Title:

SARS-CoV-2 requires acidic pH to infect cells

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37   **One sentence summary:**

38   Detailed molecular snapshots of the productive infectious entry pathway of SARS-CoV-2 into cells

ABSTRACT:

SARS-CoV-2 cell entry starts with membrane attachment and ends with spike-protein (S) catalyzed membrane fusion depending on two cleavage steps, one usually by furin in producing cells and the second by TMPRSS2 on target cells. Endosomal cathepsins can carry out both. Using real-time 3D single virion tracking, we show fusion and genome penetration requires virion exposure to an acidic milieu of pH 6.2-6.8, even when furin and TMPRSS2 cleavages have occurred. We detect the sequential steps of S1-fragment dissociation, fusion, and content release from the cell surface in TMPRRS2 overexpressing cells only when exposed to acidic pH. We define a key role of an acidic environment for successful infection, found in endosomal compartments and at the surface of TMPRSS2 expressing cells in the acidic milieu of the nasal cavity.

Significance Statement:

Infection by SARS-CoV-2 depends upon the S large spike protein decorating the virions and is responsible for receptor engagement and subsequent fusion of viral and cellular membranes allowing release of virion contents into the cell. Using new single particle imaging tools, to visualize and track the successive steps from virion attachment to fusion, combined with chemical and genetic perturbations of the cells, we provide the first direct evidence for the cellular uptake routes of productive infection in multiple cell types and their dependence on proteolysis of S by cell surface or endosomal proteases. We show that fusion and content release always require the acidic environment from endosomes, preceded by liberation of the S1 fragment which depends on ACE2 receptor engagement.

3

Main Text:

SARS-CoV-2 cell entry begins with engagement at the cell-surface and ends with deposition of the viral contents into the cytosol by membrane fusion. The first step is binding of the viral spike protein (S) with its cellular receptor, angiotensin converting enzyme (ACE2) (*1–4*). The last step delivers the viral genomic RNA in association with the nucleocapsid protein (N), which is removed for translation of the input genome (*5, 6*). Proteolytic activation of S by additional host-cell factors is necessary for it to function as a fusogen. Cleavage of S by furin in producer cells (*7*) generates the S1 receptor binding subunit non-covalently associated with the S2 fusion subunit. The S protein is cleaved by cell surface or endosomal proteases during virion entry into host cells, that activate the viral fusion machinery (*1, 8–10*). This entry associated proteolysis of S has led to the current model of two routes of infectious cell entry: fusion of viral and cellular membranes at the host-cell surface or fusion following endosomal uptake (*6*).

The cellular proteases that are involved in processing S during entry include the transmembrane serine proteases TMPRSS2 or TMPRSS4 found at the cell surface (*1, 8*), and the endosomal cathepsins that require the acidic milieu of the compartments in which they are enriched (*1, 10*). Processing of S by TMPRSS proteases or by cathepsins, at a site designated S2', depends on prior cleavage at the furin site in the producer cells (*7, 11, 12*). TMPRSS cleavage has been thought to result in infection from the plasma membrane and cathepsin cleavage, in cells lacking TMPRSS activity, with infection from endosomes (*5, 6*). Chemical inhibitors of TMPRSS or cathepsin proteases in cells in culture indeed show that infection of some cell types is more sensitive to inhibition of endosomal cathepsins whereas others are more sensitive to inhibition of TMPRSS proteases (*1, 9*). TMPRSS inhibitors such as camostat and nafamostat are in clinical development as SARS-CoV-2 therapeutics, further highlighting the need to understand how entry pathways depend upon specific proteases.

To analyze the routes of cellular uptake that lead to successful fusion and release of virion contents into the cytoplasm, we developed a set of new tools that allow direct visual tracking of the uptake of single virions and release of their contents. Using a chimeric vesicular stomatitis virus (VSV) in which SARS-CoV-2 S has replaced the endogenous glycoprotein gene (G), we modified the virus to permit the separate tracking of the S protein and the viral contents. We engineered a structural component of the replicative core of VSV, the phosphoprotein (P), to append to its amino terminus enhanced green fluorescent protein (eGFP), for tracking the virion content. This eGFP-P chimeric virus is structurally fluorescent and depends upon S for entry. We

4

95   also sparsely labelled the S protein by direct conjugation with a fluorescent dye, allowing us to

96   visualize the steps of entry, from S1 fragment release to membrane fusion, including virion content

97   release into cells during productive infection. We have found that content release requires acidic

98   pH and that it occurs principally from endosomes irrespective of the cell type and irrespective of

99   the dependence of the virus on TMPRSS2 or cathepsin-mediated processing of S. Only mild

100   acidification of the medium allows efficient entry at the plasma membrane. We correlate our

101   findings with studies of infection by several human isolates of SARS-CoV-2.

102

103   **RESULTS**

104   **Cell entry of individual virions mediated by SARS-CoV-2 S**

105   We used live-cell fluorescence microscopy to monitor uptake of single VSV-SARS-CoV-2 virions

106   that depend upon SARS-CoV-2 S for infection (Wuhan Hu-1 isolate and Delta and Omicron

107   variants, chimeras generated as described: see Methods and (*13*)) and for the Wuhan variant

108   also containing a fluorescent internal structural protein, P, fused at its amino terminus to eGFP

109   (eGFP-P) (Fig. S1). We counted the number of tagged particles internalized 1 hr post inoculation

110   at MOI 0.5 and found 50-70 particles in Caco-2, Calu-3, and Vero cells, but about three times that

111   number in Vero-TMPRSS2 cells, which overexpress TMPRSS2 (Fig. S1D, E), presumably

112   because of more efficient TMPRSS2 cleavage in the high expression cell line. Examination of the

113   labeled virions by spinning-disc confocal microscopy showed distinct, diffraction-limited puncta

114   with a single, Gaussian distribution of fluorescent intensities (Fig. S2A), consistent with the

115   presence of single virus particles and absence of virion aggregates. We also labeled the S protein

116   sparsely with the fluorescent dye Atto565 NHS ester (25-35 dyes per virion), with minimal effect

117   on particle infectivity (Fig. S2A-D). The double labeling allowed us to track the viral S protein

118   separately from the virion contents.

119

120   **SARS-CoV-2 S-mediated entry requires endocytic uptake**

121   We used live-cell volumetric, lattice-light-sheet fluorescence microscopy (LLSM) (*14*) to obtain 3D

122   views of virion cell-entry over time during their uptake into cells (Figs. 1A-E, Fig S3). We incubated

123   cells for 8 min, transferred them to the LLSM, and recorded sequential 3-D stacks from a single

124   cell, acquiring a full stack every 2 sec, for 10 min (300 stacks total). We then moved quickly to

125   an adjacent cell and recorded a similar 10 min series of 300 stacks, after which we moved finally

126   to a third neighboring cell, for 10 min. The sequence of representative 10-plane projections in

127   Fig. 1B shows that particles attached to the cell surface during the initial 8 min incubation, followed

128   by efficient internalization at later time points. The figure shows images from Vero-TMPRSS2

129   cells; we obtained similar results for Vero, Caco-2 and Calu-3 cells, as shown in Fig S3. The views

130   from 20- and 30-minutes post-inoculation also showed many examples of intracellular spots

131   labeled with eGFP-P only, representing delivery of the VSV ribonucleoprotein core (RNP) into

132   cells visualized as the separation of fluorescent eGFP-P from the membrane-bound, Atto565

133   labelled S glycoprotein.

134

135   We confirmed that the views in Fig 1B reflected the outcome of sequential internalization and

136   RNP delivery events by tracking individual particles in the volumetric time series acquired using

137   LLSM (Fig. 1C). Single virions attached to cells during the 8 min period following addition of virus,

138   and ~ 90% (1508/1692) of the attached particles had internalized during the 30 min time course,

139   as recorded in 60 time-lapse 3D videos from 20 cells. The representative 10-plane projection in

140   Fig. 1C obtained during the initial 10-minute interval shows several single-particle examples of

141   virion internalization and three events of RNP core release (for similar results with Vero, Caco-2

142   and Calu-3 cells, see Fig S3). The highlighted example in Fig. 1C and the complete ortho

143   projection in Fig. 1D show a fluorescent spot corresponding to a virus particle, first captured at

144   the cell surface, then undergoing rapid directed movement towards the cell interior, due to

145   endocytosis and intracellular traffic of virus-containing vesicles. Dissociation of the eGFP-P from

146   the Atto565 signals marks delivery of the genome into the cytosol (see also Figs. S3-S7). In a

147   total of 60 Caco-2, Calu-3, Vero and Vero-TMPRSS2 cells, we detected separation of signals at

148   an intracellular location for 138/1692 trajectories during the 30-min interval starting two minutes

149   after an initial 8-minute inoculation (Fig. 1E), and only one dissociation event at the cell surface

150   (for a Vero-TMPRSS2 cell). The fluorescent signals from eGFP-P released in these times frames

151   remained stable and punctate in the cytosol during the duration of the 10 min acquisition, and any

152   released eGFP-P in the cytosol at the outset of the second or third 10 min time series remained

153   stable for the entire 10 min recording. Moreover, we never observed uncoated (delivered)

154   particles (i.e., released eGFP-P) in the early frames of the time lapse from the first cell. These

155   observations rule out the possibility of rapid, early entry events from the cell surface or from

156   endosomes during the "blind" 8 minutes during inoculation, and we conclude that an endocytic

157   route accounted for all but one of the detected VSV-SARS-CoV-2 fusion events in these

158   experiments.

159

160   Analysis of mean square displacement (MSD) curves from three-dimensional, single-particle

161   trajectories allowed us to determine the dynamic regime of the particle at any time point after

162   attachment. The alpha coefficient (α) in the anomalous diffusion equation ($<r^2> = 6Dt^{\alpha}$)

163   corresponds to confined motion (α < 0.8) for the virus attached to the cell surface, directed motion

164   (α >1.2) for intracellular particles with co-localized Atto565-labelled S and eGFP-P labelled RNP,

165   presumably associated with endosomes, and Brownian motion (0.8< α <1.2) for eGFP-P spots

166   diffusing in the cytosol. The results shown in Fig. 1F (which corresponds to the viral particle traced

167   in Figs. 1 C and D) and the summary for all tracked particles in Fig. 1G (see also Fig. S3-S7)

168   illustrate the moment at which an RNP escapes into the cytosol (α = 1.07), while the tagged S

169   remains associated with an endosome (α = 1.93, see below).

170

171   The chimeric VSV particles are roughly 80 by 200 nm (*15*, *16*) and appear in the microscope as

172   diffraction-limited spots. Endosomes typically range in diameter from 300-1000 nm (*17*). Thus, a

173   subset of fluorescent endosomes are larger than the diffraction limit, facilitating use of an

174   increased apparent size of an Atto565 fluorescent spot as a proxy for viral membrane fusion with

175   the surrounding (larger) endosomal membrane (Fig. S8). Using this approach, we detected fusion

176   in 17 $\pm$ 4 % (n = 20 virions), 28 $\pm$ 2% (n = 61 virions), 23 $\pm$ 2% (n = 27 virions), and 27 $\pm$ 6% (n =

177   30 virions) of the traces in Vero, Vero-TMPRSS2, Caco-2 or Calu-3 cells, respectively. All the

178   events coincided with release into the cytosol of the eGFP-P labeled RNP core of VSV (Figs. 1E,

179   S3, S7). We did not observe release of the virion contents at the plasma membrane (Figs. 1E,

180   S3-S7), indicating that S-mediated infection of cells only occurred through an endocytic pathway.

181

SARS-CoV-2 S-mediated infection requires endocytosis

183   Dynamin, a large GTPase required for cargo uptake in clathrin-mediated or fast endophilin-

184   mediated endocytosis (*18*), is susceptible to interference by a dominant negative mutant, K44A

185   (*19*). To test whether dynamin-dependent endocytosis is necessary for SARS-CoV-2 S mediated

186   infection, we transiently over expressed the dominant-negative mutant in Caco-2, Calu-3, Vero

187   and Vero-TMPRSS2 cells. We monitored the effect on infection of these cells by VSV-SARS-

188   CoV-2 that expresses eGFP as a marker of infection (VSV-eGFP-SARS-CoV-2) according to the

189   scheme summarized in Fig S9A, and we quantified infection by single-cell imaging at 8 hours post

190   inoculation. In control cells, to achieve comparable infectivity we used 10 times more VSV-SARS-

191   CoV-2 for Caco-2 and Calu-3 cells than for Vero or Vero-TMPRSS2 over-expressing TPMRSS2

192   (Figs. S9B, C). Irrespective of the cell type, infection was inhibited by the dominant negative

193   dynamin mutant (Fig 1H) or by addition of dynasore-OH, a small molecule dynamin inhibitor (*20*)

194   (Fig. 1I). Dynasore-OH addition at 1h post-inoculation had no effect on viral infectivity, consistent

195   with inhibition of an early, entry-related event, but not with later inhibition of viral gene expression

196   (Fig. S9C). We previously reported that S mediated infection of cells by VSV-SARS-CoV-2

197   correlates well with infection by SARS-CoV-2 (*9*, *13*, *21*, *22*). In accord with that correlation,

198   dynasore-OH also inhibited infection of Calu-3 and Vero-TMPRSS2 cells by a SARS-CoV-2

199   Wuhan (B.1) clinical isolate (Fig 1J). These results show a critical role for endocytosis in SARS-

200   CoV-2 infection of multiple cell types in culture including cells that express TMPRSS2.

201

202   **Viral entry from endosomes**

203   Our data show that internalized virus particles reached internal membrane compartment(s) from

204   which fusion and genome entry into the cytosol occurred. To establish the identity of the(se)

205   endosomal compartments, we used live-cell LLSM to track particles entering SVG-A cells

206   ectopically expressing ACE2, or both ACE2 and TMPRSS2, and gene-edited for fluorescent

207   labeling of specific endosomal compartments. (Fig. 2A, S10). Single particle tracking of 6815

208   viruses over a 50 min period starting from 30 minutes post inoculation of cells revealed VSV-

209   SARS-CoV-2 particles trafficked from the cell surface to early endosomes marked with early

210   endosomal antigen 1 fused to the fluorescent protein Scarlett (EEA1-mScarlett) and subsequently

211   to late endosomes/lysosomes marked with a Halo-tagged version of the cholesterol transporter

212   Niemann Pick C1 NPC1-Halo-JFX646 (examples for single virions shown in Fig 2 B, C, S11-S12).

213   We only detected particles located in the interior localizing with the endosomal markers in early

214   frames of the time lapse from the first cell taken at the end of the 30 min inoculation period. Like

215   with the other cells, these observations also rule out for SVG-A cells the possibility of entry events

216   from the cell surface or from endosomes during the 30-min inoculation period.

