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**The M, E and N structural proteins of the SARS coronavirus are required for efficient assembly, trafficking and release of virus-like particles.**

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## Abstract

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27

28 Production of virus-like particles (VLPs) constitutes a relevant and safe model to  
29 study molecular determinants of virion egress. The minimal requirement for  
30 assembly of VLPs for the coronavirus responsible for severe acute respiratory  
31 syndrome in humans (SARS-CoV) is still controversial. Recent studies have  
32 described that SARS-CoV VLP formation depends on either M and E or M and N  
33 proteins. Here we show that both E and N must be co-expressed with M for  
34 efficient production and release of VLPs by transfected Vero E6 cells. This result  
35 suggests that the mechanism of SARS-CoV assembly differs from other studied  
36 coronaviruses, which only require M and E for VLP formation. When co-  
37 expressed, the native envelope trimeric S glycoprotein is incorporated onto VLPs.  
38 Interestingly, when a fluorescent protein tag is added to the C-terminal end of N or  
39 S, but not M, the chimeric viral proteins can be assembled within VLPs and allow  
40 visualization of VLP production and trafficking in living cells by state of the art  
41 imaging technologies. Fluorescent VLPs will be used further to investigate the role  
42 of cellular machineries during SARS-CoV egress.

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47 **Keywords:** SARS coronavirus, virus-like particle, virus assembly, virus budding,  
48 egress, fluorescent viral particles

49

## Introduction

50

51

52 Coronaviruses are positive-sense RNA enveloped viruses that belong to the  
53 *Coronaviridae* family in the Nidovirales order. These viruses are found in a wide  
54 variety of animals and can cause respiratory and enteric disorders of diverse  
55 severity (15, 18). In the past five years, several human and animal coronaviruses  
56 have been discovered, including the highly pathogenic virus responsible for the  
57 severe acute respiratory syndrome (SARS-CoV) (34, 58, 60, 64, 68, 69).  
58 Coronavirus particles consist of a helical nucleocapsid structure, formed by the  
59 association between nucleocapsid (N) phosphoproteins and the viral genomic  
60 RNA, which is surrounded by a lipid bilayer where three or four types of structural  
61 proteins are inserted: the spike (S), the membrane (M), the envelope (E) proteins  
62 and, for some coronaviruses only, the hemagglutinin-esterase (HE) protein (see  
63 for review: (12). Once sufficient amounts of new genomic RNA and structural  
64 proteins have been produced, assembly of particles occurs. Assembly and release  
65 of virions are the last stages of the virus life cycle.

66 The triple spanning membrane glycoprotein M drives assembly of coronavirus,  
67 which bud into the lumen of the endoplasmic reticulum-Golgi intermediary  
68 compartment (ERGIC) (32, 33, 62, 63). M is the most abundant envelope protein  
69 that sorts viral components to be incorporated into virions. M oligomerisation,  
70 mainly driven by its transmembrane domain, is believed to allow formation of a  
71 lattice of M proteins at ERGIC membranes (14, 41). S and E membrane proteins  
72 are integrated into the lattice through lateral interactions with M, whereas N and  
73 viral RNA interact with M C-terminal domain, which is exposed to the cytosol (4, 8,  
74 13, 19, 30, 36, 48, 54, 55). The coronavirus S protein, responsible for receptor

75 binding and membrane fusion, does not seem to have any major role in  
76 coronavirus assembly. Recent studies show that E is a viroporin that forms ion  
77 channels (46, 66, 67). Despite its minor incorporation into virion particles (7, 22,  
78 40), the small E protein plays an important but not fully understood role in virus  
79 morphogenesis and budding (20, 35, 70). Studies performed on coronaviruses,  
80 including the SARS-CoV, demonstrate that depletion of the E gene from  
81 coronavirus genome strongly diminish virus growth and particle formation (9, 16,  
82 35, 37, 57). The N protein self-associates and encapsidates the RNA genome for  
83 incorporation into budding virions through interactions with the M protein  
84 independently of E and S proteins (24, 52, 53, 61). For SARS-CoV, the interaction  
85 of N with M was described to be independent of viral RNA (25, 45).

86 Work on mouse hepatitis virus (MHV), bovine coronavirus (BcoV), infectious  
87 bronchitis virus (IBV) and transmissible gastroenteritis virus (TGEV) has  
88 established that E and M proteins are necessary and sufficient for assembly of  
89 virus-like particles (VLPs), which share size and morphological features with real  
90 viruses (1, 2, 7, 8, 38, 65). Nevertheless, the minimal requirement for assembly of  
91 SARS-CoV VLPs is still controversial. Huang Y. and colleagues described  
92 formation of VLPs in transfected human 293 renal epithelial cells that only required  
93 co-expression of the M and N viral proteins (29). On the contrary, other studies  
94 described that co-expressed M and E proteins were sufficient for release of  
95 sedimentable particles from transfected mammalian cells (27) or insect cells, using  
96 a baculovirus expression system (26, 50). A few groups have proposed  
97 immunization with SARS-CoV VLPs as an effective vaccine strategy. VLPs  
98 produced in insect cells or chimeric MHV/SARS-CoV VLPs produced in  
99 mammalian cells were used in these studies (42, 44).

100 Our objective was to delineate the molecular mechanisms that regulate SARS-  
101 CoV egress in mammalian cells. Here we demonstrate that whereas VLPs could  
102 hardly be recovered from culture medium of cells co-expressing combinations of M  
103 and E or M and N proteins, co-expression of both SARS-CoV E and N with M  
104 allowed release of significant levels of VLPs within one day. When co-expressed,  
105 the trimeric S protein was found in VLPs. Therefore, in apparent contrast to other  
106 coronaviruses, SARS-CoV egress strongly depends on three structural proteins:  
107 M, E and N. Addition of fluorescent tags to viral structural proteins allowed us to  
108 visualize egress of fluorescent VLPs in living cells. Monitoring of VLPs egress in  
109 living cells will be a powerful new tool to study the involvement of viral and cellular  
110 factors during the late stages of the SARS-CoV life cycle.

111

## Materials and Methods

112

113

114 **Cells and culture conditions.** The Vero E6 African Green Monkey kidney cell line  
115 was cultured in Dulbecco's modified Eagle's medium supplemented with 10%  
116 heat-inactivated foetal calf serum, 100 U of penicillin and 100 µg of streptomycin  
117 per ml, at 37°C with 5% CO<sub>2</sub>.

118

119 **Plasmid constructions.** CDNAs coding for the SARS-CoV M, E, S and N  
120 structural proteins were codon-optimized for mammalian cells and synthesized by  
121 GeneArt (Regensburg, Germany). The M cDNA was amplified by PCR and sub-  
122 cloned into the pIRES plasmid vector (BD Biosciences, San Jose, CA, USA)  
123 between the Nhe I and EcoR I restriction sites of the upstream multiple cloning  
124 site. The E cDNA was amplified by PCR and inserted either into the pcDNA3.1  
125 plasmid vector between the Kpn I and Not I restriction sites or into the pIRES  
126 plasmid vector between the Sal I and Not I restriction sites of the downstream  
127 multiple cloning site. The S cDNA was amplified by PCR and inserted either into  
128 the pcDNA3.1 plasmid vector between the Nhe I and Apa I restriction sites. The  
129 pcDNA-Nflag plasmid was constructed from the original pCRScript-Nflag  
130 (produced by GeneArt, Regensburg, Germany) using the Kpn I and Xho I  
131 restriction sites. The Flag tag was in fusion with the 3' end of N cDNA and  
132 separated from N cDNA by six nucleotides encoding two glycine residues. The  
133 cDNAs coding for the enhanced yellow (eYFP), cyan (eCFP), green (eGFP)  
134 fluorescent proteins and the monomeric red fluorescent protein (mRFP1, (5), were  
135 amplified by PCR from plasmids purchased from Clontech Laboratories (Takara  
136 Bio, Shiga, Japan) and subcloned into pcDNA3.1 or pIRES vectors. Then S, N or

137 M cDNAs were inserted in 5' of the fluorescent protein cDNAs using Cla I and Apa  
138 I sites (S and N) or Xho I and Mlu I (M) restriction sites. The two fused cDNAs  
139 were separated by two codons encoding glycines.

140

141 **Antibodies.** The M and E proteins were detected with rabbit polyclonal antibodies  
142 raised against the C-terminal extremity of each protein. The rabbit polyclonal  
143 antibody against the M C-terminal domain was purchased from ProSci  
144 Incorporated (Poway, CA, USA). The rabbit polyclonal antibody against the E  
145 protein was produced by Dr Nicolas Escriou (Institut Pasteur, Paris) using a C-  
146 terminal peptide. The Flag tag was detected with the mouse monoclonal IgG1 M2  
147 antibody purchased from Sigma-Aldrich. The mouse polyclonal antibody against  
148 the S protein was obtained by immunizing mice with purified S trimers expressed  
149 in mammalian cells as described previously (31). The mouse monoclonal antibody  
150 against the N protein was a generous gift from Dr Chan KH (Department of  
151 Microbiology, the University of Hong Kong) and produced as described in (56).

152

153 **Transient transfections and production of SARS-CoV VLPs.** Eight hundred  
154 thousand cells were plated in 75 cm<sup>2</sup> dishes, incubated over-night and transfected  
155 with plasmid constructs using the FuGENE 6 transfection reagent (Roche, Basel,  
156 Switzerland), according to the manufacturer's instructions. Briefly, 54 µl of  
157 FuGENE 6 transfection reagent were mixed with DMEM, incubated for five  
158 minutes and then 6 µg of each plasmid were added. The FuGENE 6 / plasmid  
159 mixture was incubated for 30 minutes at room temperature. Cell medium was  
160 discarded and replaced with 3 ml of warm DMEM. The FuGENE 6 / plasmid  
161 mixture was added to the cells. After 3 hours of incubation at 37°C, the medium



162 containing the transfection mixture was discarded and 10 ml of fresh medium was  
163 added. Cells were incubated for 21 or 45 hours.

