

1 **3D reconstruction of SARS-CoV-2 infection in ferrets**
2 **emphasizes focal infection pattern in the upper respiratory**
3 **tract**

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28 **Abstract**

29 The visualization of viral pathogens in infected tissues is an invaluable tool to
30 understand spatial virus distribution, localization, and cell tropism *in vivo*. Commonly,
31 virus-infected tissues are analyzed using conventional immunohistochemistry in
32 paraffin-embedded thin sections. Here, we demonstrate the utility of volumetric three-
33 dimensional (3D) immunofluorescence imaging using tissue optical clearing and light
34 sheet microscopy to investigate host-pathogen interactions of pandemic SARS-CoV-
35 2 in ferrets at a mesoscopic scale. The superior spatial context of large, intact
36 samples ($> 150 \text{ mm}^3$) allowed detailed quantification of interrelated parameters like
37 focus-to-focus distance or SARS-CoV-2-infected area, facilitating an in-depth
38 description of SARS-CoV-2 infection foci. Accordingly, we could confirm a
39 preferential infection of the ferret upper respiratory tract by SARS-CoV-2 and
40 emphasize a distinct focal infection pattern in nasal turbinates. Conclusively, we
41 present a proof-of-concept study for investigating critically important respiratory
42 pathogens in their spatial tissue morphology and demonstrate the first specific 3D
43 visualization of SARS-CoV-2 infection.

44

45 **keywords:** SARS-CoV-2, COVID-19, 3D immunofluorescence, light sheet
46 microscopy, confocal laser scanning microscopy, tissue optical clearing, host-
47 pathogen interactions, respiratory tract infection

48

49 Introduction

50 In December 2019, a novel coronavirus (2019-nCoV) associated with viral
51 pneumonia emerged in Wuhan, Hubei Province, China [1-4]. The virus was
52 subsequently designated as severe acute respiratory syndrome coronavirus 2
53 (SARS-CoV-2) [5] and identified to be the causative agent of COVID-19 (Coronavirus
54 disease 2019). Patients most commonly present with fever, cough, fatigue, and
55 dyspnea [6-9]. About one out of five patients develops severe disease [10,11]. The
56 outbreak was declared a “public health emergency of international concern” on
57 January 30, 2020, and a pandemic on March 11, 2020. As of October 12, 2020,
58 37,109,851 confirmed cases and 1,070,355 confirmed death were reported in 235
59 countries [12].

60 SARS-CoV-2 is an enveloped virus with a single-stranded RNA genome of positive
61 polarity and has been classified as a member of the *Coronaviridae* family, genus
62 *Betacoronavirus* [5]. Including SARS-CoV-2, there are currently two alpha- and five
63 betacoronaviruses associated with human disease [13]. While most result in only mild
64 respiratory illness, the three zoonotic betacoronaviruses SARS-CoV [14], SARS-
65 CoV-2 [1-4], and MERS-CoV [15] (Middle East respiratory syndrome coronavirus)
66 can cause severe respiratory disease. Bats presumably serve as natural reservoir for
67 both SARS-CoV [16,17] and MERS-CoV [18-20], whereas palm civets [21] and
68 dromedary camels [22] have been identified as the intermediate hosts for animal-
69 human transmission for SARS-CoV and MERS-CoV, respectively. Viruses closely
70 related to SARS-CoV-2 have been found in bats [2] and Malayan pangolins [23,24]
71 but no direct transmission event or intermediate host species have been identified
72 thus far.

73 Over the course of the current pandemic, tremendous research efforts have been
74 undertaken to study the virus and its disease. Consequently, critical information on
75 the virus, e.g. receptor usage [25], and necessary research tools, including reverse
76 genetics systems [26,27], became rapidly available. Furthermore, a variety of animal
77 studies to investigate susceptibility and suitability as animal models have been
78 conducted in a number of animal species (reviewed in [28]): ferrets [29-31], hamsters
79 [32-34], cats [31,35], dogs [31], raccoon dogs [36], rabbits [37], transgenic mice [38-
80 40], pigs [30,31], cattle [41], monkeys [42-44], poultry [30,31,45], and fruit bats [30].

81 Within the respiratory tract, detection of viral antigen and RNA suggested a
82 preferential replication of SARS-CoV-2 in the upper respiratory tract (URT) of ferrets
83 [29-31], whereas viral antigen was detected in both the URT und lower respiratory
84 tract (LRT) of Syrian hamsters [32,33]. In humans and non-human primates (NHPs),
85 viral antigen detection indicates virus replication in both the URT and LRT [42,46,47].
86 Thus far, almost all approaches to detect and image SARS-CoV-2 infection in tissues
87 have been based on conventional immunohistochemistry (IHC) of paraffin-embedded
88 thin sections. However, by omitting the spatial context, thin tissue sections of only
89 several micrometers in thickness bear the risk of incomplete or inaccurate
90 description, particularly for focal infections. Recent developments in the field of tissue
91 optical clearing (TOC) have facilitated the preservation of large intact tissue
92 structures by turning them optically transparent. This eliminates the need of physical
93 sectioning and allows acquisition of intact three-dimensional (3D) structures using
94 only optical sectioning, e.g. in light sheet fluorescence microscopy (LSFM) (reviewed
95 in [48]). Lately, the opportunities and advantages of TOC for virus research have
96 been demonstrated in several studies [49-54]. While two approaches to 3D imaging
97 of SARS-CoV-2-infected lung tissue have been described recently [55,56], neither of
98 them is capable of direct visualization of SARS-CoV-2 infection via virus-specific
99 antigen staining.
100 In our study, we provide a first complete 3D overview of SARS-CoV-2 infection in the
101 ferret model. By staining for the viral nucleocapsid protein (SARS-CoV-2 N), we were
102 able to directly visualize and localize SARS-CoV-2-infected foci within large volumes
103 of the ferret respiratory tract. Direct visualization further allowed detailed description
104 of the foci in their spatial context. To the best of our knowledge, this is the first report
105 of specific 3D reconstruction of SARS-CoV-2 infection as well as the first report of 3D
106 visualization of respiratory virus infection in nasal turbinates using LSFM.

107 **Materials and Methods**

108 Cells and viruses

109 VeroE6 cells (CCLV-RIE 0929, Collection of Cell Lines in Veterinary Medicine
110 [CCLV], Friedrich-Loeffler-Institut, Germany) were maintained in Minimal Essential
111 Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Biowest, France)
112 and non-essential amino acids (Gibco).

113 SARS-CoV-2 isolate 2019_nCoV Muc-IMB-1 (kindly provided by Roman Wölfel,
114 German Armed Forces Institute of Microbiology, Germany) was propagated on
115 VeroE6 cells. The complete sequence is available through GISAID under the
116 accession number ID_EPI_ISL_406862 and name "hCoV-
117 19/Germany/BavPat1/2020".

