

1 **Nuclear translocation of spike mRNA and protein is a novel pathogenic feature of SARS-**  
2 **CoV-2.**

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16 Short title: S mRNA and S protein colocalize and translocate into the nucleus

17

18

19 **Abstract**

20 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes severe pathophysiology  
21 in vulnerable older populations and appears to be highly pathogenic and more transmissible than  
22 SARS-CoV or MERS-CoV [1, 2]. The spike (S) protein appears to be a major pathogenic factor  
23 that contributes to the unique pathogenesis of SARS-CoV-2. Although the S protein is a surface  
24 transmembrane type 1 glycoprotein, it has been predicted to be translocated into the nucleus due  
25 to the novel nuclear localization signal (NLS) “PRRARSV”, which is absent from the S protein of  
26 other coronaviruses. Indeed, S proteins translocate into the nucleus in SARS-CoV-2-infected cells.  
27 To our surprise, S mRNAs also translocate into the nucleus. S mRNA colocalizes with S protein,  
28 aiding the nuclear translocation of S mRNA. While nuclear translocation of nucleoprotein (N) has  
29 been shown in many coronaviruses, the nuclear translocation of both S mRNA and S protein  
30 reveals a novel pathogenic feature of SARS-CoV-2.

31

32 **Author summary**

33 One of the novel sequence insertions resides at the S1/S2 boundary of Spike (S) protein and  
34 constitutes a functional nuclear localization signal (NLS) motif “PRRARSV”, which may  
35 supersede the importance of previously proposed polybasic furin cleavage site "RRAR". Indeed,  
36 S protein’s NLS-driven nuclear translocation and its possible role in S mRNA’s nuclear  
37 translocation reveal a novel pathogenic feature of SARS-CoV-2.

38

## 39 **Introduction**

40 The recently emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), along with  
41 SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), belong to the  
42 *Coronaviridae* virus family. The current ongoing outbreak has shown that SARS-CoV-2 is highly  
43 pathogenic and more transmissible than SARS-CoV or MERS-CoV [1]. These coronaviruses  
44 contain a positive-strand RNA genome with a few unique features: two-thirds of the viral RNA is  
45 translated into a large polyprotein, and the remainder of the viral genome is transcribed by a  
46 discontinuous transcription process into a nested set of subgenomic mRNAs [3-5]. The different  
47 subgenomic RNAs encode four conserved structural proteins (spike, S; envelope, E; membrane,  
48 M, and nucleocapsid, N) and several accessory proteins [6, 7]. The S protein of both SARS-CoV  
49 and SARS-CoV-2 interacts with the host cell receptor angiotensin converting enzyme 2 (ACE2)  
50 and triggers fusion between the viral envelope and host cell membrane to facilitate successful viral  
51 entry [8, 9]. However, the S protein of MERS-CoV binds to dipetidyl peptidase (DPP4) to  
52 facilitate entry into cells [10]. Importantly, the SARS-CoV-2 S protein is a significant pathogenic  
53 factor because of its broad tropism for mammalian ACE2 [11]. While the S protein is an attractive  
54 target for therapeutic development [12], the lack of comprehensive information on S protein  
55 expression and subcellular translocation hinders the identification of an effective S protein-  
56 targeting therapeutic to combat SARS-CoV-2 infection.

57

58 The genome sequence is generally the blueprint for detecting biological function [13]. Thus, the S  
59 protein's function is encoded in the S gene sequence. Identifying novel features in the S gene  
60 sequence, its expression and subcellular localization may shed light on the unique pathogenesis of  
61 SARS-CoV-2 compared to other pathogenic beta-coronaviruses, particularly SARS-CoV and

62 MERS-CoV. A recent study showed several SARS-CoV-2 genomic features, including novel  
63 sequence insertions and enhanced N protein nuclear localization signals (NLSs) that are thought  
64 to be responsible for the unique pathogenesis of this coronavirus [14]. There are three types of  
65 NLSs: pat4, pat7, and bipartite. The pat4 signal is a chain of 4 basic amino acids consisting of  
66 lysine or arginine or three basic amino acids, with the last amino acid being either histidine or  
67 proline. The pat7 signal begins with proline and is followed by six amino acids, which contains a  
68 four-residue sequence in which three of the four residues are basic. The bipartite signal consists of  
69 two basic amino acids with a 10-residue spacer and a five amino acid sequence in which at least  
70 three of the five amino acids are basic [15-17]. The subcellular localization of some SARS-CoV-  
71 2 proteins has been studied in vitro [18], but a comprehensive understanding of the subcellular  
72 localization of the S protein is missing.

73

74 Here, we first report the nuclear translocation of S protein and mRNA in SARS-CoV-2-infected  
75 cells. The translocation of the SARS-CoV-2 S mRNA appeared to be assisted by the S protein,  
76 which contains an NLS motif that is unique among human pathogenic beta-coronaviruses.

77

## 78 **Results**

79 **The novel NLS motif “PRRARSV” is in the S protein of SARS-CoV-2 but not SARS-CoV or**  
80 **MERS-CoV.**

81 Several groups have reported novel nucleotide insertions in the S gene of SARS-CoV-2, as  
82 indicated by a multiple sequence alignment for the S protein sequences of different coronaviruses,  
83 such as a polybasic site “PRRA” produced by a 12-nucleotide acquisition at the S1-S2 boundary

84 through multiple host-species adaptations [19, 20]. However, S protein sequence alignments  
85 between SARS-CoV-2 and SARS-CoV showed the possibility of the insertions “NSPR” [21] and  
86 “SPRR” [22] at the S1-S2 boundary. It has previously been reported that the sequence insertion at  
87 the S1-S2 boundary constitutes a furin cleavage site [23, 24]. A comprehensive understanding of  
88 the consequence of the sequence insertion at the S1-S2 boundary is still missing, possibly because  
89 research focused on understanding the differences in the pathogenicity of the different SARS-  
90 CoV-2 variants and subvariants, which emerged rapidly. To determine whether the earlier SARS-  
91 CoV-2 isolate (USA/WA-CDC-WA1/2020 isolate, GenBank accession no. MN985325) has  
92 multiple novel sequence insertions in the S protein compared to SARS-CoV (Urbani strain,  
93 GenBank accession no. AY278741), we aligned the S protein sequences of both viruses using a  
94 constraint-based alignment tool for multiple protein sequences (COBALT) [25]. We did not use  
95 MER-CoV for comparison because there is only 40% similarity between SARS-CoV-2 and  
96 MERS-CoV [26]. Similar to a previous report [21], we found sequence insertions (IS) in the  
97 SARS-CoV-2 S protein at four independent positions: IS1 “GTNGKTR”, IS2 “YYHK”, IS3  
98 “HRSY”, and IS4 “NSPR” (Fig. 1A & B). To determine whether any of these sequence insertions  
99 constituted or resembled any protein motifs, such as an NLS, we analyzed the SARS-CoV-2 S  
100 protein in silico with the PSORT II web portal for NLS prediction [27]. We found that the SARS-  
101 CoV-2 glycoprotein contained an NLS of the “pat7” motifs, one of the three NLS motifs described  
102 above (Fig. S1). To our surprise, the NLS motif “PRRARSV” was present at the proposed  
103 polybasic site and was due to the fourth sequence insertion “NSPR” (Figs. 1A & B and S1). A  
104 widely reported furin consensus cleavage site motif is the canonical four amino acid motif R-X-  
105 [K/R]-R, although R-X-X-R is the minimal cleavage site on the substrate for successful furin  
106 cleavage [28, 29]. Due to the specificity of the amino acid motif, a furin cleavage motif is not

107 expected to fulfill the characteristics of an NLS motif. However, the described furin cleavage site  
108 is constitutively within the NLS motif. Thus, whether furin cleavage destroys the function of the  
109 NLS motif is important to determine. As expected, the NLS in the S protein was unique to SARS-  
110 CoV-2 among human pathogenic beta-coronaviruses, as neither the SARS-CoV S protein nor the  
111 MERS-CoV S protein has an NLS (Fig. S1).

112

113 **NLS-driven nuclear translocation of S protein (including S mRNA) occurs only in the SARS-**  
114 **CoV-2-infected airway epithelium.**

115 Although viral glycoprotein nuclear translocation is rare, NLS-driven protein nuclear translocation  
116 has already been established in different viral infections [30, 31]. Thus, it is important to determine  
117 whether the SARS-CoV-2 S protein translocates into the nucleus in addition to its canonical cell  
118 surface localization through the ER-Golgi pathway. We hypothesized that the S protein could  
119 translocate into the nucleus in SARS-CoV-2-infected cells via the identified NLS motif [17, 30,  
120 31]. We infected highly differentiated pseudostratified airway epithelial cells (which mimics *in*  
121 *vivo* human airway epithelium) with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.1 for  
122 four days. First, we confirmed the presence of S mRNA and S protein in a 5  $\mu$ m section of  
123 formalin-fixed paraffin-embedded SARS-CoV-2-infected cells by RNAscope and  
124 immunofluorescence analysis. Despite the rarity of viral mRNA (or even positive-strand RNA  
125 virus genome) to be nuclear [32, 33], a recent study showed that SARS-CoV-2 mRNA accumulates  
126 in the nucleus of infected cells [34]. Our results showed that SARS-CoV-2 S mRNA was nuclear  
127 (Fig. 2 left panel and merged images in the right panel). To confirm the physical apposition  
128 between S mRNA and the nucleus by comparing their distributions in fluorescent images, we used  
129 the spot-to-spot colocalization function in Imaris image analysis software (Oxford Instruments).

130 We found that S mRNA was nuclear and abundant in the cytoplasm (Fig. S2, top panel, three  
131 donors). To avoid image artifacts, we imaged multiple independent slides of SARS-CoV-2-  
132 infected airway epithelium (from three independent donors) using at least two different high-end  
133 confocal microscopes. Additionally, we used at least two different image processing strategies to  
134 determine nuclear localization. Based on high-resolution imaging, we determined the subcellular  
135 distribution of S mRNA at the single-molecule and single-cell levels (Figs. 2 & 3A, S2 & S3).  
136 Importantly, we were able to determine S mRNA nuclear translocation not only inside the nucleus  
137 but also on the nuclear surface (Figs. 3A, 3C, and S3). The determination of S mRNA distribution  
138 and abundance showed that S mRNA subcellular localization spans from the inside and outer  
139 surface of the nuclear membrane to everywhere in the cytoplasm. We found that almost 90% of S  
140 mRNA was distributed in the cytoplasm, which was expected, as SARS-CoV-2 transcription and  
141 replication occur in the cytoplasm (Figs. 3 A & C, S3). Interestingly, less than 10% of S mRNA  
142 was detected at the nuclear surface, which could explain the transitional stage of S mRNA before  
143 it enters the nucleus or the novel transnuclear-membrane translocation of S mRNA, which was  
144 examined and described later (Figs. 3 A & C, S3). In approximately 1% of instances, S mRNA  
145 successfully translocated into the nucleus (Figs. 3 A & C, S3). The nuclear translocation of S  
146 mRNA is highly unusual because there have been few previous reports of S mRNA nuclear  
147 translocation and no information on the mechanism of nuclear translocation. However, we have  
148 explored how S mRNA could translocate into the nucleus.

