

1           **Tunneling nanotubes provide a novel route for SARS-CoV-2 spreading**  
2           **between permissive cells and to non-permissive neuronal cells.**

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20 **Abstract**

21 SARS-CoV-2 entry into host cells is mediated by the binding of its spike glycoprotein to the  
22 angiotensin-converting enzyme 2 (ACE2) receptor, highly expressed in several organs, but very  
23 low in the brain. The mechanism through which SARS-CoV-2 infects neurons is not  
24 understood. Tunneling nanotubes (TNTs), actin-based intercellular conduits that connect  
25 distant cells, allow the transfer of cargos, including viruses. Here, we explored the  
26 neuroinvasive potential of SARS-CoV-2 and whether TNTs are involved in its spreading  
27 between cells *in vitro*. We report that neuronal cells, not permissive to SARS-CoV-2 through  
28 an exocytosis/endocytosis dependent pathway, can be infected when co-cultured with  
29 permissive infected epithelial cells. SARS-CoV-2 induces TNTs formation between permissive  
30 cells and exploits this route to spread to uninfected permissive cells in co-culture. Correlative  
31 Cryo-electron tomography reveals that SARS-CoV-2 is associated with the plasma membrane  
32 of TNTs formed between permissive cells and virus-like vesicular structures are inside TNTs  
33 established both between permissive cells and between permissive and non-permissive cells.  
34 Our data highlight a potential novel mechanism of SARS-CoV-2 spreading which could serve  
35 as route to invade non-permissive cells and potentiate infection in permissive cells.

36

## 37 **Introduction**

38

39 COVID-19, the disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-  
40 CoV-2), has been developing into a global pandemic since the first reported events in December  
41 2019 (1,2). Although SARS-CoV-2 primarily targets the respiratory tract and the majority of  
42 COVID-19 patients present severe respiratory symptoms (3), other organs such as the intestine,  
43 liver, kidneys, heart and brain are also affected. Neurological manifestations of different gravity  
44 associated with the COVID-19 have been reported (4–7) . Interestingly the neurological  
45 symptoms can be acute and resolve with the disease or can represent a major issue in the case  
46 of long-COVID (8–10). The ability of SARS-CoV-2 to enter the central nervous system (CNS)  
47 is not surprising given that several types of coronavirus (CoV) (e.g., severe acute respiratory  
48 syndrome, SARS-CoV; Middle East respiratory syndrome, MERS-CoV) have been reported to  
49 invade and persist in the CNS (11,12) . In addition, case reports have shown that the brain tissue  
50 of patients that died following COVID-19 were positive for SARS-CoV-2 RNA (13).

51 However, how SARS-CoV-2 gains access to the CNS and how infection leads to neurological  
52 symptoms is still not clear (14–18) . SARS-CoV-2 neuroinvasion could be achieved through  
53 several routes as previously described (19), and once it reaches the CNS it could bind the  
54 angiotensin-converting enzyme 2 (ACE2) receptor exposed on neuronal cells to infect the brain  
55 (20). ACE2 receptor is the main actor responsible of the virus entry in the lower respiratory  
56 tract (13,21,22). To enter host cells the viral spike (S) proteins of coronaviruses bind the  
57 enzymatic domain of the ACE2 receptor, which is exposed on the surface of the cells forming  
58 the oral cavity and the oropharynx (23–25). While the expression of the ACE2 receptor has  
59 been well documented in many cell type and tissues (23,24), it is important to underline that in  
60 human brain the expression of the ACE2 receptor is low, with the exception of brain areas such  
61 as the thalamus and the choroid plexus (26). For this reason, it is not clear how the virus can  
62 propagate through the brain, and it is a priority to investigate how SARS-CoV-2 enters into  
63 neuronal cells in order to provide new insights in the understanding of the neurological  
64 manifestations associated with COVID-19.

65 An interesting aspect to consider is that the presence of SARS-CoV-2 RNA and proteins has  
66 been found in anatomically distinct regions of the brain of COVID-19 patients (13,27). In this  
67 respect, the spreading of SARS-CoV-2 in the CNS is reminiscent of toxic amyloid proteins in  
68 neurodegenerative disorders (NDs), that propagate in the brain according to the progression of  
69 the pathology (28,29).

70 We have previously shown that the spreading of different amyloid aggregates between cells of  
71 the CNS and from peripheral lymphoid system cells to neurons occurs mainly through  
72 Tunneling Nanotubes (TNTs), a novel mechanism of cell-to-cell communication (30–32).  
73 TNTs are thin, membranous conduits rich in actin that form contiguous cytoplasmic bridges  
74 between cells over long and short distances (33,34). Recently we set up a pipeline in correlative  
75 cryo-electron microscopy (Cryo-CLEM) to determine the ultrastructure of TNTs in neuronal  
76 cell lines, demonstrating their structural identity and differentiating them from other cellular  
77 protrusion as filopodia (35). Functionally, TNTs allow direct transport of cargos including virus  
78 between distant cells (30,34,36,37). Of specific interest, it has been documented that viruses  
79 from different families induce increased formation of TNTs or TNT-like structures, using these  
80 membranous structures to efficiently spread the infection to neighboring health cells (38–42).  
81 Here, we investigated the neuroinvasive potential of SARS-CoV-2 and whether TNTs are  
82 involved in its intercellular spreading. Since TNT-transferred virions would not necessarily be  
83 exposed outside the host cell, we hypothesize that TNT-mediated transmission can be used by  
84 the viruses to escape neutralizing antibody activity and immune surveillance, as well as to infect  
85 less permissive cells lacking the membrane receptor for virus entry, thus allowing for spreading  
86 of virus tropism and pathogenicity. Our data show that human neuronal cells, not permissive to  
87 SARS-CoV-2 through an exocytosis/endocytosis dependent pathway, can be infected when co-  
88 cultured with permissive epithelial cells, previously infected with SARS-CoV-2. We observed,  
89 in confocal microscopy, that SARS-CoV-2, induced the formation of TNTs that then could be  
90 used by the virus to efficiently spread toward uninfected permissive and non-permissive cells.  
91 Furthermore, by setting up correlative Cryo-CLEM and–tomography (35), we demonstrated  
92 that SARS-CoV-2 virions are associated to the plasma membrane of TNTs formed between  
93 permissive cells. Interestingly, we also observed virus-like vesicular structures and double  
94 membrane vesicles (DMVs) inside the TNTs, both between permissive cells and between  
95 permissive and non-permissive cells.

96 Altogether, our results shed new light on the structure of the viral particles undergoing  
97 intercellular spreading and provide important information about the molecular mechanism of  
98 SARS-CoV-2 infection and transmission. They support the role of TNTs in the viral spreading  
99 in both permissive and non-permissive cells, possibly enhancing the efficiency of viral  
100 propagation through the body.

101

102 **Results.**

103

104 **1. SARS-CoV-2 can spread among cells through an exocytosis/endocytosis independent**  
105 **pathway.**

106 The main route of SARS-CoV-2 entry into the cell is determined by the binding of the spike  
107 (S) glycoproteins, exposed on its surface, with the membrane protein ACE2 as an entry receptor  
108 (1,24,43,44). Current data indicate that this receptor is only expressed in low amounts in the  
109 brain, so the question is whether and how the virus is able to infect neuronal cells (45). To this  
110 aim we tested different cell types to verify whether they were permissive to viral infection by  
111 the receptor-mediated pathway.

112 Different cells of mammalian and human origin were plated on a 96 multi-well plate and  
113 infected with a MOI (multiplicity of infection) ranging from  $10^{-1}$  to  $10^{-5}$ . The cell-lines used in  
114 this assay included human colon epithelial cell-lines (Caco-2,), monkey kidney epithelial cell-  
115 line (Vero E6), and the human (SH-SY5Y) and murine (CAD) neuronal cells lines. After 3 days  
116 we looked for productive infection by staining the infected monolayers with a virus-specific  
117 antibody and by titrating the virus released in the supernatant. Using these two parameters we  
118 found that only the epithelial Vero E6 and Caco-2 cells were susceptible to infection with  
119 SARS-CoV-2, as previously shown (46), while both neuronal cell lines (mouse and human) did  
120 not show any sign of infection or viral production (fig. S1A, B). Consistently, the  
121 immunofluorescent signal of the viral Nucleoprotein (N) protein was evident in the monolayers  
122 of Vero E6 and Caco-2 cells after 3 days of infection with different MOI. On the contrary, no  
123 fluorescent signal was detected in CAD, and SH-SY5Y cells (fig. S1A). In addition, an aliquot  
124 of the supernatant from the MOI 0.1 infection was collected at day 2 and 3 to quantify the  
125 kinetic of virus production by titration using a semisolid plaque assay on Vero E6 cells. As  
126 shown in fig. S1 B, no viral production was detected using the supernatant derived from CAD  
127 and SH-SY5Y infection, while clear signs of cytopathic effect (CPE) were detected in Vero E6  
128 cells infected with Vero E6 and Caco-2 supernatants.

129 These data show that neuronal cells cannot be infected directly from the supernatant through a  
130 receptor-mediated mechanism.

131 The main mediator of cellular entry of SARS-CoV-2 is the ACE2 receptor (1,24,47); whilst it  
132 is highly expressed in vascular endothelial cells of the lungs (47) , it was detected at extremely  
133 low levels in the neuronal cells (45). Consistently, we were unable to detect a signal for ACE2  
134 in SH-SY5Y cells (fig. S2), confirming previous observation reporting extremely low levels of  
135 expression in neuronal cells (45). Nonetheless, emerging case reports showed that patients

136 infected with SARS-CoV-2 have common neurological manifestation (17,48–53) suggesting  
137 that the virus could invade and infect the CNS (7,53,54). Since the ACE2 receptor is not widely  
138 expressed in neuronal cells one likely possibility is that the virus can exploit intercellular  
139 communication pathways to enter neuronal cells directly from permissive cells, which would  
140 allow bypassing the main receptor mediated pathway. To investigate this, we set up co-  
141 culture experiments between permissive Vero E6 cells, routinely used as infection model of  
142 SARS-CoV-2, and non-permissive SH-SY5Y human neuronal cells. Vero E6 cells (donor cells)  
143 infected with SARS-CoV-2 at MOI 0.05 for 48 hours were co-cultured with SH-SY5Y cells  
144 (acceptor cells) previously transfected with a plasmid encoding mCherry, to be easily  
145 distinguished from donor cells (fig. S3A). After 24h and 48h of co-culture, cells were fixed and  
146 immunostained with anti-N antibody recognising SARS-CoV2 nucleoproteins, and  
147 labelled with cell mask blue to stain the whole cells (Fig. 1A-C). By using confocal  
148 microscopy, and the ICY software ([icy.bioimageanalysis.org](http://icy.bioimageanalysis.org)), we calculated the percentage of  
149 SH-SY5Y acceptor cells positive for the anti-N antibody immunostaining. After 24h of co-  
150 culture, 36,4% of acceptor cells contained in their cytoplasm spots recognised by the anti-N  
151 antibody (Fig. 1D), and this percentage increased to 62,5% after 48h (Fig. 1A-D). To further  
152 investigate the nature of the viral particles, present in acceptor neuronal cells, additional co-  
153 cultures between Vero E6 infected with SARS-CoV2 MOI of 0.05 and SH-SY5Y neuronal cells  
154 were immunostained (after 24h and 48h of co-culture) using an anti-Spike (anti-S) antibody,  
155 both alone (Fig. 1E-F) and in combination with the anti-N antibody (Fig. 1H-J). Similar to the  
156 results obtained with the N-antibody, we found that after 24h of co-culture 21,8 % of acceptor  
157 cells contained in their cytoplasm spots positive for the anti-S antibody, and after 48h of co-  
158 culture this value increased to 42,4 % (Fig. 1G). We then evaluated the co-localization of the  
159 two viral proteins N and S in the acceptor cells (Fig. 1H-J) and we found that at 48h the PCC  
160 (Pearson's coefficient) was in average 0,716, indicating that the two proteins partially  
161 colocalize. While separate signals for anti-N and anti-S antibodies could be suggestive of virus  
162 uncoating during the first step of the infection (56), colocalization of N and S proteins could  
163 correspond to mature virions inside endocytic vesicles that are entering the cell and/or to newly  
164 synthesized virions assembled in the neuronal acceptor cells. Because at 24 hours we found  
165 negligible colocalization between the two proteins, we could assume that the particles labelled  
166 with both the N and S antibodies in the neuronal cells at 48 hours correspond to virus newly  
167 assembled in the SH-SY5Y cells after transfer. Indeed at 24 hours of co-culture Vero E6 cell  
168 are already full of newly synthesized virions (fig. S3 B, C), therefore if there was direct transfer

169 of fully assembled virions we would have expected to find them in acceptor cells already at 24h  
170 of co-culture, which was not the case.

171 To directly investigate whether SARS-CoV-2 transferred from the donor cells was still  
172 functional, and able to replicate in neuronal cells, we performed an immunostaining using the  
173 anti-dsRNA (double-stranded RNA) antibody J2 that is the gold standard for the detection of  
174 dsRNA in infected cells (57). After 48h of co-culture (Vero E6 infected cells as donors and SH-  
175 SY5Y cells as acceptors), cells were fixed and immunostained to detect dsRNA and SARS-  
176 CoV-2 particles using the anti-S antibody. We found J2 positive signal both in donor Vero E6  
177 infected cells and acceptor SH-SY5Y cells (Fig. 2A-C). Interestingly, J2 signal in SH-SY5Y  
178 cells (Fig. 2B, C) corresponds to bright foci of different sizes and intensities, that could  
179 represent the replication organelles (RO) or double-membrane vesicle (DMVs) where viral  
180 RNA synthesis occurs (58,59). Furthermore, the localization of J2 spots in the perinuclear/ER  
181 region of acceptor cells could suggest an active viral replication occurring in the neuronal cells  
182 (Fig. 2B, C) (60–64). Accordingly, J2 signal was specific for infected cells in co-culture as it  
183 was not detected in non-infected Vero E6 co-cultured with SH-SY5Y mCherry (Fig. 2D), as  
184 well as, in the negative control in which the co-culture was incubated only with the secondary  
185 antibody (Fig. 4E). These data indicate that SARS-CoV-2 transferred by a cell-to-cell contact-  
186 dependent mechanism could be actively replicated in neuronal acceptor cells.

187 To support this hypothesis, as complementary approach we performed an immunostaining  
188 against the non-structural protein 3 (nsp3), an essential component of the viral  
189 replication/transcription complex. After 48h of co-culture (Vero E6 infected cells as donors and  
190 SH-SY5Y cells as acceptors), cells were fixed and immunostained to detect nsp3 and SARS-  
191 CoV-2 particles (by anti-N). We found cytoplasmic accumulations containing the viral N  
192 protein and the non-structural protein nsp3 both in donor Vero E6 and acceptor SH-SY5Y cells  
193 (Fig. 3A-D). This data confirms that SARS-CoV-2 can replicate in the acceptor neuronal cells.  
194 The above data show that neuronal cells can be infected when in co-culture with permissive  
195 cells, but do not address the mechanism. Although our data show that SH-SY5Y cells cannot  
196 be infected by the supernatant (fig S1), in order to investigate the possible contribution of the  
197 endocytic entry pathway in co-culture conditions, as control for the co-culture experiments, we  
198 performed “secretion tests” where the supernatants from Vero E6 infected cells were used to  
199 infect SH-SY5Y cells for 24h and 48h respectively (fig. S4A). We did not detect any signal for  
200 anti-N and anti-S antibodies in the acceptor cells that received the supernatants from the  
201 infected Vero E6 cells after 24h (fig. S4B, C); and a negligible signal was found at 48h (fig.  
202 S4C). On the other end, the supernatants from Vero E6 infected cells was able to infect control

203 Vero E6 cells where we could detect both anti-N and anti-S signal (fig. S4D). The infectious  
204 titer of the supernatant used in the secretion experiment in Vero E6 acceptor cells was calculated  
205 using a focus forming assay (fig. S4E). Additionally, the 48h supernatants from donor infected  
206 cells, co-culture and secretion experiments were used to assess viral production by focus  
207 forming assay titration protocol (fig. S4F).