217

218   To establish the location of the compartments from which the virion contents were released, we

219   identified the point at which the trajectory of the eGFP-P signal changed from directed (α >1.2) to

220   Brownian (0.8< α <1.2) concomitant with a loss of co-localization with endosomal markers

221   (examples for single virions shown in Fig 2 D, E, S13-S14). All entry events occurred from

222   compartments marked with either EEA1 or NPC1 in cells expressing TMPRSS2, and only from

223   NPC1-compartments in cells lacking TMPRSS2 (Fig. 2 F, S15).

224

225   **Release of S1 at the surface of cells expressing TMPRSS2**

226   Membrane fusion mediated by S depends on its proteolytic cleavage and dissociation of the S1

227   fragment (*6*). Cleavage alone does not release S; the required trigger is ACE2 binding. Addition

228   of soluble ACE2 ectodomain to VSV-SARS-CoV-2 particles activated by trypsin, to bypass

229   TMPRSS2, released about 70% of the Atto 565 label, a reasonable estimate of the fraction of S1

230   rather than S2. In live-cell imaging experiments with TMPRSS2 expressing cells, we noted a

231   partial loss of Atto 565 intensity from labeled VSV-SARS-CoV-2 particles after they attached to

232   the cell surface (Fig. S19). In these experiments, we used a microfluidics device to introduce virus

233   to cells while in the LLSM, enabling us to detect attachment directly. We interpreted the loss of

234   Atto 565 signal as S1 dissociation, and we took it as a proxy for cleavage by TMPRSS2 at the S2'

235   site on an ACE2-attached spike protein. Dissociated S1 will remain attached to ACE2 for some

236   time, and in about ~2 % of the events, we could indeed detect lateral spreading (interpreted as

237   diffusion in the plasma membrane) associated with the abrupt partial reduction of the punctate

238   Atto 565 signal. We found ~25% loss of the Atto 565 signal per particle in ~ 78% of particles at

239   the surface of Vero-TMPRSS2 cells expressing TMPRSS2 (Fig. 3 A-D) within the first 10 min of

240   LLSM single-particle tracking (Fig. 1E). In all cases the loss was in a single step with a half time

241   of 2 sec or less (Fig. 3B and Fig. S16). The signal reduction, which preceded endocytosis, was

242   never associated with RNP delivery (e.g., eGFP-P) from the cell surface (Fig. 1E, cell #1), except

243   under the special circumstances of slightly acidic medium during inoculation as described in the

244   following section (Fig. 3 E-J and Fig. 4). The signal loss at the cell surface depended strictly on

245   TMPRSS2 activity, as it was inhibited by 10 µM Camostat and was absent in Vero cells lacking

246   TMPRSS2 (Fig 3D). The decrease in punctate intensity, which we interpret as release of S1,

247   occurred in less than 5% of particles attached to Caco-2 or Calu-3 cells, which naturally express

248   TMPRSS2 (Fig. 3D). This result suggests that in these cells, cleavage at S2' by TMPRSS2 occurs

249   primarily after uptake into endosomes.

250

251   **Viral membrane fusion requires acidic pH**

252   Endosomes undergo rapid acidification, and acidic pH is a trigger that induces fusogenic

253   conformational changes in many viral envelope proteins. We therefore examined the effect of pH

254   on productive viral entry by adjusting the pH of the medium at 10 minutes post inoculation. From

255   187 traces from VERO-TMPRSS2 cells incubated at pH 6.8, we observed 84 fusion events at the

256   cell surface, as defined by loss of Atto 565 signal and by accompanying eGFP-P delivery into the

257   cytosol (Fig. 3 E-H, S17). The abrupt partial loss of punctate signal, interpreted as S1-fragment

258   release, always preceded fusion (signaled by diffusion in the plasma membrane of the remaining

259   Atto 565 signal with a half-life of about 20 sec) and by eGFP-P delivery into the cytosol, typically

260   within 10 sec (Fig. 3 E, F). We defined eGFP-P delivery into the cytosol by a change in its motion

261   from confined (α <0.8) when on the cell surface to Brownian (0.8< α <1.2) when in the cytosol

262   (Fig. 3G). In the absence of S1-release, we did not detect S1 diffusion or eGFP-P delivery into

263   the cytosol. We conclude that release of S1 and subsequent or concomitant exposure to acidic

264   pH result in release of virion contents into the cell.

265

266   Incubation of Vero-TMPRSS2 cells at pH 6.2 rather than 6.8 during inoculation prevented release

267   of the S1-fragment and hence prevented viral fusion at the cell surface (Fig. 3 E, G, and H). This

268   pH dependence is consistent with an expected loss of TMPRSS2 proteolytic activity as the pH

269   falls below 7. When cells were incubated at pH 7.4 throughout infection, we did not observe any

270   particle fusion and content release at the cell surface, even though TMPRSS2 cleavage, as

271   detected by S1-fragment release, still occurred (Fig. 3 E, G and H).

272

273   The efficiency of entry by VSV-SARS-CoV-2 tracked by visualizing content release or by extent

274   of infection increased in Vero-TMPRSS2 cells at pH 6.8 (Fig. 3H). To investigate further the

275   requirement of acidic pH for efficient SARS-CoV-2 S-mediated membrane fusion, we carried out

276   bypass experiments to initiate infection directly at the cell surface. We blocked endocytic uptake

277   into cells with dynasore-OH and exposed bound particles to different pH ranging from 6.2-7.4.

278   Infection of Vero, Caco-2 or Calu-3 cells was blocked regardless of the pH, whereas Vero cells

279   overexpressing TMPRSS2 were readily infected by acid triggering of the S protein at the cell

280   surface (Fig. 3J, S18).

281

282   These results were consistent with the observed lack of release of the S1-fragment from viruses

283   at the surface of Vero, Caco-2, or Calu-3 cells (Fig. 3D). To eliminate a requirement for TMPRSS2

284   cleavage of S, we pre-activated particles by incubation with trypsin before inoculating the cells.

285   These pre-activated particles were as infectious in Vero-E6 TM cells treated with dynasore-OH

286   and inoculated at pH < 7 as non-trypsinized particles in the same cells and conditions. Thus,

287   trypsin and TMPRSS2 cleavages were equally effective in activating the particles. With

288   progressive acidification of the medium, infection increased, reaching a maximum at pH 6.6 and

289   remaining constant to pH 6.2 (Fig. 3H). These results show that S-mediated fusion requires pH <

290   7. We obtained similar results concerning the effect of pH on infectivity of authentic SARS-CoV-

291   2 with cells in which endocytosis was blocked and genome penetration depended solely on cell-

292   surface fusion events (Fig 3I-J).

293

294   **Influence of S protein variation on infectious entry pathways**

295   We found that response to infection inhibitors and the requirement for endocytosis and proteolytic

296   cleavage of S for the VSV chimera with the Delta variant S protein were essentially the same for

297   the chimera with the S protein from the original Wuhan-Hu1 isolate (Fig. 4A-D and S20, S21).

298   Conserved events included release of the S1-fragment from receptor-bound virions attached to

299 Vero-TMPRSS2 cells (Fig. 4 B), and S1 release followed by fusion and genome penetration when

300 inoculation was at pH 6.5-6.8 (Fig. 4C). Both Wuhan-Hu1 and Delta also showed enhanced

301 infectivity of Vero-TMPRSS2 cells inoculated at pH 6.8 (Fig. E-G).

302

303 Examination of the entry pathway mediated by the S protein of Omicron varied with the cell type

304 used to produce the virus. VSV-Omicron produced in Vero-TMPRSS2 cells depended on

305 TMPRSS2 activation for infection of Vero-TMPRSS2, although somewhat fewer virions released

306 the S1 fragment at the cell surface (Fig 4B-D). VSV-SARS-CoV-2 chimeras with Omicron S

307 produced in MA104 cells could not be activated by TMPRSS2 and failed to infect or fuse from the

308 surface of Vero-TMPRSS2 cells at pH 6.8 (Fig. 4A-D, S20, S21). Infection required activation by

309 cathepsins for fusion and infection from endosomes (Fig 4 A-D, S20, S21). Trypsin treatment of

310 VSV-SARS-CoV-2-Omicron, which cleaves at both the furin and TMPRSS protease sites (23),

311 allowed infection to proceed from the cell surface at acidic pH, independent of the producer cell

312 line, even in the presence of an endocytosis inhibitor (Fig.4A-D, S20, S21).

313

314 Sequence analysis of the genomic RNA from the VSV-SARS-CoV-2 chimeric viruses confirmed

315 the presence of the TMPRSS2 and furin cleavage sites in Omicron S. As furin cleavage in

316 producing cells is essential for subsequent proteolysis by TMPRSS2 in the infecting cell, we

317 tested whether incomplete furin cleavage explained the differential susceptibility to TMPRSS2

318 activation. We found much less cleavage of Omicron S for virus produced in MA104 cells (Fig 4D)

319 than for virus produced in Vero-TMPRSS2 cells (Fig. 4D). Particles with furin cleaved S were

320 susceptible to TMPRSS2 activation (Fig 4A-D), released the S1-fragment (Fig 4A), and fused at

321 pH 6.8 (Fig. 4 B,D).

322

323 **Intranasal pH**

324 Our results suggest that an acidic environment is required for successful infection, found in

325 endosomal compartments and at the surface of TMPRSS2 expressing cells purposely exposed

326 to mildly acidic extracellular pH conditions. Since expression of TMPRSS2 appears to be highly

327 expressed in a subset of cells located in the nasopharyngeal cavity (24, 25), we asked whether

328 this milieu would be acidic. Using a pH catheter placed in the left and right nasal cavities of 17

329 healthy male and female volunteers, we found a mildly acidic pH of around 6.6 (Fig. 4 E), in

330 agreement with earlier measurements (26, 27).

DISCUSSION

331

332 We examined how SARS-CoV-2 Wuhan and its variants Delta and Omicron enter host cells using

333 a combination of high-resolution live cell 3D imaging and quantitative assays for viral infectivity.

334 Real-time tracking of single VSV-SARS-CoV-2 chimeric particles by lattice light sheet microscopy

335 allowed us to visualize directly, with a sensitivity and time resolution substantially greater than

336 any previous work, the key early steps of viral infection: virion binding, release of the S1-fragment

337 upon cleavage by TMPRSS2, viral membrane fusion, and genome penetration into the cytosol.

338 These observations revealed a previously unsuspected requirement to fuse by exposure to pH

339 between 6.5 to 6.8, even after priming by TMPRSS2 and the attendant release of S1 and the S2'

340 fragment (Figure 5).

341

342 Current understanding, derived in part from earlier work on SARS-CoV, has assumed that the

343 only requirement for low pH was for cathepsin L activity when TMPRSS2 was absent or furin

344 cleavage, a prerequisite for TMPRSS2 digestion, had failed during exit from the producing cell (*1*,

345 *11*, *12*, *28–30*). Our results instead distinguish three complementary routes of productive entry,

346 all of which involve exposure of the entering particle to pH < 6.8. The two principal routes are by

347 uptake and traffic to early endosomes, for TMPRSS2-primed virions, or to late endosomal

348 compartments, for cathepsin cleavage in cells regardless of the presence or absence of

349 TMPRSS2. A third, minor route does not require virion uptake, but instead proceeds entirely at

350 the cell surface, but only if cell attachment is at a pH range between 6.5 and 6.8. These

351 observations define the alternative, multi-step pathways of SARS-CoV-2 entry and restrict the

352 conditions for cell-surface penetration.

353

354 The released Atto 565-labeled fragment diffused laterally in the membrane, away from the labeled

355 virion, consistent with our identification of the fragment as S1, which we expect to remain attached

356 to ACE2. The release, which always preceded membrane fusion, was independent of exposure

357 to acidic pH. Its dissociation was necessary but not sufficient for S2 to undergo its full, fusion-

358 promoting conformational change. Release required S2' site cleavage, as evidenced by its

359 absence in the presence of the TMPRSS2 inhibitor, Camostat, and by its absence, even in

360 TMPRSS2 expressing cells, at pH lower than about 6.5, where TMPRSS2 is inactive. Trypsin

361 cleavage in our experiments circumvented TMPRSS2 activity, and we indeed observed fusion at

362 pH as low as 6.2.

363

364   The structure of the spike protein and the distribution of lysine residues suggest that most of the
365   Atto 565 label will be on S1 and hence, from the fraction of total label released, that about 25%
366   of the S-protein trimers will have shed S1 upon interaction with membrane bound ACE2,
367   assuming that dissociation of the 3 S1 fragments is cooperative. We estimate from the ratios of
368   stained band intensities on SDS-PAGE that the VSV chimeras have 15-20 S trimers on their
369   surface, and we infer from these numbers that on average, 3-4 spike will have lost S1, liberating
370   their S2 fragments to extend and interact with the host-cell membrane. This estimate is consistent
371   with the likely fraction of the virion surface that makes contact with the cell membrane and with
372   the dependence of S1 shedding from a trimer on its binding to ACE2. It is also consistent with the
373   number of active fusion proteins on other viruses, including VSV itself, required for fusion to
374   proceed (*31–34*).

375

376   Syncytium formation between cells expressing SARS-CoV-2 spike and cells expressing ACE2,
377   often used as a spike-mediated fusion assay, does not appear to depend upon acidic pH. But
378   the interface between the two cells will have vastly more spike protein than the interface between
379   a virus and a target cell. Thus, even low probability S1 dissociation events should be sufficient to
380   create a fusion pore, which can widen and spread across the entire cell-cell junction.

381

382   Release of S1 from a spike also detaches that spike from ACE2. Because cleavage at the S2'
383   site was complete in our experiments, any spike bound by ACE2 would probably have released
384   S1. Continued association with the host cell would thus have depended on formation of
385   alternative contacts as S1 dissociates. We propose that formation of an extended intermediate
386   and insertion of the S2 fusion peptides into the host-cell membrane creates the interactions that
387   retain the virion at the cell surface. This proposal further implies that protonation of one or more
388   S2 residues at acidic pH then enables S2 to collapse toward the folded-back, post fusion trimer
389   of hairpins and pull together the viral and host-cell lipid bilayers.

390

391   The Omicron variant is more refractory to furin cleavage than previously isolated strains. Its entry
392   pathway will thus depend on the level of furin activity in the producing cells. We indeed found
393   that when grown in Vero-TMPRSS2 cells, Omicron VSV chimeras were susceptible to S2'
394   cleavage by TMPRSS2 and entered from early endosomes or at the cell surface at mildly acidic
395   pH, but when grown in MA104 cells, they required cathepsin cleavage in late endosomes for
396   entry. With authentic SARS-CoV-2 virus, TMPRSS2 susceptibility similarly depended on the cells

397   in which the virus was propagated. These observations resolve some ambiguities in the literature

398   concerning the role of TMPRSS2 in Omicron infection (*35–37*).

399

400   Together with differential protease activities, the pH of respiratory mucosa could also influence

401   viral tropism. The pH of the airway-facing surface of the nasal cavity is between 6.2 and 6.8 (our

402   observations and (*26*). Thus, in principle, rapid entry could occur at the surface of TMPRSS2-

403   expressing cells in the nose. In other parts of the nasopharyngeal cavity and in the lung, the pH

404   is neutral (*38*), and we would not expect virus to fuse in those tissues until its endocytic uptake.

405

406   Our suggestion that at neutral pH, a relatively long-lived, extended S2 intermediate may be

407   present at the virus-cell interface bears both on potential therapeutic interventions and on the

408   availability of otherwise occluded epitopes to mucosal antibodies. Persistence of an extended

409   intermediate after gp120 release during HIV entry is thought to account for inhibition of viral

410   infectivity by the peptide fusion inhibitor entfuvirtide and for neutralization by antibodies that

411   recognize epitopes unavailable on a prefusion Env trimer. Our results are consistent with

412   observations that comparable interventions can impede SARS-CoV-2 infection in animal models

413   (*39*).