118 Antibodies and chemical reagents

119 For the detection of SARS-CoV-2 infection, a 1:1 mixture of hybridoma cell culture
120 supernatants of anti-SARS-CoV-1 N mouse monoclonal antibody clones 4E10A3A1
121 (RRID:AB_2833160) and 4F3C4 (RRID:AB_2833162) [57] at a dilution of 1:5 or a
122 polyclonal rabbit anti-SARS-CoV-1 N (RRID:AB_838838; Novus Biologicals, USA) at
123 a dilution of 1:250 were used. Alexa Fluor™ 488/568/647-conjugated antibodies
124 against mouse IgG and rabbit IgG were used as secondary antibodies (1:500;
125 Invitrogen, USA).

126 A detailed list of the chemicals and reagents used for the immunostaining and optical
127 clearing of SARS-CoV-2-infected tissue samples, and their suppliers is provided in
128 Supplementary Table S1.

129 Virus infection and immunofluorescence staining of mammalian cell cultures

130 5×10^5 VeroE6 cells were seeded on coverslips one day prior to infection with $1 \times$
131 10^6 TCID₅₀ of SARS-CoV-2 isolate 2019_nCoV Muc-IMB-1. Infected VeroE6 cells
132 were then fixed 24 h post-infection with 4% paraformaldehyde (PFA) for 20 min.
133 Following permeabilization with 0.5% Triton X-100/PBS for 15 min, cells were
134 blocked with 10% normal donkey serum in 0.1% Tween-20/PBS (PBS-T) for 30 min.
135 Primary antibodies against SARS-CoV N were applied for 1 h at room temperature in
136 1% normal donkey serum/PBS-T, followed by three washes with PBS and incubation
137 with the secondary antibody for 1 h at room temperature in 1% normal donkey
138 serum/PBS-T. Nuclei were counterstained with Hoechst33342 (Invitrogen) and
139 samples were embedded in ProLong™ Glass AntiFade Mountant (Invitrogen) for
140 analysis by confocal laser-scanning microscopy.

141 Tissue samples of SARS-CoV-2-infected ferrets

142 In a previous study on experimental transmission of SARS-CoV-2 among different
143 animal species, ferrets were inoculated intranasally with 10^5 TCID₅₀ of SARS-CoV-2

144 isolate 2019_nCoV Muc-IMB-1 [30]. Tissues were collected in 10% neutral-buffered
145 formalin and fixed for at least 21 days to ensure complete virus inactivation. In this
146 study, nasal conchae, trachea, and lung tissue samples from infected ferrets
147 euthanized on day 4 post-infection were analyzed.

148 Immunofluorescence staining of high-volume tissue sections

149 Large sections of respiratory tissues ($\geq 150 \text{ mm}^3$) were immunostained according to
150 a modified iDISCO protocol [50,58]. All incubation steps were conducted with slight
151 agitation and, if not indicated otherwise, at room temperature.

152 To this end, formaldehyde-fixed tissues were washed three times for at least 1 h
153 each in PBS. Nasal conchae were furthermore decalcified for 4-7 days in Formical-
154 2000™ (Statlab, USA). Samples were trimmed to the sizes and volumes described
155 above, and bleached overnight in 5% H₂O₂ in PBS at 4 °C. For permeabilization, the
156 tissue samples were first incubated twice for 3 h each with 0.2%Triton X-100/PBS at
157 37 °C and subsequently in 0.2% Triton X-100/20% DMSO/0.3 M glycine/PBS for 2
158 days at 37 °C. Following a blocking step with 6% normal donkey serum/0.2% Triton
159 X-100/10% DMSO/PBS for 2 days at 37 °C, primary antibodies were diluted in 3%
160 normal donkey serum/5% DMSO in PTwH (0.2% Tween-20 in PBS with 10 µg/mL
161 heparin) and applied for 4 days at 37 °C. Unbound antibody was removed by
162 washing the samples 4-5 times over the course of a day, leaving the final wash on
163 overnight. Secondary antibodies were diluted in 3% normal donkey serum/PTwH and
164 the samples were incubated for another 4 days at 37 °C. Washing was performed as
165 described for the primary antibody.

166 Ethyl cinnamate (ECi)-based tissue optical clearing

167 Immunostained tissue sections were cleared with an adjusted ECi-based protocol
168 [59]. All incubation steps were conducted with slight agitation.

169 The samples were dehydrated in a graded ethanol series (30% [v/v], 50%, 70%, and
170 twice in 100%; each for ≥ 8 h at 4 °C, diluted in aqua ad injectabilia, and pH-adjusted
171 to 9). Following a two-hour wash with *n*-hexane at room temperature [60], *n*-hexane
172 was gradually replaced with the clearing agent ECi and samples were incubated until
173 optically transparent.

174 Light sheet microscopy of optically clear tissue samples

175 Light sheet micrographs of optically clear and immunostained respiratory tissues from
176 SARS-CoV-2-infected ferrets were acquired with a LaVision BioTec Ultramicroscope
177 II (LaVision, Germany). The microscope was equipped with an Olympus MVX-10
178 zoom body (magnification range: 0.63x – 6.3x, total magnification: 1.26x – 12.6x;
179 Olympus, Japan), an Olympus MVPLAPO 2x objective (NA = 0.5), a LaVision laser
180 module with four laser lines (488 nm, 561 nm, 639 nm, and 785 nm), and a Andor
181 Zyla 5.5 sCMOS Camera (Andor Technology, UK) with a pixel size of $6.5 \mu\text{m}^2$. To
182 visualize tissue morphology, non-specific autofluorescence was excited with the 488
183 nm laser. Excitation lines 561 nm and 639 nm were used to excite Alexa FluorTM 568
184 and Alexa FluorTM 647, respectively. Channels of a high-volume 3D image were
185 acquired sequentially with a z-step size of 2 μm , a light sheet width of 100%, and a
186 light sheet thickness of 3.89 μm (NA = 0.156). Acquisition was done with ImSpector
187 (v7.0.124.0).

188 Confocal laser-scanning microscopy (CLSM)

189 Confocal images were acquired with a Leica DMI6000 TCS SP5 confocal laser-
190 scanning microscope (Leica Microsystems, Germany) equipped with a 63x/1.40 oil
191 immersion HCX PL APO objective and a 40x/1.10 water immersion HC PL APO
192 objective. Fluorescence was recorded sequentially between lines with a pinhole
193 diameter of 1 Airy unit and z-step sizes of 0.35 μm . Acquisition was done with LAS
194 AF (v2.7.3.9723).

195 For high-resolution confocal laser-scanning analysis of cleared and immunostained
196 tissue samples, they were sectioned into 1-mm-thick slices using a stainless steel
197 tissue matrix (World Precision Instruments, UK). Tissue slices were then mounted in
198 3D-printed imaging containers as described before [50]. Tissue morphology was
199 reconstructed from non-specific tissue autofluorescence via excitation with a 405 nm
200 UV laser diode.