208

## 209 **2. TNTs contribute to the SARS-CoV-2 transmission**

210 The results described above provide evidence that while SARS-CoV-2 entry into epithelial cells  
211 is mediated by the classical exo/endocytosis pathway, the spreading between permissive  
212 cells and non-permissive neuronal cells could occur in a direct cell-to-cell contact dependent  
213 manner. Recent studies have shown that viruses of many different families, including  
214 retroviruses such as HIV, and enveloped viruses like herpesviruses, orthomyxoviruses, and  
215 several others trigger the formation of TNTs or TNT-like structures in infected cells and use  
216 these structures to efficiently spread to uninfected cells (39). We therefore explored whether  
217 TNTs could be a mechanism involved in the spreading of SARS-CoV-2 to non-permissive  
218 neuronal cells. To detect TNTs between Vero E6 and SH-SY5Y cells we used cell Mask Blue,  
219 which stains the entire cell cytosol and outlines the entire cell shape. To properly identify TNTs  
220 by confocal microscopy it is crucial to distinguish them from other actin driven membranous  
221 protrusions such as filopodia (65,66). TNTs hover above the substrate and even over other cells,  
222 and unlike dorsal filopodia, TNTs are able to connect two or more distant cells. Based on these  
223 criteria, first we assessed the presence of TNTs in control co-culture between Vero E6 cells and  
224 SH-SY5Y cells expressing the NLS-GFP (Nuclear Localization Signal) and stained with  
225 rhodamine phalloidin (for cellular membrane and actin labelling). We observed heterotypic  
226 TNTs formed between Vero E6 cells and SH-SY5Y NLS-GFP (fig. S5A, B). Strikingly, we  
227 also observed TNTs between SARS-CoV-2 infected Vero E6 donor cells and mCherry-SH-  
228 SY5Y acceptor cells in co-culture that contained particles stained with anti-N antibody, which  
229 were also found inside the cytoplasm of the acceptor neuronal cells (Fig. 4 A, B). In addition,  
230 we found TNTs between SH-SY5Y acceptor cells, which also contained anti-N labelled  
231 particles (Fig. 4 C, D). Altogether the data presented above (Fig 1-4) indicate that SARS-CoV-  
232 2 infection can be transferred in a cell-to-cell contact-dependent manner (likely TNT-mediated)  
233 from permissive cells to non-permissive neuronal cells. The evidence showing N-labelled  
234 particles in TNTs between neuronal cells suggests that once reached non-permissive cells  
235 infection could be further spread among them via TNTs.



236 **3. Cryo-EM reveals viral compartments in TNTs between permissive and non-permissive**  
237 **cells.**

238 Next, we wanted to identify the nature and structure of the viral particles shared by TNTs and  
239 investigate the mechanisms allowing their TNT-mediated transfer to non-permissive cells (eg,  
240 whether the infectious particles were using TNTs as membrane bridges to surf on top, or as  
241 channels to be transferred inside the tube). To this aim we set up a correlative fluorescence and  
242 cryo-electron microscopy and tomography approach (CLEM, cryo-EM and cryo-ET) (35).  
243 These techniques allowed us to assess for the first time in correlative mode, both SARS-CoV-  
244 2 and TNTs architecture in the closest to native conditions. Vero E6 cells were infected with  
245 SARS-CoV-2 (MOI 0.05) and 48h post infection were seeded on Cryo-EM grids in co-culture  
246 with SH-SY5Y mCherry cells (Fig. 5A and 5 F). Before vitrification, the EM grids were imaged  
247 by fluorescence microscopy (FM) after labelling cells with either cell mask blue (Fig. 5A) or  
248 wheatgerm agglutinin (WGA) conjugated with a 488 fluorochrome (Fig. 5F) in order to identify  
249 TNTs (for details see material and methods). Consistent with our previous data, we detected  
250 TNTs between Vero E6 and SH-SY5Y mCherry (Fig. 5A, 5F). By using the grid finders after  
251 vitrification, we could identify precisely the TNTs position and image them at the ultrastructure  
252 level using both Glacios Cryo-TEM (Fig. 5A-E) and Titan Krios cryo-TEM (Fig. 5F-K).  
253 Strikingly, TNTs connecting Vero E6 infected cells and SH-SY5Y mCherry cells (labelled with  
254 cell mask blue) revealed the presence, inside the tube, of membranous structures of various  
255 sizes resembling DMVs (double-membrane vesicles) (Fig. 5D-E and Supplementary Movie 1).  
256 DMVs have been identified as the central hub for SARS-Cov2-RNA synthesis Klein et al, (58).  
257 Furthermore, as shown in the tomogram in figure 5I and in Supplementary Movie 2, the TNT  
258 also contained many vesicular structures (Fig. 5I-K blue arrow and Supplementary Movie 2).  
259 Of note, we never observed DMVs and such crowding of vesicular structures inside TNTs  
260 between Vero E6 and SH-SY5Y mCherry cells in control conditions (fig. S6 and Movie 3),  
261 where we could rather see isolated vesicles or organelles as in the case of the mitochondrion  
262 shown in fig. S6D and Movie 3.

263 Since SARS-CoV-2 replication is associated with proliferation of membranes and presence of  
264 DMVs where viral replication is taking place (58), it is possible that these structures represent  
265 viral replicative complexes being transferred to the acceptor cells.

266

267 **4. TNTs facilitate SARS-CoV-2 transmission in permissive Vero E6 cells**

268 Altogether, these data suggested that TNT can allow the intercellular spreading of the infection  
269 from permissive to non-permissive cells. Next, we wondered whether the TNT-mediated route

270 dedicated to invading non-permissive cells could also be used to enhance the spreading of the  
271 virus between permissive cells in addition to the endocytic route. We therefore analysed  
272 whether SARS-CoV-2 exploits TNTs for cell-to-cell spread between Vero E6 cells (Fig. 6A-  
273 C). By confocal microscopy, we observed that in uninfected Vero E6 a low percentage of cells  
274 were connected by TNTs (Fig. 6A, C), but this percentage was substantially increased already  
275 after 24h of SARS-CoV-2 infection (Fig. 6B, C). Strikingly, after immunostaining using either  
276 the anti N antibody (Fig. 6B) and anti S (Fig. 6D) alone or both anti N and anti S antibodies  
277 (Fig. 6E) we could observe particles positive for one or both antibodies (Fig. 6B-E), suggesting  
278 that also mature virions could spread through TNTs between permissive cells. To verify the  
279 hypothesis that SARS-CoV-2, can use the TNT route to spread between permissive cells we  
280 decided to block the infection through the endocytic pathway. To this aim we used a  
281 neutralizing antibody which binds to the RBD domain of the spike protein (anti-SARS-CoV-2  
282 human IgG C3 235) thus blocking binding to ACE2 receptor and the receptor-mediated entry  
283 of the virus. A preliminary experiment was set up to identify the minimal concentration of  
284 antibody sufficient to achieve neutralization of the viral stock of  $1-5 \times 10^5$  FFU/ml used to  
285 infect Vero E6 cells. The viral stock was incubated 1h at 37°C, 5% CO<sub>2</sub> with three different  
286 concentrations of IgG C3 235 (1, 10 and 100 µg/ml) and then used to infect monolayers of Vero  
287 E6 cells for 48h. Viral production was then assessed by titration of the supernatant by focus  
288 forming assay (fig. S7) both 100 and 10 µg/ml concentration of antibody were enough to elicit  
289 complete neutralization of the viral stock, resulting in no sign of viral production (fig. S7).  
290 Therefore, a concentration of 10 µg/ml was set as the minimal concentration to investigate cell-  
291 to-cell transfer in Vero E6 cells.

292 As usual, Vero E6 cells were infected with SARS-CoV-2 MOI 0,05 (donor cells) for 48 hours,  
293 but then incubated with 10 µg/ml anti-SARS-CoV-2 IgG C3 235 for 1 hour at 37°C 5% CO<sub>2</sub>,  
294 prior co-culturing in presence of the antibody, with Vero E6 cells expressing mCherry (acceptor  
295 cells), to distinguish them from the infected donor population. After 24h and 48h hours cells  
296 were fixed and immunostained with anti-N antibody. By using confocal microscopy and ICY  
297 software we evaluated the percentage of Vero E6 acceptor cells containing anti N positive viral  
298 particles both in control condition and in the presence of the blocking antibody (Fig. 7A-C).  
299 Interestingly, after 24h of co-culture in presence of the anti-S neutralizing antibody, 42,9% of  
300 acceptor cells were positive for SARS-CoV-2 detected by anti-N immunostaining, and this  
301 percentage increased to 63,8% after 48h of co-culture, compared to co-culture control  
302 conditions (not incubated with the anti-S neutralizing antibody) where respectively 95% at 24h  
303 and 96,8% at 48h of acceptor cells were positive for anti-N immunostaining (Fig. 7C). As

304 control, to verify the absence of infectious virus in the supernatant of the coculture treated with  
305 the anti-S neutralizing antibody, we challenged naïve Vero E6 cells with the supernatants of  
306 both the co-cultures (incubated/and not with the 235 Ab) (Fig. 7D-F). While 100% of cells  
307 infected with the untreated supernatant were positive for SARS-CoV-2 (Fig. 7D, F), no  
308 infection could be detected in the cells challenged with the treated supernatant (Fig. 7E, F).  
309 Furthermore, the supernatants of each condition were collected to determine the virus  
310 concentration using the focus forming assay (Fig. 7 G).

311 These data show that despite the block of the receptor-mediated virus entry, Vero E6 cells could  
312 be infected when put in co-culture with infected cells, indicating that SARS-CoV-2 can spread  
313 also between permissive cells through a secretion- independent pathway.

314

### 315 **5. Cryo-EM reveals SARS-Cov-2 on top of TNTs**

316 To uncover the nature of the particles labelled with anti-N and anti-S antibodies in TNTs by  
317 fluorescence microscopy and to discriminate if they were localised inside or on top of TNTs  
318 between permissive cells we used again correlative fluorescence cryo-EM and cryo-ET (35).  
319 Vero E6 cells were infected with SARS-CoV-2 (MOI 0.05) and 48h post infection seeded on  
320 Cryo-EM grids. After having identified by FM the exact location of the TNT connecting Vero  
321 E6 infected cells (Fig. 8A), the EM-grids were cryo-fixated and analysed by cryo-EM. High-  
322 quality 3D images using a Titan Krios Microscope revealed SARS-CoV-2 viral particles  
323 located on the surface of TNTs-connecting two Vero E6 cells (Fig. 8 D-H, fig. S8, Movie 4 and  
324 6). SARS-CoV-2 particles that decorate the TNTs' surface displayed both an ellipsoidal and  
325 spherical enveloped morphology with an average diameter ranging from 50 to 100 nm typically  
326 of a coronavirus (Fig. 8, fig. S8, Movie 4 and 6). In our tomograms and in the Supplementary  
327 Movie 4, 6 we can clearly discern the most distinctive features of the virus: the spike proteins  
328 that decorate the surface of the viral particles, together with the ribonucleoprotein complexes  
329 (RNPs) organized inside the virus (Fig. 8 D-H, fig. S8, Supplementary movie 4 and 6) in  
330 accordance with recent cryo-EM data for the virus isolated from infected cells (67–69) and  
331 cryo-FIB SEM pictures of the intracellular virus (58).

332 The presence of the virus on top of TNTs was observed only in permissive cells, and not in  
333 neuronal cells (Fig. 5). This difference could be explained by the presence of the ACE2  
334 receptor, which is only expressed on the cell surface and TNT membranes of Vero E6 cells and  
335 not on SH-SY5Y cells (fig. S2). We also observed vesicular structures (average diameter of 50-  
336 100 nm) inside TNTs connecting Vero E6 infected cells (Fig. 8D green arrowhead and Movie  
337 4), similar to the ones observed in the TNT between Vero E6 infected cells and SH-SY5Y

338 mCherry cells (Fig. 5D-F and Movie 2). As the observation inside TNTs is more challenging  
339 compared to the analysis of the TNT surface, in order to unequivocally demonstrate that these  
340 vesicular structures corresponded to the virus and/or viral compartments, we set-up a  
341 challenging correlative IF cryo-EM protocol making use of the anti-S antibody. SARS-CoV-2-  
342 infected Vero E6 cells, seeded on EM grids, were fixed and processed for an immunostaining  
343 against the Spike proteins (anti-S). Before cryo-fixing the EM grids, we selected the TNTs  
344 connecting cells that were positive for the anti-S by using confocal microscopy (Fig. 9A) and  
345 then observed in the same position at cryo-EM (Fig. 9B-D). In correspondence of the  
346 fluorescent anti-S antibody signal in TNTs coming from a Vero E6 cells (Fig 9A), we could  
347 observe structures resembling SARS-CoV-2 particles that decorate the TNTs' surface with both  
348 a spherical and an ellipsoidal enveloped morphology and spike-like structures (Fig. 9D-I and  
349 Movie 5). Note that the images here are less clear compared to figure 8 and supplementary  
350 figure 8 as in this case the grids were acquired with Glacios Cryo-TEM instead of Titan Krios  
351 cryo-TEM. Interestingly, in correspondence of the S antibody we observed multiple vesicular  
352 structures inside the TNT (Fig. 9D, H-L and Movie 5), similar to the ones observed inside TNTs  
353 between permissive and non permissive cells (Fig. 5I-K blue arrow and Supplementary Movie  
354 2). Considering that cryo-ET resolution is limited by the thickness of the sample, and the fact  
355 that TNTs described here have an average diameter of more than 500 nm, we were at the  
356 resolution limits and we were not able to discriminate the precise structures of these vesicular  
357 compartments. Therefore, besides stating the fact that these structures were found in  
358 correspondence of S antibody, we cannot be sure whether they are mature virions as the ones  
359 observed outside the TNTs.

360 Our observations are in line with the recent publication by Caldas and colleagues showing by  
361 classical scanning electron microscopy that SARS-CoV-2 particles appear surf on top of  
362 cellular protrusions (70). However, in this case the authors did not enquire whether these  
363 protrusions were TNTs and whether they allowed viral transfer. Thus, despite several reports  
364 describing virus of different families (71,72) on top of filopodia and/or cellular extension,  
365 including SARS-CoV-2 (58,70,73), our report represents the first evidence that TNTs could be  
366 one possible route for the spreading of SARS-CoV-2.

367 Our study is carried out using Vero E6 cell line as epithelial model since it has been widely  
368 employed for SARS-CoV-2 isolation, propagation, and antiviral testing, due to its high virus  
369 production and a prominent cytopathic effect (CPE) upon infection (46). Our choice of SH-  
370 SY5Y cell line as neuronal model of non-permissive cells is justified not only because these  
371 cells are human and widely used as neuronal model, but also because we have thoroughly

372 characterized their TNTs (35) and able to identify them with high reliability (35,74). An ideal  
373 neuronal model could be represented by primary neurons, however in primary neurons it is very  
374 difficult to discriminate TNT-like structures (32) and it is even more challenging to apply  
375 correlative cryo-light and electron microscopy and–tomography approach. In the future it will  
376 be important to confirm these data using a different neuronal model like iPSC-derived human  
377 neurons that are able to recapitulate the complexity of human neurons. Furthermore, as we  
378 demonstrated here that TNTs allow the passage of the virus between permissive cells this work  
379 sets the basis to investigate the potential role of TNTs in allowing the spreading of the virus  
380 from the olfactory epithelium of the nasal cavity to the olfactory sensory neurons in the central  
381 nervous system (CNS).

382 Overall, within the limitation of our study mentioned above, our report provides unique  
383 structural information of SARS-CoV-2 and how it could use TNTs for its spreading between  
384 permissive cells and non-permissive cells (e.g., neuronal cells), thus increasing both viral  
385 tropism and infection efficiency. These results also pave the way to further investigations on  
386 the role of cell-to-cell communication in SARS-CoV-2 spreading to the brain in more  
387 physiological contexts, and on alternative therapeutically approaches to impair viral spreading  
388 in addition to the current investigations mainly focused on blocking the spike-receptor  
389 interactions.

390

## 391 **Methods**

### 392 **Cell Lines and Viruses**

393 African green monkey kidney Vero E6 cell and Colorectal Adenocarcinoma human epithelial  
394 (Caco-2) cells were maintained at 37 °C in 5% CO<sub>2</sub> in Dulbecco's minimal essential medium  
395 (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1%  
396 penicillin/streptomycin.

397 Human neuroblastoma (SH-SY5Y) cells were cultured at 37 °C in 5% CO<sub>2</sub> in RPMI-1640  
398 (Euroclone), plus 10% fetal bovine serum and 1% penicillin/streptomycin. Mouse  
399 catecholaminergic neuronal cell line, Cath.a-differentiated cells (CAD) were kindly given by  
400 Hubert Laude (Institut National de la Recherche Agronomique, Jouy-en-Josas, France) and  
401 cultured at 37 °C in 5% CO<sub>2</sub> in Gibco Opti-MEM (Invitrogen), plus 10% fetal bovine serum  
402 and 1% penicillin/streptomycin.

403 The strain BetaCoV/France/IDF0372/2020 was supplied by the National Reference Centre for  
404 Respiratory Viruses hosted by Institut Pasteur (Paris, France) and headed by Pr. Sylvie van der  
405 Werf. The human sample from which strain BetaCoV/France/IDF0372/2020 was isolated has  
406 been provided by Dr. X. Lescure and Pr. Y. Yazdanpanah from the Bichat Hospital, Paris,  
407 France. Moreover, the strain BetaCoV/France/IDF0372/2020 was supplied through  
408 the European Virus Archive goes Global (Evag) platform, a project that has received funding  
409 from the European Union's Horizon 2020 research and innovation programme under grant  
410 agreement No 653316.

411

#### 412 **Viral infection to identify SARS-CoV-2 permissive cells**

413 In order to assess which cell-lines were permissive to SARS-CoV-2 infection, the different cells  
414 were plated on a 96 multiwell plate and infected with a MOI (multiplicity of infection) from  
415  $10^{-1}$  to  $10^{-5}$  in DMEM 2% FBS. The cell-lines used in this assay included Caco-2, CAD, SH-  
416 SY5Y and Vero E6. All the cells were plated at a 60% confluence. The cells were incubated in  
417 infection media for 3 days. At day 2 and 3 post infection an aliquot of the supernatant from the  
418 higher MOI was collected for titration. At day 3 p.i. the monolayers were then fixed with 4%  
419 PFA and viral infection was visualized using an immunofluorescence protocol.