414 **MATERIAL AND METHODS**

415 **Materials and Cells**

416 All materials and cells used in this study are described in detail in Supplementary Appendix S1.

417

418 **Generation of VSV-SARS-CoV-2 chimeras**

419 The generation of a replication competent recombinant VSV chimera expressing eGFP where the

420 glycoprotein G was replaced with spike (S) protein Wuhan-Hu-1 strain (VSV-eGFP-SARS-CoV-

421 2) has been described (*13*). Additional details for the generation of the VSV recombinants

422 expressing eGFP and SARS-CoV-2 spike variants for Delta (B.1.617.2) and Omicron (B.1.1.529)

423 are described in Supplementary Appendix S1.

424

425 **Generation of SVG-A cells expressing ACE2 and TMPRSS2**

426 SVG-A cells ectopically expressing ACE2 and TMPRSS2 were generated by lentivirus

427 transduction. Briefly, lentivirus encoding human ACE2 or TMPRSS2 were generated as follows:

428 HEK293T packaging cells were seeded at 3.8×10^6 cells in a 10 cm tissue culture plate and grown

429 in complete DMEM supplemented with 10% v/v FBS at 37 °C and 5% CO_2.

430

431 Transfection mixtures containing 90 µL lipofectamine 3000 (Thermo Scientific L3000001),

432 psPAX2 (1.3 pmol; Addgene #12260), pMD2.G (0.72 pmol; Addgene #12259) and

433 TMPRSS2/pLX304 or ACE2/pLJM1 (1.64 pmol; gifts from Sean Whelan) 90 µL lipofectamine

434 3000 (Thermo Scientific L3000001), 1ul psPAX2 (1.3 pmol; Addgene #12260), 0.6 ul pMD2.G

435 (0.72 pmol; Addgene #12259) and 1.2 ul TMPRSS2/pLX304 or ACE2/pLJM1 (1.64 pmol) in 5 ml

436 OptiMEM medium (Thermo Scientific 31985062) mixed by pipetting and incubated for 20 minutes

437 at room temperature. 0.7 million cells were plated in a 10 cm plate 18 hr prior to transfection; the

438 medium was then replaced with the entire transfection mixture and cells incubated for 6 hours at

439 37°C, after which the medium was replaced with 15 ml complete DMEM medium. After 12 hours,

440 this medium was replaced with 15 ml of complete DMEM medium, which was then harvested 24

441 hr later after further growth. After addition of another 15 ml of medium, cells and virus were

442 allowed to growth, ending with a second harvest of medium. The medium was cleared of debris

443 by centrifugation at 5000 x g for 5 mins at room temperature and supernatants containing

444 lentivirus stored at -80°C.

445

446 Ectopic stably expression of ACE2 and TMPRSS2 was achieved by transduction of SVG-A cells

447 (gift from Walter J. Atwood, Brown University) gene-edited to simultaneously express

15

448 fluorescently tagged early and late endosomal markers EEA1 and NPC1 fused to mScarlett or

449 Halo, respectively (*21*). Briefly, SVG-A 1x10^6 cells were seeded in a well from a 6-well plate and

450 grown overnight in MEM media with 10% FBS. Cleared medium containing ACE2 or TMPRSS2

451 lentivirus (1 mL) was added to the cells and incubated for 16 h, following replacement with fresh

452 medium cells were incubated for additional 24 hrs. Cells were allowed to grow for another 4 days

453 in the presence of 7 µg/mL puromycin to select for ACE2 expressing cells or 5 µg/mL blasticidin

454 for TMPRSS2 expressing cells. Surviving cells were grown in the absence of puromycin of

455 blasticidin for 4 days and cell stocks frozen and kept in liquid nitrogen. SVG-A cells

456 simultaneously stably expressing ACE2 and TMPRSS2 were obtained by transduction with

457 lentivirus encoding TMPRSS2 and selection with Blasticidin of cells stably expressing ACE2.

458

459 **Preparation of VSV chimeras for imaging and infection experiments**

460 All VSV-SARS-CoV-2 variants were grown in MA104 cells in 15 to 20 150-mm dishes and infected

461 at a multiplicity of infection (MOI) of 0.01 as previously described (*9*, *13*) in addition to the Omicron

462 variant also grown in Vero TMPRSS2 cells. Briefly, media containing the viruses were collected

463 72 hours post infection and clarified by centrifugation at 1,000 x g for 10 min at 4°C. A pellet with

464 virus and extracellular particles was obtained by centrifugation in a Ti45 fixed-angle rotor at

465 72,000 x g (25,000 rpm) for 2 hours at 4°C, then resuspended overnight in 0.5 mL PBS at 4°C.

466 This solution was layered on top of a 15% sucrose-PBS solution and a pellet with virions obtained

467 by centrifugation in a SW55 swinging-bucket rotor at 148,000 x g (35,000 rpm) for 2 hours at 4°C.

468 The resulting pellet was resuspended overnight in 0.4 mL PBS at 4°C, layered on top of a 15 to

469 45% sucrose-PBS linear gradient and subjected to centrifugation in a SW55 swinging-bucket rotor

470 at 194,000 x g (40,000 rpm) for 1.5 hours at 4°C. The predominant light scattering band located

471 in the lower one-third of the gradient and containing the virions was removed by side puncture of

472 the gradient tube. Approximately 0.3 ml of this solution was mixed with 25 ml of PBS and

473 subjected to centrifugation in a Ti60 fixed-angle rotor at 161,000 x g (40,000 rpm) for 2 hours at

474 4°C. The final pellet was resuspended overnight in 0.2 - 0.5 mL PBS aliquoted and stored frozen

475 at -80°C for use in subsequent imaging and infection experiments, without detectable change in

476 infectivity.

477

478 **Isolation and propagation of SARS-CoV-2**

479 A human isolate of SARS-CoV-2, Wuhan (B.1) was obtained in accordance with the protocol

480 approved by the Helsinki University Hospital laboratory research permit 30 HUS/32/2018§16.

481 Briefly, a nasopharyngeal swabs from a patient infected with COVID19 was suspended in 0.5 ml

482 of universal transport medium (UTM®, Copan Diagnostics) and used to inoculate Vero TMPRSS2*

483 cells for 1 h at 37°C, after which the inoculum was replaced with minimum essential medium

484 (MEM) supplemented with 2% heat inactivated FBS, L-glutamine, penicillin, and streptomycin and

485 virus allowed to grow for 48 hr. Virions in the supernatant (P0) were subjected to a similar second

486 round of propagation (P1), analyzed by DNA sequencing, and aliquots stored at 80°C in a solution

487 containing MEM, 2% heat inactivated FCS, 2 mM L-glutamine, and 1% penicillin-streptomycin.

488 Extent of virus replication was determined by real-time PCR (RT-PCR) using primers for SARS-

489 CoV-2 RNA-dependent RNA polymerase (RdRP) (*40*).

490

491 **VSV-eGFP-SARS-CoV-2 infection assays**

492 Infection assays for the Wuhan, Delta or Omicron VSV-eGFP-SARS-CoV-2 chimeras were done

493 at a final ~ 80% confluency of cells plated one day before the infection assay as previously

494 described (*9*) and further explained in Supplementary Appendix S1.

495

496 **SARS-CoV-2 infection assays**

497 All experiments with SARS-CoV-2 were performed in biosafety level 3 (BSL3) facilities at the

498 University of Helsinki with appropriate institutional permits. Virus samples were obtained under

499 Helsinki University Hospital laboratory research permit 30 HUS/32/2018§16. Infections were

500 carried for 16 hours at 37°C with 5% CO_2. Cells were then fixed with 4% paraformaldehyde in

501 PBS for 30 min at room temperature before being processed for immunodetection of viral N

502 protein, automated fluorescence imaging, and image analysis. The detailed protocol is outlined in

503 the Supplementary Appendix S1.

504

505 **VSV-SARS-CoV-2 Atto 565 labeling and single molecule Atto 565 dye calibration**

506 Stock solutions of VSV-SARS-CoV-2 and its variants at a concentration of ~150 µg/ml viral RNA

507 were conjugated with Atto 565-NHS ester (Sigma-Aldrich, cat. 72464) as previously described

508 (*41*). The number of Atto 565 molecules attached to a single virion was determined by comparing

509 the total fluorescence intensities associated with a given virion and the fluorescence intensity

510 associated with the last bleaching step of the same virion, as previously described (*42, 43*). A

511 brief description of these steps are outlined in the Supplementary Appendix S1.

512

513 **Trypsin cleavage of VSV-eGFP-SARS-CoV-2**

514 VSV-eGFP-SARS-CoV-2 and variants (as indicated in text) at a concentration 30 µg/mL virus

515 RNA in a total volume of 100 µL in DMEM with 25 mM HEPES, pH 7.4 were incubated for 30 min

17

516   at 37°C with 1 µg/mL trypsin (Pierce trypsin, TPCK treated from Thermo scientific cat. PI20233).

517   Trypsin activity was terminated with 10 µM Aprotinin (bovine lung, Sigma-Aldrich cat. A1153). The

518   required concentration of trypsin was determined by trypsin serial dilution, aiming for the largest

519   infectivity Vero cells whose endogenous cathepsin proteases activity had been inhibited with 20

520   µM E-64 (Figure S19). Infections were done with VSV or its variants at a concentration of 0.5

521   µg/mL (for Vero and Vero TMPRSS2) or 5 µg/mL virus RNA (for Caco-2 or Calu-3). When

522   required, the trypsin cleaved VSV's were used together with infection inhibitors or for pH bypass

523   experiments as described above.

524

525   ***In vitro* release of S1 from trypsin activated VSV-SARS-CoV-2**

526   VSV-eGFP-SARS-CoV-2-Atto 565 was incubated with trypsin at various concentrations for 30

527   min and the reaction stopped by addition of aprotinin to a final concentration of 10 µM. Virus was

528   then used to infect Vero cells with endogenous cathepsin proteases inhibited with 20 µM E-64 to

529   determine maximum concentration of trypsin required to proteolytically activate the virus.   Virus

530   either without or with trypsin cleavage, or with ACE2 bound before and after trypsin cleavage

531   were plated onto a poly-D-lysine coated glass to determine the number of Atto 565 fluorescence

532   dyes associated with each one of the single particles using spinning disc confocal microscopy. At

533   least 8,000 particles were imaged per experimental condition to guarantee the ability to distinguish

534   intensity losses of at least 20%. Every experimental condition was repeated in triplicate and the

535   intensity of the mean intensities of the peak Gaussian fit was used to determine the after and

536   deviation of Atto565 dyes per condition, Figure S19.

537

538   A computation simulation was performed to validate the ability to detect with statistical

539   significance a 20% fluorescence intensity loss approximate equivalent to the 20-30% loss

540   observed *in vivo* with virions when attached to the cell surface of Vero TMPRSS2. Experimental

541   data corresponding to 8,845 undigested control virions were used to generate a probability density

542   function from which 9 fit parameters (3 mean intensities with corresponding sigmas and the

543   relative contribution to the distribution) were obtained by fitting the sum of 3 Gaussian

544   distributions. These parameters were then used to generate 8845 random numbers of the same

545   distribution and compared to the experimental data set to illustrate the accuracy of the simulation.

546   A second set of 8845 random numbers were generated with mean intensities reduced by 20%, to

547   generate a probability density distribution representing the fluorescence intensities after ACE2

548   mediated release of S cleavage by TMPRSS2.

549

18

Nasal pH and temperature measurements

The pH and temperature of the nasal cavity, close to the under lower turbinate, from each nostril from 17 healthy volunteers, age 26-55, males and females, were determined using a Digitrapper pH 400 recorder (Medtronic) connected to a single-use Versaflex disposal dual sensor pH catheter (Medtronic) and a Beurer FT 15/1digital thermometer (Beurer), respectively. The temperature dependent pH response of the Digtrapper was corrected to consider the temperature in the nostrils determined for each volunteer.

These measurements were obtained under the ethical permit n. HUS/2502/2020 granted by the ethical committee of Helsinki and Uusimaa hospital district to Ahmed Geneid and Markku Patjas at the Helsinki University Hospital.

Preparation of glass coverslips

Infection and uptake assays of VSV-eGFP-SARS-CoV-2 and VSV-P-eGFP-SARS-CoV-2 done by spinning disc confocal microscopy visualization, were performed using 25 mm #1.5 coverslips bound with polydimethylsiloxane (PDMS) of about 1 mm in thickness and of 3 mm (infection) or 5 mm (uptake) in diameter wells punched as previously described (*9*) (see also in Supplementary Appendix S1).

Live cell spinning disc-confocal microscopy

Visualization experiments were done with an inverted spinning disc confocal microscope (*42*) following the details described in Supplementary Appendix S1.

Live cell lattice light sheet microscopy

Cells were plated in a 35 mm culture dish containing 5 mm in diameter glass coverslips to achieve 60% final confluency the day of each experiment. Immediately before lattice light sheet visualization, the cover slip was placed on top of parafilm placed in a 10 cm in diameter petri dish including wet chem wipes to maintain humidity. Approximately 10 µL of a solution containing VSV-SARS-CoV-2 in DMEM with 25 mM HEPES at pH 7.4 for the times indicated in the text, after which the coverslips were transferred to the imaging stage of the LLSM. Visualization was done using phenol red free media, (FluoroBrite™) supplemented with 5% FBS and 25 mM HEPES at the indicated pH. Imaging was done at 37°C in the presence of 5% CO_2 and 100 nM fluorescent Alexa 647 or Alexa 549 dyes added to the medium to determine the cell boundary. The LLSM was operated in sample scan mode with 0.5 µm spacing between each plane along the z-imaging

19

584   axis and samples imaged as a time series of stacks acquired 1 to 5 sec using dithered multi-

585   Bessel lattice light sheet illumination (*40*, *43*). When using gene edited SVG-A cells expressing

586   EEA1-Scarlett and NPC1-Halo, Halo was first labeled by incubation of the cells with 200 nM

587   JFX646 for 30 minutes at $37^{o}C$ and 5% CO_2 followed by three 2-minute washes with DMEM

588   containing 10% FBS, 25 mM HEPES, pH 7.4 within 2 hours prior to virus addition to the cover

589   slip. Time series containing 120-300 z-stacks, sequentially obtained every 1-5 sec were acquired

590   with ~ 10 ms exposures per channel.

591

592   The following protocol was used to determine the dwell time between binding of trypsin activated

593   VSV-SARS-CoV-2-Atto 565 Wuhan and release of the S-fragment. Briefly, trypsin activated

594   virions at 5 µg/mL viral RNA were flowed on top of Vero cells plated in a homemade microfluidic

595   flow cell one day prior to the experiment to achieve a 90% confluency. Soluble 100 nM Alexa 549

596   added to the medium was used to determine the cell outline. Samples were imaged with an AO-

597   LLSM microscope configured for sample scan imaging to acquire every 4 sec a stack with planes

598   separated by 0.6 um (0.3 um along the z-optical axis) using an exposure of 3 ms/plane.

599

600   **Single virus tracking and image analysis**

601   The 3D stacks obtained using LLSM were deskewed and the diffraction limited spots were

602   detected and tracked in three dimensions using the automated detection algorithms that uses

603   least-squares minimization numerical fitting with a model of the microscope PSF approximated

604   by a 3D Gaussian function and implemented using the MATLAB developed previously (*40*)

605   available for download (https://github.com/VolkerKirchheim/TrackBrowser_Matlab.git).