149

150 We investigated whether the S protein translocated into the nucleus in the SARS-CoV-2-infected  
151 airway epithelium. Consistent with the S mRNA data, we found that the S protein translocated into  
152 the nucleus and was abundant on the cellular surface through the cytoplasmic ER-Golgi pathway

153 (Fig. 2, middle panel and merged images in the right panel). Based on high-resolution imaging,  
154 we determined that S protein nuclear translocation included both the inside of the nucleus and the  
155 nuclear surface (Figs. 3B, 3C, and S3). Similar to S mRNA quantification, we were able to quantify  
156 the distribution and abundance of S protein in the infected airway epithelium. We found that the S  
157 protein was distributed inside and in the outer membrane of the nucleus, the cytoplasmic ER-Golgi  
158 and the cell surface. We did not determine what portion of the S protein was localized inside and  
159 outside the cell surface. However, we quantified the subcellular distribution of the S protein inside  
160 the nucleus, outside the surface of the nucleus, and in the cytoplasm, which included cell surface  
161 expression because the S protein is a type 1 transmembrane glycoprotein. We found that  
162 approximately 75% of the S protein was distributed in sites other than the nucleus, including the  
163 cell surface and the cytoplasm, which was expected, as S protein translation and protein processing  
164 occur in the cytoplasm and via cytoplasmic ER-Golgi pathway, respectively (Figs. 3 B & C, S3).  
165 Interestingly, approximately 15% of the S protein was detected at the nuclear surface, which could  
166 explain the S protein transitional stage before entering the nucleus or a novel transnuclear-  
167 membrane translocation of S protein, which was examined and described later (Figs. 3 A & C,  
168 S3). Interestingly, we found that a higher percentage of total S protein translocated into the nucleus  
169 than S mRNA (Figs. 3 A-C, S3). Although viral type-1 transmembrane glycoprotein translocation  
170 into the nucleus is rare, the NLS in the S protein is responsible for nuclear translocation. It was  
171 apparent that NLS-driven S protein nuclear translocation was SARS-CoV-2 specific, and a side-  
172 by-side infection experiment with both viruses showed that the S protein of SARS-CoV did not  
173 translocate into the nucleus (Fig. S4). As both S mRNA and S protein translocated into the nucleus,  
174 it is important to determine whether S mRNA and S protein colocalize in different subcellular  
175 sites.



176

177 **Colocalization of S mRNA and S protein in different subcellular locations in the SARS-CoV-**  
178 **2-infected airway epithelium.**

179 While we can explain S protein nuclear translocation due to the presence of an NLS motif in the  
180 amino acid sequence, we can only hypothesize that S mRNA nuclear translocation is possible due  
181 to a direct interaction between S protein and S mRNA, which can be explained by the  
182 colocalization between them. The SARS-CoV-2 N protein is an abundant RNA binding protein  
183 that is essential for viral genome packaging [35]. While the structural basis of N protein binding  
184 to single- or double-stranded RNA is known [36], there is no information about whether S protein  
185 binds to S mRNA. As we found similar intracellular distribution of both S mRNA and S protein,  
186 we hypothesized that S protein interacts with S mRNA to translocate the protein–mRNA complex  
187 to different subcellular locations, including the cytoplasm and nucleus, but not the cell surface. By  
188 examining the colocalization between the S protein and S mRNA, we could confirm the presence  
189 of the protein–mRNA complex in the SARS-CoV-2-infected airway epithelium. Here, we refer to  
190 colocalization as an association between S mRNA and S protein at different intracellular locations.  
191 Technically, two separate fluorescence molecules that emit different wavelengths of light are  
192 superimposed within an indeterminate microscope resolution. To determine whether S mRNA and  
193 S protein colocalize, we used a high-resolution imaging strategy. We quantified the colocalization  
194 on a percentage scale. We found that approximately 85% of the colocalization, which was the  
195 highest, was observed outside the nucleus (Fig. 3D). These data are consistent with the previously  
196 described spatial expression data of both S protein and S mRNA (Fig. 3A-C). As expected, lower  
197 and the lowest percentages of colocalization between the S protein and S mRNA were observed  
198 on the nuclear surface and inside the nucleus, respectively (Fig. 3D). We were able to pinpoint the

199 colocalization site by using high magnification confocal imaging followed by image processing.  
200 Representatives of S mRNA and S protein colocalization in the cytoplasm (Fig 4, top two panels),  
201 on the surface of the nuclear membrane (Fig 4, middle two panels), or inside the nucleus (Fig 4,  
202 bottom two panels) are shown. We observed S protein and S mRNA colocalization in three  
203 subcellular locations, which was confirmed at the single-cell level in SARS-CoV-2-infected cells  
204 (Fig. S5). We also observed that S mRNA inside and on the nuclear surface was associated with  
205 the S protein, in contrast to the cytoplasmic S mRNA distribution with or without colocalization  
206 with the S protein (Figs. 4 and S5). Thus, S mRNA translocates into the nucleus through the S  
207 protein-S mRNA complex and is driven by the S protein (Figs. 4 and S5).

208

209 **NLS-driven N protein nuclear translocation is common in SARS-CoV, MERS-CoV, and**  
210 **SARS-CoV-2 infections.**

211 There is no comprehensive information on SARS-CoV-2 N protein NLS motifs, and we already  
212 know that other pathogenic coronaviruses, particularly SARS-CoV [17, 37] and MERS-CoV [38]  
213 N proteins, have NLSs. Therefore, we searched for NLSs in the SARS-CoV-2 N protein by using  
214 the PSORT II. We found that the SARS-CoV-2 N protein has 7 NLSs covering all three types of  
215 NLS motifs (pat4: 2; pat7: 3, and bipartite: 2); however, the SARS-CoV N protein has 8 NLS  
216 motifs (pat4: 2; pat7: 4, and bipartite: 2) (Fig. S6). N protein translocation into the nucleus or at  
217 least the perinuclear region was confirmed (Figs. 5A and S7). However, SARS-CoV-2 N protein  
218 nuclear translocation was not as robust as SARS-CoV nuclear translocation (Figs. 5B and S7). The  
219 reduced nuclear translocation of the SARS-CoV-2 N protein is probably due to the absence of one  
220 pat7 NLS motif in the SARS-CoV-2 N protein compared to that in SARS-CoV (Figs. S7 and S8).

221

## 222 **SARS-CoV-2 S and N proteins' interactions with genomic RNA.**

223 Based on a machine learning model, the SARS-CoV-2 RNA genome and sub-genomic RNAs can  
224 be translocated in the host cells' mitochondrial matrix and nucleus [39]. Our results suggest that  
225 around 1% S mRNA translocated into the nucleus. S mRNA's (potentially SARS-CoV-2 mRNA  
226 genome) subcellular localization may play a significant role in SARS-CoV-2 pathogenesis. To  
227 determine whether the SARS-CoV-2 genome interacts with either S protein or N protein, we in  
228 silico analyzed RNA-protein interactions using the RPISeq web portal, which offers the only  
229 sequence-based prediction model [40]. We found that both S and N protein binding probability to  
230 the SARS-CoV-2 genome scored exactly 1 (Dataset S1 and S2). SARS-CoV-2 N protein is an  
231 abundant RNA binding protein essential for viral genome packaging [35]. While the structural  
232 basis of N binding to single or double-stranded RNA is known [36], we found that S mRNA  
233 nuclear translocation aids via S protein. However, the mechanism of S protein binding to the  
234 mRNA or possibly positive-strand RNA genome is yet to be determined.

235

## 236 **Discussion**

237 In the context of SARS-CoV, one of the controversies regarding the natural origin of SARS-CoV-2  
238 is that its S gene has multiple novel sequence insertions. Zhang C. et al. analyzed the report by  
239 Pradhan et al. (withdrawn) [41] on the presence of four unique novel sequences in the SARS-CoV-  
240 2 S gene and showed that these four sequence insertions were not related to the receptor-binding  
241 domain (RBD) [21]. A recent study identified S gene novel sequence insertions among several key  
242 genomic features that differentiate SARS-CoV-2 from other beta-coronaviruses, particularly

243 SARS-CoV and MERS-CoV [14]. The source and characterization of these sequence insertions  
244 have yet to be determined; however, the closest BLAST hit of these sequences is bat coronavirus  
245 RaTG13 [42]. Similar to a previous report [21], we found four multiple sequence insertions in the  
246 SARS-CoV-2 S protein: IS1 “GTNGKTR”, IS2 “YYHK”, “HRSY”, and IS4 “NSPR” (Fig. 1).  
247 Here, we showed that the fourth novel sequence insertion in the S gene was an NLS and resulted  
248 in nuclear translocation of the S protein, which not only complemented previous in silico findings  
249 [14] but also identified a novel pathogenic genomic feature of the S gene. Interestingly, the fourth  
250 significant insertion has received attention due to the description of a polybasic site “RRAR”,  
251 which may contribute to increased serin protease-driven entry of SARS-CoV-2 [19] and is  
252 implicated in broader tropism and/or enhanced viral transmissibility compared to SARS-CoV [20].  
253 However, we found that the IS4 “NSPR” created a pat7 NLS “PRRARSV” in the S protein, which  
254 was unique to SARS-CoV-2. We first reported that the S protein translocated into the nucleus in  
255 the SARS-CoV-2-infected airway epithelium, which is an appropriate lung model for studying  
256 respiratory virus infection in vitro [2, 43]. Our results confirmed that the SARS-CoV-2 S protein  
257 was a unique addition to the list of viral proteins that possess NLSs and consequently translocate  
258 into the nucleus of infected cells [17, 30, 31, 44]. Among coronaviruses, SARS-CoV-2 S protein  
259 is the first type-1 transmembrane glycoprotein that translocates into the nucleus. Vesicular  
260 stomatitis virus (VSV), which is a negative sense RNA virus, has a glycoprotein that translocates  
261 to the nucleus as well. A study by the University of Illinois at Urbana Champaign showed exactly  
262 how the glycoprotein on VSV was able to travel to the nucleus of hamster kidney cells [45].

263 NLS-driven S protein nuclear translocation is a novel pathogenic feature of SARS-CoV-2  
264 infection compared to other pathogenic coronaviruses. However, the pathogenic contribution of  
265 the S protein’s NLS motif to virus-induced pathophysiology is yet to be determined. Our results

266 suggested that the S protein translocated into the nucleus due to the NLS, which also raised two  
267 important points. First, we investigated whether the proposed polybasic site “RRAR” could itself  
268 be an NLS motif. The answer was that the proposed polybasic site was not an NLS motif because  
269 an NLS is a well characterized and predefined amino acid sequence motif [15-17]. Additionally,  
270 the amino acid sequence of the probable sequence insertion “NSPR” [21] was also not an NLS but  
271 was part of the P7 “PRRARSV” NLS. Thus, the inserted sequence creates the NLS in the S protein  
272 of SARS-CoV-2 and may make SARS-CoV-2 unique among human pathogenic coronaviruses.