420

#### 421 **Immunofluorescence protocol for Immunospot**

422 After 45 minutes incubation with 4% PFA, the monolayers were washed with PBS and  
423 incubated 5 minutes with PBS 1X-0.5% Triton at R.T. (Room Temperature); the cells were then  
424 washed and incubated for 10 minutes with PBS 1X-50mM NH<sub>4</sub>Cl. After washing, 30 minutes  
425 blocking was performed using PBS 1X-2% BSA; the monolayers were incubated with the  
426 primary antibody, a polyclonal SARS-CoV-antiN IgG, provided by Nicolas Escriou, Institut  
427 Pasteur, Paris, overnight at 4 degrees. After washing, the cells were then incubated with a goat  
428 anti-rabbit Alexafluor488-conjugated antibody for 1 hour. After washing with PBS 1X to  
429 remove the unbound antibody, the immunofluorescence was visualized using the Fluoro-X suite  
430 of a C.T.L. Immunospot® S6 Image Analyzer.

431

#### 432 **Semi-solid Plaque Assay**

433 The aliquots of supernatant collected at day 2 and day 3 were used to assess viral production  
434 through a semisolid plaque assay. Each sample underwent 1:10 serial dilutions. 250 µl of each  
435 dilution was used to infect a confluent monolayer of Vero E6 cells, in a 24 wells multiwell  
436 plate, for a total of 6 wells per sample.

437 Viral absorption was allowed for 1 hour at 37°C, and a semisolid overlay, composed by MEM  
438 1X, 10% FBS and 0.8% agarose, was then added to the infection (250 µl per well). The cells  
439 were incubated at 37°C 5% CO<sub>2</sub> for 72 hours. Finally, the infected monolayers were fixed with  
440 500 µl of 4 % PFA for 30 minutes. Afterward, the PFA was removed and the monolayers were  
441 then stained with Crystal Violet solution containing 2 % PFA, to evaluate the cytopathic effect.  
442 The reaction was stopped after 15 mins and residual Crystal violet was removed through  
443 immersion in diluted bleach, followed by washing in water.

444

#### 445 **Focus forming assay**

446 Vero E6 cells were plated in a 96 multiwell plate 2 x 10<sup>4</sup> cells per well. The monolayers were  
447 then infected with serial dilutions (1:10) of samples to be titrated. The infection was allowed  
448 for two hours at 37°C 5% CO<sub>2</sub>. Afterwards, the infection medium was removed and a semisolid  
449 overlay composed by 1.5% Carboxymethyl-cellulose and MEM 1X was added to the  
450 monolayer. The cells were incubated for 36 hours at 37°C 5% CO<sub>2</sub> to allow foci formation.  
451 The monolayers were then fixed with 4% paraformaldehyde; after 45 minutes they were washed  
452 with PBS and incubated 5 minutes with PBS 1X-0.5% Triton at R.T. (Room Temperature); the  
453 cells were then washed again and incubated for 10 minutes with PBS 1X-50mM NH<sub>4</sub>Cl. After  
454 washing, the cells were incubated 2 minutes in 0.05% PBS-tween and then incubated with the  
455 primary antibody, a polyclonal SARS-CoV-anti-N IgG, provided by Nicolas Escriou, Institut  
456 Pasteur, Paris (or alternatively with a Human SARS-CoV-2 anti-S IgG provided by Cyril  
457 Planchais from the group of Hugo Mouquet Institut Pasteur, Paris), overnight at 4 degrees. After  
458 washing the cells were then incubated with an anti-rabbit (or an anti-human) HRP-conjugated  
459 antibody for 1 hour. After washing with PBS 1X to remove the unbound antibody, the foci were  
460 visualized using a DAB staining solution in PBS with 8% NiCl<sub>2</sub>, and washed 3 times with water  
461 to stop the reaction. The foci were then visualized and counted using the Biospot suite of a  
462 C.T.L. Immunospot® S6 Image Analyzer.

463

#### 464 **Lentiviral Transduction**

465 Transduction of SH-SY5Y and Vero E6 cells with a lentiviral vector expressing pCMV-  
466 mcherry: 600.000 SH-SY5Y cells 400.000 Vero-E6 were plated in 60 mm plates. After 24 h,  
467 they were infected with 800 µl of LV- pCMV-mCherry. After 48 h, cells expressing mCherry  
468 have been validated. Transduction of SH-SY5Y cells with a lentiviral vector expressing pCMV-  
469 H2B-GFP: 600.000 SH-SY5Y cells were plated in 60 mm plates. After 24 h, they were infected  
470 with 800 µl of LV- pCMV-H2B-GFP. Transduction of SH-SY5Y cells with a lentiviral vector

471 expressing pCMV-H2B-GFP: 600.000 SH-SY5Y cells were plated in 60 mm plates. After 24 h,  
472 they were infected with 800 µl of LV- pCMV-H2B-GFP.

473

#### 474 **SARS-CoV-2 infection of Vero E6 cells for co-culture experiments and cryo-EM grids**

475 1.000.0000 of donor Vero E6 cells were infected with a MOI of 0.05 in DMEM 1% FBS for 2  
476 hours. Afterward, the infection medium was removed and substituted with fresh DMEM 10%  
477 FBS. The cells were left in incubation at 37°C with 5% CO<sub>2</sub> for 48 hours. After that time, cells  
478 were trypsinized, centrifuged (1000 rpm, 10 min), counted and seeded for the different  
479 experiments.

480

#### 481 **Co-culture preparation for SARS-CoV-2 transfer experiments and Secretion test**

482 1.000.0000 of donor Vero E6 cells were infected with a MOI of 0.05 in DMEM 1% FBS for 2  
483 hours. Afterward, the infection medium was removed and substituted with fresh DMEM 10%  
484 FBS. The cells were left in incubation at 37°C with 5% CO<sub>2</sub> for 48 hours. As acceptors were  
485 used the non-permissive SH-SY5Y cells and permissive Vero-E6 cells stably transfected with  
486 a lentivirus expressed mCherry, according to the kind of experiment. The infected donors, as  
487 well as the acceptors cells, were trypsinized centrifuged (1000 rpm, 10 min), counted and co-  
488 cultured on 24 glass coverslips 37°C with 5% CO<sub>2</sub> with 1:1 ratio (50.000 donor-50.000  
489 acceptor). After 24h and 48h, co-cultures were washed with 0.01% trypsin to remove excess of  
490 virus on top of the cell membrane and fixed for 30 min with 4% PFA, then we proceed  
491 processing the co-culture for an immunostaining for anti-Nucleoprotein and anti-Spike. After  
492 the immunostaining, cells were stained with HCS Cell Mask TM Blue Stain (Invitrogen, 1:300)  
493 in PBS1X for 30 min then 30 mounted.

494 Images were acquired on an LSM 700 confocal microscope (Zeiss) with a 40X objective.

495 After image acquisition, number of acceptor cells, which had received SARS-CoV-2 identified  
496 by the anti-N and/or anti-S immunostaining were quantified. Automated detection and  
497 quantification of the number of acceptors received SARS-CoV-2 was assessed with the open  
498 source software, ICY as described above.

499 To evaluate the possibility of SARS-CoV-2 transfer from donor to acceptor cells mediated by  
500 secretion, the supernatants from SARS-CoV-2 infected Vero-E6 cells were collected  
501 centrifuged at 1000 rpm for 10 min to remove floating cells and added on acceptor cells: SH-  
502 SY5Y mCherry. After 24h and 48h acceptor cells, acceptor cells were washed with 0.01%  
503 trypsin and fixed with 4% PFA at RT for 30 min. After image acquisition, acceptor cells were



504 counted for the presence of SARS-CoV-2 signal. Secretion test was performed in parallel to all  
505 the co-culture experiments performed in this study by following the same protocol.  
506 Additionally, the supernatants from donor infected cells were used to assess viral production  
507 by focus forming assay titration protocol.

508

### 509 **Immunofluorescence labeling**

510 Cells were fixed in 4% PFA for 30 min, quenched with 50 mM NH<sub>4</sub>Cl for 15 min,  
511 permeabilized with 0,5% Triton-100 for 5 min in PBS 1X, blocked with PBS 1X containing  
512 2% BSA (w/v) for 1 h. Cells were then incubated with primary antibody dissolved in 2% BSA  
513 in PBS-1X. The primary antibody used were: a rabbit anti-Nucleoprotein (Anti-N, gift from  
514 Nicolas Escriou, Institut Pasteur, Paris) (1:500) over night (ON); an anti-human anti-Spike (H2-  
515 162, produced by Cyril Planchais from the group of Hugo Mouquet Institut Pasteur, Paris) (1:  
516 100) ON, an anti-mouse J2 (1:50) (Scicons) ON, an anti-sheep nsp3 (1:200) (MRC PPU  
517 Reagents).

518 The day after, cells were thoroughly washed and incubated for 40 min with an anti-rabbit Alexa-  
519 Fluor 633-conjugated secondary antibody (Invitrogen), an anti-human Alexa-Fluor 488-  
520 conjugated secondary antibody (Invitrogen), goat anti-mouse Alexa-Fluor 633-conjugated  
521 secondary antibody (Invitrogen) at 1:500 in 2% BSA (w/v) in PBS 1X respectively. Cells were  
522 then carefully washed in PBS 1X and labeled with HCS Cell Mask TM Blue Stain (Invitrogen,  
523 1:300) in PBS 1X for 30 min then 30 mounted. For ACE2 Antibody (PA5-20046-Thermo  
524 Fisher Scientific) Immunostaining: cells were fixed in 4% PFA for 10 min, quenched with  
525 50 mM NH<sub>4</sub>Cl for 15 min, blocked with PBS 1X containing 2% BSA (w/v) for 1 h. Cells were  
526 then incubated with primary antibody ON dissolved in 2% BSA in PBS-1X. The day after, cells  
527 were thoroughly washed and incubated for 40 min with an anti-rabbit Alexa-Fluor 488-  
528 conjugated secondary antibody (Invitrogen). Cells were then carefully washed in PBS 1X and  
529 labeled with HCS Cell Mask TM Blue Stain (Invitrogen, 1:300) in PBS 1X for 30 min then 30  
530 mounted.

531

### 532 **Co-culture preparation for SARS-CoV-2 transfer experiments in presence of neutralizing 533 antibody**

534 Vero-E6 donor cells, infected as previously described, were put in co-culture, in a 1:1 ratio,  
535 with mCherry-Vero-E6 acceptors in presence of a SARS-CoV-2 neutralizing antibody. Briefly,  
536 infected donors were trypsinized and counted. They were then diluted at a concentration of 5 x  
537 10<sup>5</sup> cells per ml in DMEM 5% FBS, containing a concentration of 10 ug/ml of anti-SARS-CoV-

538 2 IgG C3 235 [produced by Cyril Planchais from the group of Hugo Mouquet Institut Pasteur,  
539 Paris] which it has been proved to be sufficient to elicit complete neutralization for a viral  
540 concentration of  $1-5 \times 10^5$  FFU/ml. Donor cells were incubated in presence of the antibody for  
541 1 hour at 37°C 5% CO<sub>2</sub>. Afterward, donor cells were co-cultured in a ratio 1:1 with mCherry-  
542 Vero -E6 acceptor cells in DMEM 5% FBS with 10 ug/ml of the afore mentioned neutralizing  
543 antibody. The co-cultures were incubated for 24 and 48h hours 37°C 5% CO<sub>2</sub>. Then, co-  
544 cultures were fixed in 4% PFA for 30 min and immunostained for the anti-N (protocol described  
545 above) and with HCS Cell Mask TM Blue Stain (Invitrogen, 1:300). Images were acquired on  
546 an LSM 700 confocal microscope (Zeiss) with a 40X objective. After image acquisition,  
547 number of acceptor cells, which had received SARS-CoV-2 identified by the anti-N  
548 immunostaining were quantified ICY software as before. In parallel, supernatant of each  
549 conditions was then collected to assess viral neutralization using Focus Forming Assay (FFA)  
550 titration protocol. For the secretion test, performed in parallel with the co-culture, an aliquot of  
551 the supernatant from donor was incubated with 10 ug/ml of the anti-SARS-CoV-2 IgG C3 235  
552 for 1h at 37°C, in order to neutralize the viral particles, present in the supernatant. In parallel  
553 another aliquot was left untreated, for comparison. The supernatants were then added on top of  
554 acceptors cells. Afterwards, we proceed for the analysis as before mentioned.

555

### 556 **TNT counting**

557 For quantification of TNT-connected cells, Vero-E6 cells infected (as described before) and not  
558 infected were trypsinised and counted; 50,000 cells were plated on 24 glass coverslips. After  
559 24h, cells were fixed (15 min at 37 °C in 2% PFA, 0.05% glutaraldehyde and 0.2 M HEPES in  
560 PBS, and then additionally fixed for 15 min in 4% PFA and 0.2 M HEPES in PBS). Cells were  
561 carefully washed in PBS, labeled for 20 min at RT with a 3.3 µg/µL solution of Wheat-Germ  
562 Agglutinin (WGA) Alexa Fluor®-647 nm conjugate (Invitrogen) in PBS, washed again and  
563 mounted. The whole cellular volume was imaged by acquiring 0.45 µm Z-stacks with an  
564 inverted confocal microscope (Zeiss LSM 700) using ZEN software. TNT-connected cells, cells  
565 connected by straight WGA-labeled structures that do not touch the substrate, were manually  
566 counted by ICY software using semi-automatized TNT counting tool as previously described  
567 (36,65). The 3D rendering of TNTs were performed using IMARIS software.

568

### 569 **Cell preparation for cryo-EM**

570 Carbon-coated gold TEM grids (Quantifoil NH2A R2/2) were glow-discharged at 2 mA and  
571  $1.5-1.8 \times 10^{-1}$  m bar for 1 min in an ELMO (Cordouan) glow discharge system. Grids were

572 sterilized under UV three times for 30 min at RT and then incubated at 37 °C in complete culture  
573 medium for 2h. 200,000 Vero-E6-infected cells (after 48h post infection) were counted and  
574 seed on cryo-EM grids positioned in 35 mm Ibidi  $\mu$ -Dish (Biovalley, France). For co-culture,  
575 100,000 Vero E6-infected cells (after 48h post infection) were co-cultured with 100,000  
576 mCherry-SH-SY5Y on cryo-EM grids in 35 mm Ibidi  $\mu$ -Dish (Biovalley, France). After 24h of  
577 cells resulted in 3 to 4 cells per grid square. Prior to chemical and cryo-plunging freezing, cells  
578 were labeled with WGA-Alexa-488 (1:300 in PBS) for 5 min at 37 °C. For correlative light-  
579 and cryo-electron microscopy, cells were chemically fixed in 2% PFA + 0.05% GA in 0.2 M  
580 Hepes for 15 min followed by fixation in 4% PFA in 0.2 M Hepes for 15 min and kept hydrated  
581 in PBS buffer prior to vitrification.

582 For correlative light- and cryo-electron microscopy using the anti-S primary antibody, cells  
583 were fixed with PFA 4% for 15 min at 37 °C, quenched with 50 mM NH<sub>4</sub>Cl for 15 min, and  
584 blocked with PBS containing 2% BSA (w/v) for ON at 4 °C. Cells were labeled with an anti-  
585 human AlexaFluor 488-conjugated secondary antibody (Invitrogen) at 1:500 and labelled with  
586 HCS Cell Mask TM Blue Stain (Invitrogen, 1:300). For cell vitrification, cells were blotted  
587 from the back side of the grid for 8 s and rapidly frozen in liquid ethane using a Leica EMGP  
588 system as we performed before (35).

589

### 590 **Cryo-electron tomography data acquisition and tomogram reconstruction**

591 The cryo-EM data was collected from different grids at the Nanoimaging core facility of the  
592 Institut Pasteur using a Thermo Scientific Titan Krios G3i electron microscope with a Gatan  
593 Bioquantum energy filter and K3 detector. Tomography software from Thermo Scientific was  
594 used to acquire the data. Tomograms were acquired using dose-symmetric tilt scheme (75), a  
595 +/-60 degree tilt range with a tilt step 2 was used to acquire the tilt series. Tilt images were  
596 acquired in counting mode with a calibrated physical pixel size of 3.2 Å and total dose over the  
597 full tilt series of 3.295 e- /Å<sup>2</sup> and dose rate of 39,739 e-/px/s with an exposure time of 1s. The  
598 defocus applied was in a range of -3 to - 6  $\mu$ m defocus.

599 The tomograms showed in the Figure 8 and 9 were performed on Glacios equipped with a field  
600 emission gun and operated at 200 kV (Thermo Fisher Scientific) and a Falcon 3 direct electron  
601 detector. Tilt series were recorded using Tomography software (Thermo Fisher Scientific) in  
602 counting mode and an angular range of - 60° to + 60° with a calibrated physical pixel size of  
603 3.2 Å and a and total dose over the full tilt series of 3.49 e- /Å<sup>2</sup> and dose rate 42,16 e-/px/s 3.49  
604 e- /Å<sup>2</sup> with 1 second exposure time, 70  $\mu$ m objective aperture. The defocus applied was in a  
605 range of -3  $\mu$ m defocus.