606   Estimated fluorescent intensities associated with each spot were calculated from the

607   corresponding amplitudes of the fitted 3D Gaussian and compared to those from single virions

608   bound to poly-D-lysine coated glass imaged under the same acquisition conditions and whose

609   dye content was determined by single bleaching steps (*43*).

610

611   Tracks with intensities corresponding to single virus were exported into a custom-made program

612   written in LabView for visualizing trajectories available for download

613   (https://github.com/VolkerKirchheim/TrackBrowser_LabView.git). Each virus trajectory was

614   visually examined for co-localization within a specific compartment (cell surface, EEA1 early

615   endosomes or NPC-1 late endosomes/lysosomes, cytosol) and the mean squared displacements

616   (MSDs) were calculated from all 3D coordinates for all possible time frames within that

617 compartment. A non-linear relationship between MSD and time for anomalous diffusion was fitted

618 to the MSD data according to the power law in equation:

619 $$MSD(t) = 6\,K\,t^{\alpha}$$

620 where K is the generalized diffusion coefficient and α is the anomalous exponent.

621

622 **Statistical analysis**

623 An unpaired t-test was used to determine the statistical significance in the difference between

624 control and experimental values.

FIGURE LEGENDS:

Figure 1. **SARS-CoV-2 infection requires endocytosis**

(A) Schematic of live-cell volumetric lattice-light-sheet fluorescence microscopy (LLSM) imaging experiments **(B-G)** used to obtain 3D time series of VSV-eGFP-P-SARS-CoV-2-S-Atto 565 entry into VERO TMPRSS2 cells during early stages of infection using an MOI of 2. For each experiment, three cells were consecutively imaged volumetrically every 4.7 seconds for 10 min.

(B) Maximum intensity projections showing fluorescently tagged VSV-SARS-CoV-2 within 1 um in thickness optical sections from the first frame of the time series acquired for representative cells 1, 2 and 3.

(C) Representative single virion trajectories of VSV-eGFP-P-SARS-CoV-2-S-Atto 565 within a 1 um optical slice of a time series acquired during the first 10-minutes of cell 1. Traces highlight particles at the cell surface (black) and within the cell volume after endocytosis (blue), in both cases containing colocalized eGFP-P and S-Atto 565; it also shows traces in the cytosol (green) containing eGFP-P upon its separation from the Atto565 signal (light blue). Single images highlighting these events are shown in the panels below.

(D) Orthogonal projection of the traced event highlighted in **(C)**.

(E) Representative summary of 266 virion traces analyzed during cell entry. Data from single coverslips (out of five) obtained per each cell type are shown. Vertical traces highlight the transfer of virions from the cell surface to the cell interior (assumed to be in endosomes because the colocalization of the eGFP-P with S-Atto 565 signals) or from endosomes to the cytosol (upon loss of localization of the eGFP-P and S-Atto 565 signals). Events corresponding to step wise loss of the S-Atto 565 signal at the cell surface are indicated (yellow).

(F) Representative plot illustrating the mean squared displacements (MSD) for the trajectory depicted in **(D)** when the particle is at the cell surface (black), in endosomes (blue), or in the cytosol upon separation of eGFP-P (which remains in endosomes, light blue) and S-Atto565 in the cytosol (green).

(G) Summary dot plot showing the diffusion mode (α) for 1692 virion trajectories and corresponding 139 penetration events; all penetration events occurred from endosomes except for one event at the cell surface in a single Vero TMRSS2 cell. The plot highlights the confined motion ($\alpha < 0.80$) of virions at the cell surface, trajected motion ($\alpha > 1.2$) in endosomes, and Brownian motion ($0.80 < \alpha < 1.2$) in the cytosol.

(H-J) Effect of inhibition of endocytosis in the infection by VSV-eGFP-SARS-CoV-2 **(H,I)** or a human isolate of SARS-CoV-2 **(J)**. Top panel shows examples of infection observed in representative fields of Vero TMPRSS2 over expressing or not the dominant negative dynamin

22

659   K44A mutant or treated or not with 40 µM dynasore-OH. Images in the top panels were obtained

660   using spinning disc confocal microscopy and show maximum intensity projections. Results from

661   similar infections obtained with different cell types are shown in the bottom panel. The difference

662   of results between control conditions and inhibition of endocytosis by K44A dynamin

663   overexpression or incubation with dynasore-OH incubation was statistically significant with p

664   value of <0.001 using an unpaired t-test.

665

666   **Figure 2. Endocytic entry routes of VSV-SARS-CoV-2**. VSV-eGFP-P-SARS-CoV-2 was used

667   to infect SVG-A gene-edited to express early endosomal antigen 1 fused to the fluorescent protein

668   Scarlet (EEA1-Scarlett) as an early endosomal marker and for late endosomal/lysosomal

669   compartments a Halo-tagged version of the cholesterol transporter Niemann Pick C1 (NPC1-

670   Halo) together with ectopic expression of ACE2 and TMPRSS2 and volumetrically imaged using

671   LLSM according to Fig. 1.

672   **(A)** Representative 2 µm projection from the first frame of the time series acquired 8-min after

673   inoculation.

674   **(B-E)** Representative examples of single trajectories of VSV-eGFP-P-SARS-CoV-2 highlighting

675   the extent of colocalization between eGFP-P and EEA1 or between eGFP-P and NPC-1 Halo

676   labeled with JFX646 (top panel), the orthogonal projection of the trajectory (middle panel) and

677   corresponding plots for number of VSV particles on the spot, extent of colocalizations and MSD

678   (bottom panels). Additional examples found in related Fig S11-16.

679   **(F)** Representative summary of results for 257 and 373 virion traces analyzed during cell entry

680   from single coverslips (out of a total of five) plated with SVG-A ACE2 or SVG-A ACE2 TMPRSS2.

681   Vertical and diagonal traces highlight the transfer of virions from the cell surface to its interior and

682   associated with early or late endosomes/lysosomes as defined by colocalization of eGFP-P with

683   EEA1-Scarlett or eGFP-P with NPC1-Halo, respectively.

684

685   **Figure 3. Surface entry route of SARS-CoV-2**

686   **(A-G)** VSV-eGFP-P-SARS-CoV-2-S-Atto 565 was used to infect Vero TMPRSS2 cells and used

687   to study the effect by acidic pH in the medium on the TMPRSS2 mediated surface release of the

688   S-fragment, on cellular location of fusion and genome delivery and on infectivity.

689   **(A, B)** Single virion trajectories of VSV-eGFP-P-SARS-CoV-2-Atto565 in a Vero TMPRSS2 cell

690   incubated at pH 6.8 showing in **(A)** an example of S-release at the surface without subsequent

691   fusion and **(B)** an example of S-release followed by penetration of eGFP-P to the cytosol.

692   Orthogonal views of the tracings and corresponding time-dependent fluorescent intensities for S-
693   Atto 565 and eGFP-P are shown.

694   **(C)** Representative summary from 237 virion traces analyzed during cell entry. Data from single
695   coverslips (out of five) obtained per each pH condition in the medium are shown. Vertical traces
696   of cells incubated at 6.8 highlight the efficient transfer of virions from the cell surface to the cell
697   interior (based on loss of signal colocalization between eGFP-P and S-Atto 565 and
698   corresponding change of diffusion from constrained to directed). Events of stepwise partial loss
699   of S-Atto 565 are indicated (yellow). Similar data with cells incubated at pH 6.2 shows
700   accumulation of virions in endosomes, complete absence of fusion events from the cell surface
701   and limited number of fusion events from endosomes.

702   **(D)** Data showing fraction of virions that released the S-fragment from virions at the cell surface
703   of Vero TMPRSS2 cells incubated at pH 6.8 in the absence or presence of 10 uM Camostat, or
704   of Vero E6, Caco-2 or Calu-3 cells also at pH 6.8 and in the absence of Camostat. The difference
705   of results between control and all other conditions was statistically significant with p value of
706   <0.0001 using an unpaired t-test.

707   **(E)** Cumulative plot corresponding to the dwell time between the stepwise partial drop of the S-
708   Atto 565 signal of a virion at the cell surface and fusion defined by surface spreading of the
709   remaining Atto 565 signal and transfer into the cytosol of eGFP-P. Data from 86 traces and from
710   five experiments.

711   **(F)** Effect of extracellular pH on the transfer of eGFP-P of virions from the cell surface (red) or
712   from endosomes (black) to the cytosol. Each dot represents average +/- std from 5 coverslips
713   with 3 cells imaged per coverslip and at least 600 virus tracked per condition. Line across box
714   represents the median of distribution and the top and bottom represent the quartiles.

715   **(G)** Effect of extracellular pH of the cell medium on the mode of diffusion of the eGFP-P signal
716   associated with a virion before and after delivery from the surface (red) or from endosomes (black)
717   to the cytosol.

718   **(H)** pH bypass infection experiments to test the effect of extracellular acidic pH on the extent of
719   infection of Vero or Vero TMPRSS2 cells by VSV-eGFP-SARS-CoV-2 alone or treated for 30 min
720   with 1 µg/mL trypsin; experiments carried in the absence (top panel) or presence (middle and
721   bottom panels) of 40 uM dynasore-OH. Each data point represents an experiment. In each case,
722   the values determined at pH 6.8 and 7.4 are significantly different with a p value of at least <0.0003
723   using an unpaired t-test.

724   **(I)** pH bypass infection experiment using authentic SARS-CoV-2 and Vero TMPRSS2* cells in the
725   absence or presence of 40 uM dynasore-OH. Each data point represents an experiment. No

726   statistical difference was observed in the absence of dynasore-OH between pH 6.8 and pH 7.4

727   (p = 0.13); the difference was statistically significant in the presence of dynasore-OH (p < 0.0001)

728   using an unpaired t-test.

729

730   **Figure 4. Entry routes of VSV-SARS-CoV-2 variants are conserved.**

731   **(A-C)** Effect of extracellular pH and type of cells infected with the indicated variants of VSV-eGFP-

732   P-SARS-CoV-2 on **(A)** the extent of S-fragment release from the cell surface and of **(B)** fusion

733   from the cell surface or **(C)** from endosomes.

734   **(D)** Experiments to determine the effect of extracellular pH on the extent of infection by the Delta

735   and Omicron variants of VSV-SARS-CoV-2 in the presence of 40 uM dynasore-OH. The pH

736   bypass experiments in the right panel were done with trypsin-cleaved virions. The bottom two

737   rows compare results obtained with the Omicron variant grown in MA104 or Vero TMPRSS2 cells.

738   Western Blot showing cleavage states of spike protein of VSV-SARS-CoV-2-Omicron grown in

739   different cell types. The bypass pH experiments in the left panels show statistical differences

740   between pH 6.8 and pH 7.4 (p < 0.0001) for Delta and Omicron grown in Vero TMPRRSS2 and

741   of minimal significance for Omicron grown MA 104 using an unpaired t-test. Similar analysis for

742   the experiments in the right panels show statistical differences between pH 6.8 and pH 7.4 for

743   Delta (p < 0.0001) and Omicron grown in Vero TMPRRSS2 (p < 0.0001) and also for Omicron (P

744   = 0.0015) grown in MA 104.

745   **(E)** Nasal pH values determined from 17 healthy individuals. Each dot represents a single pH

746   determination by the pH catheter at the lower turbinate of the right and left nostrils.

747

748   **Figure 5. Schematic representation of the principal entry routes SARS-CoV-2 uses for**

749   **infection.** Entry starts with membrane attachment and ends with spike-protein (S) catalyzed

750   membrane fusion releasing the viral contents into the cytosol. Fusion activity depends on two

751   proteolytic cleavage steps, one typically carried out by furin in the producing cell and the second

752   by TMPRSS2 on the cell surface on in endosomes of the target cell. Alternatively, endosomal

753   cathepsins can carry out both cleavages. Exposure of the virus to an acidic milieu is essential for

754   membrane fusion, genome penetration, and productive infection. Fusion and penetration occur

755   only in acidic early and late endosomal/lysosomal compartments but not at the cell surface, even

756   when the furin and TMPRSS2 cleavages have both occurred. Fusion and penetration can occur

757   at the cell surface of cells expressing TMPRSS2 if the extracellular pH is ~6.8.

758

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936

Author contributions:

Alex J.B. Kreutzberger carried all the experiments except those with human SARS-CoV-2 isolates. Louis-Marie Bloyet and Spencer Stumpf generated, characterized, and sequenced the recombinant virus VSV-eGFP-P-SARS-CoV-2 Whuhan. Zhuoming Liu generated, characterized, and sequenced the recombinant viruses VSV-eGFP-SARS-CoV-2 Delta and Omicron. Anwesha Sanyal generated SVGA-A ACE2 and SVGA-A ACE2 TMPRSS2, maintained the cells lines and assisted with infectivity assays. Catherine A. Doyle helped analyzed data and carried out some VSV-SARS-CoV-2 infection assays. Elliott Somerville helped with virus sample preparation for DNA sequence. Gustavo Scanavachi and Anand Saminathan helped collect LLSM imaging data. Giuseppe Di Caprio set up the single particle data analysis pipeline. Volker Kiessling developed the LabVIEW-based viewer, simulated data and produced the single-particle MSD fitting algorithm. Sean P. J. Whelan oversaw the work to generate and characterize the VSV-chimeras, participated in early discussions, and helped edit portions of the manuscript. Giuseppe Balistreri guided the work on SARS-CoV-2 and performed infection assays, imaging, and image analysis.

971 Ravi Ojha performed SARS-CoV-2 infection assays, imaging, and image analysis. Olli Vapalahti

972 coordinated the BSL3 work, provided SARS-CoV-2 and performed virus sequencing.

973 Tom Kirchhausen and Alex J.B. Kreutzberger were responsible for the overall design of the study;

974 Tom Kirchhausen drafted the manuscript with direct input from Alex J.B. Kreutzberger; all authors

975 commented on the manuscript.

976

977 **Competing interests**

978 T.K. is a member of the Medical Advisory Board of AI Therapeutics, Inc. The other authors declare

979 no competing interests.

