N), and phosphoprotein (P) (Lichty et al.,

14   2004)—among which G protein contributes to binding to the host cell surface and fusion with

15   the endosomal membrane. VSV was the first negative-strand RNA virus for which a reverse

16   genetics approach was established (Lawson et al., 1995), an approach that allowed packaging

17   of a reporter gene into the viral genome for quantitative evaluation of infectivity of

18   recombinant viruses on the basis of the reporter gene activity. It was also found that a large

19   amount of morphologically intact virus particles could be produced even in the absence of G

20   protein. In addition, coinfection of cells with VSV and other viruses results in the formation

21   of VSV pseudotyped with heterologous envelope proteins (Huang et al., 1974; Witte and

22   Baltimore, 1977). With these features, VSV-based pseudotyping is a potentially powerful tool

23   for studies of the role of envelope proteins, including S proteins of SARS-CoV and SARS-

24   CoV-2, in virus entry (Clausen et al., 2020; Hoffmann et al., 2020).

25   During the course of experiments with VSV pseudoviruses bearing S protein of

26   SARS-CoV or SARS CoV-2 described here, we found that most pseudotyped particles

27   produced by the conventional transfection protocol lack S protein, likely because S protein

28   expression at a high level in the virus-producing cells suppresses infection by parental VSV.

29   We therefore developed an improved protocol based on cell lines stably expressing S proteins.

30   This new method generates VSV pseudotyped particles decorated with S protein at high

31   efficiency and with high infectious yields compared to the conventional approach. Our

32   protocol increases the fidelity of SARS-CoV-2 entry research and should facilitate the

33   development of new antiviral agents.

4

1 **RESULTS AND DISCUSSION**

2 We initially aimed to evaluate the loading efficiency of S proteins on VSV pseudotyped virus

3 particles. According to the published protocol (Rentsch and Zimmer, 2011), HEK293T cells

4 transiently expressing SARS-CoV S protein were exposed to VSVΔG bearing G protein of

5 VSV (VSVΔG-G), and the resulting culture supernatant was harvested as a pseudovirus

6 suspension in the presence of neutralizing antibodies to VSV G protein (to eliminate parental

7 VSVΔG-G). The pseudotyped virions obtained in this manner were stained with the lipophilic

8 carbocyanine dye DiI and then subjected to immunofluorescence analysis with a rabbit

9 monoclonal antibody to SARS-CoV S protein. Confocal fluorescence imaging revealed that

10 only a small proportion of the obtained particles harbored S protein (**Figure 1A**).

11 To examine S protein expression in the pseudotyped virion-producing cells, we

12 prepared an expression vector for a fluorescent protein-tagged form of the protein. The coding

13 sequence for mCherry, a red fluorescent protein (RFP), was thus inserted upstream of the

14 NH_2-terminal signal sequence of S protein, with the expectation that the fluorescent tag would

15 be cleaved together with the signal sequence in the ER and so would not suppress virion

16 formation. Indeed, immunoblot analysis of HEK293T cells transfected with the vector for

17 mCherry-tagged S protein revealed separate fragments corresponding to mCherry and to S

18 protein (**Figure S1A**), and no red fluorescence signal was detected from the VSVΔG-

19 mCherry-S pseudoparticles produced by these cells (**Figure S1B**). Furthermore,

20 determination of focus-forming units (FFU) by counting the number of cells positive for

21 green fluorescent protein (GFP) encoded by the reporter gene packaged in the genome of

22 parental VSVΔG (Rentsch and Zimmer, 2011) did not reveal a marked difference in

23 infectivity between VSVΔG-mCherry-S and VSVΔG-S pseudoviruses in BEAS-2B cells

24 expressing ACE2, also a receptor for SARS-CoV (**Figure S1C**). This result also supported

25 the notion that the chimeric protein undergoes proteolytic cleavage and does not inhibit

26 particle formation.

27 Fluorescence imaging of HEK293T cells transfected with expression vectors for

28 mCherry-S or mCherry showed that mCherry-S expression interfered with VSVΔG-G

29 infection; the GFP signal was thus detected in cells with no or only a low level of mCherry-S

30 expression but not in highly red fluorescent cells (**Figure 1B**). Quantitative analysis revealed

31 that GFP-positive cells were indeed highly enriched in cells in which red fluorescence

32 intensity was <10,000 arbitrary units (A.U.) (**Figure 1C**), whereas a high level of mCherry

33 expression only partially suppressed VSVΔG-G infection (**Figure S1D**). These observations

1   also suggested that virus particles lacking envelope protein might be produced from cells with

2   no mCherry-S expression. Indeed, immunofluorescence analysis with antibodies to VSV M

3   protein showed that the supernatant of vector-transfected, VSVΔG-G-exposed HEK293T

4   cells contained fluorescent puncta even when the vector did not encode an envelope protein

5   (**Figure S1E**).