164

165 **Purification of SARS-CoV VLPs.** At 24 or 48 hours post-transfection, cell  
166 medium was collected and cleared by centrifugation at low speed (1000g for 10  
167 minutes) to pull-down cell debris. After passing through 0.45  $\mu\text{m}$  filter, cleared cell  
168 medium were then loaded on top of 20% sucrose cushions and ultra-centrifuged  
169 for 3 hours at 28,000 rpm using a SW41 rotor (Beckman Coulter Inc, Fullerton, CA,  
170 USA). VLP-containing pellets were resuspended in Tris HCl 50 mM NaCl 100 mM  
171 EDTA 0.5 mM pH7.4 (TNE) buffer.

172

173 **Western blot analysis of VLPs and cell lysates.** For Western blot analysis of  
174 purified VLPs, 15  $\mu\text{l}$  of resuspended pellets from ultracentrifuged culture medium  
175 were mixed with 5  $\mu\text{l}$  lithium dodecyl sulfate (LDS)-containing loading buffer and  
176 loaded on 4 to 12 % polyacrylamide gels (NuPAGE Novex Bis-Tris Mini Gels,  
177 Invitrogen, Carlsbad, CA, USA). Electrophoresis was performed with the NuPAGE  
178 MOPS SDS Running Buffer from the same manufacturer. Alternatively,  
179 resuspended pellets from 48 hours time points were loaded on top of 20 to 60%  
180 sucrose gradients and ultracentrifuged for 3.5 hours at 26,700 rpm (27). Twenty  
181 fractions were collected and analyzed by Western blot. For Western blot analysis  
182 of cell lysates, cells were washed twice with PBS 1X at 24 or 48 hours post-  
183 transfection, and lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl,  
184 20 mM Tris-HCl pH7.5 and 1 mM EDTA for 15 minutes on ice with frequent  
185 vortexing. Then lysates were cleared by centrifugation at 16100 g for 15 minutes  
186 at 4°C and analyzed by Western blot. 15  $\mu\text{l}$  of each lysate were mixed with 5  $\mu\text{l}$

187 LDS loading buffer and loaded on polyacrylamide gels. For detection of E but S,  
188 samples were treated with dithiothreitol and heated at 95°C for 5 minutes before  
189 migration on polyacrylamide gels. To detect both E and trimers of S from same  
190 preparations, samples were split and either treated or not before loading of two  
191 separate gels for E and S detection, respectively. Results for M and Nflag were  
192 similar with or without treatment.

193

194 **Electron microscopy.** For transmission electron microscopy experiments,  
195 transfected cells were harvested at 24 and 48 hours post-transfection. Cells were  
196 detached using 10 mM EDTA, fixed in 2.5 % glutaraldehyde in cacodylate buffer  
197 and post-fixed with 1% Osmium Tetroxide (OsO<sub>4</sub>) in Cacodylate buffer for 1 hour  
198 at room temperature. Then cells were embedded in 2% agarose to form cell blocks  
199 which were then dehydrated in graded series of ethanol and embedded in epoxy  
200 resin. Ultrathin sections were stained for 45 minutes with 2% aqueous uranyl  
201 acetate and for 30 minutes with Reynolds' lead citrate. For analysis of secreted  
202 VLPs, VLPs were purified from cell medium by ultracentrifugation on 20% sucrose  
203 cushion, separated on 20-60% discontinuous sucrose gradient and fraction 10 was  
204 collected and then concentrated by ultracentrifugation in TNE buffer, pH 7.4 for  
205 one hour at 28,000 rpm. 5µl of the VLP suspension, mixed with equal volume of  
206 negative stain (2% aqueous uranyl acetate and 2% phosphotungstate solution, pH  
207 7.0), was placed onto a formvar-carbon-coated copper grid for 2 minutes, excess  
208 suspension were drained. The grids were viewed and photographed with a Philip  
209 CM100 electron microscope at 80 kV.

210

211 **Fluorescence microscopy.** For fluorescence microscopy on fixed cells, Vero E6  
212 cells were grown on glass coverslips, transfected and analyzed at 24 hours post-  
213 transfection. Cells were washed with PBS, nuclei were stained with DAPI and  
214 coverslips were mounted on glass slides for analysis. Fixed cells were visualized  
215 under AxioObserver Z1 inverted motorized fluorescent microscope using the  
216 ApoTome module and piloted through the Axiovision 4.6 software and images  
217 were acquired through the MRm AxioCam high resolution CCD camera (Carl  
218 Zeiss, Germany). For fluorescence microscopy on live cells, Vero E6 cells were  
219 grown in glass-bottom culture dish (MatTech, USA), transfected and analyzed at  
220 24 hours post-transfection. Cells were washed and medium was changed to  
221 HBSS, Optimem pre-warmed culture medium. Widefield image acquisitions of live  
222 cells were obtained using the system described above. Confocal acquisitions of  
223 live cells were acquired at the Hong Kong University Core Imaging Facility using  
224 an AxioObserver Z1 inverted motorized fluorescent microscope equipped with a  
225 spinning disc confocal imaging system (UltraVIEW ERS, PerkinElmer, Shelton,  
226 CT, USA). For brefeldin A (BFA) treatment, cells were incubated with 6  $\mu\text{g}$  per ml  
227 of BFA for indicated times. To release BFA effect, cells were washed three times  
228 in PBS 1X and reincubated in normal medium for indicated times.  
229

## Results

230

231

232 **Efficient production and release of SARS-CoV VLPs are driven by co-**  
233 **expression of M, N and E structural proteins.**

234 To determine the optimal conditions for efficient SARS-CoV VLP production, we  
235 chose to co-express different combinations of structural proteins in the Vero E6  
236 African green monkey cell line, which is permissive to SARS-CoV replication (34).  
237 cDNAs encoding SARS-CoV structural proteins M, E and N were codon-optimized  
238 for expression in mammalian cells. Understanding the importance to maintain a  
239 low E/M ratio in transfected cells to ensure concomitant expression of E and M,  
240 low incorporation of E into VLPs and prevent potential formation of E-containing  
241 vesicles, we reasoned that the use of the pIRES bi-cistronic plasmid should be  
242 appropriate. This plasmid presents the double advantage to ensure co-expression  
243 of both cDNAs in transfected cell and allows a reduced rate of translation of the  
244 downstream gene by utilizing a partially disabled internal ribosome entry site  
245 (IRES) sequence. We therefore subcloned M upstream of E cDNA in the pIRES  
246 vector. N was expressed from a pcDNA plasmid with a C-terminal Flag tag. Vero  
247 E6 cells were either not transfected or transfected with pcDNA-E, pIRES-M or  
248 pIRES-M-E alone, pIRES-M plus pcDNA-Nflag and pIRES-M-E plus pcDNA-Nflag  
249 (Figure 1A). At 24 and 48 hours post-transfection culture medium and cells were  
250 harvested. Culture medium was subjected to ultracentrifugation on a 20% sucrose  
251 cushion to isolate VLPs and the SARS-CoV structural proteins assembled into  
252 VLPs were analyzed by Western blot.

253 The M protein was readily detected at both 24 and 48 hours post-transfection in  
254 lysates from Vero E6 cells transfected with pIRES-M, pIRES-M-E, pIRES-M plus

255 pcDNA-Nflag and pIRES-M-E plus pcDNA-Nflag (Figure 1A, upper panels). As  
256 described previously, three major forms of M, which correspond to different  
257 glycoforms, were detected on SDS-PAGE: two bands at ~ 18 and 28 KDa and a  
258 smear at higher molecular sizes reflecting heterogeneity of glycosylation patterns  
259 (51). As expected, higher levels of M were detected in cell lysates at 48 hours than  
260 at 24 hours post-transfection. Whereas a 10 KDa protein corresponding to E could  
261 be easily detected in cell lysates from cells transfected with pcDNA-E plasmid, its  
262 expression was much lower in cells transfected with the pIRES-M-E bi-cistronic  
263 vector. The Nflag protein was found in cell lysates from both pIRES-M plus  
264 pcDNA-Nflag and pIRES-M-E plus pcDNA-Nflag transfected cells at similar levels.  
265 A major band corresponding to a protein with an apparent molecular size of 45  
266 KDa was detected.  
267 Strikingly, the efficacy of VLP production was dramatically affected by the  
268 combination of viral proteins co-expressed (Figure 1A, lower panels). Although M  
269 was not detected in ultracentrifuged culture medium from pIRES-M, pIRES-M-E or  
270 pIRES-M plus pcDNA-Nflag transfected cells at 24 hours post-transfection,  
271 significant levels were found in preparations from pIRES-M-E plus pcDNA-Nflag  
272 transfected cells. Similarly, the N protein was only detected in ultracentrifuged  
273 culture medium from pIRES-M-E plus pcDNA-Nflag transfected cells at this early  
274 time point. Signals for E were below the limit of detection at 24 hours and  
275 detectable only at 48 hours. Finding low amounts of E in VLPs is in accordance  
276 with the minor presence of this protein in coronavirus particles, despite its  
277 important role for virion assembly and budding (9, 20, 37, 57, 65). In conclusion,  
278 only when M, E and N proteins were co-expressed, VLPs that contained the M and  
279 N proteins could be isolated from culture medium at 24 hours post-transfection.