980

981 **Data and material availability**

982 All materials and data generated in this study are available upon request. Further information and

983 requests for resources and reagents should be directed to and will be fulfilled by the lead contact,

984 Dr. Tom Kirchhausen, mailto:kirchhausen@crystal.harvard.edu. Requests for VSV-SARS-CoV-2

985 chimeras and their materials transfer agreements (MTA) should be directed to and will be fulfilled

986 by Dr. Sean Whelan, spjwhelan@wustl.edu. This study did not generate any unique large-scale

987 datasets. The LabView code for visualizing trajectories is available for download (GITHUB).
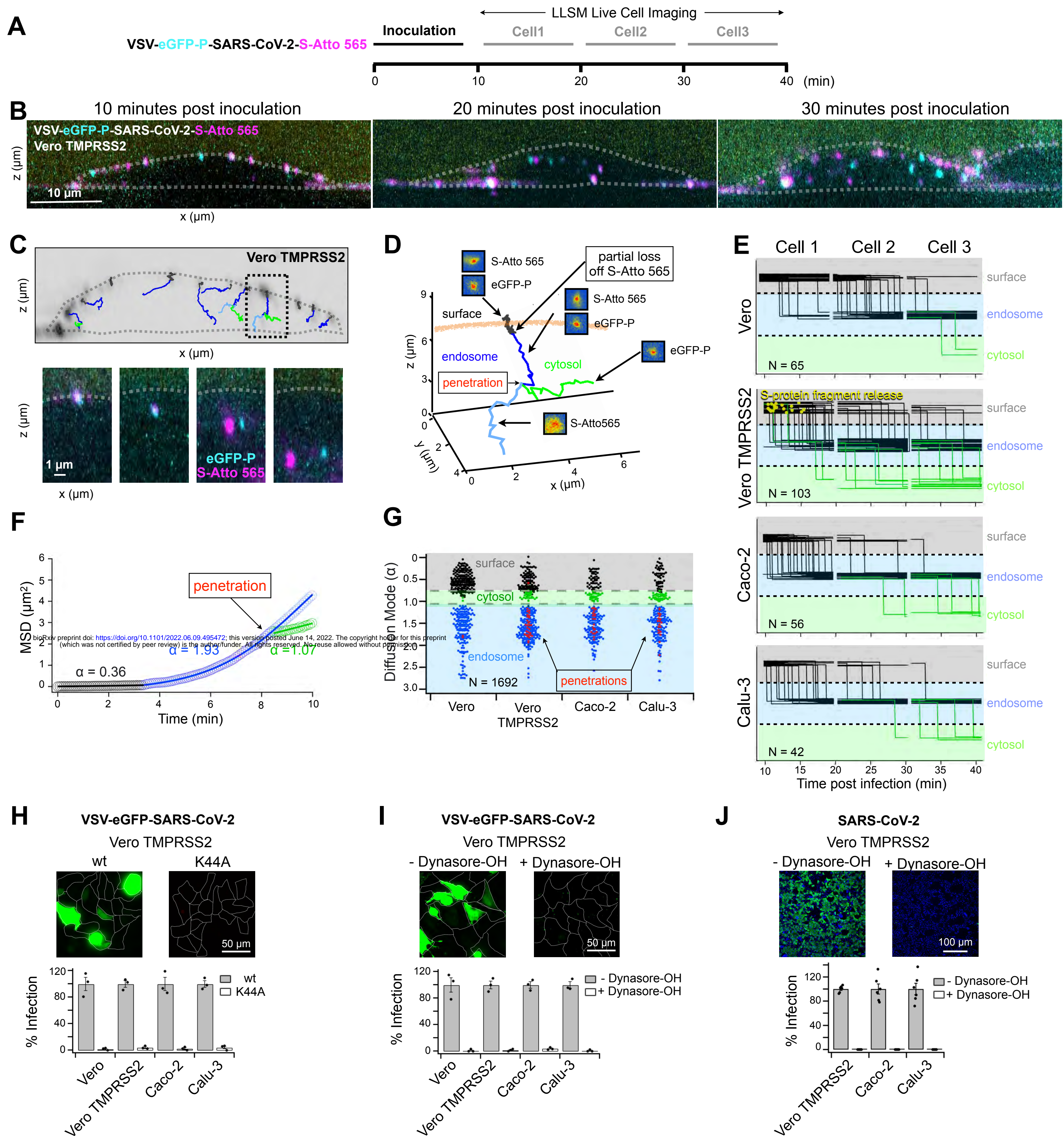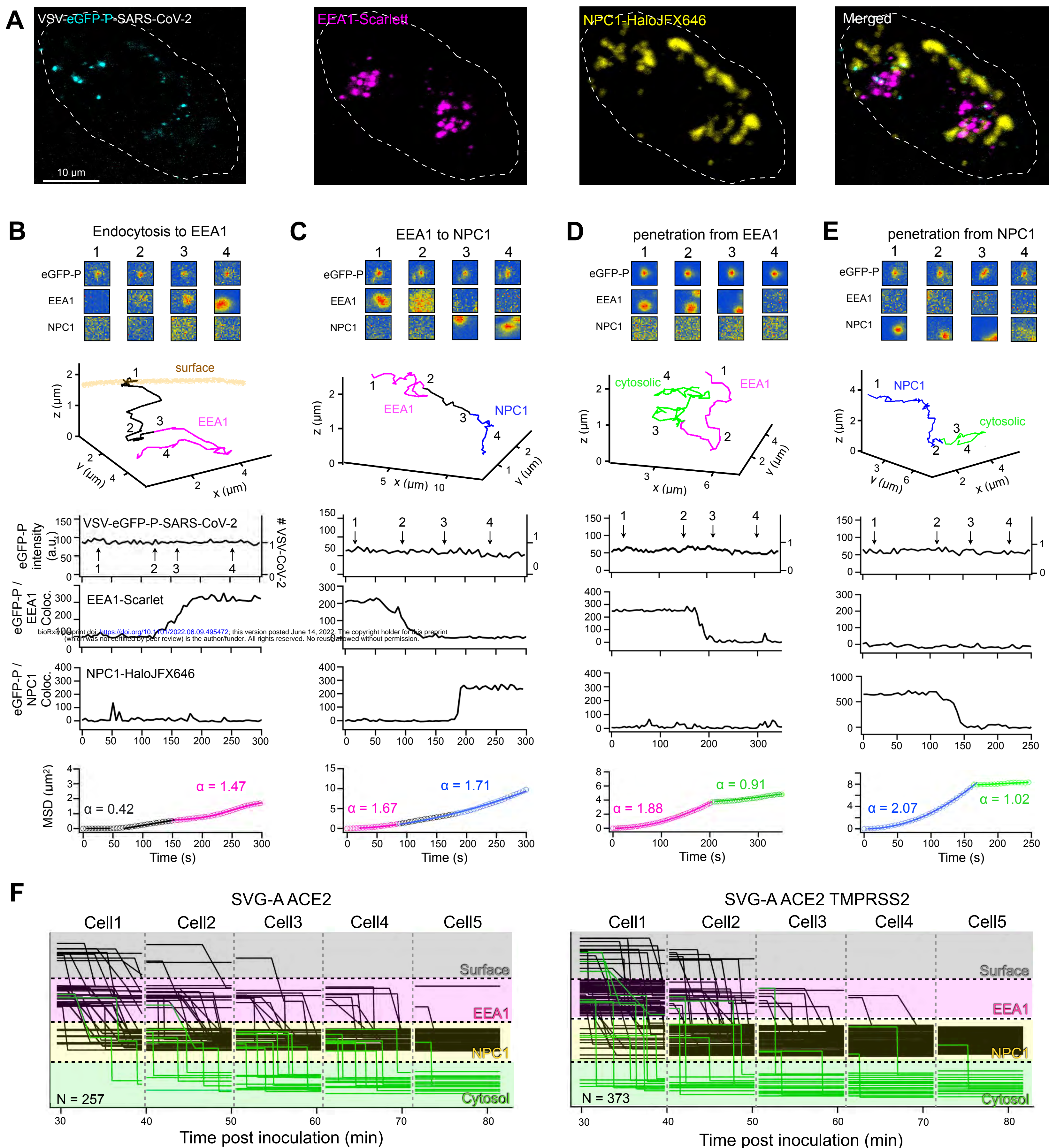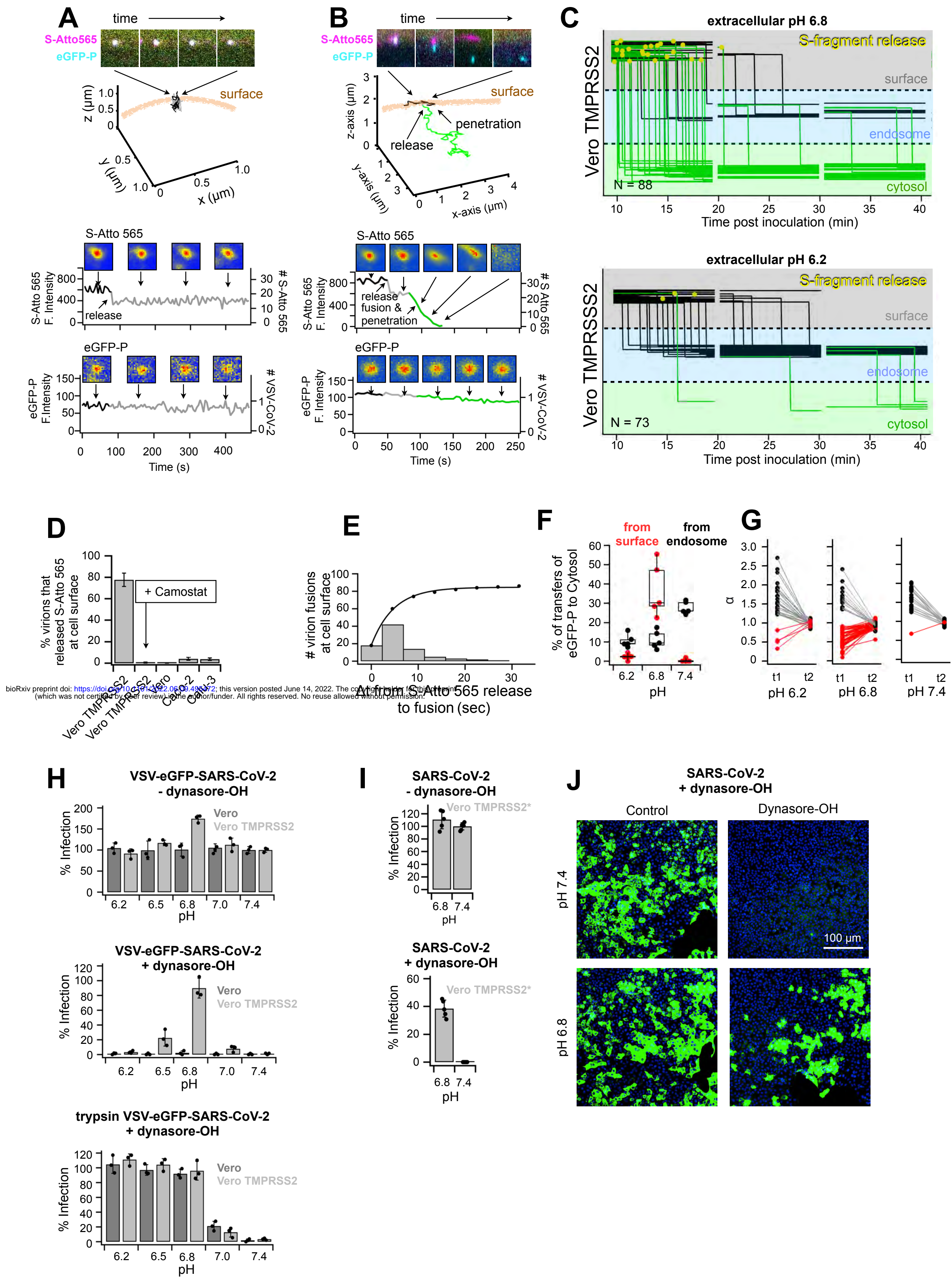
A

VSV-eGFP-P-SARS-CoV-2-S-Atto 565

LLSM Live Cell Imaging

Cell1   Cell2   Cell3

Inoculation

0   10   20   30   40   (min)

B

10 minutes post inoculation   20 minutes post inoculation   30 minutes post inoculation

VSV-eGFP-P-SARS-CoV-2-S-Atto 565
Vero TMPRSS2

z (μm)   10 μm   x (μm)

C

Vero TMPRSS2

z (μm)   x (μm)

z (μm)   1 μm   eGFP-P   S-Atto 565   x (μm)

D

S-Atto 565
eGFP-P   partial loss off S-Atto 565

S-Atto 565
eGFP-P

surface

endosome   eGFP-P

penetration   cytosol

S-Atto565

z (μm)   y (μm)   x (μm)

E

Cell 1   Cell 2   Cell 3

Vero   surface   endosome   cytosol   N = 65

Vero TMPRSS2   S-protein fragment release   surface   endosome   cytosol   N = 103

Caco-2   surface   endosome   cytosol   N = 56

Calu-3   surface   endosome   cytosol   N = 42

Time post infection (min)
10 15 20 25 30 35 40

F

MSD (μm²)

penetration

α = 1.93   α = 1.07

α = 0.36

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Time (min)

G

Diffusion Mode (α)

surface

cytosol

endosome

penetrations

N = 1692

Vero   Vero TMPRSS2   Caco-2   Calu-3

H   VSV-eGFP-SARS-CoV-2

Vero TMPRSS2

wt   K44A

50 μm

% Infection

wt   K44A

Vero   Vero TMPRSS2   Caco-2   Calu-3

I   VSV-eGFP-SARS-CoV-2

Vero TMPRSS2

- Dynasore-OH   + Dynasore-OH

50 μm

% Infection

- Dynasore-OH   + Dynasore-OH

Vero   Vero TMPRSS2   Caco-2   Calu-3

J   SARS-CoV-2

Vero TMPRSS2

- Dynasore-OH   + Dynasore-OH

100 μm

% Infection

- Dynasore-OH   + Dynasore-OH

Vero TMPRSS2   Caco-2   Calu-3

Figure 1

A

VSV-eGFP-P-SARS-CoV-2   EEA1-Scarlett   NPC1-HaloJFX646   Merged

10 μm

B Endocytosis to EEA1   **C** EEA1 to NPC1   **D** penetration from EEA1   **E** penetration from NPC1

F SVG-A ACE2   SVG-A ACE2 TMPRSS2

Figure 2

Figure 3

A VSV-eGFP-P-SARS-CoV-2

B VSV-eGFP-P-SARS-CoV-2

C VSV-eGFP-P- SARS-CoV-2

D

VSV-eGFP-SARS-CoV-2-Delta grown in Vero TMPRSS2

VSV-eGFP-SARS-CoV-2-Omicron grown in Vero TMPRSS2

VSV-eGFP-SARS-CoV-2-Omicron grown in MA104

E

Intranasal pH

Figure 4

-TMPRSS2

pH = 7.4

+TMPRSS2

pH = 7.4　　**pH = 6.8**　　**pH < 6.8**

Early
Endosome

Late
Endosome/
Lysosome

Spike　ACE2　　　　TMPRSS2
　　　　　　Cathepsin

S1

S2

Figure 5

1   **Figure S1. Virus infection and uptake in different cell lines.**

2   **(A)** Schematic of infection (fixed cell imaging with spinning disc) or virus uptake (live cell imaging

3   with LLSM) protocols.

4   **(B, C)** Example images **(B)** and quantification **(C)** of VSV-eGFP-SARS-CoV-2 infection in Caco-

5   2 (left), Calu-3 (middle left), Vero (middle right), and Vero TMPRSS2 (right). Each condition

6   contained 3 independent experiments.

7   **(D, E**) Example images and quantification of **(E)** VSV-eGFP-P-SARS-CoV-2 uptake. Each

8   condition contained 3 independent experiments.

9

10   **Figure S2. VSV-eGFP-P-SARS-CoV-2-Atto565 labeling.**

11   **(A)** Maximum intensity projection image of 5 z-planes spaced 0.27 µm of VSV-eGFP-P-SARS-

12   CoV-2-Atto 565 bound to poly-lysine coated glass collected on a spinning disc-confocal

13   microscope. Shift by 5 pixels of channels highlights colocalization of the fluorescence signals for

14   eGFP-P and S Atto 565 for each virion spot.