606 The tomograms were reconstructed using eTomo. Final alignments were done by using 10 nm  
607 fiducial gold particles coated with BSA (BSA Gold Tracer, EMS). Gold beads were manually  
608 selected and automatically tracked. The fiducial model was corrected in all cases where the  
609 automatic tracking failed. Tomograms were binned 2x corresponding to a pixel size of 0.676  
610 nm for the Titan and 0,6368 nm for the Glacios and SIRT-like filter option in eTomo was  
611 applied. For visualization purposes, the reconstructed volumes were processed by a Gaussian  
612 filter.

613

### 614 **Statistical analysis**

615 All column graphs and statistical analysis were performed by using GraphPad Prism version 7  
616 software. Unpaired t-test was applied to comparisons of two conditions presented in the figure  
617 1 and 4, and in supplementary figures. For more than two groups statistical significance was  
618 assessed by a one-way ANOVA with Tukey correction in the figure 5. Quantifications were  
619 done blind. Quantitative data depicted as ( $\pm$  SEM) mean standard deviation.

620 Graph in the Figure 1D showing the percentage of N transfer in co-culture at 24h and 48h.  
621 Mean percentage of N transfer in co-culture 24h: 36.47%  $\pm$  3.96, co-culture 48h: 62.56%  $\pm$   
622 8.28, (p=0.0468 (\*)) for co-culture 48h versus co-culture 24h; N=3). Graph in the Figure 1G  
623 showing the percentage of S transfer in co-culture at 24h and 48h. Mean percentage of S transfer  
624 in co-culture 24h: 21.84%  $\pm$  5.09, co-culture 48h: 42.44%  $\pm$  4.38, (p=0.0374 (\*)) for co-culture  
625 48h versus co-culture 24h; N=3). Graph in the Figure 6C showing the percentage of TNT  
626 connected cells between Vero E6 non-infected and SARS-CoV-2 infected. Mean percentage of  
627 TNTs connected Vero non-infected cells: 13.95%  $\pm$  2.46. Mean percentage of TNTs connected  
628 Vero SARS-CoV-2 infected cells: 44.69%  $\pm$  1.96 (p=0.0006 (\*\*\*) for Vero SARS-CoV-2  
629 versus Vero non-infected; N=3). Graph in the Figure 7C showing the percentage of N transfer  
630 in co-culture at 24h and 48h treat and not with the neutralizing antibody. Mean percentage of  
631 N transfer in co-culture 24h Control: 95.45%  $\pm$  4.29 versus co-culture 24h plus neutralizing  
632 antibody 42.91  $\pm$  4.55; p=0.0018 (\*\*)) for co-culture 24h Control versus co-culture 24h plus  
633 neutralizing antibody. Mean percentage of N transfer in co-culture 48h Control: 96.88%  $\pm$  3.12  
634 versus co-culture 48h plus neutralizing antibody 63.90  $\pm$  1.99; p=0.0104 (\*) for co-culture 48h  
635 Control versus co-culture 48h plus neutralizing antibody. p=0.9914 (ns) for co-culture 24h  
636 Control versus co-culture 48h control. p=0.0122 (\*) for co-culture 24h Control versus co-  
637 culture 48h plus neutralizing antibody. p=0.0016 (\*\*)) for co-culture 24h control antibody  
638 versus co-culture 48h plus neutralizing antibody. p=0.0496 (\*) for co-culture 24h plus  
639 neutralizing antibody versus co-culture 48h plus neutralizing antibody. Mean percentage of N

640 transfer in secretion 24h Control:  $100\% \pm 0$  versus co-culture 24h plus neutralizing antibody 0  
641  $\pm 0$ ;  $p=0.0005$  (\*\*\*) for co-culture 24h Control versus co-culture 24h plus neutralizing  
642 antibody. Mean percentage of N transfer in secretion 48h Control:  $80\% \pm 10$  versus co-culture  
643 48h plus neutralizing antibody  $0 \pm 0$ ;  $p=0.0008$  (\*\*\*) for co-culture 48h Control versus co-  
644 culture 48h plus neutralizing antibody. Graph in the Supplementary Figure 4C (left) showing  
645 the percentage of N transfer in secretion test at 24h and 48h. Mean percentage of N transfer in  
646 secretion 24h:  $0\% \pm 0$ , co-culture 48h:  $1.44\% \pm 1.44$ , ( $p=0.3739$  (ns) for secretion 48h versus  
647 co-culture 24h; N=3). Graph in the Supplementary Figure 4C (right) showing the percentage of  
648 S transfer in secretion test at 24h and 48h. Mean percentage of N transfer in secretion 24h:  $0\%$   
649  $\pm 0$ , co-culture 48h:  $1.44\% \pm 1.44$ , ( $p=0.3739$  (ns) for secretion 48h versus co-culture 24h;  
650 N=3). Pearson Correlation Coefficient (PCC) was employed to quantify colocalization between  
651 anti-S and anti-N. 20 cells were considered. PCC was calculated by using JACoP plugins in  
652 Fiji.  
653

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667

668 **Author contributions**

669 A.P conceived the experiments and wrote the manuscript; prepared the figures and image  
670 rendering; performed co-culture, TNT-counting experiments and all quantifications, prepared  
671 and plunch-freeze the cells for TEM experiments, performed all correlative, cryo-correlative  
672 light, and electron tomography experiments, tomograms reconstruction. A.P. helped S.P to  
673 infect cells. S.P. performed focus forming assay; semi-solid plaque; immunospot and set-up the  
674 concentration of the neutralizing antibody. A.P, S.P, M.V, G.B.S, C.Z, discussed the results.  
675 All authors commented on the manuscript. C.Z. conceived the project, supervised all the work,  
676 and wrote the manuscript. C.Z, M.V, G.B.S. contributed to funding acquisition.

677

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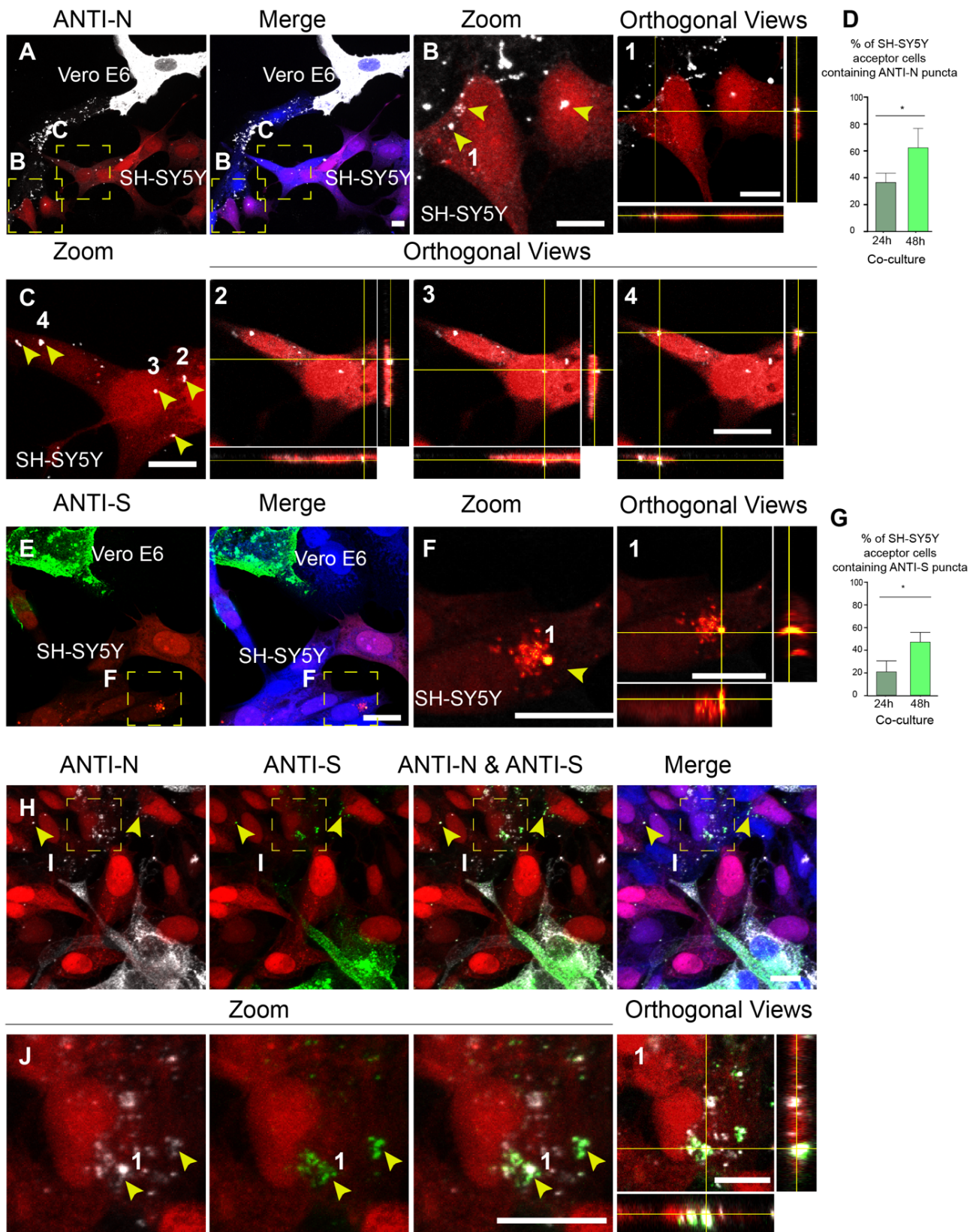
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- 925

926 **Figure 1**

Co-Culture: Vero E6 & SH-SY5Y mCherry cells



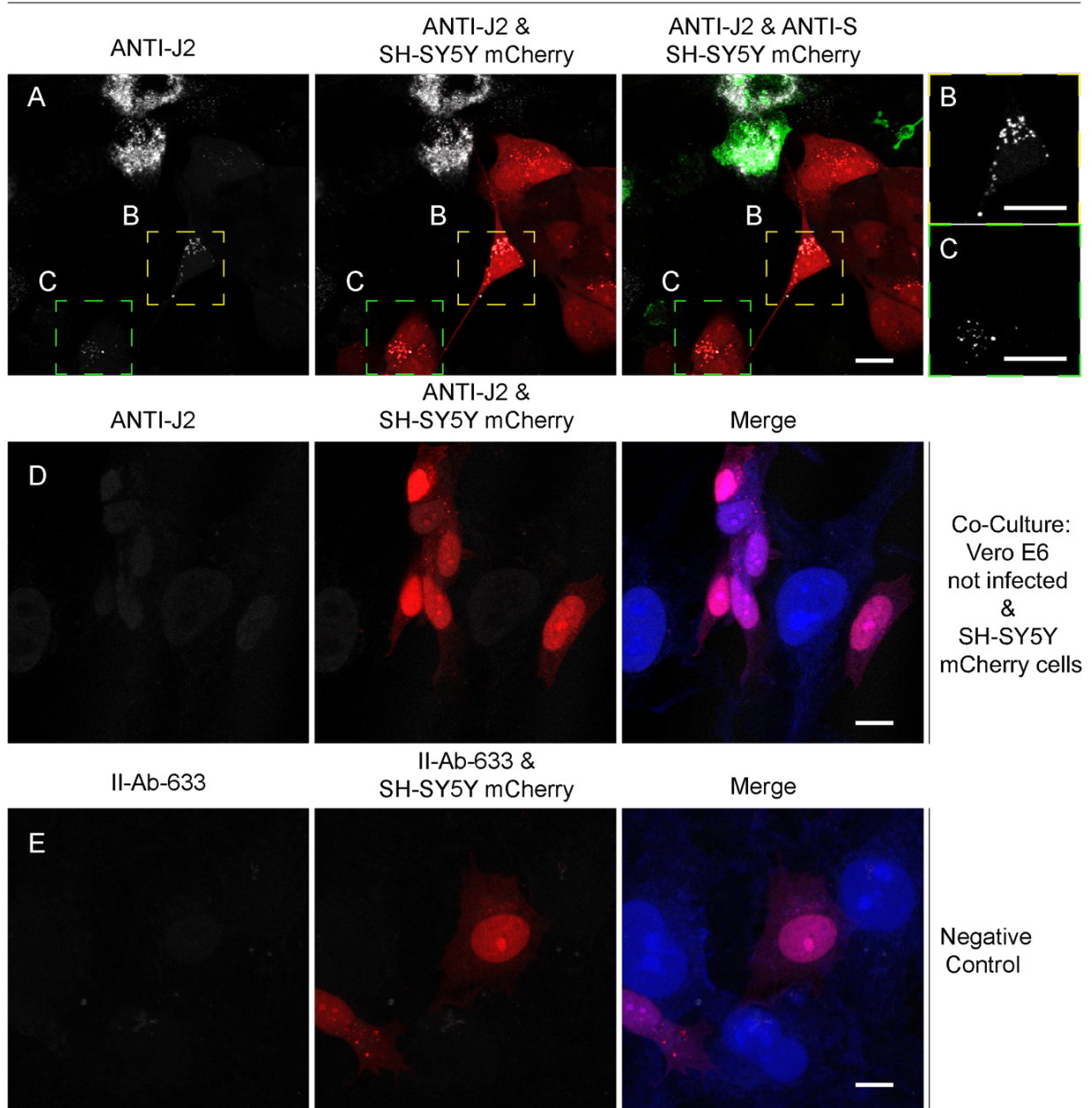
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929 **Fig.1 SARS-CoV-2 can reach SH-SY5Y neuronal cells from Vero E6 permissive cells. (A-**  
930 **D)** Infected Vero E6 cells (donor cells) were co-cultured at 1:1 ratio with SH-SY5Y neuronal  
931 cells previously stably transfected with a vector that expresses mCherry (acceptor cells) . Co-  
932 culture were fixed by using 4% PFA at 24h and 48h. **(A)** Confocal micrographs showing 48h  
933 co-culture between SARS-CoV-2 Vero E6 infected cells and SH-SY5Y mCherry cells. Anti-N  
934 antibody was used to detect SARS-CoV-2 nucleoproteins; cellular membranes were labelled  
935 with cell mask blue. **(B-C)** Enlargement of the yellow dashed squares in **(A)**, the yellow  
936 arrowheads indicate the anti-N puncta detected in the cytoplasm of acceptor cells. Numbers **(1,**  
937 **2, 3, 4)** are the orthogonal views of **(B-C)** showing the anti-N puncta inside the cytoplasm of  
938 acceptor cells. **(D)** Graph showing the mean percentage of N puncta transferred to acceptor  
939 cells after 24h and 48h of co-culture:  $36.47\% \pm 3.96$  and  $62.56\% \pm 8.28$  respectively,  
940 ( $*p=0.0468$  co-culture 48h versus co-culture 24h; N=3). **(E)** Confocal micrographs showing  
941 48h co-culture between SARS-CoV-2 cells Vero-E6 infected and SH-SY5Y mCherry cells.  
942 Anti-Spike (anti-S) antibody was used to detect SARS-CoV-2 particles; cellular membranes  
943 were labelled with cell mask blue. **(F)** Enlargement of the yellow dashed square in **(E)**, the  
944 yellow arrowhead indicates the anti-S puncta in the acceptor cells; Number **(1)** is the orthogonal  
945 views of **(F)** showing the anti-S puncta inside acceptor cells. **(G)** Graph showing the mean  
946 percentage of S puncta transferred to acceptor cells after 24h and 48h of 24h and 48h:  $21.84\%$   
947  $\pm 5.09$  and  $42.44\% \pm 4.38$  respectively ( $*p=0.0374$  co-culture 48h versus co-culture 24h; N=3)  
948 **(H-J)** Double immunostaining of co-culture using anti-S and anti-N antibodies. **(J)**  
949 Enlargement of the yellow dashed square in **(H)** showing colocalization between anti-N and  
950 anti-S puncta in SH-SY5Y mCherry acceptor cells. The Pearson's coefficient (PCC) between  
951 anti-S and anti-N was in average 0,716 (20 cells analysed). Scale bars: A-J 10  $\mu\text{m}$ .  
952