280 At 48 hours post-transfection, E was found in ultracentrifuged culture medium from  
281 cells transfected with the pcDNA-E plasmid alone. This is in agreement with  
282 previously published data that describe secretion of E proteins independently of  
283 other viral elements (7, 47, 50). At this time point, trace amounts of M were  
284 detected in ultracentrifuged culture medium from pIRES-M and pIRES-M-E  
285 transfected cells, whereas significant levels of M and N were found for pIRES-M  
286 plus pcDNA-Nflag ultracentrifuged culture medium. Independent secretion of  
287 SARS-CoV M proteins from Vero E6 cells as well as production of M-E VLPs was  
288 described by Mortola and Roy (2004) at four days post-transfection. Production of  
289 SARS-CoV M-N VLPs has already been reported by Huang Y. and colleagues in  
290 transfected 293 renal epithelial cells at 63 hours post-transfection (29). Very  
291 interestingly, in our conditions, significantly higher levels of M and N proteins were  
292 found in purified VLPs from pIRES-M-E plus pcDNA-Nflag than pIRES-M plus  
293 pcDNA-Nflag transfected cells at 48 hours post-transfection. We were also able to  
294 detect E in these conditions, but not when cells were only transfected with the  
295 pIRES-M-E vector.

296 In order to verify that inefficacy of production of M-E VLPs by cells transfected with  
297 pIRES-M-E was not due to inefficient E expression, we performed similar  
298 experiments but expressed E from a pCDNA-E plasmid and analyzed cells and  
299 medium at 48 hours post-transfection (Figure 1B). As expected, higher levels of E  
300 were detected in lysates of cells transfected with pIRES-M plus pCDNA-E and  
301 pIRES-M plus pCDNA-E plus pCDNA-Nflag than pIRES-M-E and pIRES-M-E plus  
302 pCDNA-Nflag, respectively (Figure 1B, left panel). Nevertheless, neither  
303 production of M-E VLPs nor M-E-N VLPs were improved by increase of E  
304 expression (Figure 1B, right panel). On the contrary, production of M-E-N VLPs

305 was enhanced when the bicistronic vector was used, as indicated by higher levels  
306 of Nflag, M and E proteins secreted, and the ratio E(medium)/E(lysate) was  
307 significantly higher in these conditions. This result suggests that expression of E  
308 along with M from the pIRES-M-E bicistronic vector and in combination with N is  
309 favorable to the production of SARS-CoV VLPs.

310 We then investigated co-sedimentation of secreted viral structural proteins by  
311 performing sucrose gradient fractionation on ultracentrifuged cell medium. Cells  
312 were either transfected by individual plasmids or combinations of plasmids as  
313 described previously. In this experiment we used the individual plasmid for E  
314 expression to verify potential presence of E-containing vesicles in medium when E  
315 was expressed at higher levels. At 48 hours post-transfection, cleared cell medium  
316 was ultracentrifuged on 20% sucrose cushion and pellets were resuspended in  
317 TNE buffer. As controls, viral proteins contained in cell lysates and pellets from  
318 ultracentrifuged medium were analyzed by Western blot (Figure 1C, left panel).  
319 Pellets were loaded on top of 20-60% discontinuous sucrose gradients. After  
320 another round of ultracentrifugation, 20 fractions were collected and analyzed by  
321 Western blot (Figure 1C, right panels a to e). Results were consistent with the data  
322 described in Figure 1 A and B. When M was expressed alone, very little amounts  
323 of protein were found in cell medium and traces were detected in fraction 9 (Figure  
324 1C, panel a). Coexpression with E or Nflag, allowed secretion of slightly higher  
325 levels of M in cell medium and detection of M in fractions 7 to 11, with enrichment  
326 in fraction 10 (Figure 1C, panel c) or 11 (Figure 1C, panel d), respectively. E  
327 protein was not detectable in fractions from medium of either pCDNA-E or pIRES-  
328 M plus pCDNA-E transfected cells (Figure 1C, panel b and c). Nflag cosedimented  
329 with M when co-expressed (Figure 1C, panel d). As expected, greater levels of M,

330 E and Nflag proteins were found in medium of cells expressing all three viral  
331 proteins (Figure 1C, panel e). M and Nflag were present in fractions 5 to 14 with  
332 proportional levels. E was detected in fractions 8 to 10 where M and N proteins  
333 were also enriched. Altogether, these results show that secreted viral structural  
334 proteins cosediment in sucrose gradient and therefore strongly suggest that viral  
335 proteins are associated into VLPs. Moreover, secretion of E-containing vesicles  
336 does not seem to be a major phenomenon in Vero E6 cells expressing E alone or  
337 in combination with other viral proteins in our system. Indeed, in all conditions,  
338 levels of E in cell medium were systematically low and only detected in fractions  
339 when co-expressed with M and N.

340 Altogether these data establish for the first time that all three M, E and N structural  
341 SARS-CoV proteins are important for efficient production and release of SARS-  
342 CoV VLPs.

343

#### 344 **S is incorporated onto SARS-CoV M-E-N-containing VLPs.**

345 We then investigated whether the SARS-CoV S glycoprotein could be  
346 incorporated onto secreted M-E-N VLPs. S cDNA was codon optimized and  
347 subcloned in the pcDNA vector. Vero E6 cells were transfected with pIRES-M-E  
348 plus pcDNA-Nflag plus pcDNA-S. Protein expression was verified in cell lysates at  
349 48 hours post-transfection (data not shown). To monitor association of S onto M-  
350 E-N VLPs, we performed sucrose fractionation on VLPs previously purified from  
351 culture medium (Figure 2). All S, Nflag, M and E proteins were co-sedimented and  
352 enriched in fractions 9 and 10 corresponding to ~40% sucrose. It is notable that S  
353 was mainly detected in its native trimeric form in these fractions (540 KDa),  
354 although we also detected low levels of monomers (180 KDa) and dimers (360



355 kDa), which may have resulted from sample treatment and SDS-PAGE conditions.  
356 Higher molecular weight proteins, detected with anti-S antibodies, were found in  
357 the lightest fractions 1 to 6. These forms could correspond to soluble, non-  
358 incorporated forms of S proteins that have formed aggregates. We have previously  
359 described formation of S aggregates in preparations of purified SARS-CoV S  
360 trimers from mammalian cell lysates (31). This result confirms association of all  
361 four SARS-CoV structural proteins into S-M-E-N VLPs that can be purified from  
362 culture medium of transfected cells.

363

364 **VLPs bud into a perinuclear compartment and are transported within**  
365 **vesicles to the plasma membrane.**

366 We next studied the subcellular localization of SARS-CoV VLPs by electron  
367 microscopy (Figure 3). pIRES-M-E plus pcDNA-Nflag plus pcDNA-S co-  
368 transfected Vero E6 cells were fixed at 24 and 48h post-transfection and ultrathin  
369 sections were prepared for transmission electron microscopy (Figure 3A to G).  
370 Large amounts of VLPs, with diameters ranging from 40 to 150 nm on sections,  
371 were found within the cytoplasm of positive cells. Three main patterns of  
372 subcellular localization were observed. First, VLPs were found within perinuclear  
373 compartments, which had appearance of groups of vacuoles (Figure 3A, C and D),  
374 or, alternatively but seldom observed, which presented morphological  
375 characteristics of the endoplasmic reticulum (Figure 3B). Vacuoles were observed  
376 at both 24 hours and 48 hours post-transfection with a diameter ranging from 300  
377 nm to 1.5  $\mu$ m on sections. Very interestingly, we detected several events of VLP  
378 budding at vacuole membranes (Figure 3D). Although VLPs accumulated therein,  
379 they were not highly compacted in this compartment. Moreover, VLPs appeared

380 pleiomorphic and heterogenous material was also observed (Figure 3D). Tubular  
381 structures were seen within some VLP-containing vacuoles, mainly at 24 hours  
382 post transfection (data not shown). Second, VLPs were observed in vesicles  
383 scattered in the cytoplasm (Figure 3A, B, C and E). These vesicles had a diameter  
384 ranging from 200 nm to 1  $\mu$ m on sections and VLPs were more compacted inside.  
385 They could be found at proximity of the perinuclear VLP-containing compartments  
386 up to the cortical area. VLPs contained in these vesicles looked more  
387 homogenous in size and shape. Third, VLPs were occasionally found at the cell  
388 surface (Figure 3A, F and G). Although we could not observe obvious spike-like  
389 structures surrounding the VLP envelope, spike-like protuberances were  
390 occasionally detected (Figure 3G). Moreover, binding of VLPs to cell surface  
391 suggests the presence of spikes and receptor recognition. Some cells, in which  
392 very high amounts of VLPs were found, presented characteristics of apoptosis with  
393 fragmented nucleus and disrupted membranes (data not shown). The cytoplasm of  
394 these cells was filled up with VLPs, either free or within intracellular compartments.  
395 The morphology of released VLPs was investigated further by electron microscopy  
396 on negatively stained particles that were purified from cell medium (Figure 3F).  
397 Round-shaped particles with a diameter of 80-100 nm were readily observed.  
398 Again, we could not observe the typical corona of spikes around VLPs (21) but  
399 globular structures protruding from VLPs were detected, which likely corresponded  
400 to trimers of spikes. Altogether, our data suggest that SARS-CoV VLPs bud in an  
401 intracellular compartment of Vero E6 cells and are efficiently transported within  
402 vesicles to the plasma membrane where they are released. Shape and size of  
403 secreted VLPs are in accordance with the morphological characteristics of the

404 SARS-CoV and therefore these VLPs should be a safe and appropriate model to  
405 study assembly and release of SARS-CoV virions.