201 Image processing and analysis

202 Image visualization and analysis were performed with arivis Vision4D (v3.2). If
203 necessary, channels were background corrected. CLSM-acquired image stacks of
204 subsectioned volumetric tissue samples were denoised. To quantify relations
205 between SARS-CoV-2 infection foci, they were segmented. The shortest distances
206 between foci were measured using the segment operation “Distances”. To calculate

207 the area of SARS-CoV-2-infected tissue, surface areas of the segmented objects
208 were extracted and divided by two to account only for the surface of the object facing
209 outwards. Lookup tables of multicolor images were selected for maximum
210 accessibility.

211 **Results**

212 **LSFM provides a unique insight into the spatial distribution of SARS-CoV-2 in
213 intact nasal turbinates.** By combining LSFM with optically cleared samples of the
214 ferret respiratory tract (Figure 1A), we aimed to shed light on the infection
215 environment and spatial context of SARS-CoV-2 infection. A commercially available
216 polyclonal serum (designated as #1), which has been used for SARS-CoV-2
217 detection by conventional IHC [30,42], and a mix of two monoclonal antibodies
218 (designated as #2) against SARS-CoV N were tested on virus-infected VeroE6 cells
219 and confirmed to be cross-reactive with SARS-CoV-2 N (Figure 1B). Following
220 immunostaining, ferret tissue samples, including the partly ossified nasal conchae,
221 were successfully turned optically transparent using a recently established ethyl
222 cinnamate (ECi)-based approach [59] (Figure 1C).

223 Full translucency of ferret nasal turbinates enabled LSFM acquisition of a > 200 mm³
224 (6.69 gigavoxels per channel; $\Sigma = 20.07$ gigavoxels)-sized tissue sample (Figure 2A
225 and Supplementary Movie S1). While there were some unspecific signals detectable
226 in the SARS-CoV-2 N-stained sample (individual green or magenta spots), they could
227 be clearly distinguished from specific SARS-CoV-2 detection by the absence of
228 colocalization (white) of the signals from either antibody (Figure 2B and Figure S1).
229 Within the about 4-mm-thick URT sample (4 days post-infection), multiple
230 comparatively small SARS-CoV-2 infection hot spots were visualized (Figure 2B).
231 They were detected in both the *Concha nasalis dorsalis* (Figure 2B, ROIs [region of
232 interests] 1 & 2) and the *Concha nasalis ventralis* (Figure 2B, ROI 3). Overall, these
233 data provide the proof of concept for the feasibility of TOC-assisted LSFM analysis
234 for SARS-CoV-2.

235 **SARS-CoV-2 infection in the upper respiratory tract of ferrets is characterized
236 by an oligofocal infection pattern.** To achieve a more in-depth analysis of the
237 individual SARS-CoV-2 infection foci, LSFM image stacks of infected areas were
238 acquired using a higher magnification (total magnification of 8x for Figure 3 vs. 1.26x

239 for Figure 2), thus, increasing image resolution while maintaining the complete spatial
240 context (Figure 3).

241 Virtually traveling through an image stack of the ROIs 1 & 2 from Figure 2, which was
242 acquired accordingly, corroborated the presence of three individual, well delimitable,
243 and distinguishable SARS-CoV-2 infection foci (Figure 3A, images 1 [filled-in
244 arrowhead], 5 [outlined arrowhead], and 7 [arrow]). A volumetric reconstruction of this
245 image stack was able to convey spatial relationships between the individual infection
246 spots (Figure 3B-D). Specificity of SARS-CoV-2 N detection was again confirmed by
247 colocalization of both independent antibody stainings (Figure 3B and C, right side).
248 Consequently, linear distances between foci and foci areas were quantified (Figure 4
249 and Table 1). This increased spatial context within the nasal turbinate sample is
250 further reinforced by the fact that the SARS-CoV-2 infection focus from Figure 3A7
251 (arrow) is buried deeper within the sample and cannot be seen from the frontal angle
252 in Figure 3B. However, either by virtually clipping the sample as indicated (Figure 3B
253 and C, red square) or using 3D rendering to virtually “fly through the sample”
254 (Supplementary Movie S2), the infection site can be visualized. Taken together, this
255 emphasizes the system’s flexibility to switch from broad, mesoscopic overviews to
256 detailed, resolved close-ups. By maintaining the full infection environment, we were
257 able to establish quantifiable relations between the individual SARS-CoV-2 foci and
258 highlight the oligofocal infection pattern of SARS-CoV-2 in the URT of ferrets.

259 **Table 1: Direct linear distances between, areas affected by, and volumes of**
260 **segmented SARS-CoV-2 infection foci.** Linear distances were calculated either as
261 the distance between the center of two foci or as the shortest possible distance
262 between the edges of two foci. The area affected by SARS-CoV-2 infection was
263 measured by calculating the surface area of the segmented objects and dividing the
264 resultant value by two, thus, only accounting for the surface facing outwards.

linear distance [μm]	A1	A5	A7
from the center	A1	0	777.4
	A5	777.4	0
	A7	1303.3	554.3
from the edge	A1	0	421.1
	A5	421.1	0

A7	997.5	75.5	0
area [μm^2]	90,212	75,389	181,431
volume [μm^3]	465,818	443,207	1,533,316

265

266 **CLSM acquisition of correlated regions of interest at subcellular resolution –**
267 **infection of ciliated and non-ciliated cells in the nasal epithelium.** While LSFM is
268 ideally suited to generate a mesoscopic overview to analyze, for example, large-scale
269 spatial virus distribution within virus-infected tissues, simultaneous resolution of
270 subcellular details is not possible. Thus, following LSFM acquisition, we subsectioned
271 the optically cleared high-volume tissue sample to 1-mm thick slices using a tissue
272 matrix to achieve compatibility with the limited free working distances of CLSM
273 objectives (Figure 1A).

274 Using the spatio-morphological information on the distribution of SARS-CoV-2
275 infection foci obtained from LSFM analysis, high-resolution CLSM image stacks of a
276 SARS-CoV-2 infection focus in the *Concha nasalis dorsalis* (ROI 1 from Figure 2)
277 were acquired (Figure 5). Individual SARS-CoV-2-infected cells could be resolved,
278 demonstrating cytoplasmic SARS-CoV-2 N distribution in both ciliated and non-
279 ciliated cells (Figure 5B, arrows). Notably, SARS-CoV-2 N accumulated particularly at
280 the apical side of the ciliated cells. Overall, these data demonstrate the feasibility of
281 this correlated approach to dissect cell-specific responses to SARS-CoV-2 infection
282 *in vivo* at subcellular resolution.

283 **SARS-CoV-2 detection in the LRT of ferrets.** Previous studies demonstrated a
284 preferential replication of SARS-CoV-2 in the URT of ferrets [29-31]. To assess
285 whether comprehensive LSFM analysis may uncover previously undetected SARS-
286 CoV-2 infection foci in the LRT, we looked at optically cleared high-volume lung and
287 tracheal samples.