273         The second important point was whether the NLS motif was functional in the context of  
274 the described polybasic site at the S1/S2 boundary. All type-1 transmembrane glycoproteins are  
275 processed through the ER-Golgi pathway before signal peptide-driven cellular surface localization.  
276 The proposed polybasic site was functional (availability to proteases) when the S protein was on  
277 the virion for host cell entry. A fully posttranslationally processed S protein surface translocation  
278 could also provide a polybasic site to be processed by furin cleavage. However, there is no  
279 information on the availability or usability of the S protein’s polybasic site by furin proteases in  
280 the cytoplasm before virus assembly. Thus, the NLS is functional in SARS-CoV-2-infected cells,  
281 and the polybasic site only functions during the viral entry step. The NLS is obviously functional  
282 in infected cells, and no furin cleavage at the polybasic site is necessary other than for viral entry.  
283 Our results confirmed that the S protein NLS motif was functional in SARS-CoV-2-infected cells.  
284 Although mutating the polybasic site (which also mutated the NLS) may impact viral S protein  
285 function in vitro, the result will not confirm or deny that one is more important than the other  
286 between the polybasic site and the NLS. While our result does provide direct evidence for the  
287 presence of the NLS motif and nuclear translocation of the S protein, our results do not confirm  
288 nor deny that the NSPR sequence has a natural origin. Instead, our results showed that the inserted

289 sequence NSPR was a functional NLS motif, which increased the intracellular distribution of the  
290 S protein, including novel nuclear translocation. The novel nuclear translocation of the SARS-  
291 CoV-2 S protein suggests that: 1. the nuclear translocation of the S protein reduces its surface  
292 expression, but whether it contributes to evading host immune recognition remains to be  
293 determined; and 2. the colocalization of the S protein with S mRNA suggests that the S protein has  
294 an RNA binding motif, which remains to be determined. One of the important ways of confirming  
295 a functional NLS motif is to use site-directed mutational analysis. Plasmid-driven transient  
296 expression of S protein in the human lung airway A549 cell line and primary normal human  
297 bronchial epithelial cells showed robust S expression but was toxic to the cells. Therefore, the  
298 success of site-directed mutational analysis of the S protein in a transient expression system is  
299 doubtful and the characterization of NLS by a mutational analysis is yet to be determined. Thus,  
300 our novel findings emphasize further research on the NLS motif of the SARS-CoV-2 S protein.

301 One of the most important findings in our study was the simultaneous detection of the  
302 different spatial distributions of S protein and S mRNA at the single-molecule level in a single  
303 infected cell. We confirmed that S mRNA translocated into the nucleus by image analysis of the  
304 colocalization of S mRNA with nuclear staining. The SARS-CoV-2 N protein has already been  
305 shown to bind to RNA [46]. There was no information available confirming whether the S protein  
306 could bind to S mRNA for nuclear translocation. Our results revealed that S mRNA nuclear  
307 translocation was mediated by the S protein because S mRNA nuclear translocation was always  
308 associated with the S protein. For example, S mRNA colocalized with the S protein inside and  
309 outside the surface of the nucleus. Although the primer-probe was designed to target S mRNA, the  
310 SARS-CoV-2 positive-strand RNA genome (whole or partial) can be targeted by the same probe  
311 due to the sequence similarity between S mRNA and the whole or partial genome. Thus, our results

312 lack sufficient detail contributing to the discussion of the controversial scientific topic of whether  
313 there is any possibility of SARS-CoV-2 genome integration into the host DNA [47, 48].  
314 Additionally, one of the significant differences in the S protein sequences of SARS-CoV and  
315 SARS-CoV-2 is the pat7 NLS motif. Whether S protein expression by the current vaccine  
316 platforms causes suboptimal expression of S protein on the cell surface due to the NLS remains to  
317 be determined [49].

318 In conclusion, the SARS-CoV-2 S protein has a functional pat7 NLS “PRRARSV”, that  
319 results in one out of four S proteins translocating into the nucleus in infected cells. S Protein  
320 appears to shuttle S mRNA (possibly the genome) into the nucleus as well. Thus, the NLS of the  
321 S protein may contribute to the evasion of the host immune response and is a novel pathogenic  
322 feature of SARS-CoV-2.

## 323 **Materials and Methods**

324 **Cells and viruses.** Primary normal human bronchial epithelial (NHBE) cells from healthy adults  
325 and high-risk adults (deidentified) were obtained from Dr. Kristina Bailey at the University of  
326 Nebraska Medical Center (UNMC) (Omaha, NE) under an approved material transfer agreement  
327 (MTA) between the University of North Dakota (UND) and UNMC (Omaha, NE). The protocol  
328 for obtaining cells was reviewed by the UNMC IRB and was determined to not constitute human  
329 subject research (#318-09-NH). In this study, we used cells from five donors: nonsmoker healthy  
330 adults (donors #1 and #2) and adult with chronic obstructive pulmonary disease (COPD) (donor  
331 #3). The protocols for subculturing primary NHBE cells were published previously [2, 43, 50].  
332 SARS-CoV-2 (USA/WA-CDC-WA1/2020 isolate, GenBank accession no. MN985325; kindly  
333 provided by CDC), SARS-CoV (Urbani strain, GenBank accession no. AY278741; kindly

334 provided by Rocky Mountain Laboratories (RML), NIAID, NIH), and MERS-CoV (GenBank  
335 accession no. NC\_019843.3; kindly provided by the Department of Viroscience, Erasmus Medical  
336 Center, Rotterdam, The Netherlands) were used for *in vitro* infections described below.

337

338

339 **In silico analysis.** We have used open-source web portals for different *in silico* analyses. 1.  
340 Constraint-based alignment tool for multiple protein sequences (COBALT)  
341 ([https://www.ncbi.nlm.nih.gov/tools/cobalt/re\\_cobalt.cgi](https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi)) was used for multiple sequence  
342 alignment. 2. PSORT II (<https://psort.hgc.jp/form2.html>) was used for NLS prediction. 3. The  
343 RPIseq web portal (<http://pridb.gdcb.iastate.edu/RPISeq/>) was used for RNA–protein interactions.

344

345 **Highly differentiated pseudostratified bronchial airway epithelium.** The protocols for  
346 differentiating primary NHBE cells to form a pseudostratified bronchial airway epithelium were  
347 published previously [2, 43, 50]. Briefly, Transwells (6.5 mm) with 0.4- $\mu$ m-pore polyester  
348 membrane inserts (Corning Inc.) were coated with PureCol (Advanced BioMatrix) for 20 min  
349 before cell seeding. NHBE cells ( $5 \times 10^4$ ) suspended in 100  $\mu$ l of complete airway epithelial cell  
350 (cAEC) medium [AEC medium (Promocell) + SupplementMix (Promocell) + 1%  
351 penicillin–streptomycin (V/V) (Thermo Fisher Scientific) + 0.5% amphotericin B (V/V) (Thermo  
352 Fisher Scientific)] were seeded in the upper chamber of the Transwell. Then, 500  $\mu$ l of cAEC  
353 medium was added to the lower chamber of the Transwell. When the cells formed a confluent  
354 layer on the Transwell insert, the cAEC medium was removed from the upper chamber, and in the  
355 lower chamber, the cAEC medium was replaced with complete ALI medium [PneumaCult-ALI



356 basal medium (Stemcell Technologies Inc.) + with the required supplements (Stemcell  
357 Technologies) + 2% penicillin–streptomycin (V/V) + 1% amphotericin B (V/V)]. The complete  
358 ALI medium in the lower chamber was changed every day. The upper chamber was washed with  
359 1x Dulbecco’s phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) once per week  
360 initially but more frequently when more mucus was observed during later days. All cells were  
361 differentiated for at least four weeks at 37°C in a 5% CO<sub>2</sub> incubator. We observed motile cilia in  
362 the differentiated airway epithelium similar to previously described [50].

363

364 **Viral infection.** All viral infection experiments were conducted in the high biocontainment facility  
365 at RML, NIAID, NIH, Hamilton, MT. After approximately 3 weeks, the differentiated airway  
366 epithelium on Transwells was shipped to RML in an optimized transportation medium [2, 50], and  
367 the recovered cells were maintained in complete ALI medium for approximately one week before  
368 infection. For infection, the airway epithelium on Transwells was washed with 200 µl of 1x PBS  
369 to remove mucus and were infected on the apical site with SARS-CoV-2, MERS-CoV, or SARS-  
370 CoV at a MOI of 0.1 in 100 µl 1x PBS for 1 hour (at 37°C with 5% CO<sub>2</sub>). For mock infection, the  
371 Transwells were similarly incubated with 100 µl 1x PBS without virus. The viral inoculum was  
372 then removed, and the epithelium on the Transwell was washed twice with 200 µl of 1x PBS.  
373 Complete ALI medium (1000 µl) was added to the lower chamber of each Transwell, and the upper  
374 chamber was kept empty. Mock-infected and virus-infected Transwells were incubated for 4 days  
375 at 37°C in an incubator with 5% CO<sub>2</sub> [2].

376

377 **Paraformaldehyde (PFA) fixation and paraffin embedding.** At 4 days postinfection (DPI), 200  
378  $\mu$ l of 1x PBS was added to the apical site of the Transwell for washing before PFA fixation. In the  
379 basal side of the Transwell inserts was 200  $\mu$ l of 1x PBS. For PFA fixation, 200  $\mu$ l of 4% PFA  
380 (Polysciences) was added to the upper chamber of the Transwells and incubated for 30 min, and  
381 the Transwells were further maintained overnight in 4% PFA prior to removal from high  
382 biocontainment. The PFA fixation protocol was approved as an inactivation method for  
383 coronaviruses by the RML Institutional Biosafety Committee. The PFA-fixed airway epithelium  
384 was paraffin-embedded and sectioned at a thickness of 5  $\mu$ m for slide preparation as previously  
385 described [43].

386

387 **Simultaneous detection of S mRNA and S protein.** Slides with 5  $\mu$ m sections were first  
388 deparaffinized by incubation in a Coplin jar as follows: 1. Histo-Clear for 5 min, two times; 2.  
389 100% ethanol for 5 min, three times; 3. 95% ethanol for 5 min, 4. 70% ethanol for 5 min, and 5.  
390 distilled water for 5 min. The deparaffinized slides were immediately incubated in 0.5% Triton X-  
391 100 in 1x PBS for 30 min. The slides were washed three times with 1X PBST (1x PBS with Tween  
392 20) or 1x PBS for 5 min. A hydrophobic barrier was drawn around the 5  $\mu$ m section on the slides  
393 by using an Immedge Hydrophobic Barrier Pen. To reduce nonspecific antibody binding, the  
394 section was blocked with 10% goat serum (Vector Laboratories) in 1x PBST for 2 hours at 4°C.  
395 The slides were then incubated with viral protein-specific primary antibody solution in 1x PBST  
396 (e.g., SARS-CoV/SARS-CoV-2 S protein-specific rabbit polyclonal antibody at a 1:100 dilution,  
397 SARS-CoV/SARS-CoV-2 N protein-specific mouse monoclonal antibody at a 1:100 dilution, or  
398 MERS-CoV N protein-specific mouse monoclonal antibody at a 1:100 dilution) overnight at 4°C.  
399 The slides were then incubated with the corresponding secondary antibody solution (anti-mouse

400 or anti-rabbit AF488 or AF647, Thermo Fisher Scientific) in 1x PBST for 2 hours at room  
401 temperature. We then stained the nuclei with DAPI reagent (Advanced Cell Diagnostics) or used  
402 RNAscope multiplex V2 to detect SARS-CoV-2 S mRNA (Probe V-nCoV2019-S) according to  
403 the manufacturer's instructions (Advanced Cell Diagnostics). The sections were mounted on Tech-  
404 Med microscope slides (Thomas Fisher Scientific) using ProLong-Gold antifade mounting  
405 medium (Thermo Fisher Scientific).

406

407 **Imaging and image analysis.** The images were taken under an Olympus FluoView laser scanning  
408 confocal microscope (Olympus FV3000) enabled with a 60X objective (Olympus), a Leica  
409 Stellaris confocal microscope (Leica Microsystem) using a 63x oil objective or a Leica DMI8  
410 epifluorescence microscope (Leica Microsystem). The images were then deconvolved using  
411 Huygen Essential deconvolution software (Scientific Volume Imaging). The surface rendering  
412 function of Imaris image processing software (Oxford Instruments) was used. The images were  
413 also analyzed for spot-to-spot colocalization by Imaris. Where applicable, images taken under a  
414 Leica DMI8 microscope were processed using 3D deconvolution and 3D view modules in LASX  
415 software (Leica Microsystem). For figure preparation, Prism version 9 (GraphPad) and Adobe  
416 Photoshop (Creative Cloud) software were used.