406

407 **Assembly and release of VLPs that incorporate fluorescently-tagged**  
408 **structural viral proteins.**

409 In order to visualize in real time, the assembly, trafficking and release of SARS-  
410 CoV VLPs, we engineered plasmid constructs that allow expression of viral  
411 proteins in fusion with fluorescent proteins. The pIRES-MmRFP1-E, pcDNA-  
412 NeCFP and pcDNA-SeYFP constructs were developed and expression of fusion  
413 viral proteins was analyzed in individually transfected or co-transfected cells by  
414 fluorescence microscopy (Figure 4). We did not include any construct coding for a  
415 fluorescently tagged SARS-CoV E protein because it does not tolerate fluorescent  
416 protein tags (C. Chan et al., unpublished data). All chimeric viral proteins could be  
417 readily observed by fluorescent microscopy. In cells individually transfected with  
418 the pIRES-MmRFP1-E, the MmRFP1 fusion protein was mainly present in a  
419 perinuclear compartment, most likely the ERGIC/Golgi apparatus (Figure 4, panels  
420 a,b). We also found MmRFP1 within the cytoplasm and occasionally at the plasma  
421 membrane. As expected, the SeYFP protein was observed both in the  
422 ERGIC/Golgi apparatus, and at the plasma membrane of individually transfected  
423 cells (Figure 4, panels c,d). These expression patterns are similar to what we  
424 observed previously for MeGFP and SeGFP fusion proteins at 15 hours post-  
425 infection with Semliki Forest Virus expression vectors (51). In most of the cells,  
426 individually expressed NeCFP protein formed bright cytosolic patches, suggesting  
427 aggregation of the protein in the cytosol in absence of functional viral protein  
428 partners (Figure 4 e,f). This pattern may reflect large inclusions of nucleocapsids,

429 which have been described to accumulate late in the infection of cells with HCoV  
430 and MHV-JHM and SARS-CoV (6, 17, 23) or may constitute a cytosolic reservoir  
431 of protein supposed to feed the viral budding system. Interestingly, when plasmids  
432 were co-transfected, all three MmRFP1, SeYFP and NeCFP fluorescent proteins  
433 presented similar intracellular distributions, co-localizing in the cytoplasm and at  
434 the plasma membrane (Figure 4 panels g,i,k,m and h,j,l,n). When co-expressed  
435 with MmRFP1, E and SeYFP, the subcellular distribution of the NeCFP protein  
436 was dramatically changed and bright cytosolic patches were rarely found (Figure  
437 4, panels e,f and k,l). Trafficking of NeCFP to the cell surface in co-transfected  
438 cells suggests that interactions with other viral proteins have occurred leading to  
439 NeCFP translocation. Nevertheless no VLPs were detected in medium from co-  
440 transfected cells (data not shown). Altogether, these results suggest that although  
441 MmRFP1, SeYFP, NeCFP and E are likely to interact when co-expressed in Vero  
442 E6 cells, they are not released in the form of VLPs in cell medium.

443 We then reasoned that fluorescent VLPs could be produced by including only one  
444 plasmid coding for one of the fluorescently tagged viral protein per co-transfection.

445 In these conditions, we investigated protein expression and release of VLPs by  
446 Vero E6 cells at 48 hours post-transfection (Figure 5A). As positive control, we  
447 monitored VLP release from pIRES-M-E, pcDNA-Nflag, pcDNA-S transfected  
448 cells. As expected, S, Nflag, M and E proteins were detected in both cell lysate  
449 and VLP preparation (Figure 5A, lane 2 and 7). Although the MmRFP1 fusion  
450 protein was readily observed by fluorescent microscopy, it could not be detected  
451 by Western blot. Most likely, the rabbit polyclonal antibody directed against the M  
452 C-terminal domain cannot recognize its epitope when it is fused to the mRFP1  
453 fluorescent tag. Although the E, Nflag and S proteins were detected on cell lysate

454 from pIRES-MmRFP1-E plus pcDNA-Nflag plus pcDNA-S transfected cells (Figure  
455 5A, lane 3), no protein corresponding to purified VLPs were found in medium  
456 (Figure 5A, lane 8). We concluded that fusion of the mRFP1 protein at the C-  
457 terminal end of M inhibits VLP production. On the contrary, both pIRES-M-E plus  
458 pcDNA-NeCFP plus pcDNA-S, as well as pIRES-M-E plus pcDNA-Nflag plus  
459 pcDNA-SeYFP transfected cells were able to release VLPs in cell medium, as  
460 indicated by the presence of viral proteins (figure 5A, lanes 9 and 10). Under these  
461 conditions, the NeCFP protein was detected by a mouse monoclonal antibody  
462 directed against N (56) and migrated to an apparent molecular size of 70 KDa.  
463 To confirm that NeCFP or SeYFP viral proteins are correctly incorporated into  
464 VLPs, we analyzed purified VLPs by fractionation on a 20-60% sucrose gradient  
465 (Figure 5B). Both NeCFP and SeYFP co-sedimented with other viral proteins,  
466 indicating that they were incorporated in purified VLPs (Figure 5B, upper and lower  
467 panels, respectively). M-E-NeCFP-S VLPs were concentrated in fractions 10 and  
468 11. The E protein was not detected, most likely because M-E-NeCFP-S VLPs  
469 were less abundant in culture medium than M-E-Nflag-S VLPs for which E levels  
470 were already low. M-E-Nflag-SeYFP VLPs were more efficiently produced and  
471 concentrated in fractions 9 and 10 although high levels were also found in  
472 fractions 6, 7 and 8. Considering the high levels of M and Nflag detected in these  
473 fractions, incorporation of SeYFP seems less efficient than S (cf. Figure 2 with  
474 Figure 5B, lower panel). Altogether, our results demonstrate that fluorescent VLPs  
475 can be readily produced in Vero E6 transfected cells by incorporating either a  
476 tagged N or S fusion protein.  
477

478 **Visualization of M-E-NeC/GFP-S VLP production and transport in living Vero**  
479 **E6 cells.**

480 We then investigated formation and transport of fluorescent SARS-CoV VLPs in  
481 living transfected cells by fluorescent microscopy. Knowing that S is expressed all  
482 along the secretory pathway in both single- and co-transfected cells, we reasoned  
483 that N would be a better marker for monitoring of SARS-CoV VLPs assembly.  
484 Furthermore, having demonstrated that M, E and N are the crucial factors for  
485 assembly and egress of SARS-CoV VLPs, and anticipating that S-bearing VLPs  
486 could be internalized back into producer cells by ACE-2 driven endocytosis, we  
487 decided to omit S. Therefore, the pcDNA-NeCFP plasmid was either transfected  
488 alone or co-transfected with pIRES-M-E. Vero E6 cells were analyzed at 24 hours  
489 post-transfection. In most pcDNA-NeCFP transfected cells, eCFP signals were  
490 very bright and concentrated in large aggregates in the cytosol at the periphery of  
491 the nucleus (Figure 6A, panel a). Inversely and very interestingly, a different  
492 pattern was observed in most of cells co-transfected with pIRES-M-E and pcDNA-  
493 NeCFP. In these cells, eCFP fluorescence was more diffuse in cell cytosol:  
494 medium-size bright vesicles were concentrated in close proximity to the nucleus,  
495 smaller and dimmer vesicles were scattered in the cytoplasm and occasionally  
496 bright dots were enriched at the cell cortical area (Figure 6A, panels b). Few bright  
497 dots were also found outside cells in the surrounding medium. This difference in  
498 NeCFP distribution pattern, suggests that NeCFP assembles with co-expressed M  
499 and E viral proteins to form VLPs and traffics from the perinuclear assembly  
500 compartment to the cell surface where fluorescent VLPs are released into the  
501 medium.

502 We then further analyzed the trafficking dynamics of a fluorescently tagged N  
503 protein, co-expressed with M and E envelope proteins in living Vero E6 cells. In  
504 this aim, images were acquired using a spinning disc confocal microscope coupled  
505 to a CCD camera suitable for high-speed and high-resolution imaging. In these  
506 experiments we replaced the NeCFP by a NeGFP fusion protein, which is  
507 excitable by the Argon laser the microscope was equipped with. We could  
508 consistently identify three types of NeGFP containing vesicles, where fluorescent  
509 signal intensities, sizes and movements differ (Figure 6B and corresponding  
510 videos provided as supplementary data). First, the largest and brightest vesicles  
511 were found close to the nucleus and were static. Second, smaller and dimmer  
512 vesicles were trafficking actively, most of the time in a multidirectional way, making  
513 transient interactions with other vesicles. Occasionally these vesicles moved in  
514 rapid, unidirectional way and for longer distances, most likely along microtubules.  
515 Third, some cells presented an accumulation of bright dots at the cortical area,  
516 which may correspond to smaller secretory vesicles and released VLPs. Few  
517 bright dots were found outside cells, which probably correspond to fluorescent  
518 VLPs that have been released from producer cells. These results illustrate that  
519 fusion of a fluorescent tag to the C-terminal end of the nucleocapsid viral protein  
520 makes monitoring of SARS-CoV VLPs' egress possible.