15   **(B)** Single molecule photobleaching steps of Atto 565 dye attached to virus taken at 1 second

16   exposure with the step intensity distribution of the last step of 727 VSV-eGFP-P-SARS-CoV-2-

17   Atto565 virus shown in inset.

18   **(C)** The linear relationship between exposure times and fluorescence intensity allows the step

19   size taken at 1 second exposure to be used to calibrate the number of dyes per virus when taken

20   at 20 ms exposures when the dye single on each virus spot is not (*1*). This was used to determine

21   an average distribution of ~30 dyes per VSV-SARS-CoV-2-Atto 565.

22   **(D)** Labeling VSV-eGFP-SARS-CoV-2 with Atto 565 showed little inhibition of viral infection.

23

24   **Figure S3. VSV-eGFP-P-SARS-CoV-2 uptake in Vero E6, Caco-2 and Calu-3 cells**.

25   Trajectories overlayed onto a 2 µm projection though the x-axis of a cell imaged using LLSM

26   microscopy with whole cell volumes collected every 4.7 seconds. Over the course of 10 minutes

27   virus was observed to be on the cell surface (black trajectories), internalize into the cell with both

28   eGFP-P and Atto 565 dye co-localizing (blue trajectories), with some eGFP-P observed to

29   separate from Atto 565 signal (green). Example images of boxed trajectories shown below with

30   complete orthogonal projections underneath. The intensity of the eGFP-P and the intensity of

31   Atto 565 co-localizing with the eGFP-P signal are showing as well as the MSD analysis of the

32   trajectory calculated localize when the particles are co-localized to the surface, inside the cell

33   following the trajectory of the Atto 565 signal or following the trajectory of the eGFP-P signal after

34   loss of co-localization with Atto 565.

1

2 **Figure S4. VSV-eGFP-P-SARS-CoV-2 surface colocalized trajectories.** Example events of

3 VSV-eGFP-P-SARS-CoV-2-Atto-565 co-localized to the cell surface from Vero E6 **(A)**, Vero E6

4 TM **(B)** Caco-2 (**C**) and Calu-3 **(D)** cells. A complete orthogonal projection (left), the intensity of

5 eGFP-P and co-localization with Atto 565 spots (middle left), the MSD analysis (middle right), and

6 the alpha values of every virus trajectory during their colocalization at the cell surface in the

7 respective cell type.

8

9 **Figure S5. VSV-eGFP-P-SARS-CoV-2 endocytosis trajectories.** Example events of VSV-

10 eGFP-P-SARS-CoV-2-Atto-565 co-localized to the cell surface and subsequent endocytosis into

11 Vero E6 **(A)**, Vero TMPRSS2, **(B)** Caco-2 **(C)** and Calu-3 **(D)** cells. A complete orthogonal

12 projection (left), the intensity of eGFP-P and co-localization with S Atto 565 spots (middle left),

13 the MSD analysis (middle right), and the alpha values (right) of every trajectory when it was co-

14 localized to the cell surface then to the alpha value after it entered the cell volume for every virus

15 with this behavior in the respective cell type.

16

17 **Figure S6. VSV-eGFP-P-SARS-CoV-2 inside endosomes.** Example events of VSV-eGFP-P-

18 SARS-CoV-2-Atto-565 co-localized with eGFP-P and Atto565 colocalized inside Vero E6 (A),

19 Vero TMPRSS2 (B) Caco-2 (C) and Calu-3 (D) cells. A complete orthogonal projection (left), the

20 intensity of eGFP-P and co-localization with S Atto 565 spots (middle left), the MSD analysis

21 (middle right), and the alpha values (right) of every trajectory exemplifying this behavior in the

22 respective cell type.

23

24 **Figure S7. VSV-eGFP-P-SARS-CoV-2 fusion events from endosomes.** Example events of

25 VSV-eGFP-P-SARS-CoV-2-Atto-565 inside the cell with initial colocalization and subsequent

26 separation of the eGFP-P and Atto 565 signals from Vero E6 **(A)**, Vero TMPRSS2, **(B)** Caco-2,

27 **(C)** and Calu-3 **(D)** cells. A complete orthogonal projection (left), the intensity of eGFP-P and co-

28 localization with Atto 565 spots (middle left), the MSD analysis (middle right), and the alpha values

29 (right) of every trajectory exemplifying this behavior in the respective cell type.

30

31 **Figure S8. The spread of the S associated Atto 565 signal after fusion between the viral**

32 **envelope and endosomal membrane.** Cross section showing three representative examples of

33 the intensity distribution of the S associated Atto 565 signal from VSV-eGFP-P-SARS-CoV-2-Atto

1   565 before (black) and after (blue) separation of eGFP-P. Insets show images of the events used

2   for the intensity profiles.

3

4   **Figure S9. Chemical inhibition of dynamin inhibits an early step of VSV-SARS-CoV-2**

5   **infection.** Dynasore at 40 uM was incubated with the respective cell type for **(A)** beginning 1

6   hour prior to addition of VSV-SARS-CoV-2 throughout the experiment, **(B)** beginning 1 hour prior

7   to addition of VSV-SARS-CoV-2 until 1 hour after virus was removed, and **(C)** beginning 1 hour

8   after the washing away of unbound VSV-SARS-CoV-2.

9

10   **Figure S10. VSV-eGFP-SARS-CoV-2 infection of SVG-A cells endogenously expressing**

11   **EEA1-Scarlett and NPC1-Halo**.

12   **(A)** Max intensity projections of example images taken with 1 μm spacing for 20 μm.

13   **(B)** Quantification of VSV-eGFP-SARS-CoV-2 infection in parental (right), ACE2 expressing

14   (middle right), TMPRSS2 expressing (middle left), and ACE2 combined with TMPRSS2

15   expressing (left) SVG-A cells.

16   **(C)** Max intensity projection of 10 planes taken with 0.27 μm spacing of VSV-eGFP-P-SARS-CoV-

17   2 uptake and quantification

18   **(D)** Whole cell volumes were taken with each data point representing number of virus per cell for

19   parental (right), ACE2 expressing (middle right), TMPRSS2 expressing (middle left), and ACE2

20   combined with TMPRSS2 expressing (left) SVG-A cells.

21

22   **Figure S11. Endocytosis and arrival of VSV-eGFP-P-SARS-CoV-2 to early endosomes. (A,**

23   **B)** Example events with orthogonal projections (top), insets centered around the trajectory

24   coordinates at discrete time points, eGFP-P fluorescence, and co-localization intensities for the

25   EEA1-Scarlett and NPC1-HaloJFX646 channels, and the MSD analysis of the event. **(A)** Depicts

26   endocytic uptake of virus and accumulation in an EEA1 positive compartment over ~2 minutes

27   after internalization. **(B)** Depicts virus internalization and accumulation in an EEA1 positive

28   compartment with subsequent fusion with another EEA1-Scarlett positive endosome.

29

30   **Figure S12. Trafficking of VSV-eGFP-P-SARS-CoV-2 from early to late endosomes. (A, B)**

31   Example events with orthogonal projections (top), insets centered around the trajectory

32   coordinates at discrete time points, eGFP-P fluorescence, and co-localization intensities for the

33   EEA1-Scarlett and NPC1-HaloJFX646 channels, and the MSD analysis of the event. **(A)** An

34   event depicting virus in an early endosome with subsequent loss of EEA1-Scarlett over a 1-minute

1 time period and the rapid increase in NPC1-HaloJFX646 indicative of a fusion event with a late

2 endosome. (B) A virus particle in an early endosome showing loss of EEA1 concomitant with

3 merging with a late endosomal, NPC1-HaloJFX646 positive compartment. This particle then

4 diffuses and undergoes a subsequent fusion event with a NPC1-HaloJFX646 positive endosome

5 that contains eGFP-P corresponding to ~2 viruses.

6

7 **Figure S13. Release of VSV-EGFP-P-SARS-CoV-2 from an early endosome.**

8 **(A, B)** Example events with orthogonal projections (top), insets centered around the trajectory

9 coordinates at discrete time points, eGFP-P fluorescence, and co-localization intensities for the

10 EEA1-Scarlet and NPC1-HaloJFX646 channels, and the MSD analysis of the event. Both

11 examples show a single virus in EEA1-Scarlett positive endosome.

12

13 **Figure S14. Release of VSV-eGFP-P-SARS-CoV-2 from a late endosome.**

14 **(A, B)** Example events with orthogonal projections (top), insets centered around the trajectory

15 coordinates at discrete time points, eGFP-P fluorescence, and co-localization intensities for the

16 EEA1-Scarlett and NPC1-HaloJFX646 channels, and the MSD analysis of the event. **(A)** Shows

17 a release event of a single particle from an NPC1-HaloJFX646 positive late endosome. **(B)** Shows

18 a NPC1-HaloJFX646 positive late endosome with fluorescence corresponding to roughly 4

19 viruses. The signal corresponding to 1 virus releases from the endosome and exhibits Brownian

20 motion while the signal from the remainder continues to traffic with the late endosome.

21

22 **Figure S15: Analysis of the location of VSV-eGFP-P-SARS-CoV-2 throughout the**

23 **endosomal pathway.**

24 **(A)** Discrete timepoint in 10-minute intervals of the location of VSV-eGFP-P-SARS-CoV-2 in

25 gene-edited SVG-A EEA1-Scarlett NPC1-HaloJFX646 cells imaged using LLSM from the data in

26 Fig 2. The average localization of the virus in either early or late endosomes, and the fraction of

27 viral contents released into the cytosol is shown for cells expressing ACE2 (left) or ACE2 and

28 TMPRSS2 (right). Each point is an average of 5 cells at that timepoint with errors being S.E.M.

29 **(B)** All observed release events from a single coverslip spanning 5 cells imaged consecutively

30 were pooled to examine the number of observed fusion events in EEA1 positive early endosome

31 or NPC1 positive late endosomes/lysosomes. Each condition is an average of 5 coverslips where

32 5 cells were imaged consecutively for 10 minutes from 30 to 80 minutes post inoculation. Error

33 bars are S.E.M.

34

1   **Figure S16: Loss of S-tagged Atto565 is TMPRSS2 dependent.**

2   **(A, B**) Intensity profile and orthogonal projections of single VSV-eGFP-P-SARS-CoV-2-Atto 565

3   on the surface of Vero TMPRSS2 cells showing a characteristic stepwise loss of Atto 565 signal.

4   **(C, D)** As in A, B except that cells were pretreated for 1h with 10 µM camostat mesylate, and

5   particles showed no stepwise loss in Atto 565 signal.

6

7   **Figure S17. TMPRSS2 dependent surface fusion in the presence of slightly acidic**

8   **extracellular media.**

9   **(A, B)** Orthogonal projections (top) show location of VSV-eGFP-P-SARS-CoV-2-S-Atto 565 on

10 the cell surface (black) where eGFP-P is released into the cytosol (green). Example images of a

11 particle before and after the fusion event evident by separation of the eGFP (light blue) from the

12 Atto565 (magenta) on the surface with subsequent spread of the Atto565 signal on the plasma

13 membrane of a Vero TMPRSS2 cell at extracellular pH 6.8. Intensity of the events over time are

14 shown where the spread and decay of the S Atto 565 signal occurs following the stepwise drop

15 indicating release of S1 from the S2 of the spike protein.

16

17 **Figure S18. Proteolytic activation of S and the pH dependence of VSV-eGFP-SARS-CoV-2**

18 **infection in different cell types.**

19 **(A)** Schematic representation of a pH bypass experiment comparing the behavior of VSV-eGFP-

20 SARS-CoV-2 with or without trypsin activation.

21 **(B)** Vero, Vero TMPRSS2, Caco-2 and Calu-3 cells were treated with 40 uM dynasore-OH for 1

22 hr prior to exposure to VSV-eGFP-SARS-CoV-2 at an MOI of ~ 0.5 in media of varying pH. The

23 relative level of infection is shown compared to infection in the absence of dynasore-OH (left

24 graph). Pretreatment of virus with trypsin (right graph) rescues infection in all cell types in the

25 presence of dynasore-OH.

26

27 **Figure S19. Reconstitution of S1 release from VSV-eGFP-SARS-CoV-2-Atto 565 particles.**

28 **(A)** VSV-eGFP-SARS-CoV-2-Atto 565 infects Vero cells in the presence of the host cell cathepsin

29 protease inhibitor (20 µM E-64) when viral particles are pretreated with trypsin for 30 min at 37°C

30 (followed by inhibiting trypsin with Aprotinin).

31 **(B-D)** Calibration of the fluorescence intensity of single Atto 565 molecules using photobleaching

32 and then imaging thousands of VSV-eGFP-SARS-CoV-2-Atto 565 that were allowed to adsorb

33 on poly-lysine coated coverslips showed that trypsin cleaved virus needs to bind ACE2 to release

34 the S1 fragment. **(B)** Shows example images of a max intensity projection of 10 planes spaced

1 every 0.27 µm obtained by spinning-disc confocal microscopy of virus treated with ACE2 alone

2 (left) or first treated with trypsin and then incubated with ACE2. **(C)** Number of Atto 565 per virus

3 of an experiment with and without trypsin cleavage. A numeric simulation was run to determine

4 the distribution of a data set with similar labeling with and without a 20% loss in signal to mimic

5 what was observed in for Atto 565 signal loss in the LLSM experiments (Figure 3, S16, S17). **(D)**

6 Fluorescence intensity distribution of Atto 565 signal shows that the addition of ACE2 only causes

7 a loss of Atto 565 signal if the virus was pretreated with trypsin. All histograms are single

8 experiments with distributions representing more than 8,000 per experiment. Each experiment

9 was done in triplicate with the peak intensity of distributions determined to be 38.2 ± 3.4, $39.7 \pm$

10 4.1, 38.5 ± 1.6, and 9.2 ± 2.4 Atto 565 molecules per virus for control, trypsin, ACE2, or trypsin

11 followed by ACE2 treatments respectively. **(E)** Trypsin cleaved VSV-SARS-CoV-2-Atto 565 was

12 rapidly added to Vero cells (lacking any surface protease) using a microfluidic flow cell and after

13 binding a stepwise drop corresponding to ~25% signal was observed after a delay (F) which was

14 measured for 130 virus binding events where most step drops occurred between 8-30 seconds

15 after virus binding.

16

17 **Figure S20. Drug sensitivities of VSV-SARS-CoV-2 spike variants show that furin cleavage**

18 **determine proteolytic entry route.**

19 **(A-D)** VSV-eGFP-SARS-CoV-2 infection of spike variants including Wuhan **(A)**, Delta **(B)**, and

20 Omicron **(C, D)** with different levels of furin cleavage where virus grown in MA104 cells **(C)** had

21 defective furin cleavage while virus grown in Vero TMPRSS2 cells **(D)** were where furin cleaved

22 (Fig. 4). Cells were treated with indicated drugs for 1 hour, infected with virus for 1 hr, and then

23 washed while maintaining the concentration of the drug constant. After 8 hours cells were fixed,

24 stained with WGA-Alexa647, and imaged for eGFP expression using a spinning-disc confocal

25 microscope. The sensitive to TMPRSS2 was determined by treatments with 10 µM camostat

26 mesylate or 100 nM nafamostat mesylate. The sensitivity to cathepsins was determined with 20

27 µM E-64 while the endosomal trafficking route necessary for cathepsin dependent entry was

28 targeted with 100 nM of the inhibitor for PIKfyve, apilimod. Combinations of 10 µM camostat

29 mesylate and 20 µM E-64 or 100 nM nafamostat mesylate and 100 nM apilimod completely

30 abolished infection in all cell types as previously described (*2, 3*). Cells were infected at an MOI

31 of ~ 0.5 which was 0.5 µg/mL virus RNA for all spike variants in Vero and Vero TMPRSS2 and 5

32 µg/mL in Calu-3 cells, except for the furin defective VSV-eGFP-SARS-CoV-2-Omicron **(C)** which

33 required 30 µg/mL viral RNA to achieve and MOI of 0.5 in Calu-3 cells.