417

418 **Fig 1. Only the SARS-CoV-2 S protein had an NLS motif “PRRARSV” due to a novel**  
419 **sequence insertion. A.** Full-length SARS-CoV-2 genome (nucleotide) (USA/WA-CDC-  
420 WA1/2020 isolate, GenBank accession no. MN985325) and open reading frames (ORF) are shown  
421 at the top. The SARS-CoV-2 S protein amino acid sequence was aligned with SARS-CoV (Urbani

422 strain, GenBank accession no. AY278741) by NCBI's constraint-based multiple alignment tool  
423 COBALT [25], and the relative positions of four novel sequence insertions (ISs) are shown in the  
424 S protein ORF as follows: IS1: "GTNGKTR", IS2: "YYHK", IS3: "HRSY", and IS4: "NSPR".  
425 The fourth IS (NSPR) created a pat7 NLS "PRRARSV" in the S protein (shown in the red  
426 rectangle). **B.** The S protein ORF sequences between SARS-CoV-2 and SARS-CoV were aligned,  
427 and the Lined rectangles highlight the four novel insertions: IS1, IS2, IS3, and IS4. The IS4  
428 "NSPR" created a pat7 NLS "PRRARSV" in the S protein (shown in the black rectangle).

429

430 **Fig 2. The intracellular distribution of S mRNA and S protein suggests nuclear translocation.**

431 Four-week **highly differentiated** pseudostratified airway epithelium was infected with SARS-  
432 CoV-2 at a MOI of 0.1 for four days, paraformaldehyde-fixed, paraffin-embedded, and sectioned  
433 at a thickness of 5  $\mu\text{m}$  for immunohistochemistry (IHC) and slide preparation [43, 50]. A combined  
434 protocol of RNAscope and IHC was used to simultaneously detect S mRNA and S protein in the  
435 SARS-CoV-2-infected airway epithelium. S mRNA (red) was detected using a SARS-CoV-2 S  
436 mRNA probe for RNAscope, and S protein (cyan) was detected by an S protein-specific rabbit  
437 polyclonal antibody and a corresponding anti-rabbit secondary antibody for immunofluorescence  
438 (IFA) analysis. The nucleus (blue) was detected by DAPI staining. The images were taken under  
439 an Olympus confocal microscope using a 60x oil objective. The images represent multiple  
440 independent technical replicates from two independent experiments with different donors  
441 (experiment 1: donors 2 and 3 and experiment 2: donor 1). The scale bar is 10  $\mu\text{m}$ .

442

443 **Fig 3. The nuclear translocation of S protein and S mRNA includes both the outer surface**  
444 **and inside of the nucleus.** Separate slides (see Fig. 2) were imaged under a Leica Stellaris  
445 confocal microscope (Leica) using a 63x oil objective. The images were then deconvolved using  
446 Huygen Essential deconvolution software (Scientific Volume Imaging). Using the surface  
447 rendering function of an image processing IMARIS software. **A.** S mRNA (red) on the nuclear  
448 surface (top) and inside the nucleus (bottom). White arrows indicate S protein on the nuclear  
449 surface (top image) or inside the nucleus (bottom image). **B.** S protein (green) on the nuclear  
450 surface (top image) and inside the nucleus (bottom image). White arrows indicate S protein on the  
451 nuclear surface (top image) or inside the nucleus (bottom image). **C.** The total distribution of S  
452 mRNA and S protein in the cells. The data were obtained by combining multiple images from an  
453 independent experiment. **D.** The total colocalization between S mRNA and S protein in the cells.  
454 The data were obtained by combining multiple images from an independent experiment.

455

456 **Fig 4. Colocalization between S mRNA and S protein inside infected cells.** The images (see  
457 Fig. 3) were analyzed by using the surface rendering and colocalization features of IMARIS. S  
458 protein and S mRNA distribution and colocalization in the cytoplasm (top panel), on the nuclear  
459 surface (middle panel) and inside the nucleus (bottom panel). The specific region of colocalization  
460 is indicated by a white spot. Scale bar 0.5  $\mu\text{m}$ .

461

462 **Fig 5. The nucleoproteins of SARS-CoV, MERS-CoV, and SARS-CoV-2 translocate into the**  
463 **nucleus.** **A.** Four-week pseudostratified airway epithelium was infected with SARS-CoV-2 at a  
464 MOI of 0.1 for four days, fixed, paraffin-embedded, and sectioned at a thickness of 5  $\mu\text{m}$  for

465 immunohistochemistry slide preparation. Simultaneous detection of S mRNA (shown in red) and  
466 N protein (shown in cyan) on the same slide was performed by a combined detection protocol in  
467 RNAscope-based mRNA and immunofluorescence-based protein detection. An S mRNA-specific  
468 probe was used for RNAscope, and an N protein-specific rabbit polyclonal antibody and the  
469 corresponding anti-rabbit secondary antibody were used. The nucleus (shown in blue) was detected  
470 by DAPI staining. The images were taken under an Olympus confocal microscope using a 60x oil  
471 objective. The images represent multiple independent technical replicates from two independent,  
472 healthy donors (top row: donor #1 and bottom row: donor #2). The scale bar is 10  $\mu$ m. **B.** Four-  
473 week pseudostratified airway epithelium was infected with SARS-CoV-2, SARS-CoV, or MERS-  
474 CoV at an MOI of 0.1 for four days. SARS-CoV-2 or SARS-CoV N protein was detected by an N  
475 protein-specific rabbit polyclonal antibody and corresponding anti-rabbit secondary antibody.  
476 Similarly, the MERS N protein was detected by MERS N protein-specific primary and  
477 corresponding secondary antibodies. The nucleus (shown in blue) was detected by DAPI staining.  
478 The images represent multiple independent technical replicates from one experiment (donor #1).

479

480 **Fig S1. NLS prediction in the S protein of pathogenic coronaviruses.** All categories of NLS  
481 motifs were searched in the S protein sequence using the web-based program PSORT II  
482 (<https://psort.hgc.jp/form2.html>) [27] for the S protein ORF amino acid sequence of SARS-CoV-  
483 2 (USA/WA-CDC-WA1/2020 isolate, GenBank accession no. MN985325) (Query 1), SARS-CoV  
484 (Urbani strain, GenBank accession no. AY278741) (Query 2), or MERS-CoV (GenBank accession  
485 no. NC\_019843.3) (Query 3).

486

487 **Fig S2. Detection of the nuclear translocation of S mRNA and S protein.** Confocal images of  
488 SARS-CoV-2-infected airway epithelium (described in Fig 2) were analyzed for spot-to-spot  
489 colocalization using Imaris image analysis software (Oxford Instruments). The left panel shows  
490 the confocal images, the middle panel shows spot-to-spot colocalization, and the right panel shows  
491 merged confocal images and spot-to-spot colocalization. Spot-to-spot colocalization between the  
492 nucleus and S protein or S mRNA is indicated by a different color. The images represent multiple  
493 independent cross sections of the SARS-CoV-2-infected airway epithelium (from 3 independent  
494 donors).

495

496 **Fig S3. The translocation of S mRNA and S protein includes both the inside and outer surface**  
497 **of the nucleus.** From the images shown in Fig 3, the signals of S mRNA and S protein were plotted  
498 in the graph by Imaris image analysis software. The distance and intensity of all S mRNA or S  
499 protein from the nuclear surface (considered 0) were plotted. A negative value indicates that S  
500 mRNA or S protein resides inside the nucleus. The higher the negative value is, the farther the  
501 distance from the nuclear surface. In contrast, a positive value indicates that S mRNA or S protein  
502 resides on the nucleus surface and beyond in the cytoplasm. The higher the positive value is, the  
503 farther the distance from the nuclear surface.

504

505 **Fig S4. The SARS-CoV S protein does not translocate into the nucleus.** A four-week  
506 pseudostratified airway epithelium was infected with SARS-CoV at an MOI of 0.1 for four days,  
507 fixed, paraffin-embedded and sectioned at a thickness of 5  $\mu\text{m}$  for immunohistochemistry slide  
508 preparation. S protein (shown in cyan) was detected by immunofluorescence-based protein

509 detection using SARS-CoV/SARS-CoV-2 S specific rabbit polyclonal primary antibody and anti-  
510 rabbit secondary antibody. The confocal image was analyzed for spot-to-spot colocalization using  
511 Imaris image analysis software. The left panel shows a confocal image, the middle panel shows  
512 spot-to-spot colocalization, and the right panel shows merged confocal images and spot-to-spot  
513 colocalization. The images represent multiple independent cross sections of SARS-CoV-infected  
514 airway epithelium (at least two donors).

515

516 **Fig S5. S mRNA and S protein colocalization was spatially evident in all possible ways inside**  
517 **the infected cell.** The confocal images shown in Figs. 3 & 4 were further visualized at a higher  
518 magnification to detect S mRNA and S protein colocalization spatially. S protein and S mRNA  
519 distribution and colocalization in the cytoplasm (top panel), on the nuclear surface (middle panel)  
520 and inside the nucleus (bottom panel). The specific region of colocalization is indicated by a white  
521 spot. The colors were made translucent to show colocalization. Scale bar 0.2  $\mu\text{m}$ .

522

523 **Fig S6. NLS motif prediction in the N protein of pathogenic coronaviruses.** All categories of  
524 NLS motifs were searched in the N protein sequence using the web-based program PSORT II  
525 (<https://psort.hgc.jp/form2.html>) [27] for the N protein ORF of SARS-CoV-2 (USA/WA-CDC-  
526 WA1/2020 isolate, GenBank accession no. MN985325) (Query 1), SARS-CoV (Urbani strain,  
527 GenBank accession no. AY278741) (Query 2), or MERS-CoV (GenBank accession no.  
528 NC\_019843.3) (Query 3).

529



530 **Fig S7. Nuclear translocation of the N protein of pathogenic coronaviruses.** Four-week  
531 pseudostratified airway epithelium was infected with SARS-CoV-2, SARS-CoV, or MERS-CoV  
532 at an MOI of 0.1 for four days, fixed, paraffin-embedded and sectioned at a thickness of 5  $\mu$ m for  
533 immunohistochemistry slide preparation. SARS-CoV-2 or SARS-CoV N protein (green) was  
534 detected by a SARS-CoV/SARS-CoV-2 N protein-specific antibody. Similarly, the MERS N  
535 protein (green) was detected by the MERS N protein-specific antibody. The nucleus (shown in  
536 blue) was detected by DAPI staining. The confocal images were analyzed for spot-to-spot  
537 colocalization. The left panel shows a confocal image, the middle panel shows spot-to-spot  
538 colocalization, and the right panel shows merged confocal images and spot-to-spot colocalization.  
539 Spot colocalization between the nucleus and N protein is indicated by a different color. The images  
540 represent multiple independent technical replicates from at least one independent experiment for  
541 one donor (donor #1).

542

543 **Fig S8. NLS motif distribution in the N protein in different pathogenic coronaviruses.** The  
544 sequences of the N protein of the SARS-CoV-2 N protein (nCoV-WA1-2020, GenBank accession  
545 no. MN985325), SARS-CoV N protein (Urbani Strain, GenBank accession no. AY278741), and  
546 MERS-CoV N protein (HCoV-EMC/2012, GenBank accession no. NC\_019843) by NCBI's  
547 constraint-based multiple alignment tool COBALT [25]. All categories of NLS motifs are shown  
548 in the colored rectangle box: pat4: green; pat7: blue; bipartite 1: black; bipartite 2: orange.