6   Our results together suggested that a low and persistent level of S protein expression

7   might be required for the production of pseudotyped virus particles that uniformly bear S

8   protein. We therefore attempted to establish cell lines that stably express S protein. However,

9   although HEK293T cells are used in the standard protocol for VSV-based pseudotyped virus

10 production, we found that HEK293T cells expressing mCherry-S manifested a shrunken

11 morphology compared with those expressing mCherry alone (**Figure S1F**). We next

12 introduced the expression vector for mCherry-S into VeroE6 cells, which have been widely

13 adopted for SARS-CoV research. Fluorescence imaging showed that the morphology of

14 VeroE6 cells expressing mCherry-S was similar to that of those expressing mCherry alone

15 (**Figure 2A**). To generate stable cell lines, we isolated transfected VeroE6 cells by treatment

16 with trypsin and subjected them to puromycin selection. After incubation for 24 h,

17 multinucleated cells, indicative of cell-cell fusion resulting from S protein activation by

18 trypsin cleavage, were apparent for the cells expressing mCherry-S (**Figure 2B**). To avoid

19 cleavage of S protein, we used enzyme-free cell-dissociation solutions for cell isolation.

20 Treatment with such solutions did not induce cleavage of S protein (**Figure S2A**) or

21 multinucleated cells (**Figure 2C**), thus allowing the generation of cell lines stably expressing

22 S protein (**Figure 2D**).

23   We next examined whether such a cell line was susceptible to VSVΔG-G infection for

24 production of pseudoviruses harboring S protein. Fluorescence microscopy and quantitative

25 analysis of the virus-exposed cells revealed that the mCherry fluorescence intensity of all

26 cells was <10,000 A.U. (the desired upper limit as determined in **Figure 1C**), and that most

27 cells were positive for GFP, indicative of uniform VSVΔG-G infection (**Figures S2B** and

28 **S2C**). The virus particles produced by the cells were then stained with DiD and adsorbed on a

29 polyethylenimine-coated glass-based dish to determine the amount of particles per unit

30 volume by measurement of the area of DiD-positive puncta with a confocal microscope. The

31 amount of particles produced by VeroE6 cells stably expressing mCherry-S was ~10% of that

32 of those produced by HEK293T cells transiently expressing mCherry-S (**Figures S3A** and

33 **S3B**). BEAS-2B cells expressing ACE2 were then exposed to the pseudoviruses for 1 h at

34 4°C (to allow for virus attachment) and subjected to immunofluorescence analysis with

6

1   antibodies to SARS-CoV S protein and to VSV M protein in order to allow visualization of S

2   protein and all pseudovirus particles, respectively. Confocal imaging showed that, in contrast

3   to the virus particles produced by transiently transfected HEK293T cells, essentially all virus

4   particles produced by the stably transfected VeroE6 cell line were positive for S protein

5   (**Figure 3A**). Indeed, quantitative analysis revealed that the fraction of S protein-positive

6   particles generated by the stable cell line was about seven times that generated by the

7   transiently transfected cells (**Figure 3B**). These results thus demonstrated the efficient

8   production of pseudoviruses that were essentially all loaded with S protein by the stable cell

9   line.

10   We further examined whether the titer of pseudovirus particles generated by the stable

11   cell line was higher than that of particles generated by the conventional method. There was no

12   significant difference between the FFU values obtained for the two types of pseudoviruses

13   (**Figure S3C**). To normalize infectivity, we prepared dilutions of the virus suspensions (down

14   to 1.0 FFU/µl) and plotted FFU against the area of puncta. A linear relation was apparent for

15   each type of pseudovirus, although the slopes of the regression lines differed (**Figure S3D**).

16   The FFU value normalized by the amount of viruses produced by the stable cell line was 6.4

17   times the corresponding value for the viruses produced by the transiently transfected cells

18   (**Figure 3C**), indicating that the modified method established in this study results in a

19   substantial improvement in infectious pseudovirus particle production.