34

1   **Figure S21. Inhibition of endosomal acidification blocks VSV-SARS-CoV-2 infection for all**

2   **variants with a shift to a lower affinity in the presence of TMPRSS2 protease.** Cells where

3   incubated with indicated concentration of bafilomycin A1 for 1 hour, indicated VSV-eGFP-SARS-

4   CoV-2 spike variant was added for 1 hour, unbound virus was then washed away, and 7 hrs later

5   cells were labeled with WGA-Alexa647, fixed, and imaged for eGFP expression using a spinning-

6   disc confocal. Bafilomycin A1 was kept constant throughout the time course of the infection

7   assay.

8

1 **MATERIAL AND METHODS**

2 **Materials**

3 The following reagents were purchased as indicated: Dulbecco modified Eagle's medium(DMEM)

4 supplemented with 4.5 g/liter glucose, L-glutamine, and sodium pyruvate (catalog number 10-

5 013-CV; Corning, Inc.), minimum essential media (MEM) with L-glutamine (catalog number 10-

6 010-CV; Corning, Inc.), Media 199 (catalog number 11150067, Thermo Scientific), OptiMEM

7 media (catalog number 31985070, Life Technologies), FluoroBrite™ DMEM media (catalog

8 number A18967-02, Life Technologies), trypsin/EDTA (catalog number 25-053-CI, Mediatech),

9 fetal bovine serum (FBS) (catalog number S11150H; Atlanta Biologicals), a 100x solution of

10 penicillin-streptomycin (catalog number 45000-652; VWR), HEPES (catalog number HOL06,

11 Caisson Labs) camostat mesylate (catalog number SML0057; Sigma-Aldrich), E-64 (catalog

12 number sc-201276; Santa Cruz Biotechnology), nafamostat mesylate (catalog number 14837;

13 Cayman Chemical Company), apilimod (catalog number HY-14644;MedChem Express), dimethyl

14 sulfoxide (DMSO) (catalog number 26855; Sigma-Aldrich), Toluene (catalog number 34866,

15 Sigma-Aldrich), dichloromethane (catalog number 270997, Sigma-Aldrich), ethanol (catalog

16 number TX89125172HU, VWR), round cover glass number 1.5 (25 mm, catalog number C8-1.5H-

17 N; Cellvis), polydimethylsiloxane (PDMS) (Sylgard, catalog number DC4019862; Krayden),

18 isopropyl alcohol (catalog number 9080-03/MK303108; VWR), potassium hydroxide (catalog

19 number 484016; Sigma-Aldrich), TPCK treated trypsin (catalog number PI20233, Thermo

20 Fischer), Aprotinin from bovine lung (catalog number A1153, Sigma-Aldrich), wheat germ

21 agglutinin (WGA)-Alexa Fluor647 (catalog number W32466; Invitrogen), paraformaldehyde

22 (catalog number P6148; Sigma-Aldrich), di-basic sodium phosphate (catalog number BP329-1;

23 Thermo Fisher Scientific), monobasic potassium phosphate (catalog number P5379; Sigma-

24 Aldrich), sodium chloride (catalog number SX0420-5; EMD Millipore), poly-D-lysine (catalog

25 number P6403, Sigma-Aldrich), potassium chloride (catalog number P217-500; Thermo Fisher

26 Scientific), Tris (catalog number T-400-500; Goldbio), EDTA (catalog number E5134; Sigma-

27 Aldrich), sucrose (catalog number S0389;Sigma-Aldrich), fetal calf serum (FCS) (catalog number

28 SH30073.03; HyClone), bovine serum albumin (GE Healthcare), Triton X-100 (catalog number

29 28314; Thermo Fisher), Hoechst DNA stain (catalog number62249; Thermo Fisher), L-glutamine

30 (catalog number G7513; Sigma), Hoechst DNA dye (catalog numberH6021; Sigma-Aldrich), and

31 Alexa Fluor 647fluorescently labeled goat anti-rabbit antibody (catalog number A32733; Thermo

32 Fisher). IRDye 800CW Goat anti-rabbit IgG secondary antibody (catalog number 926-32211; Li-

33 COR). Hydroxy Dynasore (Dynasore-OH) outsourced by the Kirchhausen laboratory, Hydroxy

34 Dynasore (Dynasore-OH) (catalog number 5364, Tocris).

1

2 **Cells**

3 Vero (ATCC CRL-1586), Caco-2 (ATCC HTB-37), Calu-3 (ATCC HTB-55) were purchased from

4 ATCC. Vero TMPRSS2 cells ectopically expressing TMPRSS2 cells were a gift from Siyuan Ding

5 (Washington University St. Louis) (*4*). Vero TMPRSS2* were as described in in (*5*) Vero, Vero

6 TMPRSS2, Vero TMPRSS2* and Caco-2 cells, were grown in 10% CO_2 and at 37°C in DMEM

7 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 25 mM HEPES, pH

8 7.4. Calu-3 cells were grown in 10% CO_2 and at 37°C in DMEM supplemented with 10%

9 (Kirchhausen laboratory) or 20% (Balistreri laboratory) fetal bovine serum, 1% penicillin-

10 streptomycin, and 25 mM HEPES, pH 7.4. Vero, Vero TMPRSS2 and Caco-2 cells grown to

11 ~90% confluency were split at a ratio of 1:10 every 3 to 5 days. Vero TMPRSS2* cells grown to

12 70% confluency were split at a ratio of 1:10 every 3 days. Calu-3 cells at ~90% confluency were

13 split at a ratio of 1:3 every 5-8 days. MA104 cells (ATCC CRL-2378.1) were grown in 5% CO_2

14 and at 37°C in medium 199 supplemented with 10% FBS and 1% penicillin-streptomycin and

15 upon reaching ~90% confluency were split at a ratio of 1:3 every 3 days. SVG-A cells were grown

16 in 5% CO_2 and at 37°C in MEM supplemented with 10% fetal bovine serum, 1% penicillin-

17 streptomycin, and 25 mM HEPES, pH 7.4. HEK293T cells used for lentivirus production were

18 grown in DMEM supplemented with 10% FBS and grown at 37°C and 5% CO_2.

19

20 **Generation of VSV-SARS-CoV-2 chimeras**

21 The generation of a replication competent recombinant VSV chimera expressing eGFP where the

22 glycoprotein G was replaced with spike (S) protein Wuhan-Hu-1 strain (VSV-eGFP-SARS-CoV-

23 2) has been described (*13*). Additional VSV recombinants expressing eGFP and SARS-CoV-2

24 spike variants were prepared as follows. Briefly, spike genes of Delta (B.1.617.2), Omicron

25 (B.1.1.529) were truncated to remove the C-terminal 63 nucleotides and were then cloned into an

26 infectious molecular clone of VSV-eGFP in place of the native VSV G gene. To generate VSV-

27 eGFP-P-SARS-CoV-2 the open reading frame of the glycoprotein in plasmid pVSV1(+)-eGFP-P

28 (PMID 16339901) was replaced by a codon-optimized version of the coding sequence of the

29 Wuhan strain of the SARS-CoV-2 spike with the last 21 amino acid deleted.

30

31 To rescue the recombinant VSVs, BSRT7/5 cells were infected with vaccinia virus encoding the

32 bacteriophage T7 RNA polymerase (vTF7-3) (PMID 3095828), as a source of transcriptase for

33 expression of VSV N, P, L, and G, and the full-length antigenomic RNA of VSV from individual

34 plasmids. Cell culture fluids were harvested ~72 h post-infection, clarified by centrifugation (5 min

1 and 1,000 × g), and the supernatant filtered through a 0.22-µm filter. Viruses were isolated by

2 plaque assay on Vero TMPRSS2 cells. Individual plaque isolates of virus were amplified on

3 MA104 cells at an MOI of 0.01 in Medium 199 containing 2% FBS and 20 mM HEPES pH 7.7

4 (Millipore Sigma) at 34°C. Viral supernatants were harvested upon extensive cytopathic effect

5 and clarified of cell debris by centrifugation at 1,000 x g for 5 min. Viral stock RNA was extracted

6 using RNeasy Mini kit (QIAGEN), and S was amplified using OneStep RT-PCR Kit (QIAGEN) and

7 sequence verified by Sanger sequencing (GENEWIZ). Aliquots of sequence verified virus were

8 maintained at −80°C.

9

10 **VSV-eGFP-SARS-CoV-2 infection assays**

11 Infection assays for the Wuhan, Delta or Omicron VSV-eGFP-SARS-CoV-2 chimeras were done

12 at a final ~ 80% confluency of cells plated one day before the infection assay as previously

13 described (*3*). Briefly, cells were incubated for one hour with the appropriate VSV-eGFP-SARS-

14 CoV-2 chimera suspended in DMEM containing 25 mM HEPES at the indicated pH, at 37°C, and

15 10 %CO_2. Cells were then rinsed three times and further incubated for 7 hours in the same

16 medium, followed by chemical fixation and spinning disc confocal microscopy imaging (see below)

17 to score extent of infection by visualization of eGFP expressing cells.

18

19 The effect of inhibitors on infection was determined in cells pre-incubated with medium containing

20 the appropriate inhibitor for one hr, followed by its presence during both the inoculation period

21 and during the 7-hr duration of the infection assay. A pH bypass experimental design was used

22 to test the effect of pH on infection. Briefly, cells always kept at 37°C and 10% CO2 were

23 incubated for 1 hour in DMEM containing 25 mM HEPES, pH 7.4 and 40 µM dynasore-OH),

24 followed by incubation of the cells for another hour with the appropriate VSV-eGFP-SARS-CoV-

25 2 variant using the same medium including dynasore-OH and the required acidic pH. Cells were

26 then rinsed three times and incubated for another 7 hours in DMEM containing 25 mM HEPES,

27 pH 7.4 and dynasore-OH.

28

29 At the end of this 7-hr incubation, medium was changed to PBS containing 5 µg/mL WGA-

30 Alexa647 and cells incubated for 30 sec to highlight their surface, then chemically fixed with 4%

31 PFA in PBS for 15 minutes at room temperature, rinsed 3x with PBS and imaged within 24 hours

32 using a spinning-disk confocal microscope equipped with a Zeiss 40x oil NA=1.4 objective (1

33 pixel, 0.33 µm). Random fields, containing a Z-stack of 20 consecutive optical planes taken 1 µm

34 apart (*6*) were obtained and successful infection scored if the cytosolic eGFP fluorescence signal

1   from maximum Z-projections was 1.4 times higher than that of uninfected cells imaged using the

2   same visualization conditions.

3

4   **SARS-CoV-2 infection assays**

5   All experiments with SARS-CoV-2 were performed in biosafety level 3 (BSL3) facilities at the

6   University of Helsinki with appropriate institutional permits. Virus samples were obtained under

7   Helsinki University Hospital laboratory research permit 30 HUS/32/2018§16. Virus titers were

8   determined by a plaque assay in Vero TMPRSS2* cells. Cells in DMEM, supplemented with 10%

9   FBS, 2 mM l-glutamine, 1% penicillin-streptomycin, and 20 mM HEPES (pH 7.2), were seeded 48

10 h before treatment at a density of 15,000 cells per well in 96-well imaging plates (catalog number

11 6005182; PerkinElmer). Dynasore-OH (Tocris) at indicated concentration or DMSO control were

12 added to cells 60 minutes before infection at an MOI of 0.5 PFU per cell with virus added at

13 indicated pH. After 2 hours of infection, cells were washed removing unbound virus and dynasore-

14 OH. Infections were carried for 16 hours at 37°C with 5% CO_2. Cells were then fixed with 4%

15 paraformaldehyde in PBS for 30 min at room temperature before being processed for

16 immunodetection of viral N protein, automated fluorescence imaging, and image analysis. Briefly,

17 viral NP was detected with an in-house-developed rabbit polyclonal antibody (Cantuti-Castelvetri),

18 counterstained with Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody, and nuclear

19 staining was done using Hoechst DNA dye. Automated fluorescence imaging was done using a

20 Molecular Devices Image-Xpress Nano high-content epifluorescence microscope equipped with

21 a 20× objective and a 4.7-megapixel CMOS (complementary metal oxide semiconductor) camera

22 (pixel size, 0.332 µm). Image analysis was performed with CellProfiler-3 software

23 (www.cellprofiler.org). Automated detection of nuclei was performed using the Otsu algorithm

24 inbuilt in the software. To automatically identify infected cells, an area surrounding each nucleus

25 (5-pixel expansion of the nuclear area) was used to estimate the fluorescence intensity of the viral

26 NP immunolabeled protein, using an intensity threshold such that <0.01% of positive cells were

27 detected in noninfected wells.

28

29 **VSV-SARS-CoV-2 Atto 565 labeling**

30 Stock solutions of VSV-SARS-CoV-2 and its variants at a concentration of ~150 µg/ml viral RNA

31 were conjugated with Atto 565-NHS ester (Sigma-Aldrich, cat. 72464) as previously described

32 (7). Aliquots of Atto 565 NHS ester resuspended in anhydrous DMSO (Sigma) were dried down

33 under house vacuum to a final yield of 0.25 µg/tube stocks and stored at -20°C. A sample of 100

34 µL of virus stock was mixed with 10 µL of 1 M $NaHCO_3$ pH 8.3 and then added to one such tube

1 containing dried Atto 565 NHS ester and incubated in the dark for 1 hour at room temperature;

2 the reaction was ended by addition of 20 µL of 1 M Tris pH 8.0. Free Atto 565 dye was removed

3 from labeled virus using a 0.5 mL Pierce desalting Zeba spin column of 40 kDa molecular weight

4 cut off according to the manufacturer's instructions, and labeled virus stored at 4°C in the dark

5 was used within one week of labeling.

6

7 **VSV-SARS-CoV-2 Atto 565 labeling**

8 Stock solutions of VSV-SARS-CoV-2 and its variants at a concentration of ~150 µg/ml viral RNA

9 were conjugated with Atto 565-NHS ester (Sigma-Aldrich, cat. 72464) as previously described

10 (*7*). Aliquots of Atto 565 NHS ester resuspended in anhydrous DMSO (Sigma) were dried down

11 under house vacuum to a final yield of 0.25 µg/tube stocks and stored at -20°C. A sample of 100

12 µL of virus stock was mixed with 10 µL of 1 M NaHCO$_3$ pH 8.3 and then added to one such tube

13 containing dried Atto 565 NHS ester and incubated in the dark for 1 hour at room temperature;

14 the reaction was ended by addition of 20 µL of 1 M Tris pH 8.0. Free Atto 565 dye was removed

15 from labeled virus using a 0.5 mL Pierce desalting Zeba spin column of 40 kDa molecular weight

16 cut off according to the manufacturer's instructions, and labeled virus stored at 4°C in the dark

17 was used within one week of labeling.