549

550 **S1 dataset 1.** Prediction of SARS-CoV-2 S protein and genome interaction

551 **S2 dataset 2.** Prediction of SARS-CoV-2 N protein and genome interaction

552

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566

567 **Author contributions.** M.M. conceived the project and designed all the experiments. K.B.  
568 provided the primary cells. F.F. performed the viral infection work. K.J., S.S. and M.M. performed  
569 all staining for detection. M.M. and S.S. generated the microscopic images. J.K. and M.M.  
570 processed and quantified images. M.M. analyzed (in silico) the viral genome and protein  
571 sequences. M.M. prepared the figures and wrote and edited the manuscript.

572

573 **Conflicts of interest.** The authors declare no conflicts of interest.

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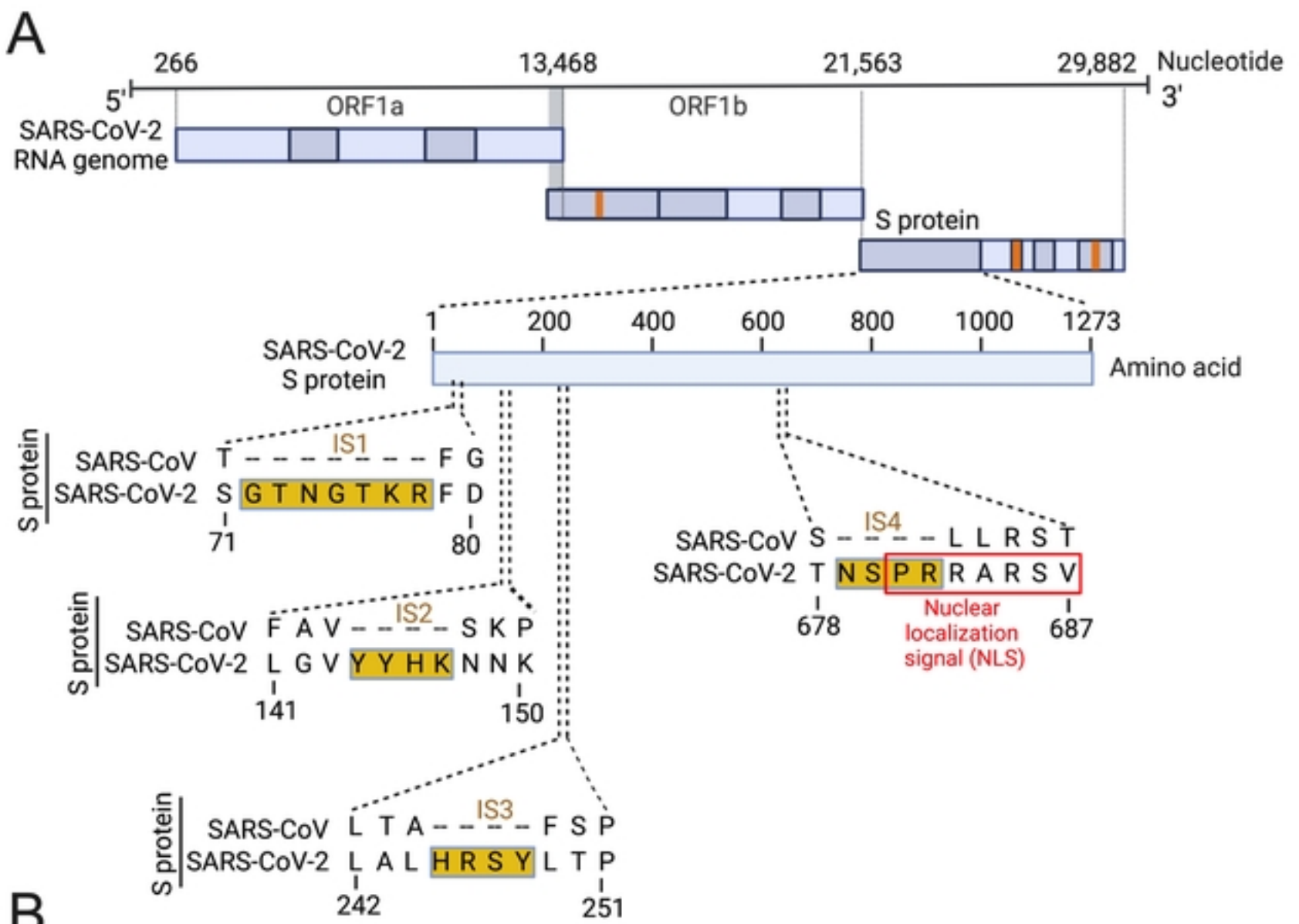
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**B**