288 As before, some unspecific fluorescence signals could be seen in both lung (Figure
289 6) and tracheal tissue (Figure S2 and Supplementary Movie S3). At a first glance, no
290 specific SARS-CoV-2 infection foci could be identified. However, hidden within an
291 airway of the large lung tissue volume, a 172 μm by 102 μm -sized spot of colocalized
292 antibody signals was detected (Figure 6B and Supplementary Movie S4). Contrary to
293 the SARS-CoV-2 infection foci in the ferret nasal turbinates (Figures 2 and 3), the

294 signal was localized above the epithelial cell layer (Figure 6B, single plane). This
295 suggested detection of debris-associated antigen, which was mostly likely inhaled
296 from the URT. Overall, while we were able to detect an $8.6 \times 10^{-5} \text{ mm}^3$ ($86,000 \mu\text{m}^3$)
297 spot of debris-associated antigen within a $> 80 \text{ mm}^3$ volume, we did not identify
298 additional sites of infection within the LRT of ferrets, which corroborates preferential
299 replication of SARS-CoV-2 in the URT of ferrets.

300 **Discussion**

301 While conventional immunohistochemistry studies have been used to assess the
302 presence or absence of SARS-CoV-2 antigen in human and animal tissues [29-
303 33,42,46,47], none of them were able to provide a greater spatial context of the
304 infection site. By combining TOC with LSFM, we acquired large intact volumes of
305 SARS-CoV-2-infected respiratory tissues from ferrets (Figure 1). The direct 3D
306 visualization of virus infection via SARS-CoV-2 N staining established a
307 comprehensive and mesoscopic overview of the infection in its full spatial context
308 (Figures 2-6 and Supplementary Movies 1-4). Moreover, the determination of
309 morphological parameters, e.g. focus-to-focus distances or the area of virus-infected
310 tissue, not only allowed the characterization of individual SARS-CoV-2 infection foci
311 but also provided a first quantitative insight into virus distribution within the spatio-
312 morphological context of ferret nasal turbinates (Figure 4 and Table 1).

313 Here, we employed an ECi-based TOC approach [59] and adjusted it to visualize
314 immunostained SARS-CoV-2 infection in large tissue samples of the respiratory tract
315 of ferrets. While two 3D imaging approaches to SARS-CoV-2 infection in lung tissue
316 have been reported, they have an entirely different scope: as both represent virtual
317 histopathology strategies, they are meant to assess pathophysiology and associated
318 tissue damage, but inherently cannot map and visualize specific SARS-CoV-2
319 infection. For the first study, Eckermann et al. [55] developed and demonstrated the
320 utility of a phase contrast x-ray tomography concept to investigate unstained lung
321 tissue. The second study describes the use of fluorescent H&E-analog stains (TO-
322 PRO-3 for nuclear contrast and Eosin-Y for cytoplasmic/stromal contrast) to achieve
323 “3D pseudo-histological imaging” [56]. Consequently, our study constitutes the first
324 report of direct 3D visualization of SARS-CoV-2 infection via LSFM. While unspecific
325 antibody signals were detected for both the polyclonal serum and the monoclonal mix
326 directed against N of SARS-CoV, particularly on the outer surface of the tissue

327 blocks, specificity for SARS-CoV-2 was ensured via colocalization of either staining
328 (Figures 2, 3, 5, and 6) and absence of colocalization in naïve animals (Figure S1).
329 Further optimization of the immunostaining protocol or the availability of SARS-CoV-
330 2-specific antibodies will likely aid in reduction of background staining and
331 improvement of virus detection.

332 In addition to the specific 3D reconstruction of SARS-CoV-2 infection within its spatio-
333 morphological environment, the implementation of quantitative image analysis
334 following accurate quantification of interrelated 3D parameters (Figure 4 and Table 1)
335 represents a pronounced advantage of 3D immunofluorescence imaging over
336 conventional IHC. To achieve a somewhat comparable yet more artifact-prone 3D
337 reconstruction from thin sections, exceedingly laborious and time-consuming image
338 registration pipelines following serial thin sectioning are necessary [61]. For instance,
339 the nasal turbinate section from Figures 2-4 alone would require processing of
340 around 800 sections (at 5 µm thickness), making it de facto impossible with 2D IHC.

341 When compared to previous studies [29-31], the spatial visualization of SARS-CoV-2
342 in the ferret respiratory tract confirmed preferential infection of the URT (Figures 2-4).
343 Furthermore, our data indicate and emphasize a distinct oligofocal infection pattern of
344 SARS-CoV-2 within nasal turbinates (Figures 2-4). Within a > 200 mm³ section of
345 nasal turbinate tissue, only four SARS-CoV-2 infection foci (with a combined volume
346 of 5.17x10⁻³ mm³) were detected, three of which accumulated in the *Concha nasalis
347 dorsalis* and exhibited a maximum linear distance of 1.3 mm to each other (Figure 4
348 and Table 1). It is important to note that tissues inevitably shrink during the fixation,
349 dehydration, and clearing process. For the EtOH-ECi-based TOC approach used
350 here, a 50% volume reduction, equaling to a change of about 20% in tissue
351 diameters, has been determined [59]. The limited degree of infection is particularly
352 interesting in view of the amounts of infectious virus and genome copies that can be
353 isolated from the URT of ferrets [29-31] and other animal species, like Syrian
354 hamsters [32-34] and rhesus macaques [42-44]. Clustering of SARS-CoV-2 infection
355 foci in narrow areas of the URT might also have implications for the likelihood of
356 isolation of infectious virus and the detection of viral RNA from nasal swabs in
357 comparison to nasal washes from ferrets and possibly other animal models.
358 Accordingly, a high degree of variation in viral copy numbers can be observed from
359 nose or throat swabs in comparison to bronchoalveolar lavages from SARS-CoV-2-

360 infected rhesus macaques [42]. However, because of the proof-of-principle character
361 of our study and the limited availability of SARS-CoV-2-infected material, further
362 studies have to corroborate the clustering and focal infection pattern of SARS-CoV-2
363 in the URT.

364 Alongside complex quantitative 3D image analysis, volumetric imaging has the
365 potential to discover rare events, as demonstrated by the detection of cancer
366 metastases in sentinel lymph nodes, which had not been found via conventional IHC
367 [62]. While preferential replication of SARS-CoV-2 in the URT of ferrets has been
368 demonstrated via viral RNA and antigen detection [29-31], Kim et al. also detected
369 some SARS-CoV-2-positive cells in the LRT. This is in contrast to the two other ferret
370 susceptibility studies, which detected either no [31] or only low amounts [30] of viral
371 RNA in the LRT, but neither found any SARS-CoV-2 antigen at this location. It is
372 conceivable that scarce LRT infection had been overlooked in previous 2D IHC
373 studies because of the focal character of SARS-CoV-2 infection in the tissue or that
374 the detected viral RNA originated from URT-derived aspirated material. Using this
375 high-volume imaging approach, we aimed to screen the tissue for rare SARS-CoV-2
376 infection foci in the LRT. While we did not detect any infection spots in tracheal tissue
377 (Figure S2), we did visualize an individual, only 86,000 μm^3 -sized SARS-CoV-2 N-
378 positive structure inside a lung airway (Figure 6B). However, spatial analysis
379 revealed that the signal, contrary to the SARS-CoV-2 infection foci in the nasal
380 turbinate epithelium (Figures 2 and 3), was detected above the airway epithelial
381 layer. This strongly suggested that the structure most likely represents aspirated
382 virus-containing debris as the result of localized cell or tissue damage at infected
383 URT sites. Accordingly, this example emphasizes the suitability of this volumetric 3D
384 LSFM approach to identify rare and highly localized pathogen-related events.