953 **Figure 2**

Co-Culture: Vero E6-SARS-CoV-2 & SH-SY5Y mCherry cells



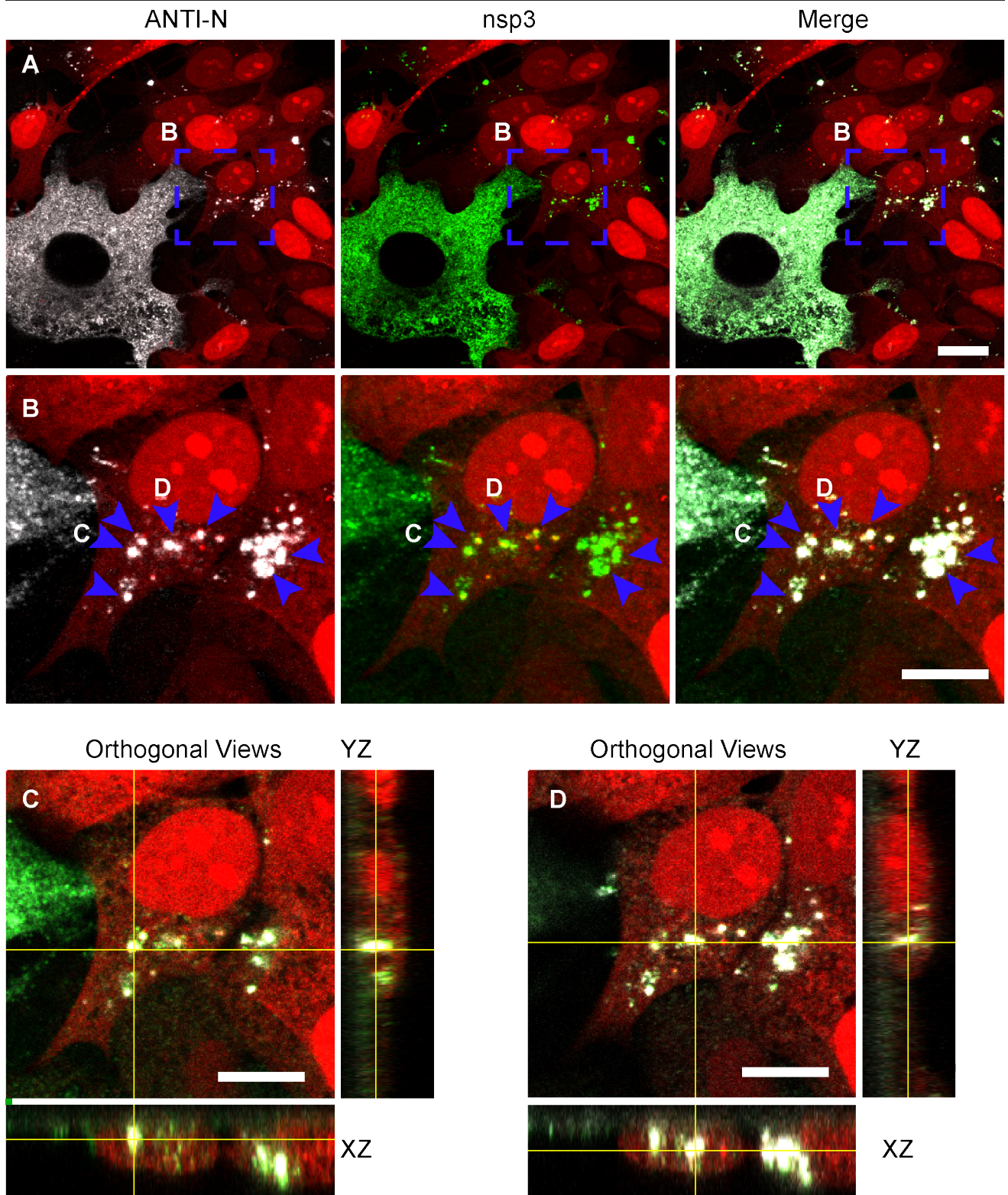
954

955

956 **Fig. 2. Anti-dsRNA (double-stranded RNA) antibody J2 is detected in SH-SY5Y cells co-**  
957 **cultured with SARS-CoV-2 Vero E6 infected cells. (A)** SARS-CoV-2 infected Vero E6 cells  
958 (donor cells) were co-cultured for 48h with SH-SY5Y mCherry acceptor cells. Confocal  
959 micrographs showing the staining with anti-J2 antibody used to detect dsRNA and an anti-S  
960 antibody is used to detect SARS-CoV-2 particles. **(B, C)** Enlargements of the yellow and green  
961 dashed squares in A showing J2 signal detected in acceptor cells. **(D)** Confocal micrographs  
962 showing not infected Vero E6 cells co-cultured with SH-SY5Y mCherry cells. The co-culture  
963 was immunostained with anti-J2 antibody and cell mask blue. **(E)** Negative control. The co-  
964 culture was immunostained with the secondary antibody conjugated with 633 fluorochrome and  
965 cell mask blue. Scale bars, 10  $\mu\text{m}$ .  
966

967 **Figure 3**

48h Co-Culture: Vero E6 & SH-SY5Y mCherry cells

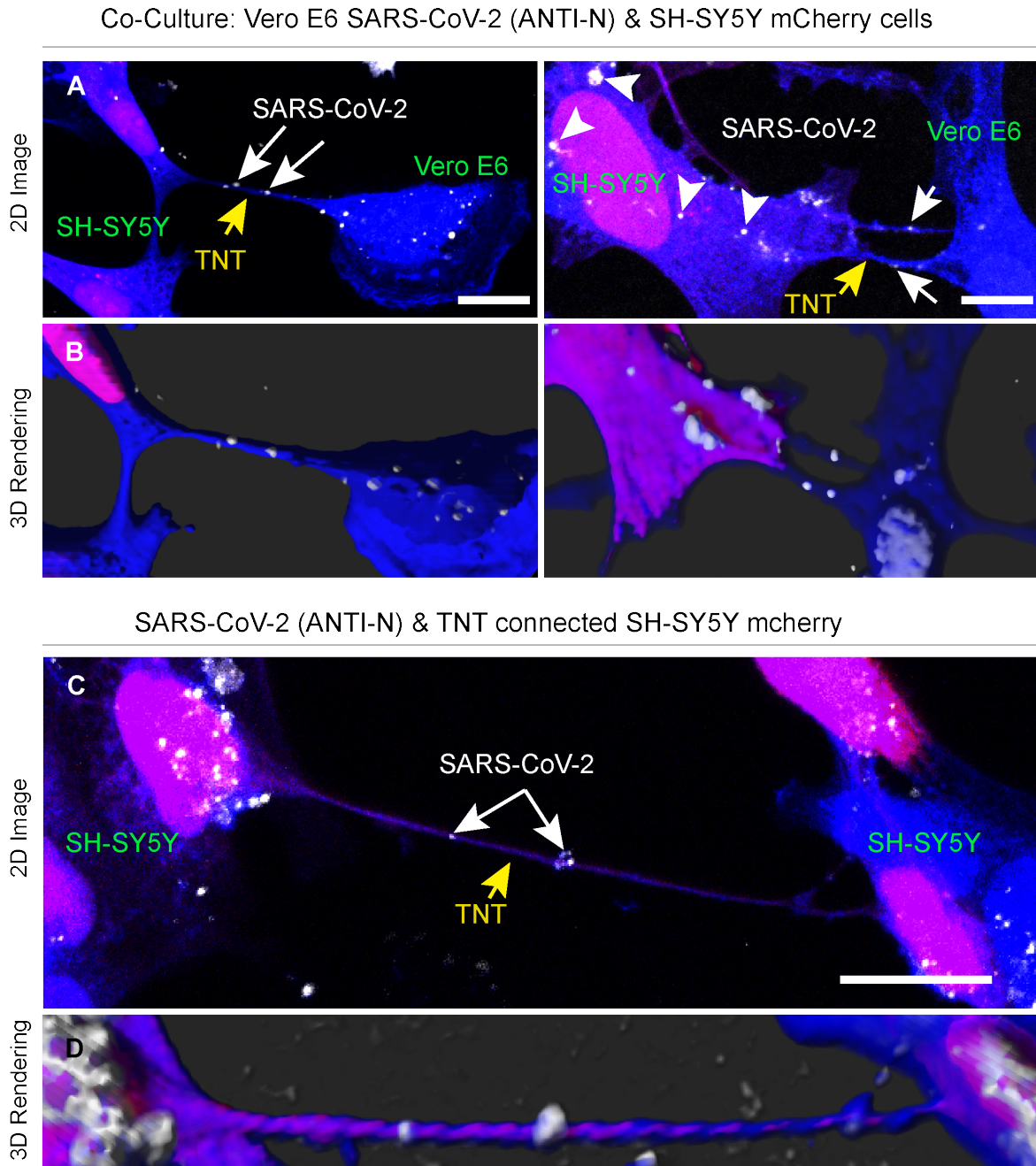


968

969

970 **Fig. 3. The non-structural protein 3 (nsp3) is detected in SH-SY5Y cells co-cultured with**  
971 **SARS-CoV-2 Vero E6 infected cells.** (A) Confocal micrographs showing 48h co-culture of  
972 SARS-CoV-2 Vero E6 infected cells (donor) and SH-SY5Y mCherry cells (acceptor) stained  
973 by using anti-nsp3 and anti-N antibodies. (B) Enlargement of the blue dashed square in (A)  
974 showing puncta positive for both anti-nsp3 and anti-N in acceptor cells (C-D) Confocal  
975 micrographs representing the orthogonal views of (B) showing anti-nsp3 and anti-N puncta in  
976 the cytoplasm of acceptor cells. Blue arrowheads indicate anti-nsp3 and anti-N signal in  
977 acceptor cells. Scale bars, A, 10  $\mu\text{m}$ , B-D, 10  $\mu\text{m}$ .  
978

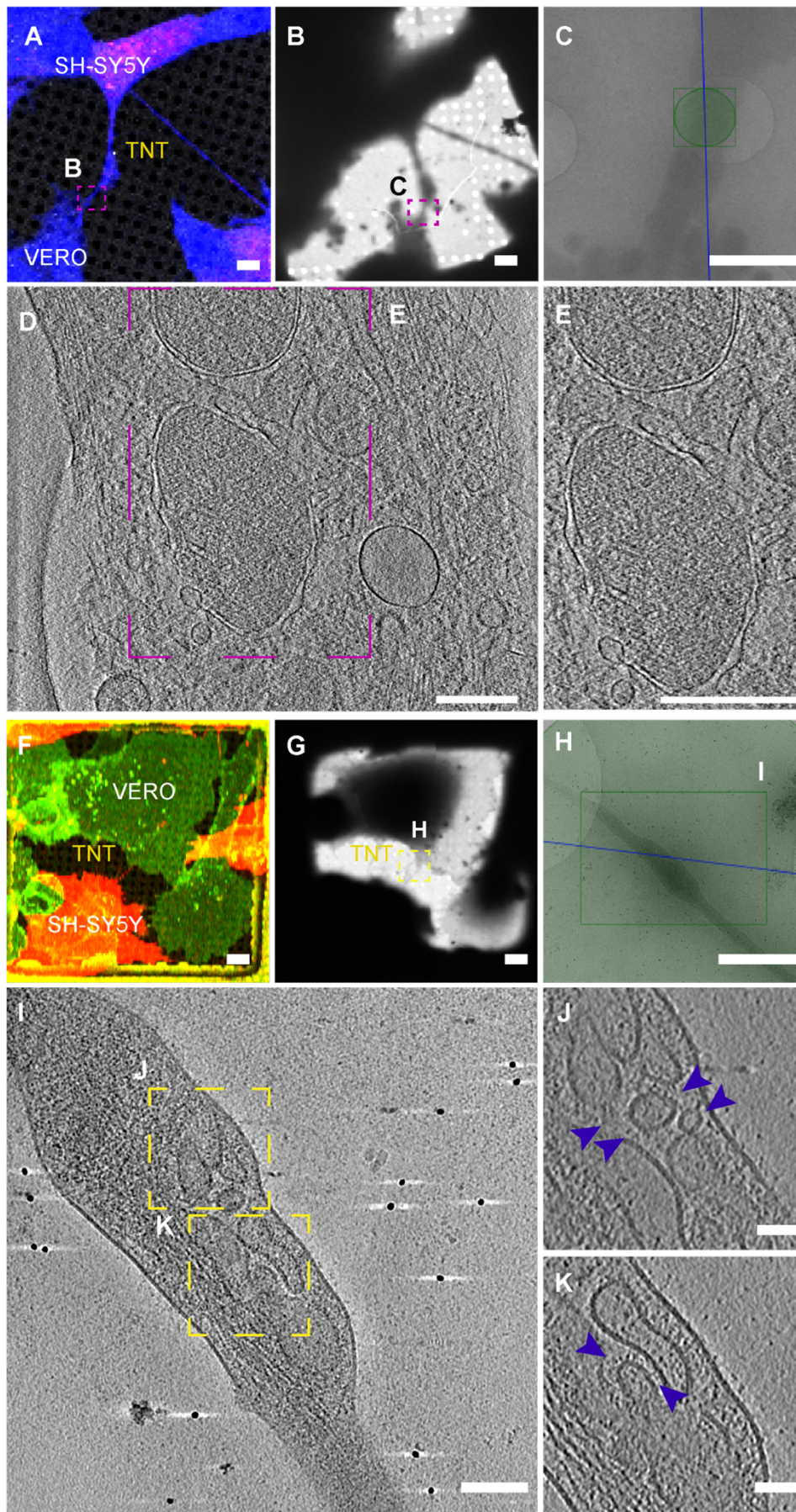
979 **Figure 4**



980  
981

982 **Fig. 4. SARS-CoV-2 spread through TNTs from permissive infected Vero E6 to non-**  
983 **permissive SH-SY5Y mCherry cells. (A, B)** SARS-CoV-2 infected Vero E6 cells (donor  
984 cells) were co-cultured with SH-SY5Y mCherry cells (acceptor cells). Co-culture were fixed at  
985 24h (left top) and 48h (right top) and stained with the anti-N antibody to detect the virus. 2D  
986 confocal micrograph (A) and 3D rendering performed by using IMARIS software in (B)  
987 showing a TNT connecting SARS-CoV-2 infected Vero E6 cells and a SH-SY5Y mCherry  
988 cell; the yellow arrow points the TNT between Vero E6 and SH-SY5Y mCherry cells; the grey  
989 arrow indicates SARS-CoV-2 N signal inside TNT and in the acceptor cells. (C, D) 2D confocal  
990 micrograph (C) and 3D rendering performed by using IMARIS software (D) showing a TNT  
991 connecting two SH-SY5Y mCherry cells, co-cultured with Vero E6 infected cells. The yellow  
992 arrow points the TNT between the SH-SY5Y mCherry cells; the grey arrow indicates SARS-  
993 CoV-2 inside TNT. Scale bars A 10  $\mu\text{m}$ , C, 15  $\mu\text{m}$ .  
994

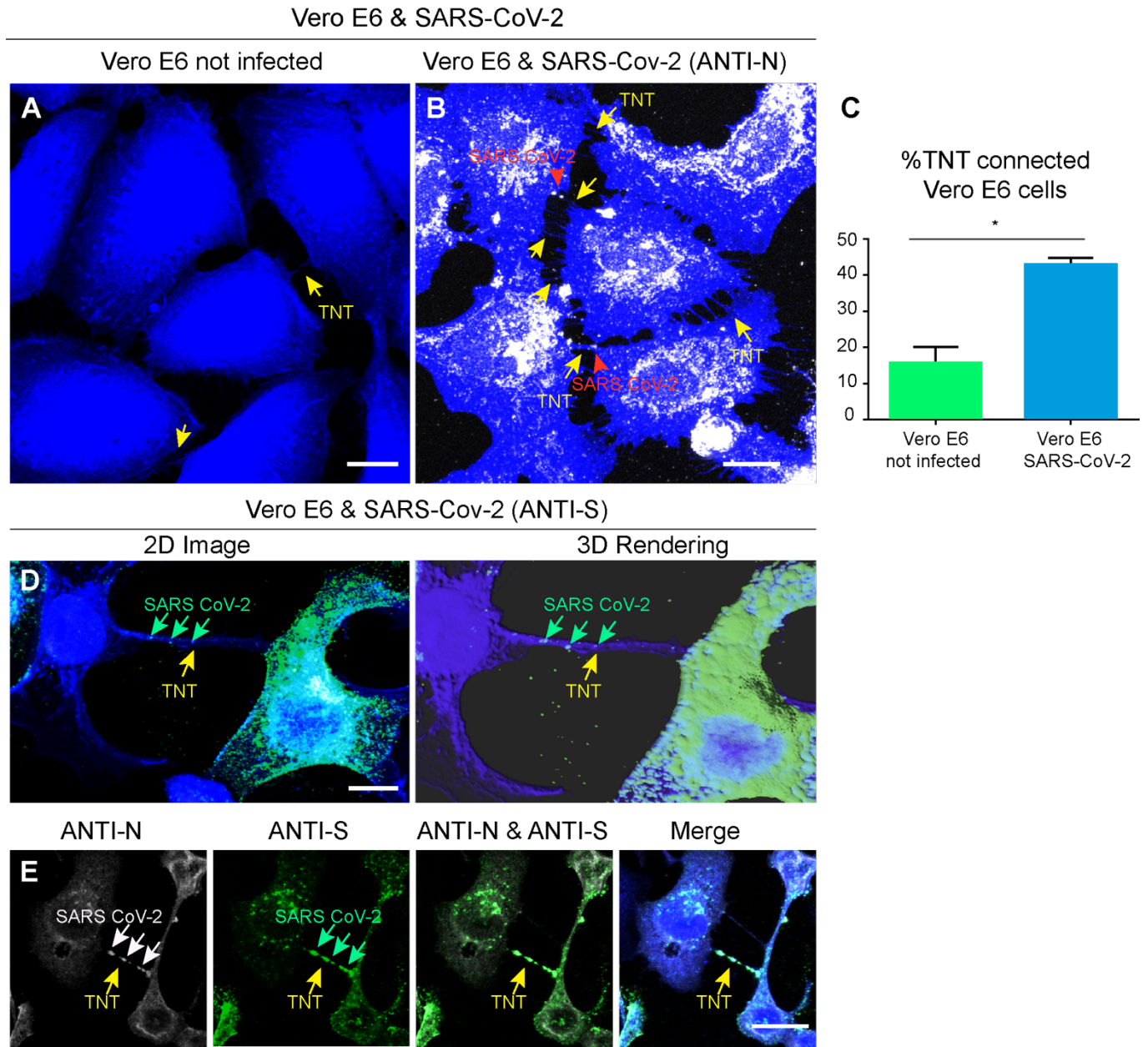
995 **Figure 5**



996 **Fig. 5. Ultrastructural analysis reveals SARS-CoV-2 viral compartments inside TNT**  
997 **between permissive Vero E6 cells and non-permissive SH-SY5Y neuronal cells. (A)**  
998 Confocal micrographs showing a TNT connecting SARS-CoV-2 infected Vero E6 cells and  
999 SH-SY5Y mCherry cells stained with cell Mask blue. **(B)** Low and **(C)** intermediate  
1000 magnification of an electron micrograph displaying TNT in **(A)**. Green square in **(C)**  
1001 corresponds to the high-magnification of cryo-tomogram slices in **(D)** showing a TNT  
1002 containing vesicular compartments and double membrane vesicles (DMV). **(E)** Enlargement of  
1003 cryo-tomogram slices in **(D)**. **(F-K)** Cryo- EM grids were prepared using Vero E6 infected cells  
1004 co-cultured with SH-SY5Y mCherry cells and stained with WGA-488. **(F)** TNT between  
1005 SARS-CoV2 infected Vero E6 cells and SH-SY5Y mCherry cells acquired by confocal  
1006 microscopy **(F)** low **(G)** and intermedia **(H)** magnification TEM. **(I)** Slices of tomograms of  
1007 TNT in the green square in **(H)** showing vesicular compartments inside TNT. **(J, K)** High-  
1008 magnification cryo-tomography slices corresponding to the yellow dashed squares showing  
1009 vesicular compartments inside TNT. The blue arrowheads indicate the vesicles inside TNT.  
1010 Scale bars: A-C 2 $\mu$ m, D 200 nm, E 300 nm, F, G, H, 2 $\mu$ m, I 100 nm, J, K 50 nm.  
1011