521 We then investigated the effect of Brefeldin A (BFA), a fungal metabolite that has  
522 multiple effects upon the organelles of the secretory pathway, including inhibition  
523 of trafficking from ER to the Golgi apparatus, fusion of the cisternae of the Golgi  
524 with the ER and fusion of the trans-Golgi network (TGN) with endosomes (59).  
525 BFA has been used in previous study for analysis of viral protein transport and  
526 virus assembly (10, 49). Time-lapse images of living Vero E6 cells transfected with

527 M-E plus NeGFP were acquired at 24 hours post-transfection (Figure 6C; for  
528 video, see supplementary data). In a first time, cells were either non-treated (panel  
529 a) or treated with 6  $\mu\text{g}$  / ml BFA for 4 hours (panel d) or over-night (panel g). After  
530 several minutes of acquisition in these conditions, medium was changed to either  
531 BFA-containing medium (panels b and c) or normal medium (panels e, f and h, i),  
532 respectively. New sequences of images were acquired and fluorescence  
533 redistribution was analyzed. Upon BFA treatment we observed fusion of scattered  
534 fluorescent vesicles into large cytosolic fluorescent clusters accumulating at the  
535 center of the cell and diminution of puncta (panels a, b and c, supplementary  
536 data). After treatment of cells for 4 hours with BFA, most of the fluorescence was  
537 present in large bright patches surrounding the nucleus (panel d). Vesicles could  
538 still be observed in the cytosol, although less numerous than in non-treated cells.  
539 Following BFA wash, we could not observe any obvious reversibility of the  
540 massive redistribution of fluorescence (compare panels d and e, panel f). After  
541 over-night incubation with BFA, fluorescence was associated with large cytosolic  
542 clusters and endoplasmic reticulum (panel g) and no drastic change was observed  
543 after BFA wash and 1:30 hours recovery (panel h), although membrane dynamics  
544 seem to increase at the edge on the reticular compartment where tubules  
545 elongated from (panel h, arrow; supplementary data). Three hours recovery after  
546 over-night BFA treatment partially restore trafficking of fluorescent vesicles in  
547 transfected cells (panel i). The recovery process after BFA treatment was slow and  
548 the dynamics could not be followed for several hours, as repetitive exposure to  
549 laser induced apoptosis. Altogether, our results suggest 1/ that BFA induce  
550 reorganization of NeGFP associated compartments and alters vesicle formation  
551 and transport, 2/ that several hours of recovery after BFA wash are necessary to



552 restore dynamics of NeGFP associated compartment and vesicles trafficking.  
553 Further studies are required in order to clarify the subcellular compartments VLPs  
554 are associated with and delineate the dynamics of egress in association with  
555 cellular structures.  
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## Discussion

557

558

559 The minimal molecular requirement for efficient assembly and egress of SARS-  
560 CoV virions is still controversial. Here we describe the development of a plasmid-  
561 based transfection method for efficient production and release of SARS-CoV VLPs  
562 in permissive Vero E6 green monkey kidney epithelial cells. In contrast to reports  
563 on other coronaviruses, we demonstrate that all three M, E and N SARS-CoV  
564 structural proteins are required for efficient assembly and release of VLPs by  
565 transfected cells. When the S viral envelope glycoprotein is co-expressed with M,  
566 N and E, trimers of S are incorporated onto VLPs. Of particular interest is the  
567 finding that incorporation of a fluorescently tagged N protein into VLPs allows  
568 visualization of transport of *de-novo* formed particles in producer cells. Fluorescent  
569 VLPs constitute a new powerful model to study the mechanisms of SARS-CoV  
570 egress in living cells and specific roles of cellular machineries by fluorescence  
571 imaging techniques.

572 Other research groups have described formation of SARS-CoV VLPs in either  
573 insect (26, 44, 50) or mammalian cells (27-29), using various expression systems.

574 Huang Y. and colleagues were the first to report generation of SARS-CoV VLPs in  
575 human 293 renal epithelial cells (29). Human codon optimized genes encoding for  
576 M, E, N and S proteins were subcloned into mammalian expression vector and  
577 VLP formation was monitored by transmission electron microscopy on transfected  
578 cells at 63 hours post-transfection. The authors show that, in their experimental  
579 conditions, M and N are necessary and sufficient for formation of intracellular  
580 VLPs, independently of E and S, but secretion of VLPs was not efficient.  
581 Moreover, they described S as an important viral factor for maturation and egress

582 of VLPs from cells, but release of M-N-S VLPs in culture medium was still  
583 inefficient. By contrast, in our system we detected sedimentable M and N proteins  
584 in culture medium from M plus N expressing cells, suggesting that M-N VLPs can  
585 form and be secreted (Figure 1). Furthermore, co-expression of E greatly boosted  
586 levels of VLPs detected in culture medium (Figure 1) and addition of S did not  
587 influence the rate of VLP production (Figure 2 and data not shown). In contrast,  
588 Hsieh and colleagues have described that co-expressed E and M proteins are  
589 released four days post-transfection in culture medium of Vero E6 cells in the  
590 absence of S and N proteins. In their study, Vero E6 cells, previously infected with  
591 a recombinant vaccinia virus harboring the T7 polymerase gene, were co-  
592 transfected with plasmids encoding MycHis or V5His-tagged S, M, E and N  
593 structural proteins. Nevertheless, sedimentable E and M proteins were also found  
594 in culture medium even when they were individually expressed and very high  
595 amounts of E were found in VLP preparations in comparison to other viral proteins,  
596 suggesting formation of E-containing vesicles. Others have described that MHV  
597 and IBV E protein expressed alone results in assembly of E-protein-containing  
598 vesicles, with a density similar to that of VLPs (7, 47). We too did find  
599 sedimentable SARS-CoV E proteins in culture medium from individually pcDNA-E  
600 transfected cells, suggesting secretion of E vesicles, but we could not detect them  
601 in any fraction after sucrose gradient fractionation, suggesting a low production  
602 rate (Figure 1). We could also detect M-E VLPs at 48 hours post-transfection by  
603 coexpressing both E and M proteins, albeit at very low levels (Figure 1A, B and C).  
604 In our study, we took advantage of the pIRES bicistronic vector to ensure  
605 concomitant expression of E and M and maintain a low expression level for E. We  
606 show that use of this vector system, in combination with expression of N, ensures

607 slightly better levels of M-E-N VLPs in medium and great incorporation of E  
608 (Figure 1B). Higher levels of E were expressed when an individual pcDNA-E  
609 vector was used for E expression and resulted in increased E/M and E/N ratio in  
610 cell medium (Figure 1B). We could not conclude whether this was due to higher  
611 level of E incorporation into VLPs or to simultaneous release of E-containing  
612 vesicles and VLPs. Analysis of viral protein in cell medium by sucrose gradient  
613 fractionation revealed that E was only released at significant levels when co-  
614 expressed with M and N proteins, with which it cosediments (Figure 1C, panels b,  
615 c and e). We have observed efficient M-E-N-S VLP formation and release in 293T,  
616 HeLa and Huh-7 human cell lines (data not shown). Consistently, formation and  
617 release of M-E-N-S VLPs from 293T cells has been shown in another study  
618 reported by Huang C. and colleagues, in which authors have used a pCAGGS  
619 mammalian expression plasmid-based transfection system (28). Huang C. and  
620 colleagues were able to generate approximately 1.3  $\mu\text{g}$  of SARS-CoV VLPs from  
621  $2 \cdot 10^7$  293T cells (42). Recently, we have measured quantity of S incorporated into  
622 envelope of M-E-N-S VLPs purified from the VLP-containing sucrose fractions in  
623 our system. Approximately 1  $\mu\text{g}$  and 28  $\mu\text{g}$  of S were present in VLP preparations  
624 from  $10^7$  VeroE6 and 293T cells, respectively (data not shown).

625 We also investigated formation and secretion of SARS-CoV VLPs in transfected  
626 cells by transmission electron microscopy and negative staining of purified  
627 secreted particles (Figure 3). We were able to identify budding events in  
628 cytoplasmic perinuclear compartments. VLP-containing vesicles were scattered  
629 within the cytoplasm and found beneath the plasma membrane. Occasionally,  
630 secreted VLPs were bound to the cell surface. The VLPs-associated  
631 compartments that we observed in transfected Vero E6 cells share significant

632 similarities with the virions-containing compartments described in SARS-CoV  
633 infected Vero E6 cells at 3-5 days post infection (23). VLPs were readily detected  
634 in sedimented fractions from culture medium by negative staining and electron  
635 microscopy (Figure 3H). Globular structures protruding from VLP envelope, and  
636 which should correspond to spike peplomers, were occasionally detected, but we  
637 could not identify any particle displaying a typical corona-like structure attesting for  
638 optimal incorporation of spike trimers on virion envelope.

639 Interestingly, we found that a C-terminal Flag or eYFP tag affects levels of S  
640 trimers incorporation onto VLPs (data not shown and Figure 5, respectively).  
641 However Sflag and SeYFP proteins could still be incorporated into VLPs and tags  
642 can be used as markers. Similar results had been obtained for MHV where S  
643 sequence had been extended by fusion with a GFP fluorescent protein (3). These  
644 data can be explained by several factors: the 30 KDa fluorescent protein may  
645 cause geometrical constraints; M-S interactions, which are important for S  
646 incorporation may be affected by the tag; S retention to the site of viral assembly  
647 may be disturbed (43, 48). We also tried to produce VLPs containing the mRFP1  
648 fluorescent protein fused to the C-terminal end of M (Figure 4 and 5A). Although  
649 MmRFP1 was expressed in transfected cells, production of VLPs was abrogated.  
650 The M endodomain is crucial for M-N, M-E and M-S interactions and VLP  
651 formation (8, 11, 13, 19) and its fusion to a fluorescent protein tag may affect its  
652 structure and/or availability for interaction with other partners. This hypothesis is  
653 reinforced by the complete loss of recognition of the chimeric MmRFP1 by a rabbit  
654 serum raised against a C-terminal peptide of M (Figure 5A, left panel).