385 Ferrets are a standard model for human respiratory infection (reviewed in [63]).
386 However, they recapitulate only mild SARS-CoV-2 infection and do not develop
387 severe respiratory disease [11,29-31]. In contrast, both URT and LRT are strongly
388 affected by SARS-CoV-2 infection in Syrian hamsters, including overt signs of
389 disease [32,33]. This is closer to human disease, where SARS-CoV-2 antigen is
390 found in the URT and LRT, as corroborated by the NHP model rhesus macaques [42-
391 44]. While this proof-of-principle study is focused on ferret samples, it may serve as

392 blueprint for further analyses in other animal models and even human clinical
393 samples.

394 Independent of sample origin, volumetric imaging of cleared samples enables
395 discovery and detection of rare infection events, as demonstrated earlier. This
396 facilitates the investigation of the involvement of other organs outside of the
397 respiratory tract in SARS-CoV-2 infection. For instance, viral antigen has been
398 detected in the intestine of ferrets [29], hamsters [32,33], and rhesus macaques [42].
399 While viral RNA has been detected in human clinical brain samples [64], infection of
400 cells of the central nervous system could thus far only be demonstrated
401 experimentally in 3D human brain organoids [65]. Except for genetically modified
402 mice expressing human angiotensin-converting enzyme 2 (ACE2) [39], the presence
403 of SARS-CoV-2 antigen in animal or human brain samples has yet to be shown.
404 Previous studies with other viral pathogens demonstrated that volumetric 3D imaging
405 using TOC and LSFM is a highly valuable tool to assess the comprehensive
406 distribution of virus infection *in vivo* [49,52]. Additional immunostaining against tissue-
407 specific cell markers may further facilitate the investigation of the global SARS-CoV-2
408 cell tropism in affected tissues. Combining and correlating this with high-resolution
409 CLSM analysis of SARS-CoV-2 hot spots identified via LSFM has the potential to
410 dissect subcellular infection processes of SARS-CoV-2 *in vivo* with unparalleled
411 detail. These results form the basis for research on larger sample sizes of both
412 respiratory and non-respiratory tissues from SARS-CoV-2 animal models and human
413 clinical samples using volumetric 3D LSFM of immunostained and cleared tissues.

414 Overall, we demonstrate the proof of concept for the utility of volumetric 3D
415 immunofluorescence of critically important respiratory pathogens like SARS-CoV-2
416 using TOC and LSFM. The ability to analyze interrelated morphological parameters,
417 like inter-foci distances and SARS-CoV-2-affected areas, and to put them into global
418 perspective to the spatial tissue morphology provides unprecedented insight into
419 SARS-CoV-2 infection in the respiratory tract of ferrets. In the future, this approach
420 will be a crucial tool to understand the mesoscopic scale of host-pathogen
421 interactions of SARS-CoV-2 but also other respiratory and non-respiratory
422 pathogens, including, for example, influenza A and henipaviruses.

423 **Author contributions**

424 Conceptualization, L.M.Z. and S.F.; methodology, L.M.Z.; formal analysis, L.M.Z.;
425 investigation, L.M.Z., D.S. and M.M.; resources, J.S., D.H., M.B. and A.B.; writing—
426 original draft preparation, L.M.Z. and S.F.; writing—review and editing, L.M.Z., D.S.,
427 J.S., M.M., D.H., M.B., E.M.A., T.C.M., A.B. and S.F.; visualization, L.M.Z.;
428 supervision, E.M.A. and S.F.; project administration, S.F.; funding acquisition, S.F
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441 **Conflict of Interest**

442 The authors declare no competing interests.

443 **Figure legends**

444 **Figure 1: Workflow for correlative LSFM-CLSM of SARS-CoV-2-infected ferret**
445 **tissues. (A)** Nasal conchae and lungs tissue from SARS-CoV-2-infected ferrets were
446 collected, trimmed, and immunostained against SARS-CoV-2 N protein. Fully
447 dehydrated and optically transparent samples were acquired *in toto* with a light sheet
448 microscope and subsequently subsectioned to 1 mm-thick sections for correlative
449 confocal laser-scanning microscopy. **(B)** Representative immunostaining for SARS-
450 CoV-2 N in infected VeroE6 cells using a commercially available polyclonal anti-
451 SARS-CoV N serum (#1, green) and a monoclonal anti-SARS-CoV N mix (#2,
452 magenta) confirms antibody specificity. Blue: Hoechst33342. Scale bars = 15 µm. **(C)**
453 Representative ferret respiratory tract samples before (left) and after (right)

454 immunostaining and ECi-based optical clearing. The photographs from the lung
455 sections (bottom) show two independent samples. Edge length of grid square: 1 mm.

456 **Figure 2: LSFM is able to visualize SARS-CoV-2 infection in nasal turbinates**
457 **within a high spatial context.** **(A)** The tissue structure of the nasal conchae (> 200
458 mm^3 ; 4 days post-infection) was reconstructed using tissue autofluorescence (cyan)
459 and is depicted as volumetric projection from three viewing angles. Anatomical terms
460 of location are provided for orientation. Edge length of grid squares = 2 mm. Total
461 magnification = 1.26x. **(B)** Maximum intensity projections of the regions of interest (1-
462 3) highlighted in (A). SARS-CoV-2 infection is characterized by colocalization of both
463 SARS-CoV-2 N stainings (#1, green; #2, magenta) and results in white coloring
464 (inset). Four distinct SARS-CoV-2 infection foci are highlighted (filled-in arrowhead
465 [A1], outlined arrowhead [A5], arrow [A7], and asterisk). Foci will hereafter be
466 referred to via their respective indicator or designation in square brackets. Ranges of
467 the MIPs in the z-dimension are provided above the respective image. MIP =
468 maximum intensity projection. Scale bar = 1 mm.