1012 **Figure 6**  
1013



1014  
1015  
1016

1017 **Fig. 6. SARS-CoV-2 infection increases TNTs between infected Vero-E6 cells.**

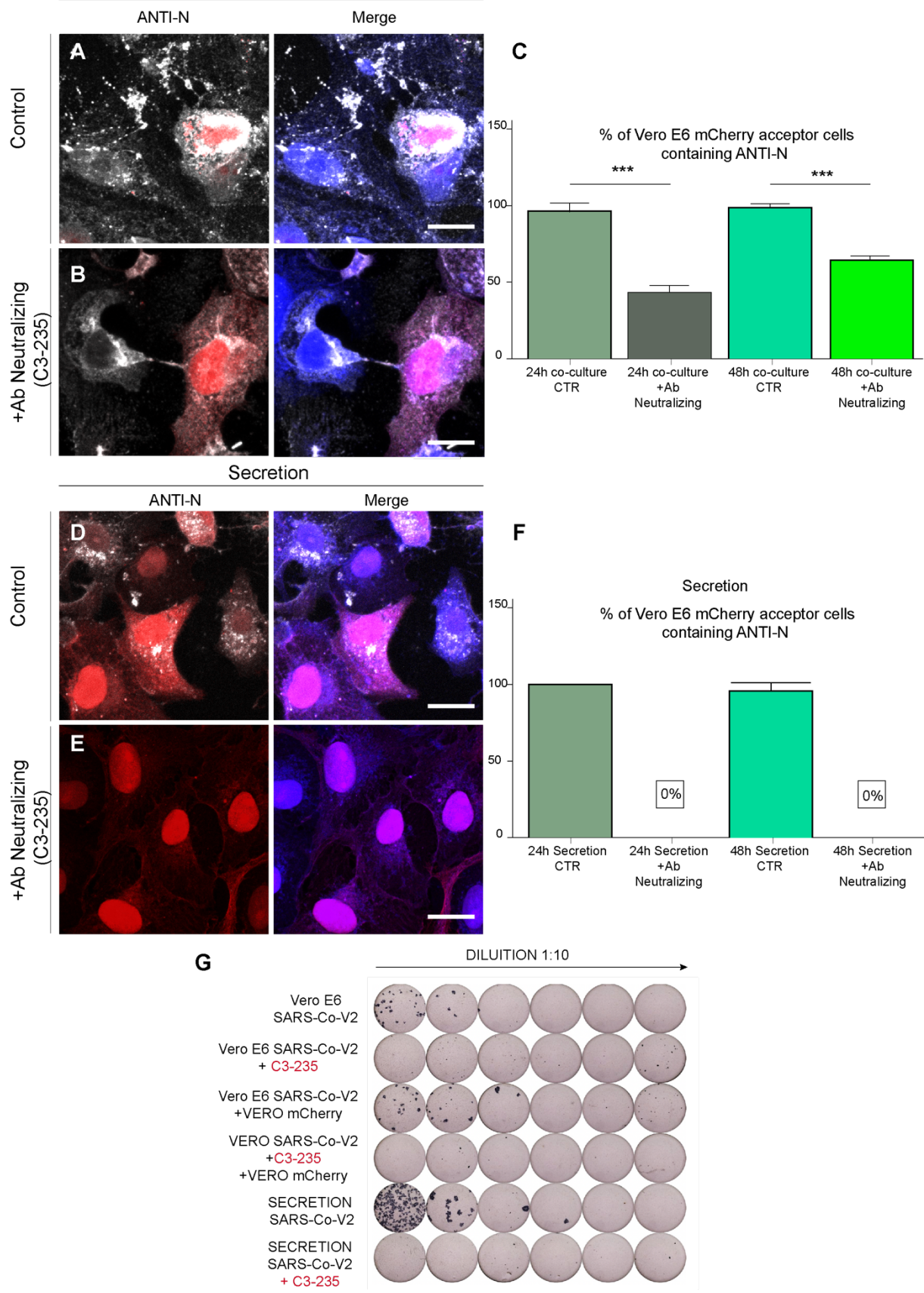
1018 **(A)** Confocal micrograph showing TNTs between non-infected Vero E6 cells. **(B)** Confocal  
1019 micrograph showing TNTs between Vero E6 cells SARS-CoV-2 infected cells. Anti-N  
1020 immunostaining is performed to detect SARS-CoV-2. The yellow arrows indicate TNTs  
1021 between VeroE6 cells; the red arrowheads indicate SARS-CoV-2 signal associated to TNTs.  
1022 **(C)** Graph showing the percentage of TNT-connected cells between Vero E6 cells non-infected  
1023 and SARS-CoV2-infected. Mean percentage of TNT-connected Vero E6 non-infected cells:  
1024 13.95%  $\pm$  2.46. Mean percentage of TNT-connected SARS-CoV-2-infected Vero E6 cells:  
1025 44.69%  $\pm$  1.96 (\*\*p=0.0006 for SARS-CoV-2-infected Vero E6 cells versus Vero E6 non-  
1026 infected; N=3). **(D)** Confocal micrograph and 3D rendering showing TNTs between SARS-  
1027 CoV-2-infected Vero E6 cells; an anti-S immunostaining was performed to detect SARS-CoV-  
1028 2. The yellow arrow indicates a TNT between Vero E6 infected cells; the green arrows indicate  
1029 SARS-CoV-2 associated to a TNT. **(E)** Confocal micrograph showing TNTs between Vero E6  
1030 cells SARS-CoV-2 infected cells labelled with cell Mask blue. Anti-N (633) and anti-S (488)  
1031 immunostaining was performed to detect SARS-CoV-2. The yellow arrows indicate a TNT  
1032 between infected VeroE6 cells; the white and the green arrows indicate SARS-CoV2 particles  
1033 inside TNTs. Scale bars A, B, E 15  $\mu$ m, C 10  $\mu$ m.

1034

1035

**Figure 7**

Co-Culture: Vero E6 & Vero E6 mCherry cells

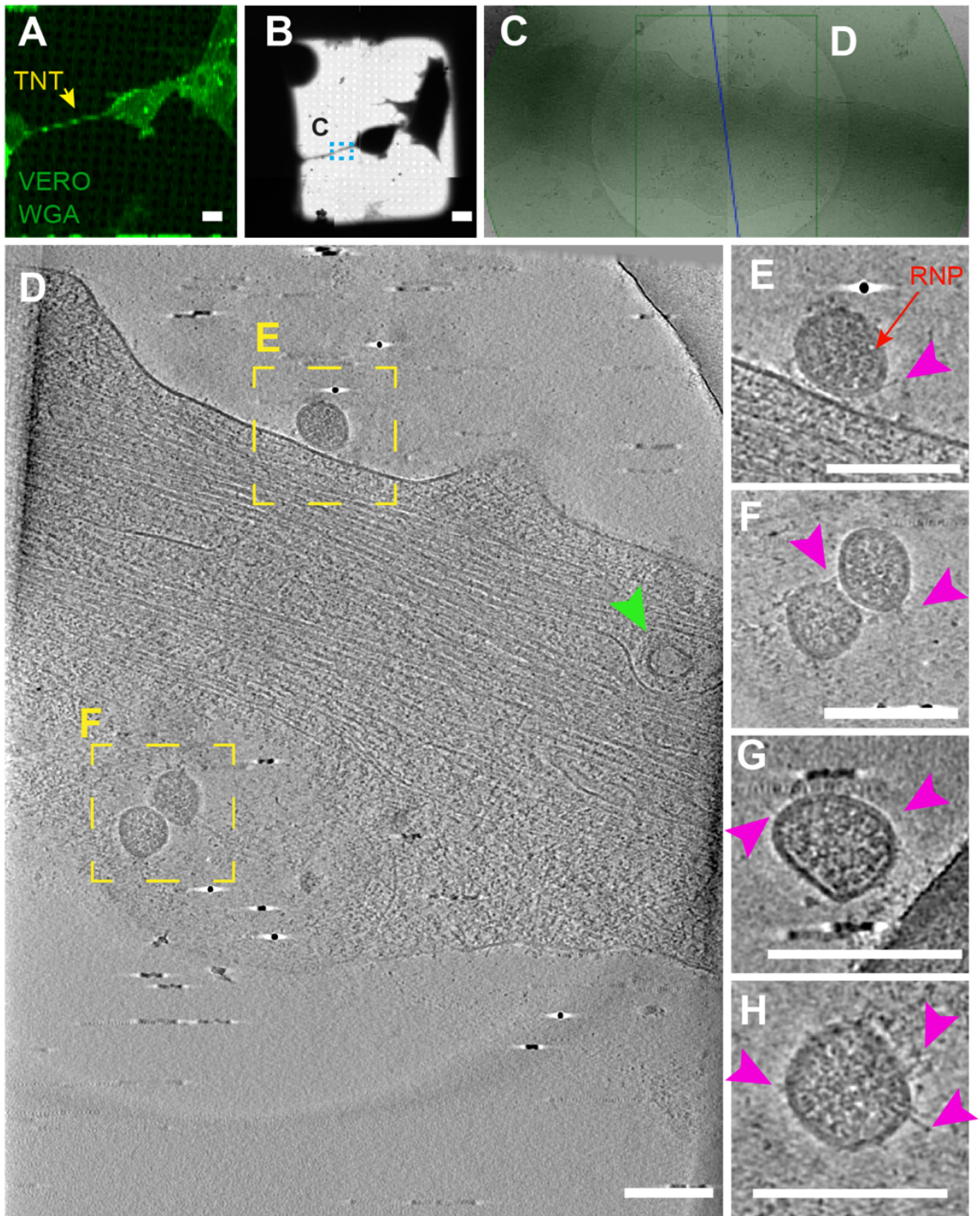


1036  
1037

1038 **Fig. 7. SARS-CoV-2 spread through TNTs between permissive cells. (A)** Vero E6 infected  
1039 donor cells were put in co-culture at 1:1 ratio with Vero E6 mCherry acceptors in control  
1040 conditions (without neutralizing antibody) and (B) in neutralizing conditions. **(B)** Donor Vero-  
1041 E6 infected cells were incubated with 10 µg/ml of anti-SARS-CoV-2 IgG C3 235 for 1 hour at  
1042 37°C 5% CO<sub>2</sub> before to be co-cultured with Vero E6 mCherry acceptors cells. The co-cultures  
1043 were fixed after 48h of incubation at 37°C 5% CO<sub>2</sub> and immunostained with anti-N antibody  
1044 to detect SARS-CoV-2. **(C)** Graph showing the mean percentage of N puncta transferred in co-  
1045 culture at 24h and 48h, treated and not with the neutralizing antibody. 24h co-culture control:  
1046 95.45% ± 4.29 versus 24h co-culture plus neutralizing antibody: 42.91 ± 4.55 (\*\*p=0.0018 24h  
1047 co-culture control versus 24h co-culture plus neutralizing antibody). 48h co-culture control:  
1048 96.88% ± 3.12 versus 48h co-culture plus neutralizing antibody: 63.90 ± 1.99 (\*p=0.0104 48h  
1049 co-culture control versus 48h co-culture plus neutralizing antibody). **(D, E)** Secretion test; **(D)**  
1050 Vero E6 mCherry cells were incubated with the supernatant deriving from donor Vero E6  
1051 infected cells. **(E)** The supernatant from donor Vero E6 infected cells was incubated with 10  
1052 µg/ml of the anti-SARS-CoV-2 IgG C3 235 for 1h at 37°C, in order to neutralize the viral  
1053 particles, before to be added on top of Vero E6 mCherry acceptor cells. After 48h of incubation  
1054 at 37°C 5% CO<sub>2</sub>, the secretion samples were fixed and immunostained for anti-N. **(F)** Graph  
1055 showing the mean percentage of N puncta contained in acceptor cells in the secretion  
1056 experiments at 24h and 48h, treated or not with the neutralizing antibody. 24h control: 100% ±  
1057 0 versus 24h plus neutralizing antibody: 0% ± 0 (\*\*p=0.0005 24h control versus 24h plus  
1058 neutralizing antibody). 48h control: 80% ± 10 versus 48h plus neutralizing antibody: 0% ± 0  
1059 (\*\*p=0.0008 48h control versus 48h plus neutralizing antibody). **(G)** Supernatant of each  
1060 condition was then collected to assess viral neutralization using Focus Forming Assay (FFA)  
1061 titration protocol. Scale bars A-E 20 µm.

1062

1063 **Figure 8**

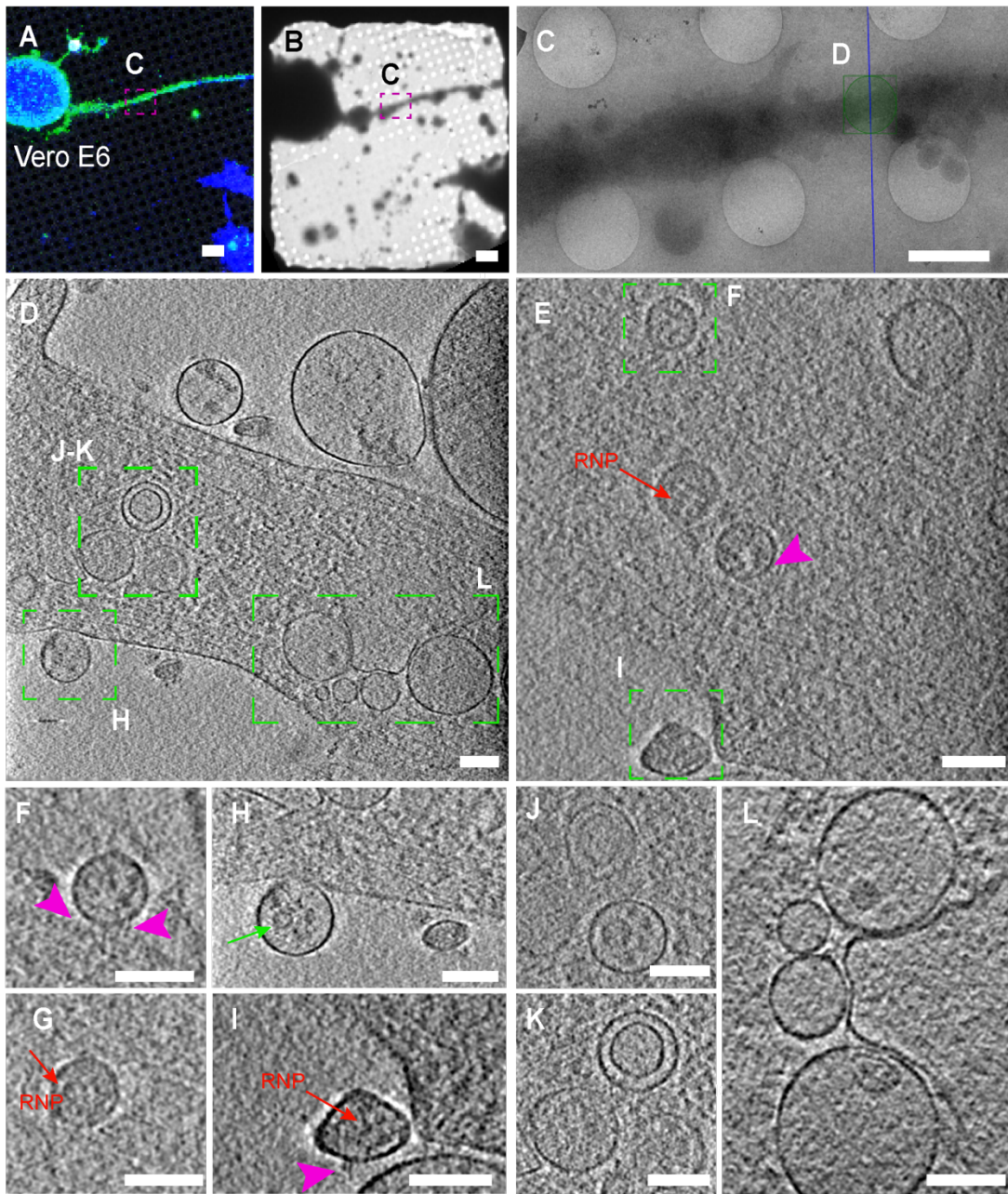


1064

1065

1066 **Fig. 8 Correlated light and cryo-electron microscopy strategies reveal SARS-CoV-2 on top**  
1067 **of TNTs between infected Vero E6 cells. (A)** TNT-connected Vero E6 infected SARS-CoV-  
1068 2 cells stained with WGA (green) and acquired by confocal microscopy (A) low (B) and  
1069 intermediate (C) magnification TEM. (D) Slices of tomograms of TNT in green square in (C)  
1070 showing extracellular SARS-CoV-2 on top of the TNT connecting Vero E6 cells. (E-F) High-  
1071 magnification cryo-tomography slices corresponding to the yellow dashed squares in (D)  
1072 showing SARS-CoV-2; RNP proteins and Spike are observed. Pink arrowheads indicate the  
1073 spike; red arrow points the RNP proteins. (E, F, G, H) High-magnification cryo-tomography  
1074 slices showing the extracellular virions on TNT. Scale bars, A, B, J, K 10  $\mu\text{m}$ , C, I 2 $\mu\text{m}$ , D-H  
1075 150 nm, L 200 nm, M 50 nm.  
1076