655 Lastly, a fusion NeCFP protein could be expressed and assemble into VLPs with  
656 M, E and S, although levels of VLPs detected in culture medium were significantly

657 reduced (Figure 5). Interestingly, whereas individually expressed NeCFP or  
658 NeGFP were found in the cell cytosol, often accumulating in the perinuclear area,  
659 the tagged N protein had a tendency to display a vesicular distribution pattern  
660 when co-expressed with M and E (Figure 6). We analyzed both distribution and  
661 trafficking of the NeGFP protein in transfected live cells by confocal microscopy  
662 (Figure 6B). When cells were co-transfected with pIRES-M-E and pcDNA-NeGFP,  
663 the NeGFP proteins were often found in static compartment near the nucleus, in  
664 trafficking vesicles scattered in the cytosol and moving actively, making transient  
665 interactions with other vesicles, and as small dot scattered at the cortical area  
666 beneath the plasma membrane, often enriched in cell projections. Fluorescent  
667 dots were also detected in the cell medium surrounding living cells. Subcellular  
668 distribution of NeGFP is consistent with our data of transmission electron  
669 microscopy. Most likely perinuclear static compartments and trafficking vesicles  
670 identified by fluorescence microscopy correspond to perinuclear vacuoles, at  
671 which membranes VLP budding events were observed, and to VLP-containing  
672 vesicles found by electron microscopy, respectively. The fluorescent dots  
673 observed at the cell cortical area may correspond to smaller secretory vesicles  
674 containing fewer VLPs – vesicles containing only one VLP were found by electron  
675 microscopy – and/or to released VLPs, which are bound to the cell surface.  
676 Interestingly, transport of fluorescent vesicles was affected by the drug BFA, well  
677 known to affect membrane transport in the secretory pathway (39, 59). BFA  
678 treatment induced backward trafficking of fluorescent vesicles and fusion into  
679 bright perinuclear clusters (Figure 6C panels a, b and c). Long lasting BFA  
680 treatment resulted in suppression of vesicle trafficking and no fluorescent puncta

681 were found at plasma membrane (Figure 6C panel g) and recovery of vesicle  
682 trafficking was restored after several hours in normal medium (Figure 6C panel i).  
683 Altogether, our data demonstrate that M, E and N structural proteins are key  
684 molecules for assembly and egress of the SARS-CoV. Production and analysis of  
685 fluorescent M-E-NeGFP SARS-CoV VLPs in living cells allowed us to identify  
686 three subcellular structures with different velocity characteristics along the  
687 secretory pathway. Molecular and cellular determinants of SARS-CoV assembly  
688 and egress will be investigated further using advanced fluorescence microscopy  
689 techniques.

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690

691

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- 939

940 **Figure legends**

941

942 **Figure 1. Production of SARS-CoV VLPs by transfected Vero E6 cells. A.**

943 Coexpression of M, E and N is necessary for efficient secretion of SARS-CoV  
944 VLPs by Vero E6 cells at 24 and 48 hours post-transfection. Monolayers of Vero  
945 E6 cells were transfected with plasmids driving the expression of SARS-CoV  
946 structural proteins M, E and Flag-tagged N as specified on top of each lane.  
947 Protein expression in cell lysates and in VLPs isolated from culture medium was  
948 analyzed by Western blot at 24 and 48 hours post-transfection, as indicated below  
949 corresponding panels. Samples were heat-denatured and reduced with  
950 dithiothreitol before loading. The M and E proteins were detected with rabbit  
951 polyclonal antibodies produced against the C-terminal extremity of each proteins.  
952 The N protein was detected with the M2 monoclonal antibody recognizing the Flag  
953 tag. Blots were exposed for 1 minute for signal detection except for detection of E  
954 contained in pellets from ultracentrifuged culture medium (bottom panels), for  
955 which blots were exposed for 10 minutes. The molecular mass (in kDa) and  
956 migration of protein standards are shown between blots. **B.** Use of the bicistronic  
957 pIRES-M-E vector restrains E expression level and favors production of M-E-N  
958 VLPs. Vero E6 were transfected with indicated plasmid combinations and cell  
959 lysates and medium were analyzed at 48 hours post-transfection as in A. To  
960 ensure better detection of E, VLPs were concentrated four times more than in A.  
961 Blots were exposed for 10 seconds for signal detection except for detection of E  
962 contained in pellets from ultracentrifuged culture medium (right bottom panel) for  
963 which the blot was exposed for 1 minute. **C.** Secreted viral structural proteins  
964 cosediment in sucrose gradient. Three 75 cm<sup>2</sup> dishes of Vero E6 cells were

965 transfected with plasmids driving the expression of SARS-CoV structural proteins  
966 M, E and Flag-tagged N either individually or in combination. Protein expression in  
967 cell lysates and in pellets from culture medium ultracentrifuged on 20% sucrose  
968 cushion was controlled by Western blot at 48 hours post-transfection (left panel).  
969 Resuspended pellets from ultracentrifuged cell medium were then loaded on a 20-  
970 60% discontinuous sucrose gradient and subjected to fractionation by  
971 ultracentrifugation. Twenty fractions of 600  $\mu$ l were collected (1-20, lightest to  
972 heaviest). Nature of viral proteins associated with each fraction was determined by  
973 Western blot (right panels, a to e). 15  $\mu$ l of samples were heat-denatured and  
974 reduced with dithiothreitol before loading. The molecular mass (in kDa) and  
975 migration of protein standards are shown on the right side of the blots. Samples  
976 from pIRES-M, pIRES-E plus pcDNA-E, pIRES-E plus pcDNA-E plus pcDNA-  
977 Nflag and pcDNA-E, pIRES-M plus pcDNA-Nflag have been generated in two  
978 separate experiments.  
979 Ctrl: non-transfected cells; E: pcDNA-E; M: pIRES-M; M-E: pIRES-M-E; M +  
980 Nflag: pIRES-M plus pcDNA-Nflag; M-E + Nflag: pIRES-M-E plus pcDNA-Nflag; M  
981 + E: pIRES-M + pcDNA-E; M + E + Nflag: pIRES-M plus pcDNA-E plus pcDNA-  
982 Nflag.

983

984 **Figure 2. S glycoprotein trimers are incorporated onto SARS-CoV M, E and**  
985 **N-containing VLPs.** pcDNA-S plasmid was co-transfected with pIRES-M-E and  
986 pcDNA-Nflag vectors. Culture medium was harvested at 48 hours post-  
987 transfection, ultracentrifuged on 20% sucrose cushion and pellets were  
988 resuspended in TNE buffer and ultracentrifuged on a 20-60% discontinuous  
989 sucrose gradient. Twenty fractions were collected (1-20, lightest to heaviest) and

990 analyzed by Western blot. Samples were either heat-denatured and reduced with  
991 dithiothreitol before loading for detection of Nflag, M and E or not heated and not  
992 reduced for analysis of S. Blots were exposed for 10 seconds for signal detection,  
993 except for E for which blots were exposed for 10 minutes. The highest levels of S,  
994 M, N and E structural proteins were found in fractions 9 and 10 corresponding to  
995 40% sucrose. S protein was detected with mouse polyclonal antibodies raised  
996 against purified S trimers. Arrows indicate bands that correspond to trimeric and  
997 monomeric forms of S. The molecular mass (in kDa) and migration of protein  
998 standards are shown on the right side of the blots.

999

1000 **Figure 3. Structural analysis and intracellular distribution of SARS-CoV**

1001 **VLPs.** Vero E6 cells were co-transfected with pIRES-M-E, pcDNA-Nflag and  
1002 pcDNA-S. At 24 and 48 hours post-transfection cells were fixed and ultrathin  
1003 sections were analyzed by electron microscopy (panels A to G). **A.** VLPs were  
1004 found in intracellular vacuoles (vc), in vesicles (vs) scattered in the cytosol and  
1005 bound to the plasma membrane (pm). The arrow points VLPs attached to the cell  
1006 surface. **B.** Large amount of VLPs within the lumen of the endoplasmic reticulum  
1007 (er) and within a cytoplasmic vesicle. n: nucleus. **C.** Presence of VLPs within  
1008 vacuoles and vesicles. Arrows point to small VLPs-containing vesicles beneath the  
1009 plasma membrane. **D.** Magnification of a VLP-containing vacuole. Black arrows  
1010 point to budding events. **E.** Compacted VLP-containing vesicles were found  
1011 beneath the plasma membrane. **F.** VLPs bound to the surface of a producer cell.  
1012 Two membrane-bound VLPs are shown by arrows. **G.** VLPs bound to a cell  
1013 filopodia. Spike-like protuberances were visible on VLP surface (arrows). **H.**  
1014 Electron microscopy images of negatively stained VLPs purified from cell medium

1015 at 48 hours post-transfection. A scale bar is indicated for each picture. A-D and F-  
1016 G panels correspond to cells fixed at 48 hours post-transfection. Image E was  
1017 taken from cells fixed 24 hours post-transfection. n: nucleus, er: endoplasmic  
1018 reticulum, vc: vacuole, vs: vesicle, pm: plasma membrane.

1019

1020 **Figure 4. Expression and subcellular distribution of viral structural proteins**  
1021 **tagged with fluorescent proteins.** Vero E6 cells grown on glass coverslips were  
1022 either transfected with single plasmids (left panels) or co-transfected (right panels)  
1023 with the three plasmids encoding the MmRFP1, E, SeYFP and NeCFP proteins. At  
1024 24 hours post-transfection, cells were processed for nuclear staining with DAPI  
1025 dye, fixed and analyzed under a fluorescent microscope equipped with an  
1026 ApoTome device to acquire images of optical sections. a, b, g, h: MmRFP1; c, d, i,  
1027 j: SeYFP; e, f, k, l: NeCFP; m, n: merged images. For all conditions, two  
1028 representative images are shown side-by-side.