469 **Figure 3: 3D detail views highlight oligofocal SARS-CoV-2 infection pattern in**
470 **nasal turbinates at 4 days post-infection.** **(A)** Tomographic representation of three
471 individual SARS-CoV-2 foci (filled-in arrowhead [A1], outlined arrowhead [A5], and
472 arrow [A7]) from ROIs 1 & 2 in Figure 2 along a length of 1,658 μm . The relative
473 distance to plane #1 is indicated in the bottom left corner. Cyan = autofluorescence;
474 green = SARS-CoV-2 N #1; magenta = SARS-CoV-2 N #2. Scale bar = 400 μm .
475 Total magnification = 8x. **(B,C)** Volumetric projections of the detail view from two
476 angles. Clipping at the indicated plane (red) reveals the third SARS-CoV-2 foci (C,
477 arrow), which is hidden behind nasal turbinate tissue in (B). Single channel views
478 further emphasize the colocalizing pattern of both SARS-CoV-2 N stainings (#1,
479 green; #2, magenta). Cyan/grayscale = autofluorescence (AF). **(D)** Close-ups of the
480 three individual infection foci. The angle of the respective image is indicated in the
481 bottom left corner.

482 **Figure 4: Virtual segmentation of SARS-CoV-2 infection foci at 4 days post-**
483 **infection.** **(A)** xz-view of the magnified nasal turbinate view from Figure 3, clipped at
484 the indicated plane (red square). Segmented SARS-CoV-2 infection foci (A1: yellow,
485 A5: red; A7: light blue) are visible through the autofluorescence reconstruction of the
486 tissue morphology (cyan). Once they are uncovered by the clipping plane, they are

487 highlighted with their respective indicator. **(B)** Detail and alternate viewing angle of
488 segmented infection foci. A slightly darker sphere represents the respective foci
489 center. The direct linear distances between the centers of each foci (from Table 1)
490 are highlighted.

491 **Figure 5: High-resolution CLSM analysis of SARS-CoV-2 infection foci in the**
492 **concha nasalis dorsalis of a SARS-CoV-2-infected ferret at 4 days-post**
493 **infection. (A)** 3D maximum intensity projection (MIP) of a SARS-CoV-2 infection
494 focus from ROI 1 in Figure 2. The image stack was acquired with a 40x/1.1 water
495 immersion objective. Cyan = autofluorescence; green = SARS-CoV-2 N #1; magenta
496 = SARS-CoV-2 N #2. Edge length of grid square = 40 μm . **(B)** MIPs from ROIs 1 and
497 2 in (A). Individual cells can be analyzed at subcellular resolution, highlighting
498 infection of ciliated and non-ciliated cells (arrows). Scale bar = 100 μm (overview)
499 and 5 μm (detail).

500 **Figure 6: Only debris-associated SARS-CoV-2 antigen was detectable in ferret**
501 **lung tissue at 4 days post-infection. (A)** Volumetric projection of a large lung tissue
502 section. While some background staining is detectable for the monoclonal antibody
503 mix (#2, magenta), no signal overlap with the polyclonal antibody (#1, green) is
504 visible. Cyan/grayscale = autofluorescence. Edge length of grid squares = 800 μm .
505 Total magnification = 1.6x. **(B)** Alternate viewing angles reveal a spot inside an
506 airway where both signals colocalize (white box in (A)). Contrary to the SARS-CoV-2-
507 associated foci in Figures 2 and 3, the overlapping signal is detected lying on top the
508 epithelial layer, suggesting that it is most likely cell debris inhaled from the URT.