20   Finally, we attempted to produce pseudotyped virus particles with envelopes bearing

21   the S protein of SARS-CoV-2 by our newly established protocol. We thus established a

22   VeroE6 cell line that stably expresses SARS-CoV-2 S protein (**Figure S4A**). The fraction of

23   SARS-CoV-2 S protein-positive puncta (**Figures 4A** and **4B**) and FFU normalized by the area

24   of DiD-positive puncta (**Figures 4C, S4B** and **S4C**) were five and seven times as high,

25   respectively, for viruses produced from the VeroE6 cell line as were those for viruses

26   produced from transiently transfected HEK293T cells. Our procedure was thus shown to be

27   highly efficient with regard to pseudovirus production for studies of the entry of SARS-CoV

28   or SARS-CoV-2.

29   In this study, we unexpectedly found that a large proportion of pseudotyped virus

30   particles produced by the conventional protocol (Hoffmann et al., 2020; Rentsch and Zimmer,

31   2011) did not bear coronavirus S proteins. This finding raised the concern that S protein–free

32   virions might affect the entry process of, or cellular responses to, S protein–bearing particles

33   and thereby confound experimental outcomes. We therefore established a modified protocol

34   based on stable cell lines in order to generate pseudoviruses for which a large proportion of

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1   the virus particles harbor heterologous envelope proteins. Alternative methods that are free of

2   such issues relating to virus particles lacking envelope protein, including reverse genetic

3   systems for in vitro virus assembly from SARS-CoV-2 full-length cDNA (Torii et al., 2021;

4   Xie et al., 2020), have been developed. However, given that the VSV-based pseudotyped

5   virus systems have been adopted by many researchers, the modification described here should

6   be readily incorporated into current protocols and lead both to a better understanding of virus

7   entry and to accelerated vaccine and therapeutic development for SARS-CoV-2.

8

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16

AUTHOR CONTRIBUTIONS

18   Conceptualization, Y.F. and Y.O.; Methodology, Y.F. and S.K.; Formal analysis, Y.F. and

19   S.K.; Investigation, Y.F., S.K., A.Y., A.O.S., and M.F.; Resources, Y.F., S.K., A.Y., A.O.S.,

20   and M.F.; Writing–original draft, Y.F. and Y.O.; Writing–review and editing, Y.F., M.A.,

21   Y.Y., and Y.O.; Visualization, Y.F. and Y.O.; Supervision, Y.Y. and Y.O.; Project

22   administration, Y.O.; Funding acquisition, Y.F. and Y.O.

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DECLARATION OF INTERESTS

25   Y.F. and Y.O have a patent pending that includes claims relating to the present study. Other

26   authors declare no competing interests.

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20

1   **STAR Methods**

2   **Contact for reagent and resource sharing**

3   Further information and requests for resources and reagents should be directed to and will be

4   fulfilled by the Lead Contact, Yusuke Ohba (yohba@med.hokudai.ac.jp).

5   **Cell lines**

6   HEK293T (ATCC CRL-11268), VeroE6 (CRL-1586), and BEAS-2B (CRL-9609) cells were

7   obtained from American Type Culture Collection (ATCC; Manassas, VA) and were

8   maintained under a humidified atmosphere of 5% CO_2 at 37°C in Dulbecco's modified

9   Eagle's medium (DMEM) (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% fetal

10   bovine serum (FBS) (Capricorn Scientific, Ebsdorfergrund, Germany). BHK21/G43 were

11   kindly provided by S. Pöhlmann (Leibniz Institute for Primate Research, Göttingen,

12   Germany). Expression plasmids were introduced into cells by transfection for 24 h with the

13   use of Polyethylenimine "Max" (PEI MAX; Polysciences, Warrington, PA). The absence of

14   mycoplasma contamination was confirmed with the use of a PCR Mycoplasma Test Kit

15   (Takara, Shiga, Japan).