1029

1030 **Figure 5. Production of fluorescent VLPs by transfected Vero E6 cells. A.**  
1031 Determination of optimal plasmid combinations for production of SARS-CoV  
1032 fluorescent VLPs. Viral proteins from cell lysates (left panel) and sedimented VLPs  
1033 from medium (right panel) were analyzed by Western blot. VLPs could be  
1034 produced when either N or S (lanes 9 and 10) but not M (lane 8) were tagged with  
1035 fluorescent proteins. **B.** Production of fluorescent VLPs and efficiency of  
1036 incorporation of NeCFP and SeYFP fusion proteins into VLPs. Cells were co-  
1037 transfected with the specified plasmid combinations (corresponding to lanes 9 and  
1038 10 of panel A) and purified VLPs from medium were analyzed by sucrose gradient.  
1039 Tagging S (lower blot) resulted in a greater yield of VLP production when

1040 compared to tagged N (upper blot). However, the eYFP tag greatly reduced S  
1041 incorporation onto VLPs (lower blot). Arrows indicates spike trimers and  
1042 monomers.

1043

1044 **Figure 6. Tracking of fluorescent SARS-CoV VLPs in living cells. A.** Widefield  
1045 fluorescence microscopy images showing accumulation of fluorescent VLPs at the  
1046 plasma membrane of pIRES-M-E plus pcDNA-NeCFP co-transfected cells (panels  
1047 b), whereas a strong perinuclear staining was observed in Vero E6 expressing  
1048 NeCFP alone (panel a). **B.** Confocal microscopy of living cells expressing M-E-  
1049 NeGFP VLPs. Four categories of fluorescent signals were observed: a bright and  
1050 static large perinuclear compartment (white encircling lines), smaller and dimmer  
1051 actively trafficking vesicles (orange encircling lines), bright dots accumulating at  
1052 the cell cortex (yellow encircling lines) and dots in the medium surrounding  
1053 transfected cells (yellow dots). Videos are available as Supplemental Material. **C.**  
1054 Treatment of transfected cells with brefeldin A (BFA) alters trafficking of  
1055 fluorescent vesicles. Vero E6 cells were transfected with pIRES-M-E plus pcDNA-  
1056 NeGFP plasmids. Cells were either non-treated (a) or treated with 6 $\mu$ g/ml of BFA  
1057 for either 4 hours (d) or overnight (g). BFA was then added to non-treated cells  
1058 and time-lapse acquisitions were performed. Panels b and c show the same cell  
1059 than in panel a but after 25 and 70 minutes incubation with the drug. Alternatively,  
1060 BFA was washed out and recovery after BFA treatment was analysed (panels e, f  
1061 and h, i). Panels a, b and c, d and e, g and h show same cells at different time  
1062 points. Video illustrating panels b, d, f, h and I are provided as supplementary  
1063 data.