1077 **Figure 9**



1078

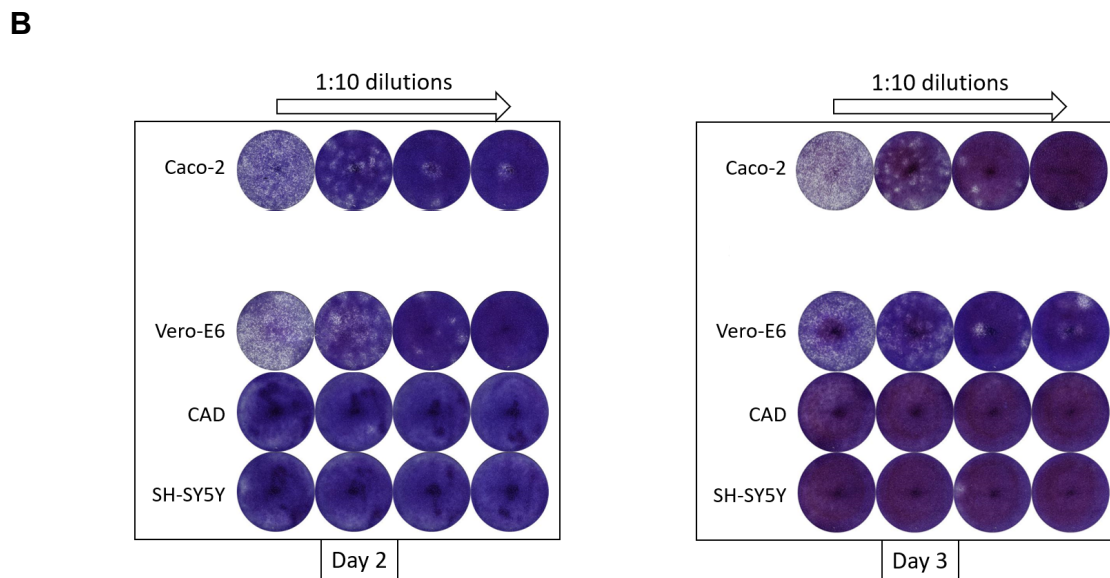
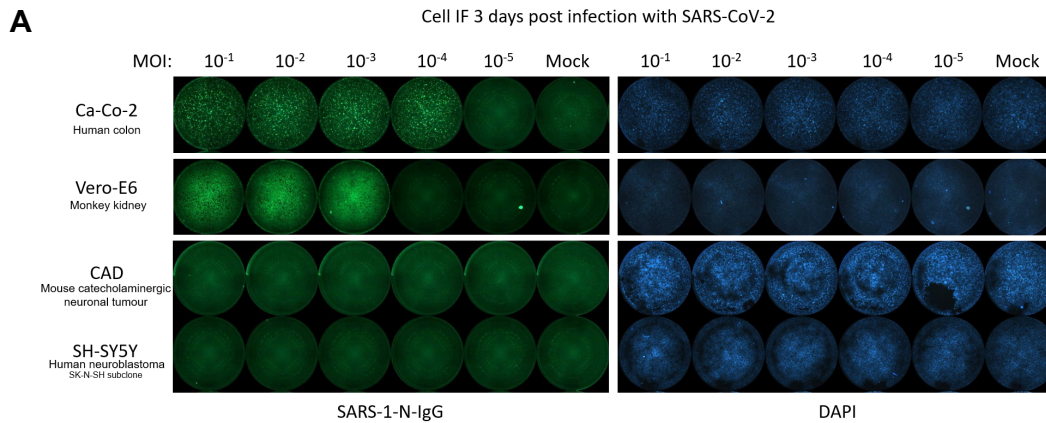
1079

1080 **Fig. 9. Correlative IF cryo-EM strategies to discriminate SARS-CoV-2 localization in**  
1081 **TNTs.** (A-L Cryo-EM grids were prepared using Vero E6 infected cells (A) Confocal  
1082 micrograph showing TNT connecting infected Vero E6 cells stained with anti-S antibody  
1083 (green) and cell mask blue. Low (B) and intermediate (C) magnification TEM of (A). (D-E)  
1084 High-magnification cryo-tomography slices showing vesicular compartments in  
1085 correspondence of anti-S signal and SARS-CoV-2 like structure on TNT surface. (F-I)  
1086 Enlargement of the high-magnification cryo-tomography slices (D-E) showing SARS-CoV-2  
1087 like structure on TNT surface (J-L) Enlargement of the high-magnification cryo-tomography  
1088 slices (D-E) showing viral like structure inside TNT. RNP proteins and Spike are observed.  
1089 Pink arrowheads indicate the spike; red arrow points the RNP proteins, green arrow indicates  
1090 SARS-CoV-2 like structure inside vesicles. Scale bars, A, B 10  $\mu\text{m}$ , C, 2 $\mu\text{m}$ , D-L 100 nm.  
1091



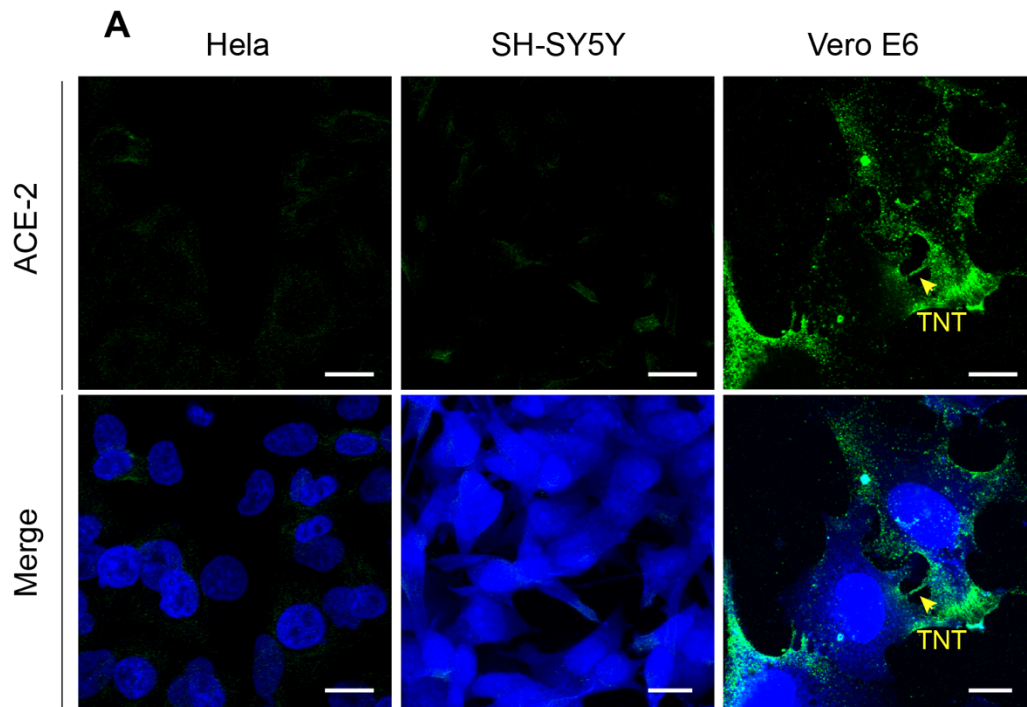


## 19 Supplementary Figure 1



20  
21 **Supplementary Fig. 1 Screening of cell lines for susceptibility to SARS-CoV-2.** (A) Caco-  
22 2, Vero E6, CAD, SH-SY5Y cells were plated on a 96 multi-well plate and infected with SARS-  
23 CoV-2 MOI from 10<sup>-1</sup> to 10<sup>-5</sup> in DMEM 2% FBS. At 3 days post infection the monolayers were  
24 fixed with 4% PFA and viral infection was visualized using an anti-N immunostaining and  
25 DAPI to stain the nuclei and then visualized using the Fluoro-X suite of a C.T.L. Immunospot®  
26 S6 Image Analyzer. (B) At day 2 and 3 post infection of the cells: Caco-2, Vero E6, CAD, SH-  
27 SY5Y cells, an aliquot of the supernatant from the higher MOI was collected for titration.  
28

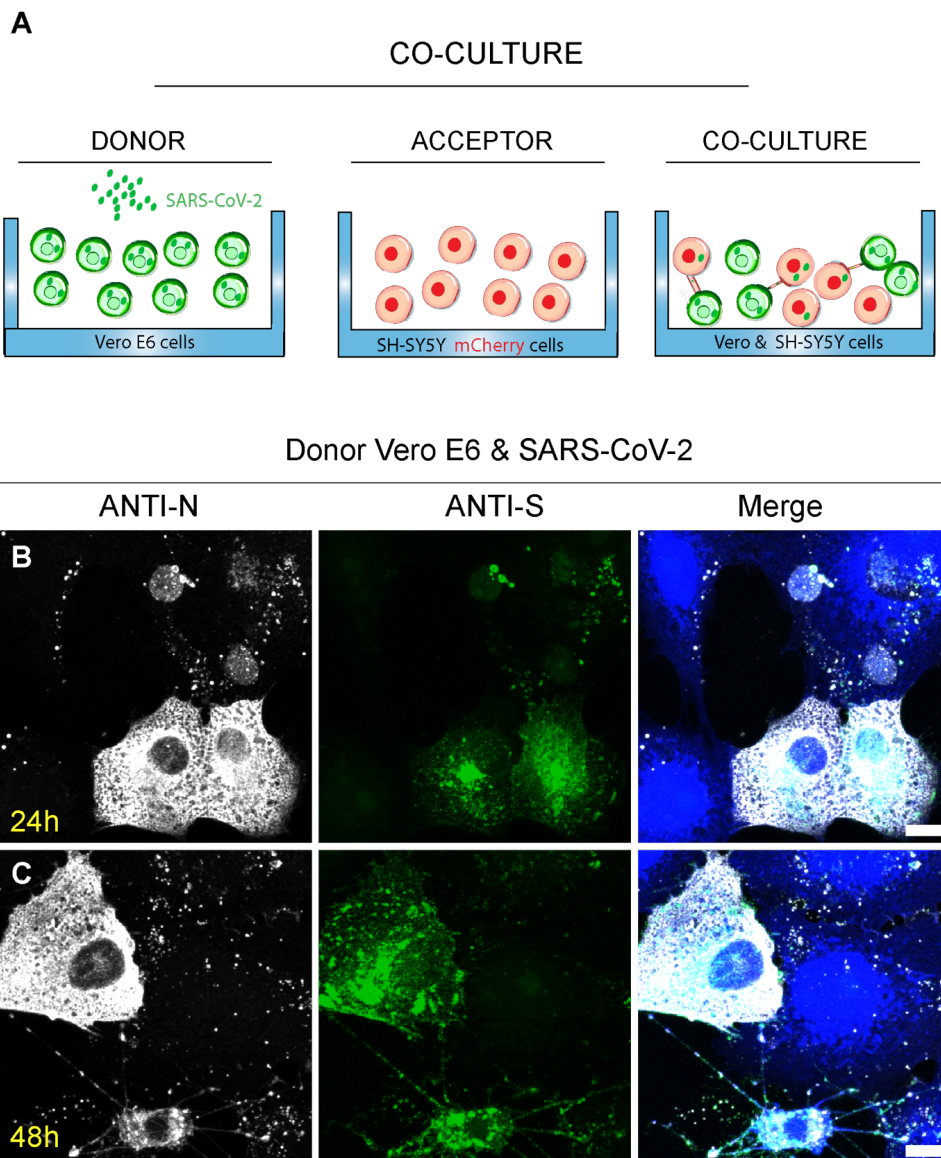
29 **Supplementary Figure 2**



30  
31 **Supplementary Fig. 2. ACE2 receptor expression.** (A) Confocal micrograph displaying Hela,  
32 SH-SY5Y and Vero E6 cells labeled with an anti-ACE2 antibody (green) and cell mask blue.  
33 Scale bars: a 15  $\mu$ m.

34

35 **Supplementary Figure 3**



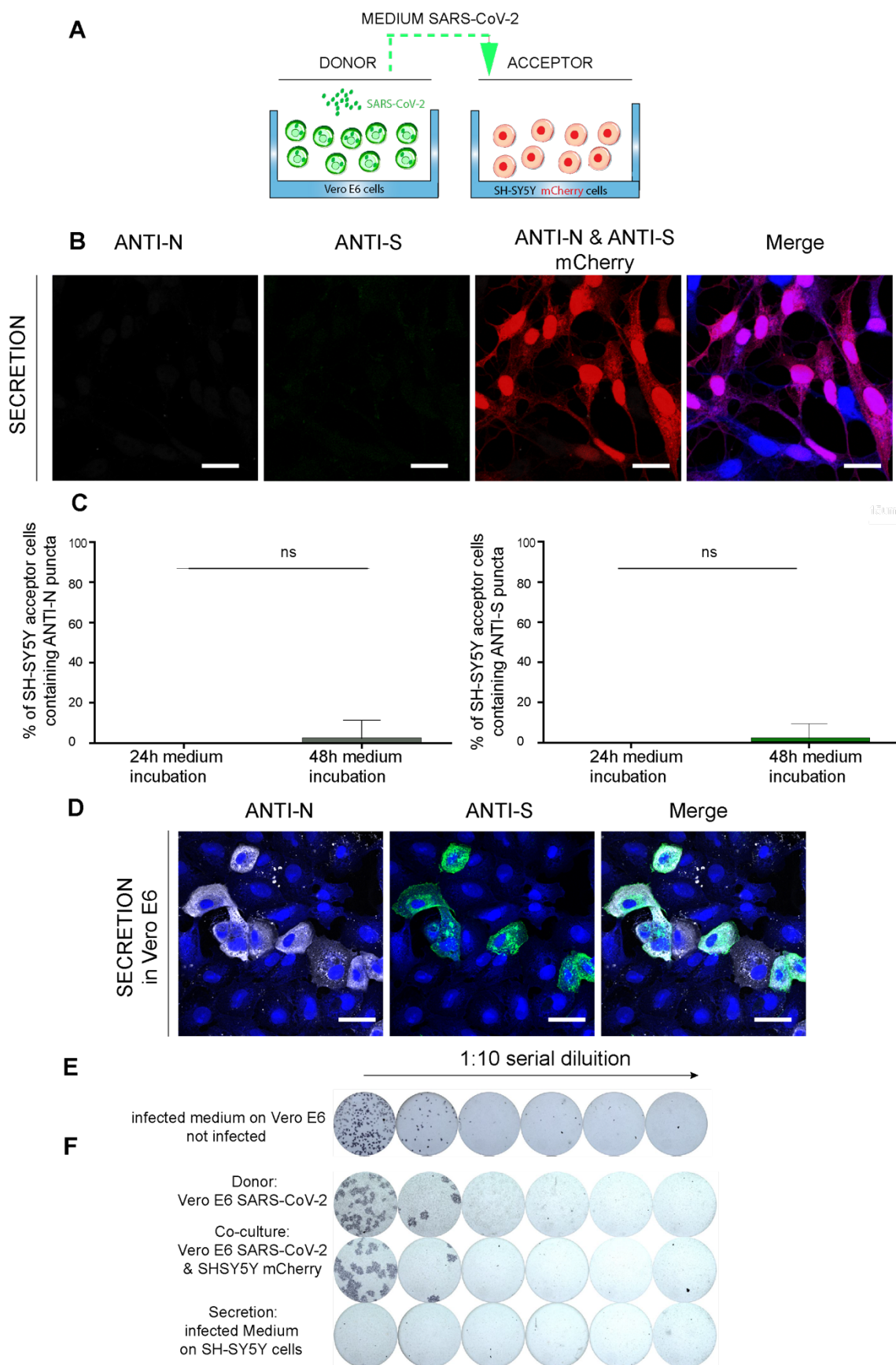
36

37 **Supplementary Fig. 3. Co-culture pipeline and SARS-CoV-2 detection in Vero E6 donor**  
38 **cells.**

39 **(A)** Description of co-culture experiments: Donor Vero E6 cells were infected with SARS-  
40 CoV-2 MOI of 0.05 for 48h. After 48h, donor cells were co-cultured with the acceptor SH-  
41 SY5Y mCherry cells and incubate for additional 24h and 48h before to be fixed. **(B-C)** Confocal  
42 micrographs showing only donor SARS-CoV-2 infected Vero E6 cells used to perform a 24h  
43 and 48h co-culture with SH-SY5Y cells (co-culture not showed). Donor cells were fixed at 24h  
44 and 48h and immunostained with anti-N and anti-S antibodies to detect SARS-CoV-2. B, C  
45 10µm.