16   **Reagents and antibodies**

17   Antibodies to SARS-CoV S protein (#40150-R007), to RFP (#PM005), to VSV G protein

18   (#EB0010), and to VSV M protein (#MABF2347) were obtained from Sino Biological

19   (Beijing, China), Medical & Biological Laboratory (Nagoya, Japan), Kerafast (Boston, MA),

20   and Merck (Darmstadt, Germany), respectively. Hoechst 33342, carbocyanine dyes (DiO, DiI,

21   and DiD), and Alexa Fluor 647– or Alexa Fluor 488–labeled antibodies to rabbit or mouse

22   immunoglobulin G were obtained from Thermo Fisher Scientific (Carlsbad, CA). Horseradish

23   peroxidase (HRP)–conjugated goat secondary antibodies were obtained from Jackson

24   ImmunoResearch Laboratories (West Grove, PA). Trypsin was obtained from FujiFilm-Wako

25   (Osaka, Japan), and cell-dissociation solutions were from Thermo Fisher Scientific and

26   Biological Industries (Beit-Haemek, Israel). Mifepristone was obtained from Sigma-Aldrich.

27   **Plasmids**

28   Expression vectors for the S protein of SARS-CoV or SARS-CoV-2 (pCG1-SARS-CoV-S

29   and pCG1-SARS-CoV-2-S) (Hoffmann et al., 2020) were kindly provided by S. Pöhlmann.

30   The coding sequence for mCherry was cleaved out of pFX-Tom20-mCherry (Kashiwagi et al.,

31   2019a) by digestion with *Bam*HI and *Bgl*II, and was then subcloned into the *Bam*HI sites of

32   the expression vectors for the S proteins to obtain pCG1-mCherry-SARS-CoV-S and pCG1-

12

1   mCherry-SARS-CoV-2-S. The pBABE-puro plasmid encoding a puromycin resistance gene

2   was obtained from Addgene.

3   **Generation of stable cell lines**

4   pCG1-mCherry-SARS-CoV-S or pCG1-mCherry-SARS-CoV-2-S was linearized with *Puv*I

5   and introduced together with pBABE-puro into HEK293T or VeroE6 cells by transfection for

6   24 h with the use of PEI MAX. The cells were then cultured in DMEM supplemented with

7   10% FBS and puromycin (10 µg/ml), and puromycin-resistant colonies were collectively

8   harvested. Expression of the mCherry-tagged S proteins was confirmed by

9   immunofluorescence analysis.

10   BEAS-2B cells stably expressing human ACE2 were generated by lentivirus-mediated

11   gene transfer. HEK293T cells were thus transfected with pLVX-ACE2-IRES-BLD (Daly et

12   al., 2020), pCAG-HIVgp, and pCMV-VSVG-RSV-Rev (Miyoshi et al., 1998) for 48 h, after

13   which the culture supernatant was collected. BEAS-2B cells were exposed to the recombinant

14   lentivirus–containing supernatant for 1 h at 35°C, cultured for 2 days at 35°C in DMEM

15   supplemented with 10% FBS, and further cultured for 14 days in DMEM supplemented with

16   10% FBS and blasticidin (20 µg/ml) (Funakoshi, Tokyo, Japan). The resulting blasticidin-

17   resistant colonies were collectively harvested, and the constituent cells were maintained in

18   DMEM supplemented with 10% FBS and blasticidin (15 µg/ml).

19   **Preparation of VSV pseudoviruses**

20   Pseudotyped viruses were generated according to an established protocol (Rentsch and

21   Zimmer, 2011) or its modified version developed in the present study. In brief, for preparation

22   of VSVΔG-G, BHK21/G43 cells (which stably express VSV G protein) were incubated with

23   mifepristone (10 nM) for 6 h and then infected with VSVΔG at a multiplicity of infection

24   (MOI) of 100,000 FFU per cell for 16 h at 35°C. For generation of VSVΔG-S particles, cells

25   expressing S protein of SARS-CoV or SARS-CoV-2 (either stably or transiently) were

26   exposed to VSVΔG-G at an MOI of 3 FFU per cell for 16 h at 35°C in the presence of

27   antibodies to VSV G in order to neutralize the parental viruses. The culture supernatants were

28   passed through a filter (pore size of 0.45 µm) and subjected to ultracentrifugation, and the

29   virus pellets were resuspended in phenol red-free DMEM/F12 (Thermo Fisher Scientific) and

30   stored at –80°C until use. Given that the enhanced GFP (EGFP) gene is packaged in the

31   genome of the pseudoviruses in this system (Rentsch and Zimmer, 2011), the titer of

32   infectious viruses in culture supernatants can be determined by counting GFP-positive cells

33   with use of a fluorescence microscope.