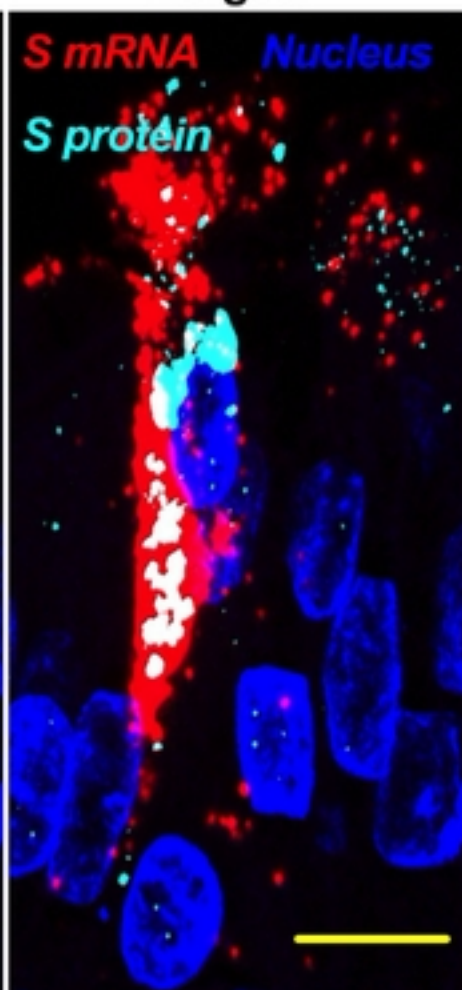
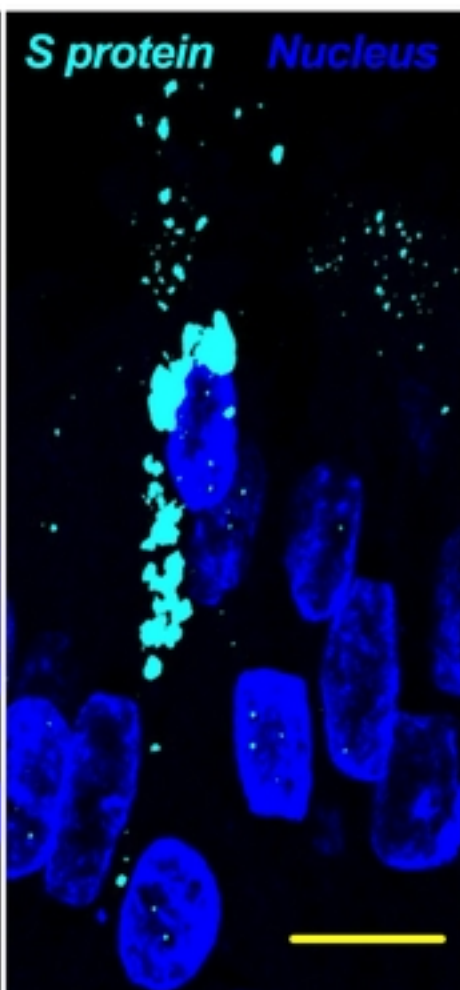
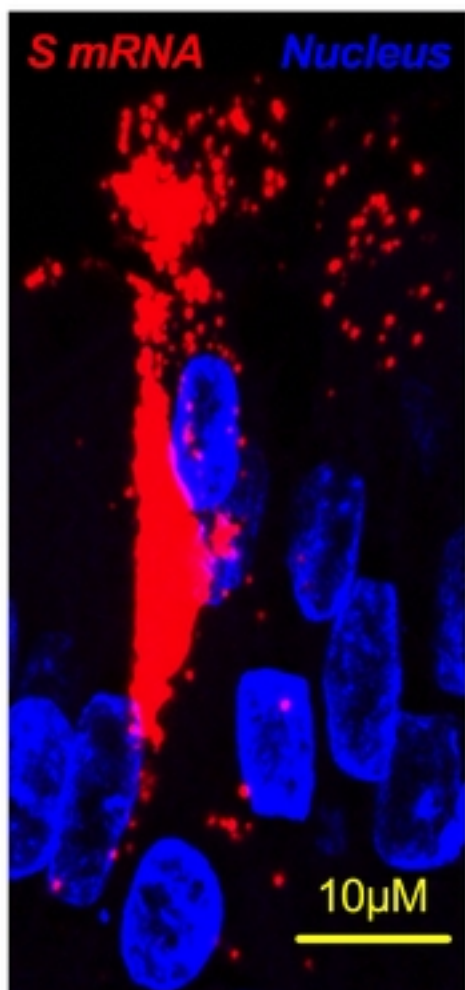
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SARS-CoV	MFIFLLFLTLTSGSDLDRCTTFDDVQAPNYTQHTSSMRGVYYPDEIFRSDTLYLTDQLFLPFYSNVTGFHTINHT	75
SARS-CoV-2	MFVFLVLLPLVSSQCVNLTTRTQ--LPPAYTN--SFRTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWFHAIHVS	76
SARS-CoV	--FGNPVVPFKDGIYFAATEKSNVVRGWVFGSTMNKSQSVIIINNSTNVVIRACNFELCDNPFAY--SKPMGTQTH	149
SARS-CoV-2	KRFDNPVLPFNDGVYFASTEKSNIRGWIFGTTLDSTKQSLLIWNATNVVIVKVECFQFNDPFLGYYYHKNKSWMESE	156
SARS-CoV	TMIFDNANCTFEYISDAFSLDVSEKSGNFKHLREFVFNKDGFLVYKGYQPIDVVRDLPSGFNTLKPFIKPLGINIT	229
SARS-CoV-2	FRVYSSANNCTFEYVSQPFMDLEGKQGNFKNLRREFVFNKIDGYFKIYSKHTPINLVRDLPQGFSALEPLVDLPIGINIT	236
SARS-CoV	NFRAILTA--FSPAQDI--WGTSAAAYFVGYLKPTTFMLKYDENGITDAVDCSQNPLAELKCSVKSEIDKGIYQTS	303
SARS-CoV-2	RFQTLALHRSYLTTPGDSSSGWTAGAAAYVGYLQPRFTLLKYNENGTITDAVDCALDPLSETKCTLSFTVEKGIYQTS	316
SARS-CoV	NFRVVPVSGDVRFPNITNLCPFGEVFNATKFPVSVYAWERKKISNCVADYSVLVNSTFFSTFKCYGVSATKLNLDLCSNVY	383
SARS-CoV-2	NFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLVNSASFSTFKCYGVSPTKLNLDLCTNVY	396
SARS-CoV	ADSFVVKGDVVRQIAPGQTGVIADYNYKLPDDFMGCVLAWNTRNIDATSTGNVNYKYRYLRHGKLRPFERDISNVPFSPD	463
SARS-CoV-2	ADSFVIRGDEVVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNLDLSDKVGNYNYLYRLFRKSNLKPFERDISTEIQAG	476
SARS-CoV	GKPCTP--PALNCYWPLNDYGFYTTTGIGYQPYRVVLSFELLNAPATVCGPKLSTDLIKNQCVNFNFNGLTGTGVLTPSS	542
SARS-CoV-2	STPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTESN	556
SARS-CoV	KRFQPFQFGRDVSDFDTSVRDPKTSEILDISPCSFGGVSVITPGTNASSEVAVLYQDVNCTDVSSTAIHADQLTPAWRIY	622
SARS-CoV-2	KKFLPFQFGRDIADTTDAVRDPQTLIILDITPCSFGGVSVITPGTNTSNQVAVLYQDVNCTEVPVAIHADQLTPTWRVY	636
SARS-CoV	STGNVVFQTAGCLIGAHEVDTSYECDIPIGAGICASYHTVS--LLRSTSQKSIVAYTMSLGADSSIAYSNNTIAIPT	698
SARS-CoV-2	STGSNVFQTRAGCLIGAHEVWNSYECDIPIGAGICASYHTQ--NSPRARSVASQSIAYTMSLGAENSVAYSNNIAIPT	716
SARS-CoV	NFSISITTEVMPVSMAKTSVDCNMYICGDSTECANLLLQYGSFCTQLNRALSGIAAEQDRNTREVFAQVKQMYKTPTLKY	778
SARS-CoV-2	NFTISVTTEILPVSMTKTSVDCNMYICGDSTECANLLLQYGSFCTQLNRALSGIAAEQDKNTQEVFAQVKQIYKTPIKD	796
SARS-CoV	FGGFNFSQILPDPLKPTKRSFIEDLLFNKVTLADAGFMKQYGECLGDINARDLCAQKFNGLTVLPPLTDDMIAAYTAA	858
SARS-CoV-2	FGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDCLGDIAARDLCAQKFNGLTVLPPLTDEMIAQY TSA	876
SARS-CoV	LVSGTATAGWTFGAGAALQIPFAMQMAYRFNGIGVTVQNVLYENQKQIANQFNKAISQIQESLTTTSTALGKLQDVVNQNA	938
SARS-CoV-2	LLAGTITSGWTFGAGAALQIPFAMQMAYRFNGIGVTVQNVLYENQKLIANQFNKAISQIQESLTTTSTALGKLQDVVNQNA	956
SARS-CoV	QALNTLVKQLSSNFGAISSVLNDILSRDLKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQ	1018
SARS-CoV-2	QALNTLVKQLSSNFGAISSVLNDILSRDLKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQ	1036
SARS-CoV	SKRVDFCGKGYHLMSPQAAPHGVVFLHVTYVPSQERNFTTAPAICHEGKAYFPREGVVFVNGTWSFITQRNFFSPQIIT	1098
SARS-CoV-2	SKRVDFCGKGYHLMSPQASPHGVVFLHVTYVPAQEKNFNTTAPAICHGKAHFPREGVVFVNGTWSFITQRNFFSPQIIT	1116
SARS-CoV	TDNTFVSGNCDVVIGIINNTVYDPLQPELDSFKEELDKYFNHTSPDVLGDISGINASVWNIQKEIDRLNEVAKNLNES	1178
SARS-CoV-2	TDNTFVSGNCDVVIGIINNTVYDPLQPELDSFKEELDKYFNHTSPDVLGDISGINASVWNIQKEIDRLNEVAKNLNES	1196
SARS-CoV	LIDLQELGKYEQYIKWPYVWLGFIAGLIAIVMVTILLCCMSTCCSCLKGACSCGSCCKFDEDDSEPVKGVKLYHT	1255
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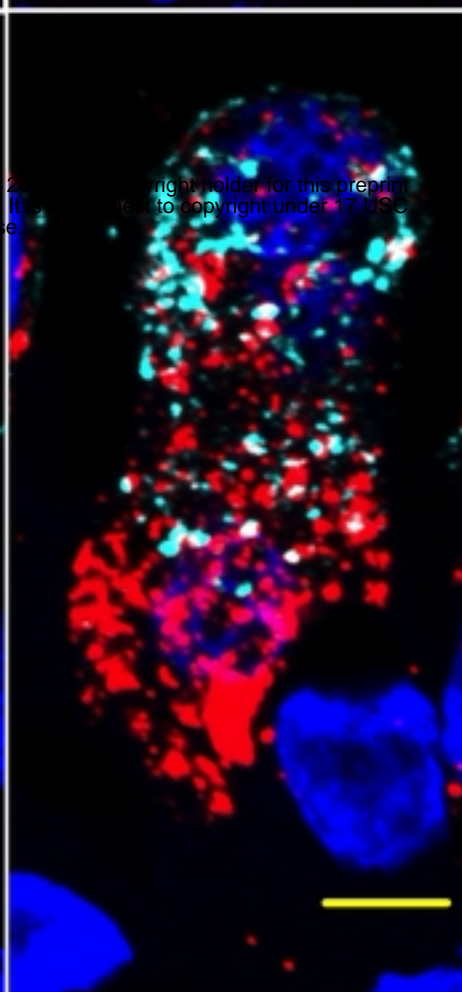
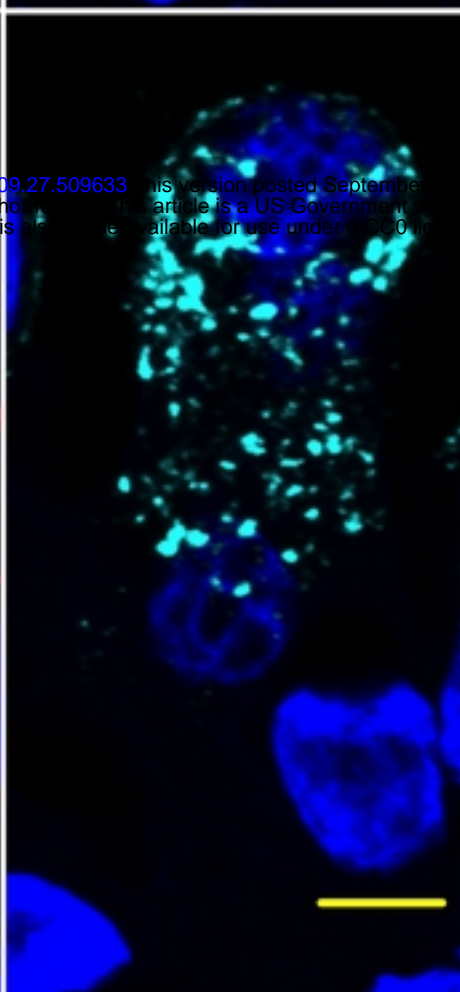
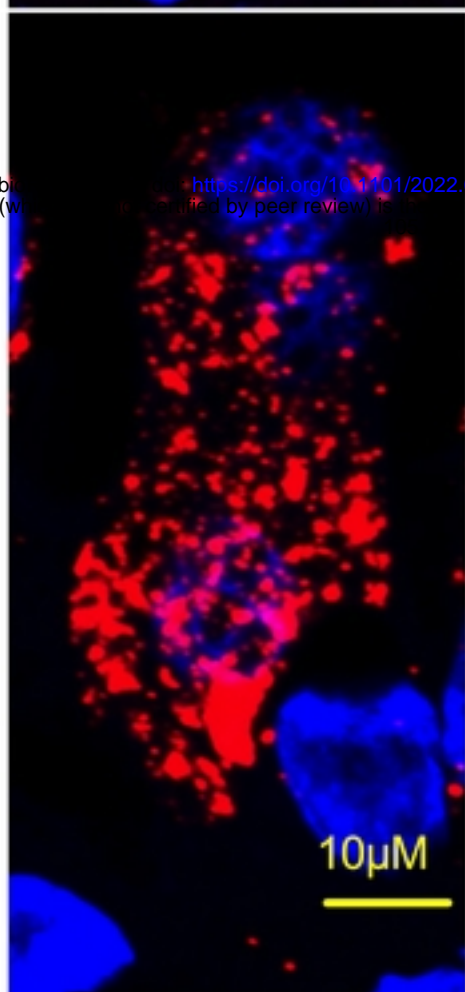
**Fig 1**

Fig 2

SARS-CoV-2 infected airway epithelium  
(donor #1) at an MOI = 0.1 for 4 days



SARS-CoV-2 infected airway epithelium  
(donor #2) at an MOI = 0.1 for 4 days



SARS-CoV-2 infected airway epithelium  
(donor #3) at an MOI = 0.1 for 4 days

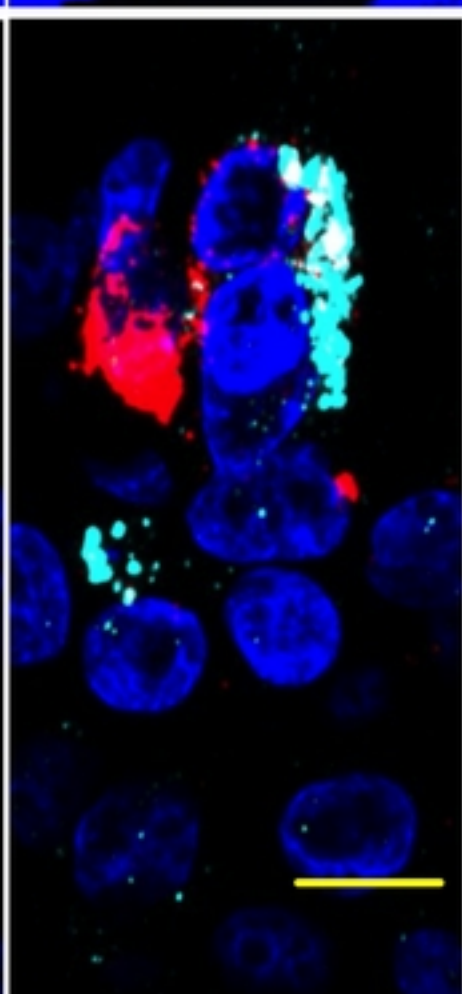
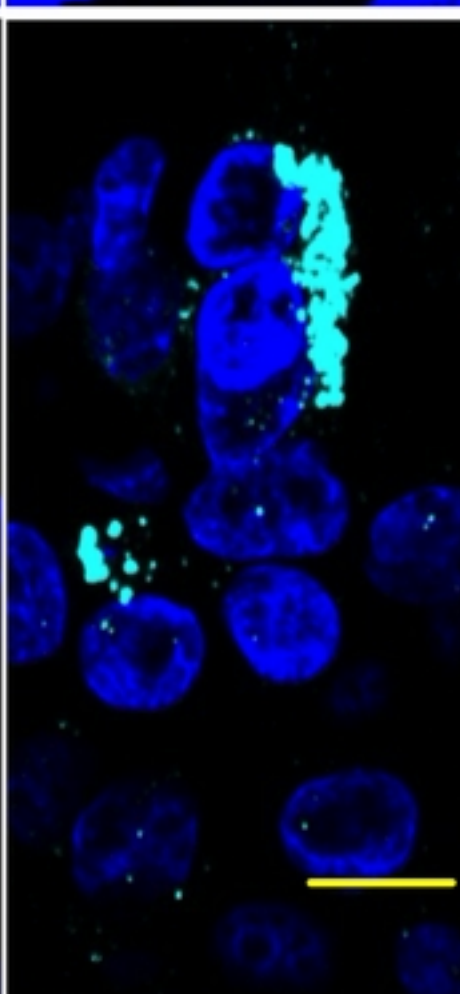
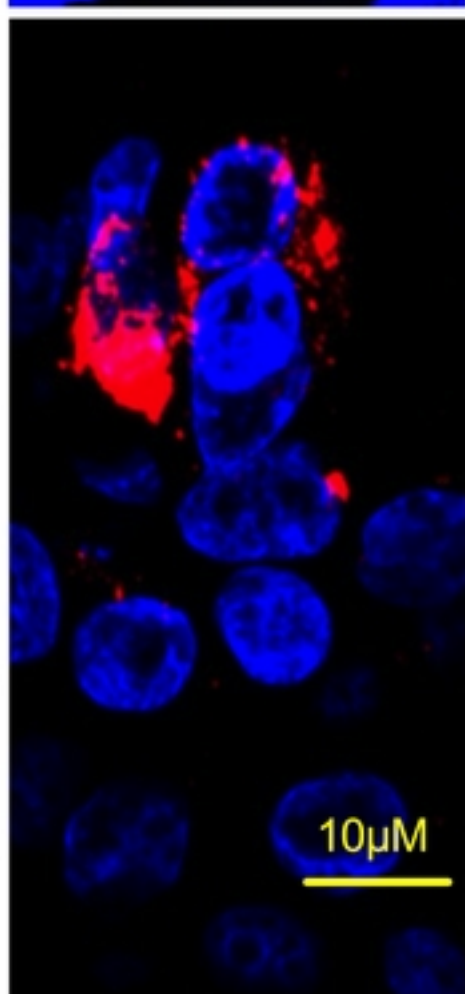