1064

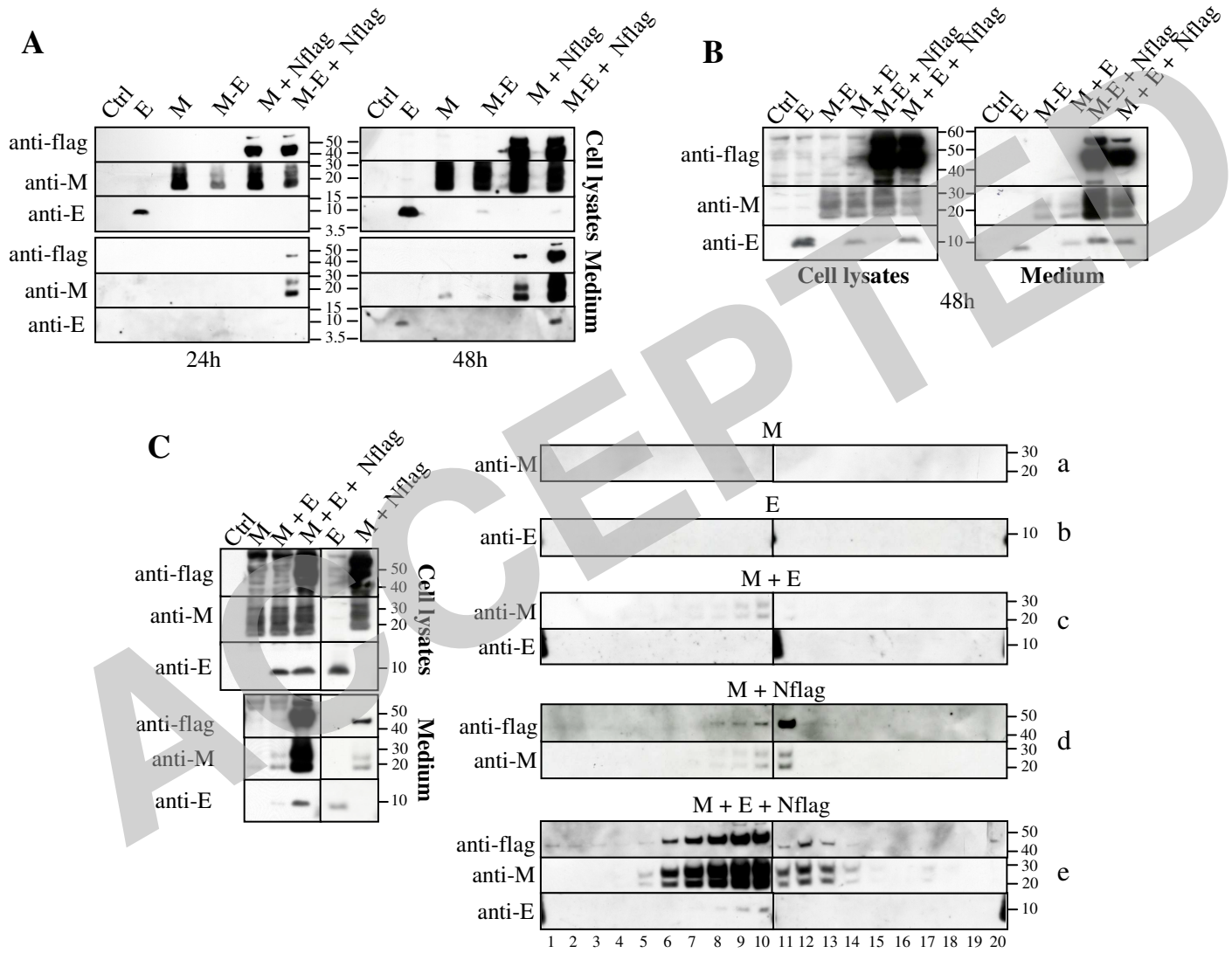


Figure 1



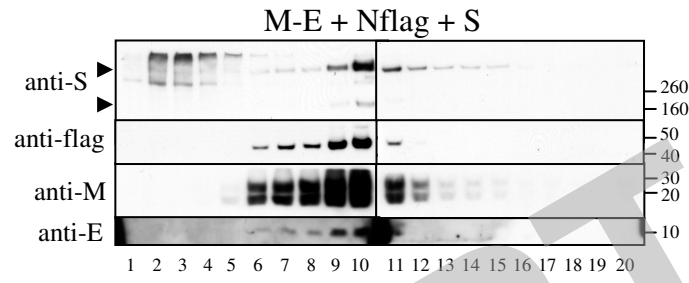


Figure 2

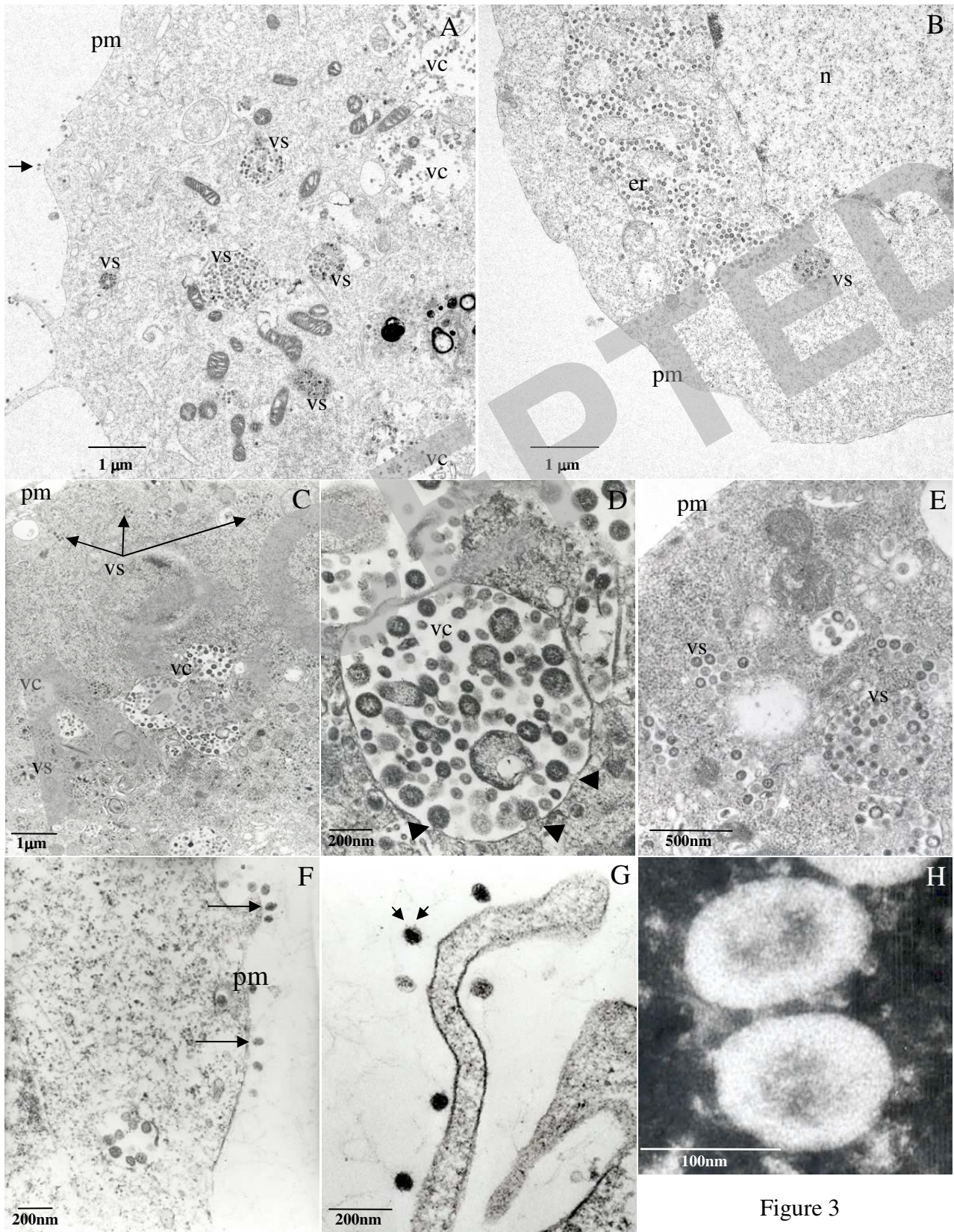


Figure 3

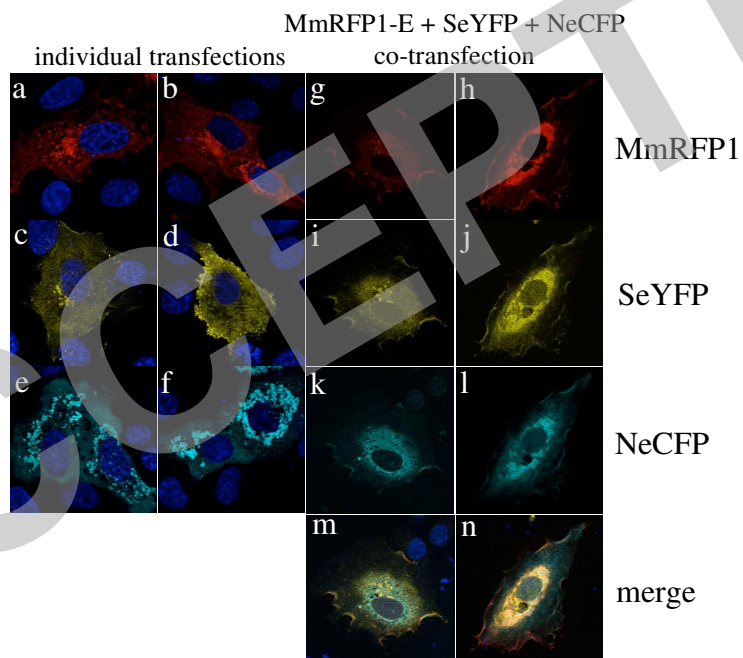


Figure 4

**A**

	1	2	3	4	5	6	7	8	9	10
M-E	-	+	-	+	+	-	+	-	+	+
MmRFP1-E	-	-	+	-	-	-	-	+	-	-
Nflag	-	+	+	-	+	-	+	+	-	+
NeCFP	-	-	-	+	-	-	-	-	+	-
S	-	+	+	+	-	-	+	+	+	-
SeYFP	-	-	-	-	+	-	-	-	-	+

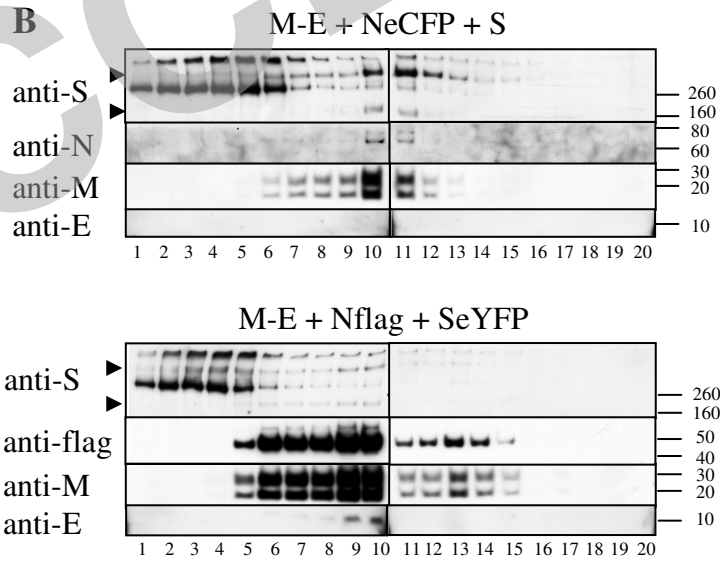
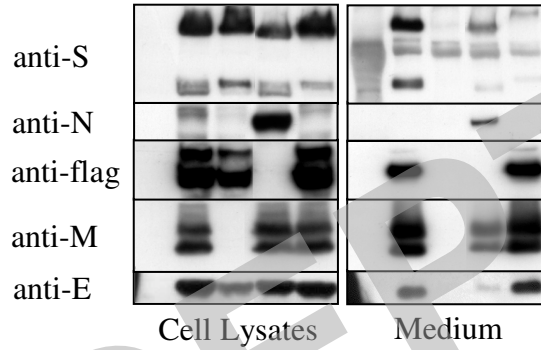


Figure 5

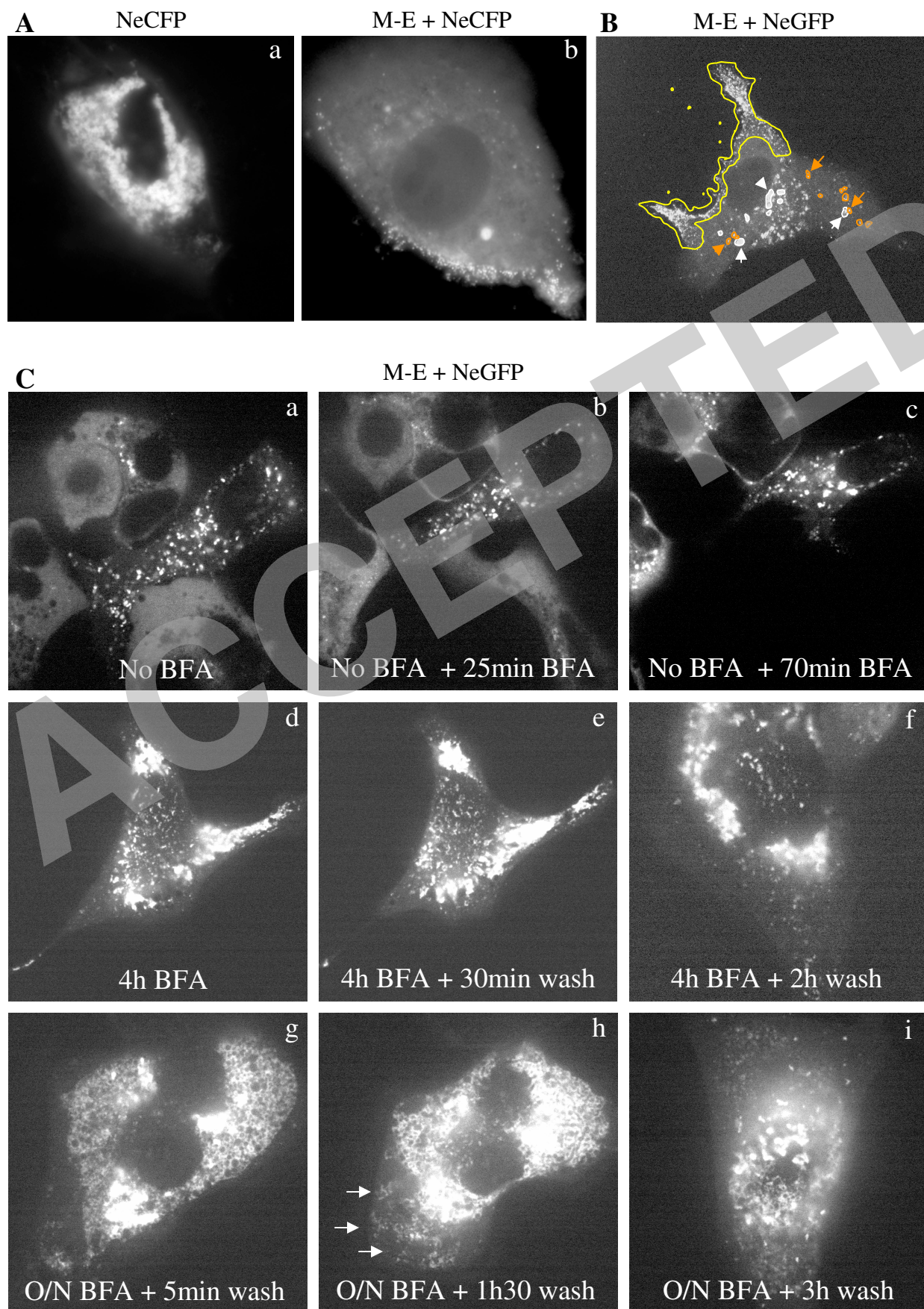


Figure 6