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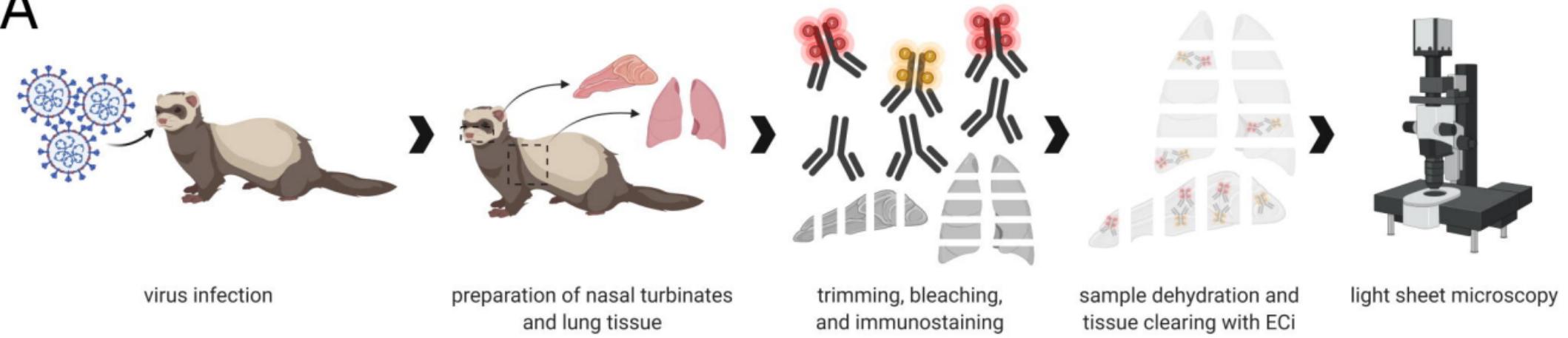
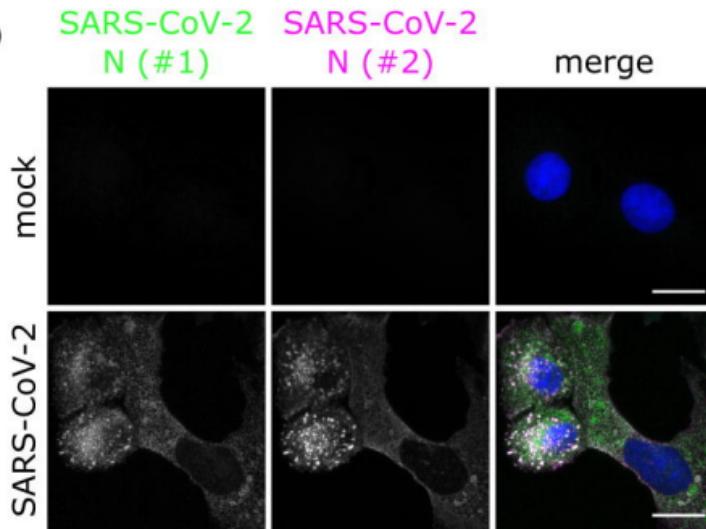
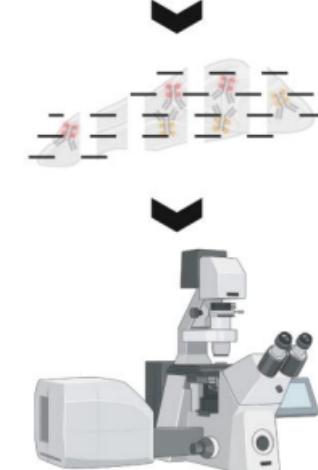
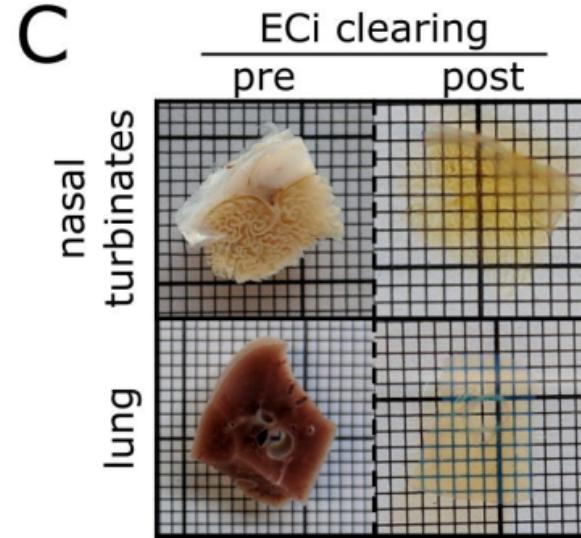
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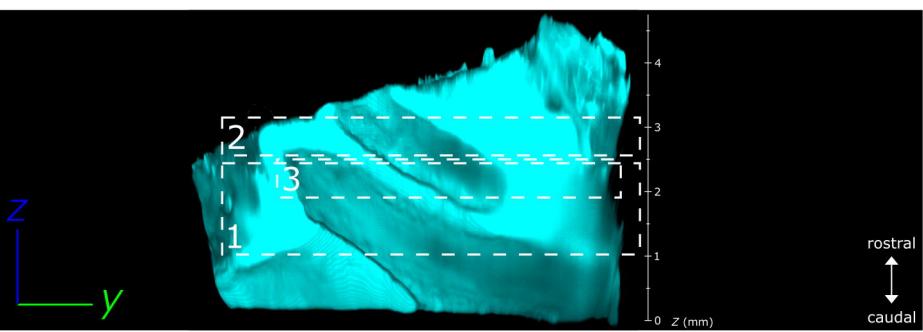
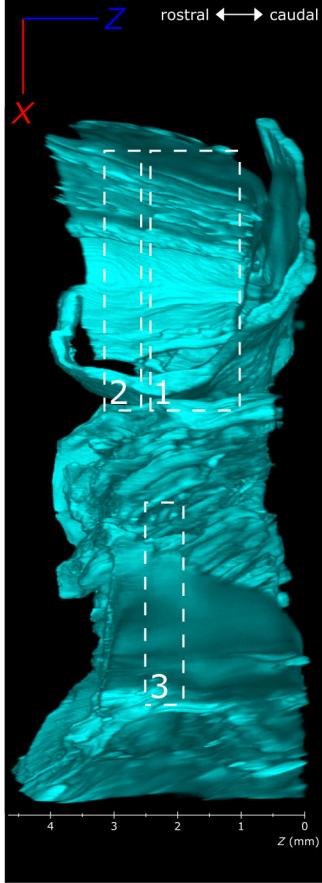
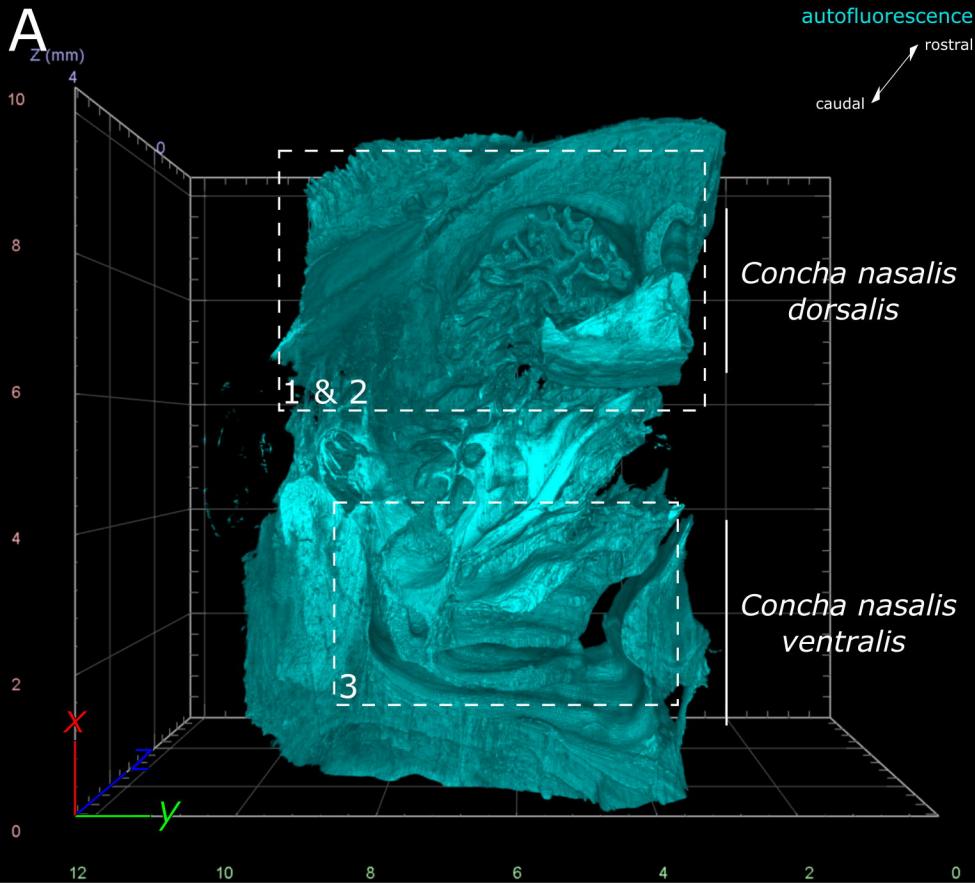
A**B****C**