1 **Determination of FFU**

2 BEAS-2B cells expressing ACE2 were incubated with pseudotyped viruses for 2 h at 35°C,

3 and the culture supernatants were then removed and the cells cultured in DMEM for 16 h at

4 35°C before staining with Hoechst 33342 for 15 min and examination with a fluorescence

5 microscope. The FFU value of the pseudovirus suspension was determined by counting the

6 number of GFP-positive cells with the use of the "Multi-wavelength cell scoring" module of

7 MetaMorph software (Molecular Devices, CA).

8 **Fluorescence microscopy**

9 Fluorescence imaging and data analysis were performed essentially as described previously

10 (Fujioka et al., 2019; Kashiwagi et al., 2019b). In brief, cells transfected with mCherry-CoV-

11 S or mCherry-CoV-2-S vectors were stained with Hoechst 33342 for 15 min and placed in a

12 stage-top incubation chamber maintained at 37°C on a Nikon Ti2 microscope (Nikon, Tokyo,

13 Japan) equipped with a Zyla5.5 scientific complementary metal oxide semiconductor camera

14 (Oxford Instruments, Belfast, UK). The cells were illuminated with an X-Cite turbo system

15 (Excelitas Technologies, Waltham, MA) through a GFP HQ, Cy3 HQ, or DAPI-U HQ

16 excitation filter (Nikon).

17 **Fluorescent labeling of pseudoviruses**

18 For preparation of fluorescently labeled pseudoviruses, 1 ml of pseudovirus suspension was

19 incubated for 1 h at room temperature with 6 µl of 100 µM DiD, DiI, or DiO stock solution in

20 the dark (Fujioka et al., 2018; Nanbo et al., 2010). The stained particles were adsorbed to a

21 PEI MAX–coated 96-well glass-based plate and observed with a confocal microscope.

22 **Immunofluorescence analysis**

23 BEAS-2B cells expressing ACE2 were incubated with pseudotyped viruses for 1 h at 4°C to

24 allow for virus attachment. The cells were then fixed with 3% paraformaldehyde for 15 min at

25 room temperature and incubated with 1% bovine serum albumin to block nonspecific binding

26 of antibodies. They were further incubated overnight at 4°C with primary antibodies (1:1000

27 dilution), after which immune complexes were detected by incubation for 1 h at room

28 temperature in the dark with Alexa Fluor 488- or Alexa Fluor 647-conjugated secondary

29 antibodies (1:250 dilution). Nuclei were visualized by staining with Hoechst 33342. Images

30 were acquired with an IX83 microscope (Olympus, Tokyo, Japan) with X-lightV3

31 (CrestOptics, Rome, Italy).

14

1 **Immunoblot analysis**

2 Transfected HEK293T cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM

3 NaCl, 1%Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, cOmplete

4 Protease Inhibitor Cocktail (Sigma-Aldrich)] for 30 min on ice. The lysates were centrifuged

5 at $20,000 \times g$ for 10 min at 4°C, and the resulting supernatants were subjected to SDS-

6 polyacrylamide gel electrophoresis. The separated proteins were transferred to a

7 polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and subjected to immunoblot

8 analysis. Immune complexes were detected with HRP-conjugated secondary antibodies, ECL

9 Western Blotting Detection Reagent (Cytiva, Tokyo, Japan) and a MIIS imaging system

10 (Givetechs, Sakura, Japan).

11 **Quantification and statistical analysis**

12 Quantitative data are presented as means + SEM from at least three independent experiments

13 and were compared with Student's t test (parametric test between two conditions) or by one-

14 way analysis of variance (ANOVA) followed by the Tukey honestly significant difference

15 (HSD) post hoc test (among multiple conditions). No statistical methods were applied to

16 predetermine sample size. Experiments were performed unblinded. A p value of <0.05 was

17 considered statistically significant, and all statistical analysis was performed with JMP Pro

18 software (version 15.0.0).

15

FIGURE LEGENDS

Figure 1. Pseudotyped virus particles produced from cells transiently expressing SARS-CoV S protein are not loaded with S protein

(**A**) HEK293T cells were transfected with an expression vector for SARS-CoV S protein as well as infected with VSVΔG-G. The culture supernatant was collected and stained with DiI as well as subjected to immunofluorescence staining with antibodies to SARS-CoV S protein and Alexa Fluor 488–conjugated secondary antibodies. The virus particles were then adsorbed onto a polyethylenimine-coated glass-based plate and observed with a confocal fluorescence microscope. Representative images are shown. Bars, 10 μm.

(**B**) HEK293T cells were transfected with an expression vector for mCherry-tagged SARS-CoV S protein and infected with VSVΔG-G for 16 h. They were then observed with a fluorescence microscope for detection of mCherry (red) and GFP (green) fluorescence. Representative images are shown. The boxed region in the lower right panel is shown at higher magnification in the other panels. Bars, 10 μm.