Fig 2

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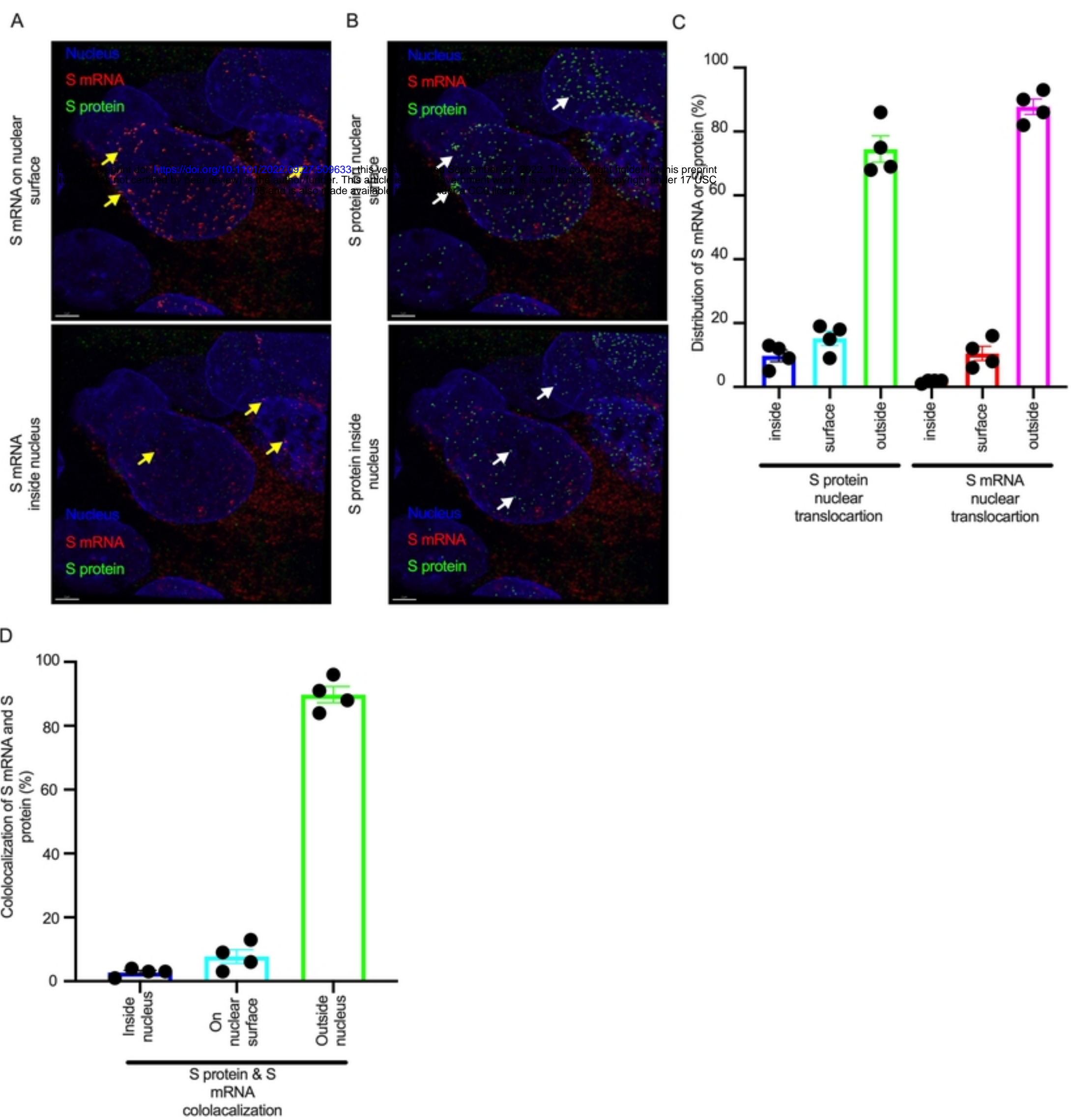