46

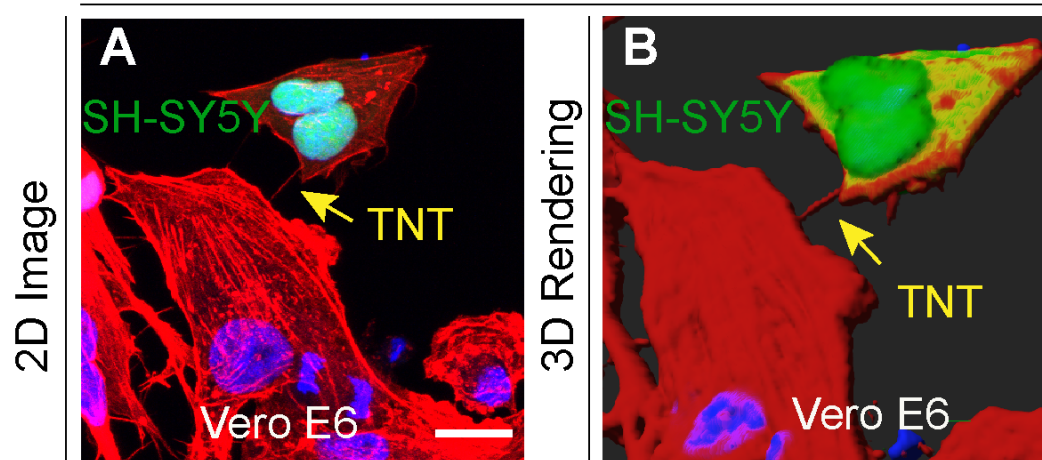
47 **Supplementary Figure 4**



49 **Supplementary Fig. 4. Secretion test.** (A) Description of secretion experiments: the medium  
50 from Vero E6 infected with SARS-CoV-2 MOI of 0.05 was centrifuged and incubated for 24h  
51 and 48h with non-permissive SH-SY5Y neuronal cells. (B) Confocal micrograph showing SH-  
52 SY5Y cells incubated with infected medium from Vero E6 cells. Cells were fixed after 48h of  
53 incubation at 37° and 5% CO<sub>2</sub> and immunostained with an anti-N antibody and anti-S antibody  
54 to detect SARS-CoV-2. (C) Left graph showing the percentage of N transfer in secretion test at  
55 24h and 48h. Mean percentage of N transfer in secretion 24h: 0% ± 0, co-culture 48h: 1.44% ±  
56 1.44, (p=0.3739 (ns) for secretion 48h versus co-culture 24h; N=3). Right graph showing the  
57 percentage of S transfer in secretion test at 24h and 48h. Mean percentage of S transfer in  
58 secretion 24h: 0% ± 0, co-culture 48h: 1.44% ± 1.44, (p=0.3739 (ns) for secretion 48h versus  
59 co-culture 24h; N=3). (D) Confocal micrograph showing not-infected Vero E6 cells incubated  
60 with infected medium from SARS-CoV-2 infected Vero E6 cells. Cells were fixed after 48h of  
61 incubation at 37° and 5% CO<sub>2</sub> and immunostained with an anti-N antibody and anti-S antibody  
62 to detect SARS-CoV-2 particles. (E) The infectious titer of the supernatant used to infect Vero  
63 E6 cells was calculated using a focus forming assay. (F) The 48h supernatants from donor  
64 infected cells, from co-culture and from secretion test were used to assess viral production by  
65 focus forming assay titration protocol. Scale bars: **B, D** 20 μm.  
66

67 **Supplementary Figure 5**

**Co-Culture: Vero E6 & SH-SY5Y cells**



68

69 **Supplementary Fig. 5. TNTs are formed between Vero E6 cells and SH-SY5Y cells. (A)**

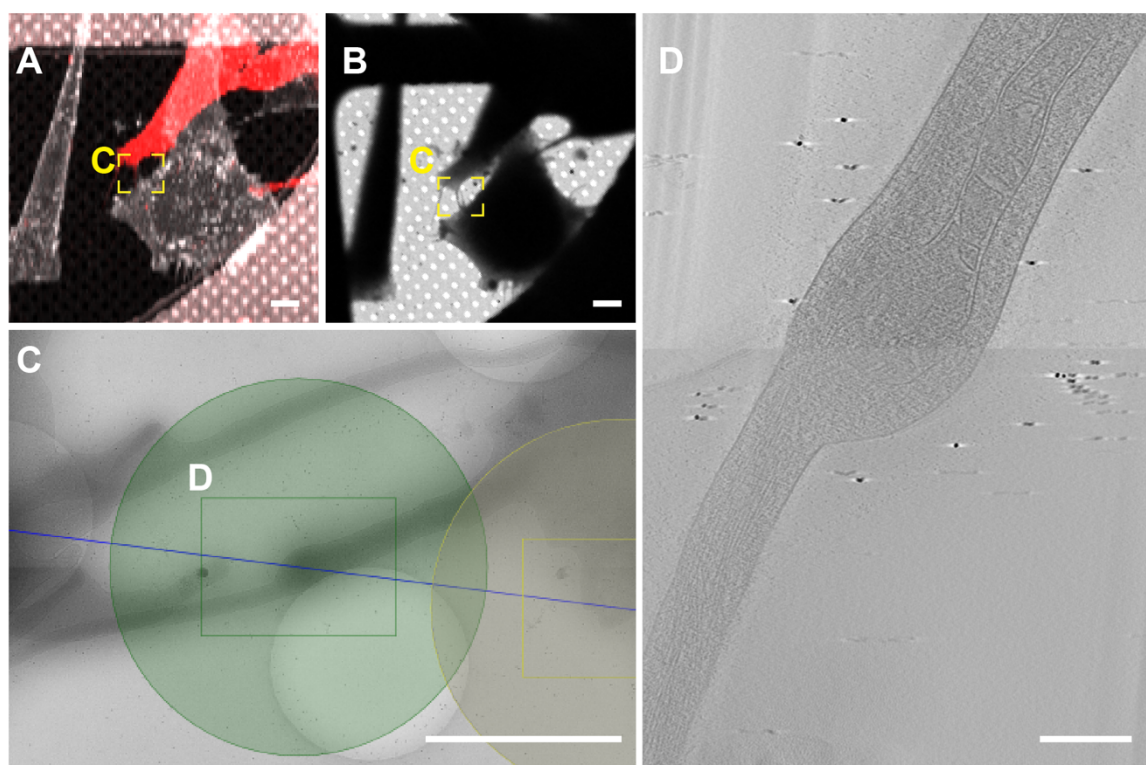
70 Representative confocal micrograph displaying TNT between Vero E6 cells and GFP NLS SH-

71 SY5Y cells. Cells were stained with Phalloidin Rhodamine (red) to label the plasma membrane

72 and TNTs, and DAPI (blue) to label the nuclei. **(B)** 3D rendering of **(A)**. Scale bars: **A**, 15  $\mu$ m.

73

74 **Supplementary Figure 6**



75

76

77 **Supplementary Fig. 6. Correlative Cryo-EM on TNT-connected not-infected Vero E6 and**  
78 **SH-SY5Y mCherry cells. (A)** Confocal micrograph shows TNT-connected naïve Vero E6  
79 cells and SH-SY5Y mCherry cells. **(B and C)** Low magnification (B) and intermediate  
80 magnification (C) of electron micrograph of TNT-connected naïve Vero E6 cells and SH-SY5Y  
81 mCherry cells. **(D)** High magnification cryo-ET slice corresponding to the green rectangle in  
82 (C). Scale bars, A, B 10µm; C, 2 µm; D, 100 nm.

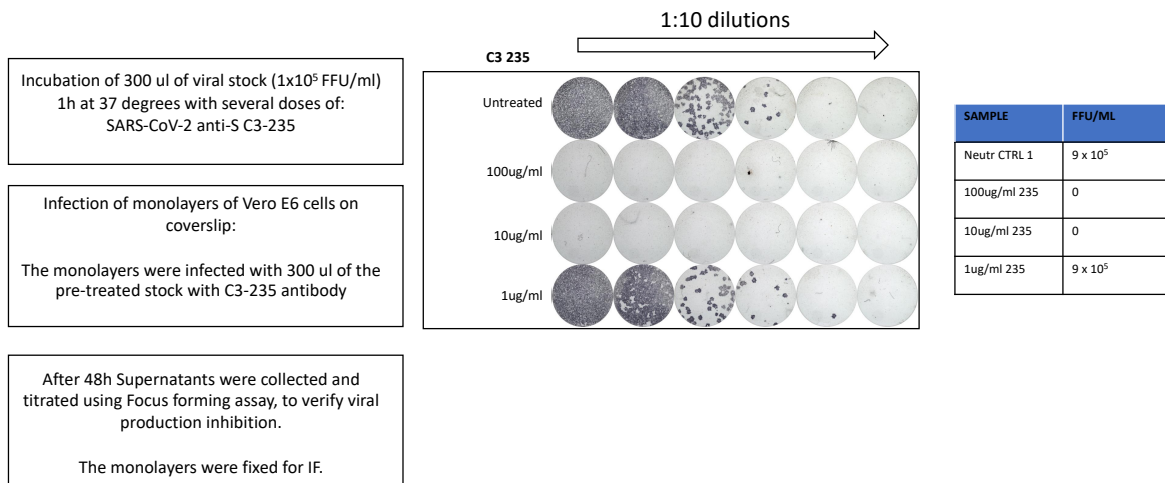
83



84 **Supplementary Figure 7**

**A**

NEUTRALIZATION TEST 235



85

86 **Supplementary Fig. 7. Experimental set-up to identify the minimal concentration of**

87 **SARS-CoV-2 IgG C3 235 sufficient to have a complete neutralization of the viral stock of**

88  **$1-5 \times 10^5$  FFU/ml. (A) Three different concentrations of human SARS-CoV-2 IgG C3 235 (1,**

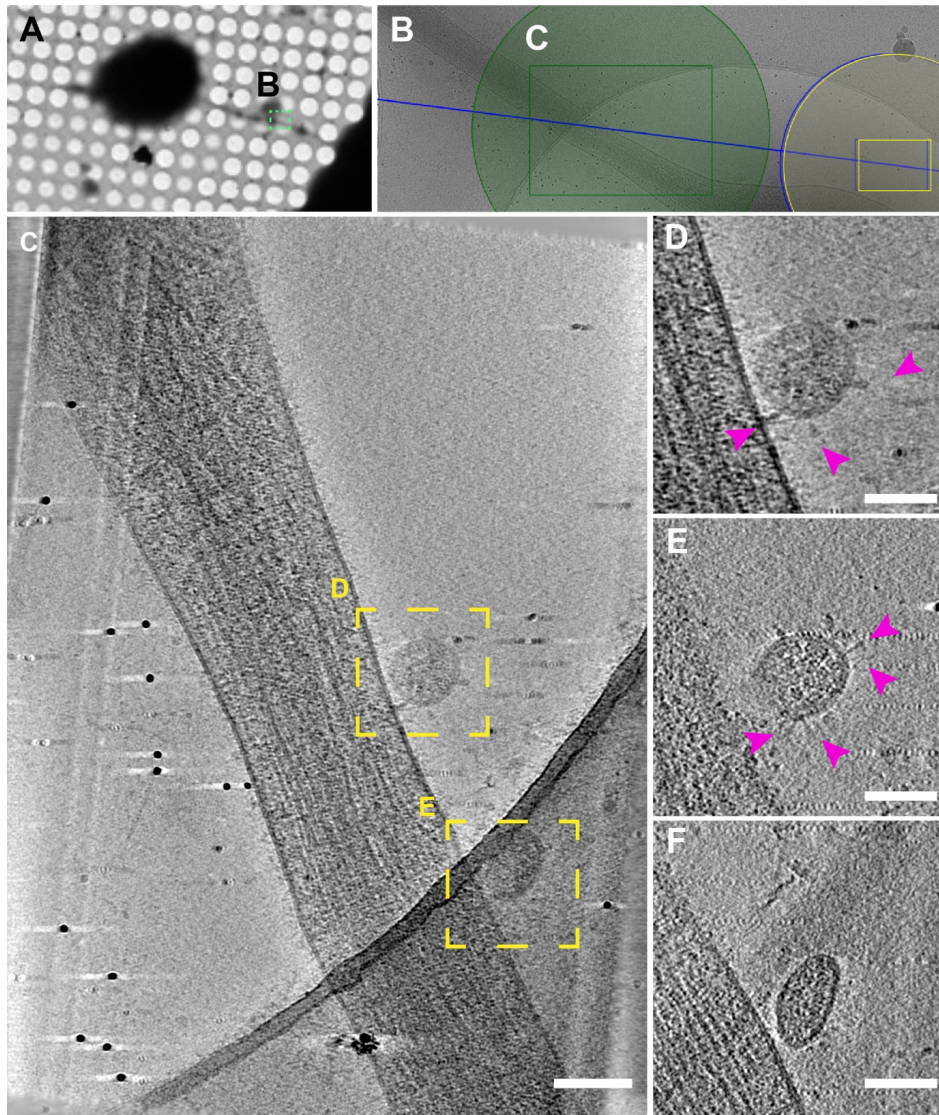
89 **10 and 100 ug/ml) were incubated 1h at 37°C, 5% CO<sub>2</sub> and then used to infect monolayers of**

90 **Vero E6 cells for 48h. Viral production was then assessed directly by processing the monolayers**

91 **and titration of the supernatant by focus forming assay.**

92

93 **Supplementary Figure 8**



94  
95

96 **Supplementary Fig. 8. SARS-CoV-2 on TNTs connected Vero E6 infected cells. (A and B)**  
97 Low (A) and intermediate (B) magnification of an electron micrograph of TNT-connected  
98 SARS-CoV-2 infected Vero E6 cells. (C) High magnification cryo-ET slice corresponding to  
99 the green rectangle in (B). (D, E and F) High-magnification cryo-tomography slices showing  
100 the extracellular virions on TNT. Pink arrowheads indicate the spike on SARS-CoV2. Scale  
101 bars, (A), 10 $\mu$ m; (B), 1  $\mu$ m; (C), 150 nm, (D), (E), (F) 100 nm.

102

103 **Description of Supplementary Files**

104

105 **File Name:** Supplementary Movie 1

106 **Description: Description:** Representative slices of a reconstructed tomogram displaying  
107 TNT between SARS-CoV-2 Vero E6 infected cells and mCherry SH-SY5Y cells shown in  
108 Fig. 5D. These slices display Double Membrane Vesicles (DMV) inside TNTs. Scale bar:  
109 200nm.

110

111 **File Name:** Supplementary Movie 2

112 **Description: Description:** Representative slices of a reconstructed tomogram displaying  
113 TNT between SARS-CoV-2 Vero E6 infected cells and mCherry SH-SY5Y cells shown in  
114 Fig. 5I. These slices display several vesicular compartments inside TNTs. Scale bar: 100nm.

115

116 **File Name:** Supplementary Movie 3

117 **Description:** Representative slices of a reconstructed tomogram displaying TNT between not-  
118 infected Vero E6 cells and mCherry SH-SY5Y cells shown in Supplementary Fig. 6D. These  
119 slices display mitochondria inside TNTs. Scale bar: 100nm.

120

121 **File Name:** Supplementary Movie 4

122 **Description:** Representative slices of a reconstructed tomogram displaying TNT connecting  
123 two SARS-CoV-2-infected Vero E6 cells shown in Fig. 8 Scale bar: 200nm.

124

125 **File Name:** Supplementary Movie 5

126 **Description:** Representative slices of a reconstructed tomogram displaying TNT arise from  
127 SARS-CoV-2 Vero E6 infected cells positive for the anti-Spike antibody containing vesicles  
128 compartments and SARS-CoV-2 like structure on the surface. Scale bar: 200nm

129

130 **File Name:** Supplementary Movie 6

131 **Description:** Representative slices of a reconstructed tomogram displaying TNT arise from  
132 SARS-CoV-2-infected Vero E6 cells show in Supplementary Fig. 8. Scale bar: 200nm

133