(**C**) Fluorescence intensities of GFP and mCherry in individual cells as in (**B**).

See also **Figure S1**.

Figure 2. Trypsin treatment induces cell-cell fusion of S protein-expressing cells

(**A**) VeroE6 cells transfected with an expression vector for mCherry or mCherry-tagged S protein of SARS-CoV (mCherry-S) that had been linearized with *Puv*I were stained with Hoechst 33342 and observed by fluorescence microscopy. Representative images of mCherry (magenta) and Hoechst 33342 (blue) fluorescence are shown. Bars, 10 μm.

(**B** and **C**) Cells prepared as in (**A**) were harvested by exposure to trypsin (**B**) or to enzyme-free cell dissociation solutions from Biological Industries (BI) or Gibco (**C**). They were then transferred to cell culture dishes and cultured for 24 h before fluorescence microscopic analysis as in (**A**). Representative images are shown. Bars, 10 μm.

(**D**) Cells transfected with the mCherry-S vector (or the corresponding empty vector) as in (**A**) were cultured in the presence of puromycin for 14 days, stained with Hoechst 33342, and subjected to immunofluorescence analysis with antibodies to SARS-CoV S protein and Alexa Fluor 488–conjugated secondary antibodies. Representative fluorescence microscopic images of S protein immunostaining (green) as well as of mCherry (magenta) and Hoechst 33342 (blue) fluorescence are shown. Bars, 10 μm.

See also **Figure S2**.

Figure 3. A large proportion of pseudotyped virus particles produced from VeroE6 cells stably expressing SARS-CoV S protein are loaded with S protein.

(**A**) VeroE6 cells stably expressing or HEK293T cells transiently expressing SARS-CoV S protein were infected with VSVΔG-G for 16 h, and equal numbers of pseudoviruses released into the culture supernatants were added to ACE2-expressing BEAS-2B cells for 1 h at 4°C. The latter cells were then fixed, stained with Hoechst 33342 (blue), subjected to immunofluorescence analysis with antibodies to SARS-CoV S protein and VSV M protein, and observed with a confocal microscope. Representative images are shown. The boxed

16

regions in the right panels are shown at higher magnification in the panels to the left. Bars, 10 μm.

(**B**) Quantification of the fraction of S protein-positive puncta among all M protein–positive puncta for images as in (**A**). Data are means + SEM from three independent experiments. *, $p < 0.001$ (Student's t test).

(**C**) BEAS-2B cells stably expressing ACE2 were exposed to pseudotyped viruses prepared as in (**A**) for determination of the number of GFP-positive cells and calculation of FFU of virus suspension. FFU was normalized by the amount of pseudotyped viruses, which was determined by the area of puncta of DiD-stained viruses on a polyethylenimine-coated glass-based plate. Data are means + SEM from three independent experiments. *, $p < 0.004$ (one-way ANOVA with Tukey's HSD post hoc test).

See also **Figure S3**.

Figure 4. Production of pseudoviruses loaded with S protein of SARS-CoV-2 by a stable cell line

(**A**) BEAS-2B cells expressing ACE2 were exposed for 1 h at 4°C to pseudotyped viruses produced from a VeroE6 cell line stably expressing or HEK293T cells transiently expressing S protein of SARS-CoV-2. The cells were then fixed, stained with Hoechst 33342 (blue), subjected to immunofluorescence staining with antibodies to SARS-CoV S protein and VSV M protein, and examined with a confocal microscope. Representative images are shown. The boxed regions in the bottom panels are shown at higher magnification in the panels above. Bars, 10 μm.

(**B**) Quantification of the fraction of S protein-positive puncta among all M protein–positive puncta in images as in (**A**). Data are means + SEM from three independent experiments. *$p < 0.001$ (Student's t test).

(**C**) FFU normalized by the area of puncta for the pseudoviruses produced as in (**A**) was determined as in **Figure S4**. Data are means + SEM from three independent experiments. *, $p < 0.004$ (one-way ANOVA with Tukey's HSD post-hoc test).

See also **Figure S4**.

A

Anti-SARS-CoV S (Alexa Fluor 488) DiI Merged

B

S protein (mCherry) VSVΔG-G (EGFP)

Merged

C

Y. Fujioka, et al., Figure 1

Y. Fujioka, et al., Figure 2

Y. Fujioka, et al., Figure 3

Y. Fujioka, et al., Figure 4