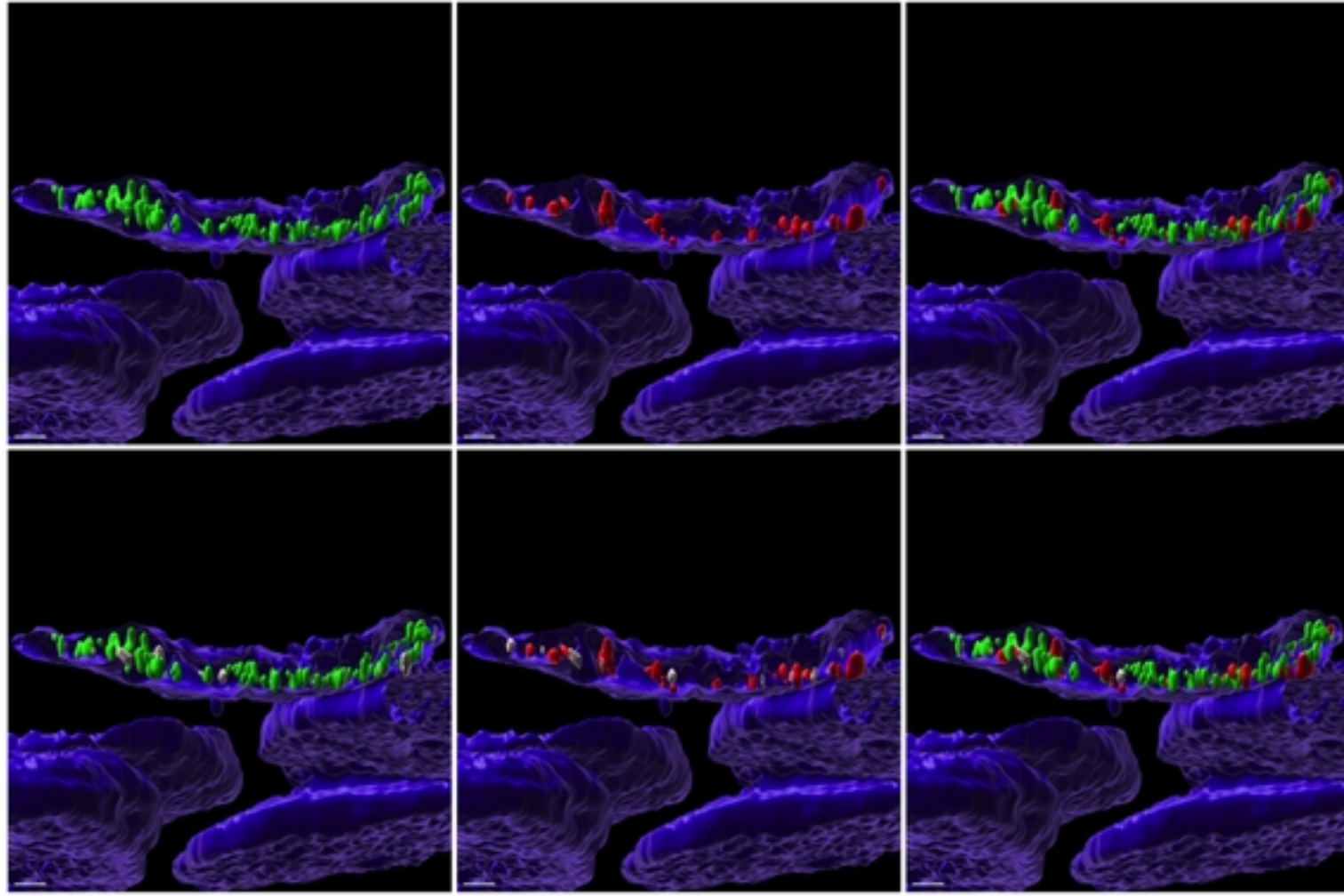
Fig 3

S mRNA & S protein colocalization

Inside nucelus

Colocalization spot

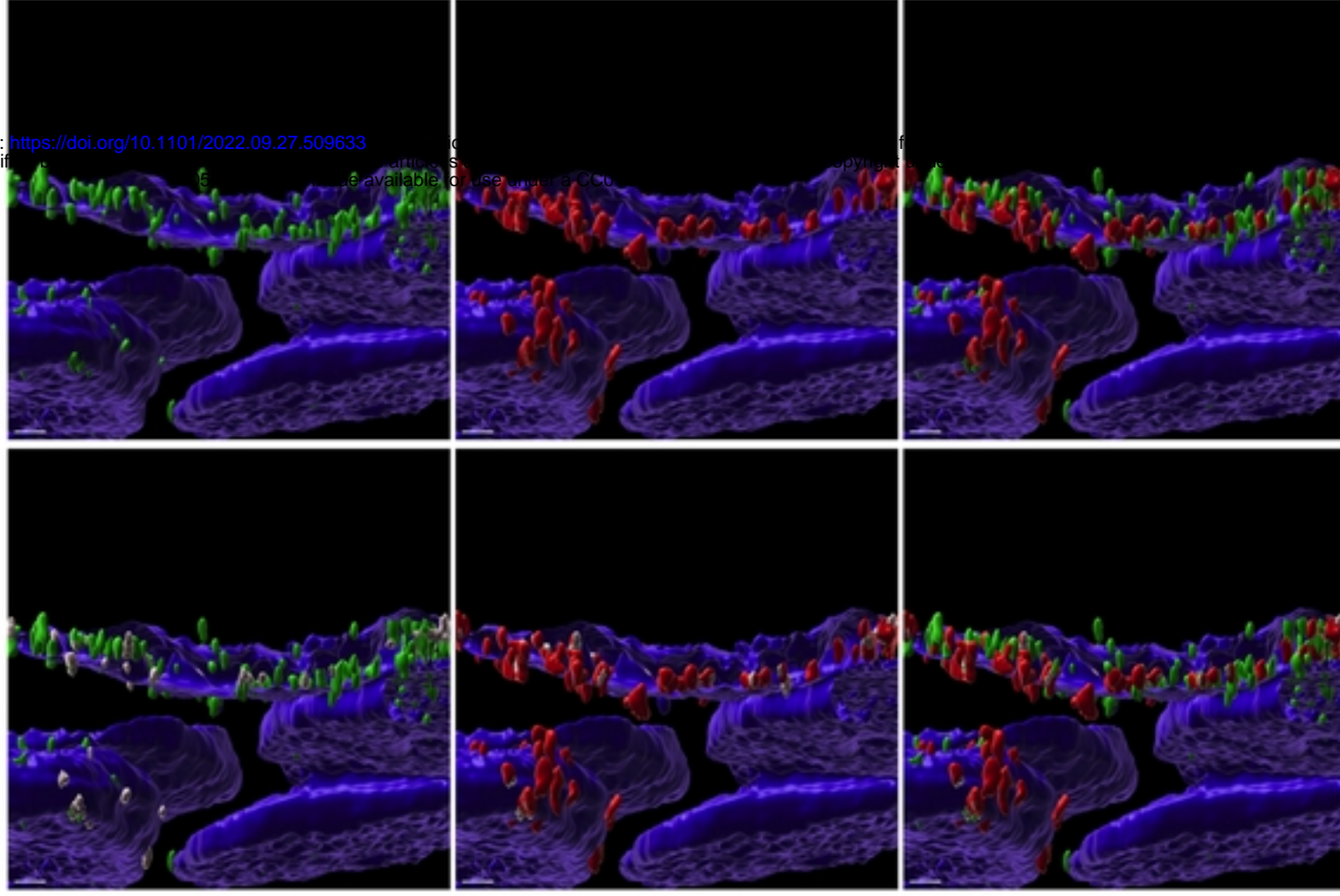
Detection



Nuclear surface

Colocalization spot

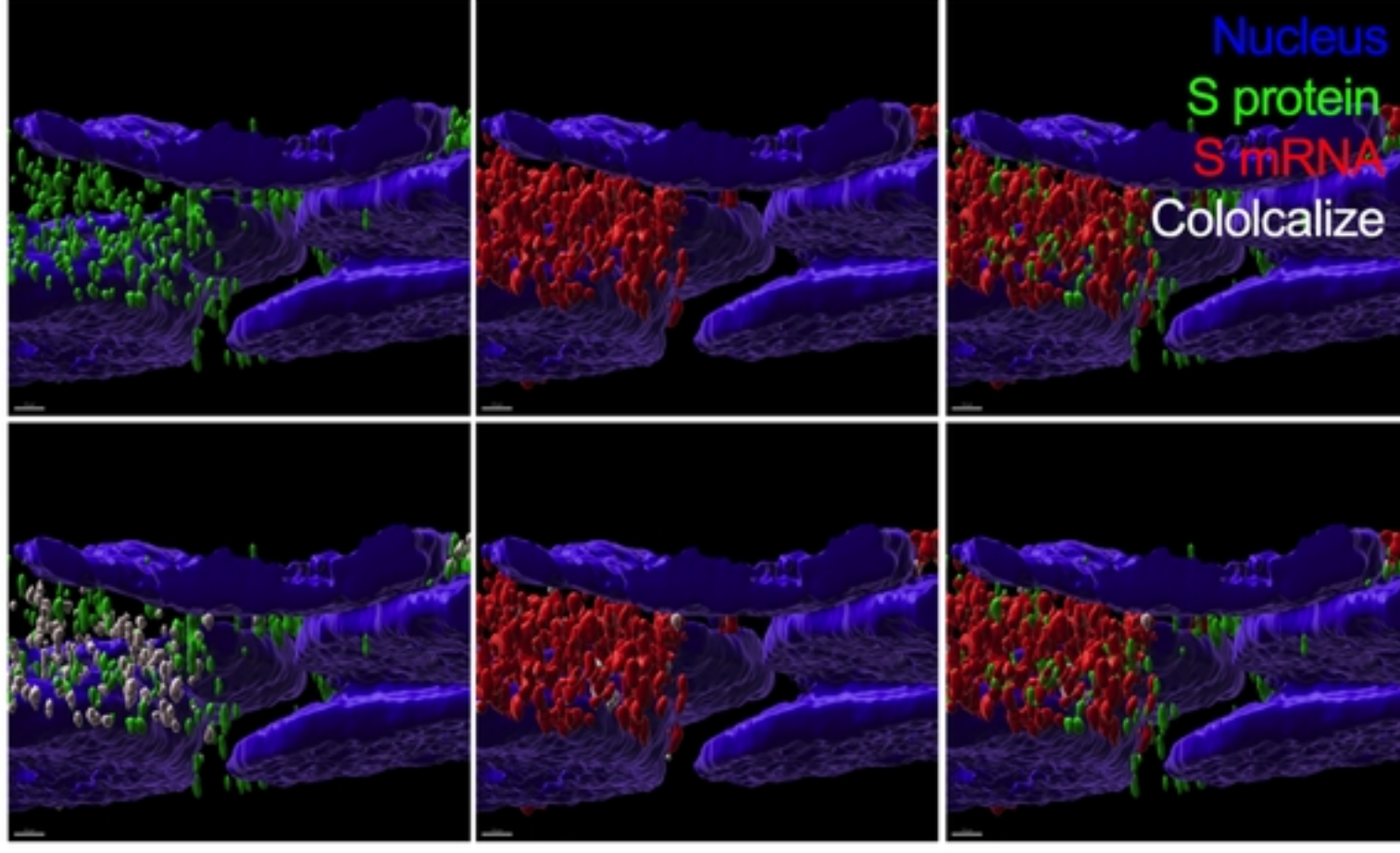
Detection



Cytoplasm

Colocalization spot

Detection



S protein

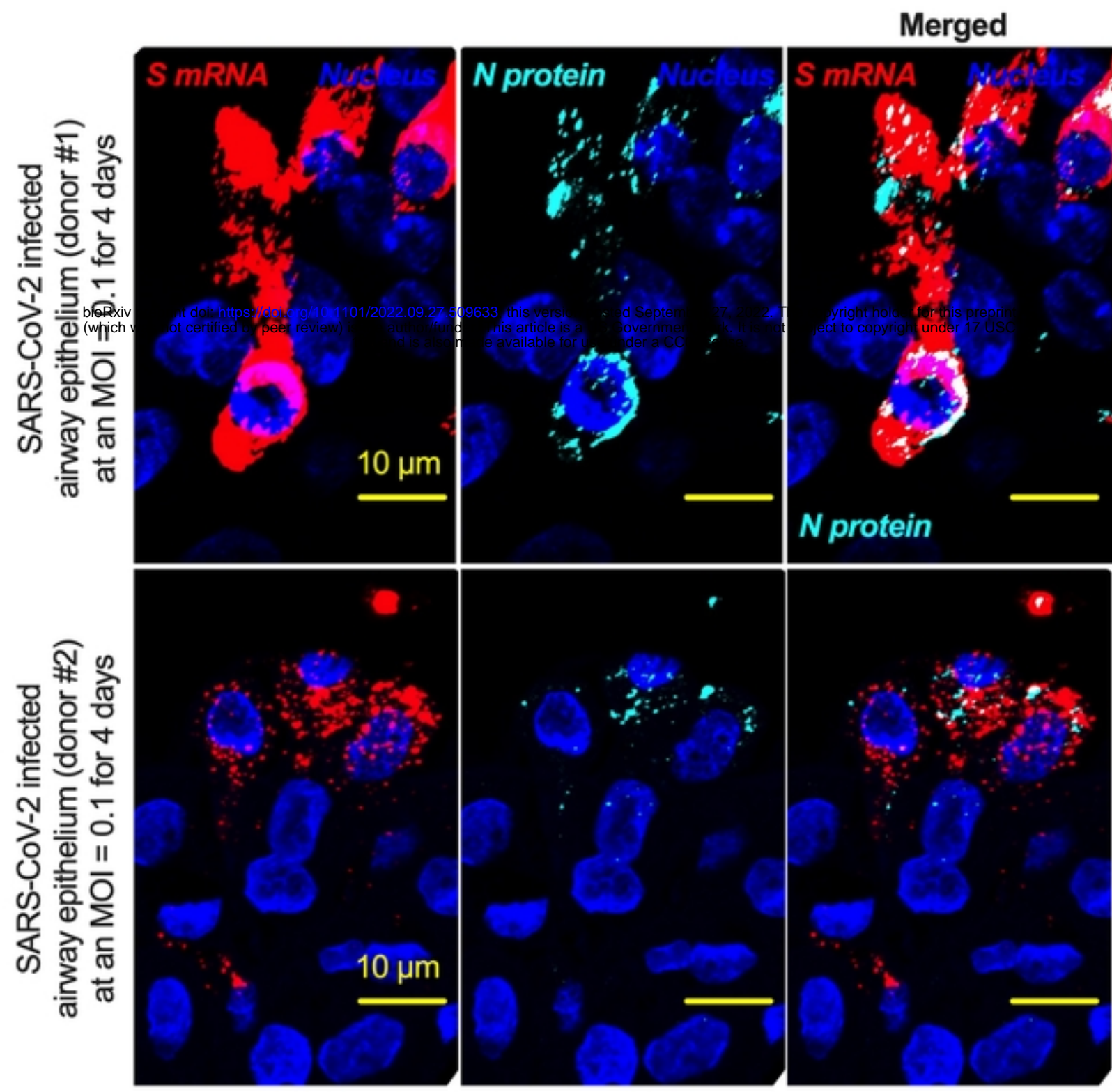
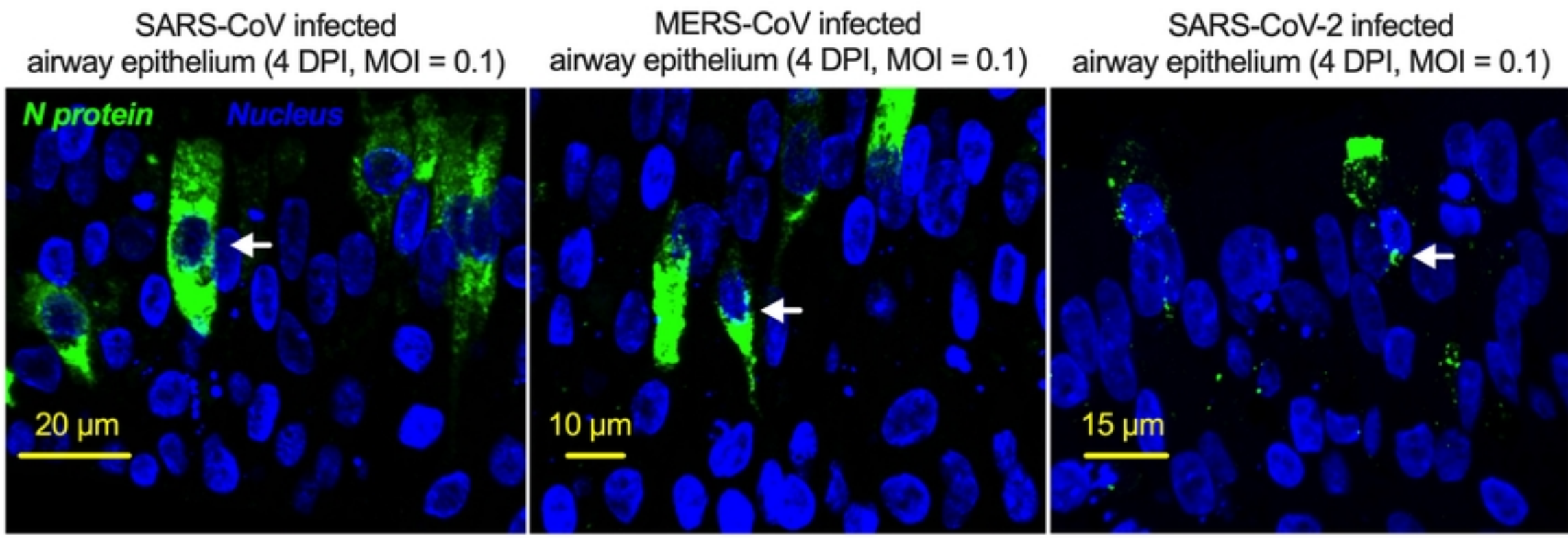
S mRNA

Merged

Nucleus  
S protein  
S mRNA  
Colocalize

Fig 4

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**A****B****Fig 5**