18

19 **VSV-SARS-CoV-2 Atto 565 labeling**

20 Stock solutions of VSV-SARS-CoV-2 and its variants at a concentration of ~150 µg/ml viral RNA

21 were conjugated with Atto 565-NHS ester (Sigma-Aldrich, cat. 72464) as previously described

22 (*7*). Aliquots of Atto 565 NHS ester resuspended in anhydrous DMSO (Sigma) were dried down

23 under house vacuum to a final yield of 0.25 µg/tube stocks and stored at -20°C. A sample of 100

24 µL of virus stock was mixed with 10 µL of 1 M NaHCO$_3$ pH 8.3 and then added to one such tube

25 containing dried Atto 565 NHS ester and incubated in the dark for 1 hour at room temperature;

26 the reaction was ended by addition of 20 µL of 1 M Tris pH 8.0. Free Atto 565 dye was removed

27 from labeled virus using a 0.5 mL Pierce desalting Zeba spin column of 40 kDa molecular weight

28 cut off according to the manufacturer's instructions, and labeled virus stored at 4°C in the dark

29 was used within one week of labeling.

30

31 **Single molecule Atto 565 dye calibration**

32 The number of Atto 565 molecules attached to a single virion was determined by comparing the

33 total fluorescence intensities associated with a given virion and the fluorescence intensity

34 associated with the last bleaching step of the same virion, as previously described (*1, 8*). This

1   strategy corrects for local variations in the imaging conditions of the microscope and allows direct

2   use of data from calibration results obtained i*n vitro* with the total fluorescence intensity acquired

3   in cells REF. Briefly, fluorescently tagged VSV chimeras (0.1 µg/mL viral RNA) dissolved in PBS

4   were adsorbed for 10 min to glass that had been extensively washed in 3x PBS after coated for

5   30 minutes and at room temperature with 0.1 mg/mL of poly-D-lysine. After removing unbound

6   virus by 3 consecutive washes with PBS, the sample was imaged using spinning disc confocal

7   microscopy using a 63x NA 1.4 oil objective or LLSM in scanning mode. Automated location of

8   the fluorescent virions in the coverslip and corresponding fluorescence intensity determined by

9   Gaussian fitting of images was done using the 3D CME package (*9*). To determine the number

10   of Atto 565 dyes associated per each virus particle, the exposure was increased to one second

11   and consecutive images acquired on the plane associated with the maximum original intensity.

12   This continuous illumination protocol, conducent to extensive bleaching and ending with

13   resolvable single bleaching steps, was used to determine the fluorescence of a single Atto 565

14   dye with the aid of a step detecting algorithm encoded in MATLAB (*10*).

15

16   **Western Blot analysis**

17   Western blot analysis of VSV-SARS-CoV-2 samples were done as follows. 20 µL of virion

18   sample at 10 µg/mL virus RNA was mixed with an equal volume of 2x concentrated Laemmli

19   sample buffer, boiled for 10 minutes at 95°C and subjected to SDS-PAGE separation using

20   Mini-PROTEAN any-kD 4-20% precast gels and electrically transferred to a nitrocellulose

21   membrane. Transfer efficiency was verified using A ponceau staining. Nonspecific binding

22   antibody to the membranes was prevented by incubation with 5% milk powder in TBS-T (50 mM

23   TRIS, 150 mM NaCl, 0.1% Tween) for 1 hour a room temperature. The membranes were

24   incubated overnight at 4°C with a solution containing 5% milk powder TBS-T and antibody

25   specific for S used diluted 1 to 1000 from 1 µg/mL. Membranes were washed 3x with TBS-T,

26   then incubated for one hour at room temperature with a 1 to 20,000 dilution of 1 mg/mL IRDye

27   800CW Goat anti-rabbit IgG secondary antibody in 5% milk powder TBS-T. Membranes were

28   then washed 3x with what and scanned using a Licore Odyssey scanner.

29

30   **Preparation of glass coverslips**

31   Infection and uptake assays of VSV-eGFP-SARS-CoV-2 and VSV-P-eGFP-SARS-CoV-2 done

32   by spinning disc confocal microscopy visualization, were performed using 25 mm #1.5 coverslips

33   bound with polydimethylsiloxane (PDMS) of about 1 mm in thickness and of 3 mm (infection) or

34   5 mm (uptake) in diameter wells punched as previously described (*9*). The punched PDMS sheets

1   were attached onto the top surface of glass coverslips previously cleaned by sonication, first in

2   isopropanol for 20 minutes and then in 0.5 M KOH for 20 minutes, followed by extensive washing

3   in MilliQ water and ending with drying for 30 minutes at 90°C. The glass was then firmly pressed

4   to the PDMS sheet, after exposure of their surfaces to air plasma in a PDC-001 plasma cleaner

5   (Harrick Plasma) operating at 750 mtorr and 30W for 2 min. The bonded glass/PDMS was heated

6   at 90°C for 20 min, followed by sterilization by incubation with 70% ethanol for 10 min immediately

7   before plating of cells.

8

9   Single virus tracking experiments done using LLSM experiment were done using round #1.5 glass

10   coverslips of 6 mm in diameter that had been cleaned by sequential 20-min steps of sonication,

11   first in toluene, then in dichloromethane, followed by 100 % ethanol, ending in MilliQ water; the

12   glass slides were stored in 70% ethanol until cells were plated. These glass coverslips were re-

13   used at the culmination of the imaging experiments by following the same previous cleaning steps.

14

15   **Live cell spinning disc-confocal microscopy**

16   Visualization experiments were done with an inverted spinning disc confocal microscope (*42*).

17   Cells were plated one day prior to the experiment into 5 mm in diameter homemade PDMS wells

18   to achieve 70% confluency during visualization. VSV-eGFP-P-SARS-CoV-2 was added to the

19   well in DMEM containing 25 mM HEPES pH 7.4 for 1 hour. Cells where then labeled with 5 µg/mL

20   of WGA-Alexa647 in FluoroBrite™ DMEM media containing 25 mM HEPES, pH 7.4 for 30

21   seconds followed by 3 rapid washes in the same media. Cells were then immediately imaged

22   using a 63x objective on a spinning-disc confocal microscope taking the entire cell volume with

23   0.27 µm spacing between each optical plane. In parallel with the experiment in cells, VSV-eGFP-

24   P-SARS-CoV-2 was bound to poly-D-lysine coated cover slips and imaged under the same

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8

A

VSV-eGFP-SARS-CoV-2

Live Cell Imaging   Fix and Image

0   1   2   3   4   5   6   7   8
              Time (hrs)

VSV-eGFP-SARS-CoV-2

B

5 µg/mL viral RNA (MOI 0.5)   0.5 µg/mL viral RNA (MOI 0.5)

Caco-2   Calu-3   Vero   Vero TMPRSS2

50 µm

C

% Infection

VSV-eGFP-SARS-CoV-2 RNA (µg/mL)

VSV-eGFP-P-SARS-CoV-2

D

5 µg/mL viral RNA (MOI 0.5)   0.5 µg/mL viral RNA (MOI 0.5)

Caco-2   Calu-3   Vero   Vero TMPRSS2

10 µm

E

Virus Per Cell

VSV-eGFPP—SARS-CoV-2 RNA (µg/mL)

Figure S1

A VSV-eGFP-P-CoV-2-S-Atto 565

10 µm

B S-Atto 565 Bleach Step

C Atto 565 Per Virus

D VSV-eGFP-CoV-2-S-Atto 565

unlabeled

Atto 565 labeled

P = 0.1043

Figure S2

A

Vero
10 minutes post inoculation

Caco-2
10 minutes post inoculation

Calu-3
10 minutes post inoculation

eGFP-P
S-Atto 565

Figure S3

A

Vero

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

α = 0.27

Diffusion Mode

B

Vero TMPRSS2

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

α = 0.71

Diffusion Mode

C

Caco-2

Fluorescence Intensity

Mean Squared Displacement

α = 0.46

Diffusion Mode

D

Calu-3

Fluorescence Intensity

Mean Squared Displacement

α = 0.42

Diffusion Mode

Figure S4

Vero

A

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

α = 0.62   α = 1.74

Diffusion Mode

surface   endosome

Vero TMPRSS2

B

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

α = 0.48   α = 1.61

Diffusion Mode

surface   endosome

Caco-2

C

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

α = 0.23   α = 2.11

Diffusion Mode

surface   endosome

Calu-3

D

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

α = 0.44   α = 1.91

Diffusion Mode

surface   endosome

Figure S5

A

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

Diffusion Mode

α = 1.62

Vero TMPRSS2

B

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

Diffusion Mode

α = 1.40

Caco-2

C

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

Diffusion Mode

Calu-3

D

XYZ Trajectory

Fluorescence Intensity

Mean Squared Displacement

Diffusion Mode

Figure S6

A   Vero

XYZ Trajectory

eGFP-P fluor. Intensity   # VSV-CoV-2

S-Atto 565 Colocalization   # S-Atto 565 Molecules

Mean Squared Displacement

α = 1.44

α = 1.09

Diffusion Mode

α

endosome   cytoplasm

B   Vero TMPRSS2

XYZ Trajectory

eGFP-P fluor. Intensity   # VSV-CoV-2

S-Atto 565 Colocalization   # S-Atto 565 Molecules

Mean Squared Displacement

α = 1.35

α = 0.91

Diffusion Mode

α

endosome   cytoplasm

C   Caco-2

XYZ Trajectory

eGFP-P fluor. Intensity   # VSV-CoV-2

S-Atto 565 Colocalization   # S-Atto 565 Molecules

Mean Squared Displacement

α = 2.15

α = 1.01

Diffusion Mode

α

endosome   cytoplasm

D   Calu-3

XYZ Trajectory

eGFP-P fluor. Intensity   # VSV-CoV-2

S-Atto 565 Colocalization   # S-Atto 565 Molecules

Mean Squared Displacement

α = 1.64

α = 0.83

Diffusion Mode

α

endosome   cytoplasm

Figure S7

Figure S8

A

VSV-eGFP-SARS-CoV-2

Dynasore-OH

Time (hrs)

Control   Dynasore-OH

Vero E6

50 μm

Vero E6 TMPRSS2

Caco-2

Calu-3

B

VSV-eGFP-SARS-CoV-2

Dynasore-OH

Fix Image

Time (hrs)

Control   Dynasore-OH

Vero E6

Vero E6 TMPRSS2

Caco-2

Calu-3

C

VSV-eGFP-SARS-CoV-2

Dynasore-OH

Fix Image

Time (hrs)

Control   Dynasore-OH

Vero E6

Vero E6 TMPRSS2

Caco-2

Calu-3

Figure S9

A

VSV-eGFP-SARS-CoV-2

Dynasore-OH

Time (hrs): -1 0 1 2 3 4 5 6 7 8

Fix Image

Control | Dynasore-OH

Vero E6 — 50 µm

Vero E6 TMPRSS2

Caco-2

Calu-3

B

VSV-eGFP-SARS-CoV-2

Dynasore-OH

Time (hrs): -1 0 1 2 3 4 5 6 7 8

Fix Image

Control | Dynasore-OH

Vero E6

Vero E6 TMPRSS2

Caco-2

Calu-3

C

VSV-eGFP-SARS-CoV-2

Dynasore-OH

Time (hrs): -1 0 1 2 3 4 5 6 7 8

Fix Image

Control | Dynasore-OH

Vero E6

Vero E6 TMPRSS2

Caco-2

Calu-3

Figure S9

VSV-eGFP-SARS-CoV-2

A

0.5 µg/mL viral RNA (MOI 0.5) in SVG-A Cells

SVG-A   SVG-A ACE2   SVG-A TMPRSS2   SVG-A ACE2 TMPRSS2

B

% Infection

0 0.01 0.05 0.5 1 5 10
VSV-eGFP-SARS-CoV-2
RNA (µg/mL)

VSV-eGFP-P-SARS-CoV2

C

0.5 µg/mL viral RNA (MOI 0.5) in SVG-A Cells

SVG-A   SVG-A ACE2   SVG-A TMPRSS2   SVG-A ACE2 TMPRSS2

VSV-P-eGFP-SARS-CoV-2

D

Virus Per Cell

400
300
200
100
0

0.05 0.5 5 10
VSV-eGFP-ARS-CoV-2
RNA (µg/mL)

E

VSV-eGFP-SARS-CoV-2

0.5 µg/mL viral RNA (MOI 0.5)

Vero   Vero TMPRSS2   SVG-A ACE2   SVG-A ACE2 TMPRSS2

% Infection

control camostat E-64 camostat/E-64

Figure S10

Figure S11

Figure S12

Figure S13

A

B

Figure S14

A

SVG-A ACE2

SVG-A ACE2 TMPRSS2

B

Release Site

Figure S15

A VSV-eGFP-P-SARS-CoV-2-S-Atto 565 Vero TMPRSS2
no Camostat Mesylate

partial loss off S-Atto 565

B VSV-eGFP-P-SARS-CoV-2-S-Atto 565 Vero TMPRSS2

partial loss off S-Atto 565

C VSV-eGFP-P-SARS-CoV-2-S-Atto 565 Vero TMPRSS2
10 µM Camostat Mesylate

D VSV-eGFP-P-SARS-CoV-2-S-Atto5 65 Vero TMPRSS2 10 µM Camostat Mesylate

Figure S16

A Vero E6 TMPRSS2 pH 6.8

eGFP-P
S-Atto 565

1 µm

z (µm)

x (µm)

surface

z (µm)

y (µm)

x (µm)

eGFP-P fluor. Intensity

VSV-CoV-2

Time (minutes)

S-Atto 565 Colocalization

S-Atto 565 Molecules

partial loss off S-Atto 565

spread / fusion

Time (minutes)

B Vero E6 TMPRSS2 pH 6.8

1 µm

surface

z (µm)

y (µm)

x (µm)

eGFP-P fluor. Intensity

VSV-CoV-2

Time (minutes)

S-Atto 565 Colocalization

S-Atto 565 Molecules

partial loss off S-Atto 565

spread / fusion

Time (minutes)

Figure S17

A

pH 6.8

pH 7.4

VSV-SARS-CoV-2

+ TMPRSS2 at surface

+ TMPRSS2 at surface

no TMPRSS2 at surface

no TMPRSS2 at surface

Trypsin activated
VSV-SARS-CoV-2

+ TMPRSS2 at surface

+ TMPRSS2 at surface

no TMPRSS2 at surface

no TMPRSS2 at surface

B

VSV-eGFP-SARS-CoV-2
dynasore-OH

Vero
Vero TMPRSS2
Caco-2
Calu-3

trypsin activated
VSV-eGFP-SARS-CoV-2
dynasore-OH

Figure S18

A 20 μM E-64 in Vero Cells

B ACE2 / trypsin then ACE2

10 μm

C

D

E

F

$\Delta t_{Binding\ to\ S\text{-}Release}$ (s)

Figure S19

A VSV-eGFP-SARS-CoV2-Wuhan grown in MA104

B VSV-eGFP-SARS-CoV-2 Delta grown in MA104

C VSV-eGFP-SARS-CoV-2 Omicron grown in MA104

D VSV-eGFP-SARS-CoV-2-Omicron grown in Vero TMPRSS2

Figure S20

VSV-eGFP-SARS-CoV-2-Wuhan   VSV-eGFP-SARS-CoV-2-Delta   VSV-eGFP-SARS-CoV-2-Omicron

grown in MA104   grown in Vero TMPRSS2

Figure S21