sample dehydration and tissue clearing with ECi

light sheet microscopy

slicing of cleared samples to 1 mm thickness

confocal laser scanning microscopy



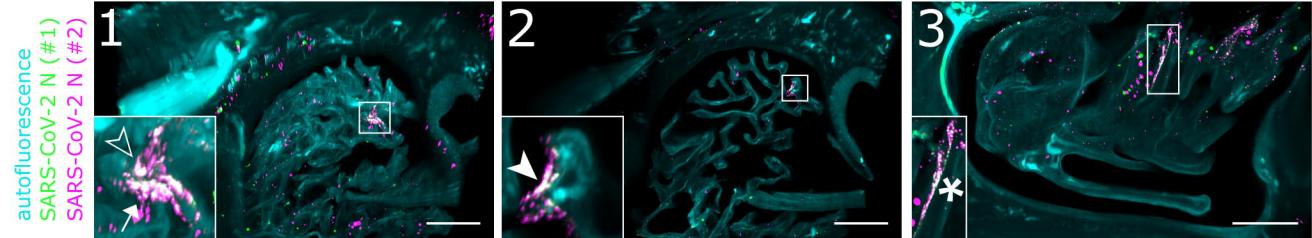
B

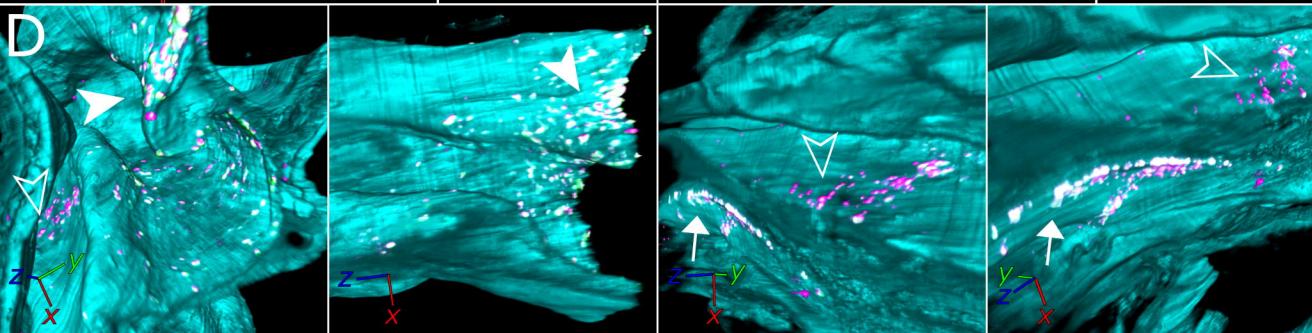
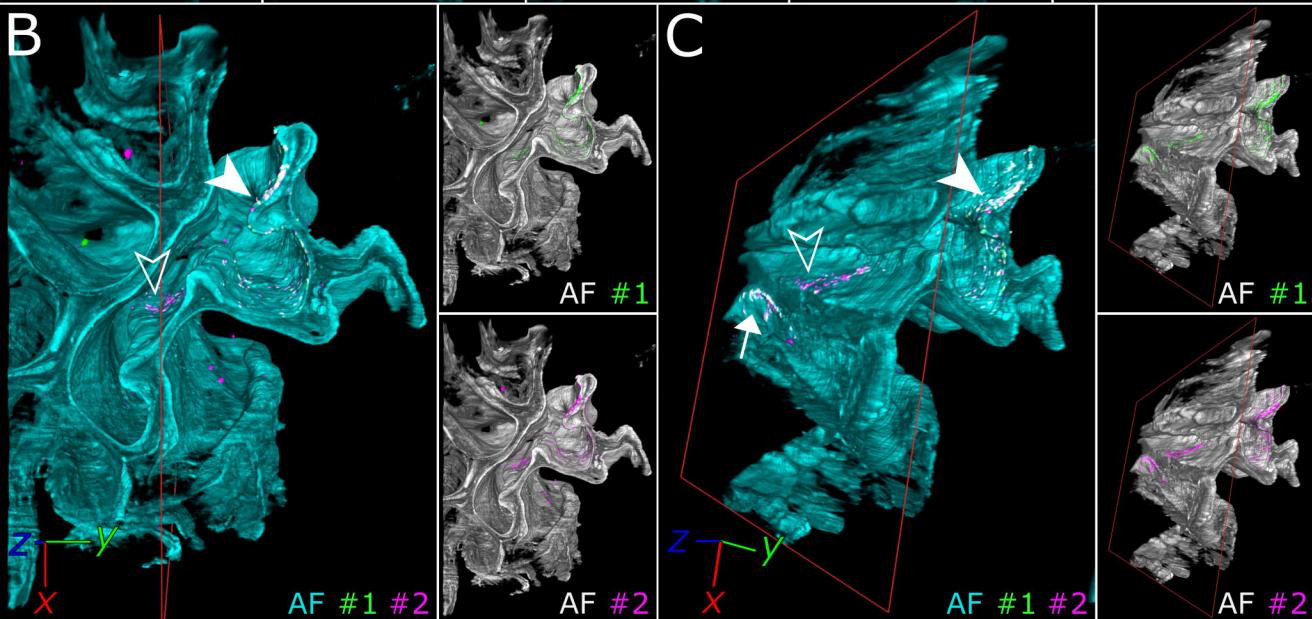
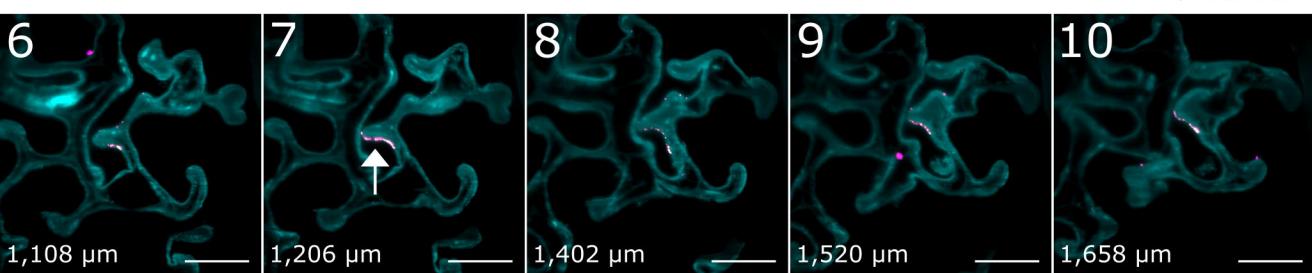
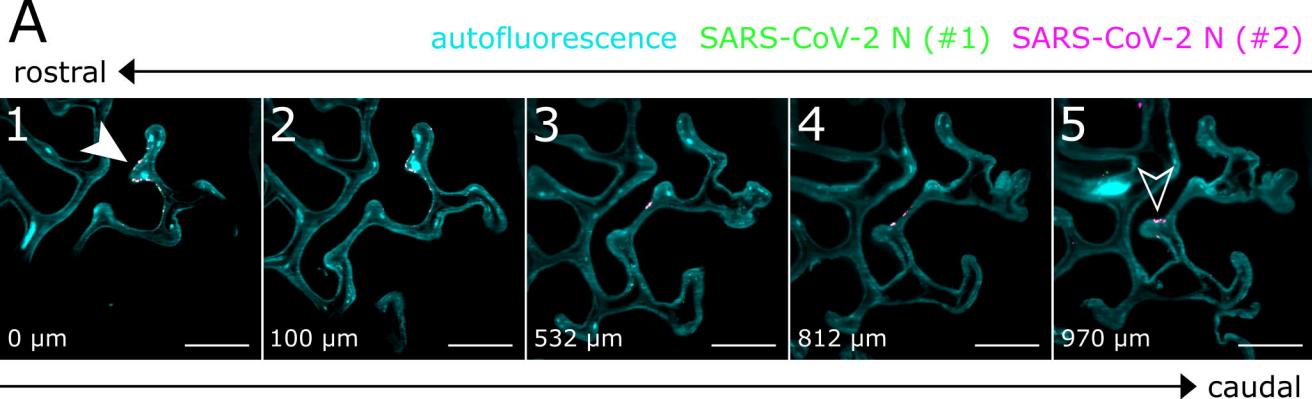
MIPs from regions of interest

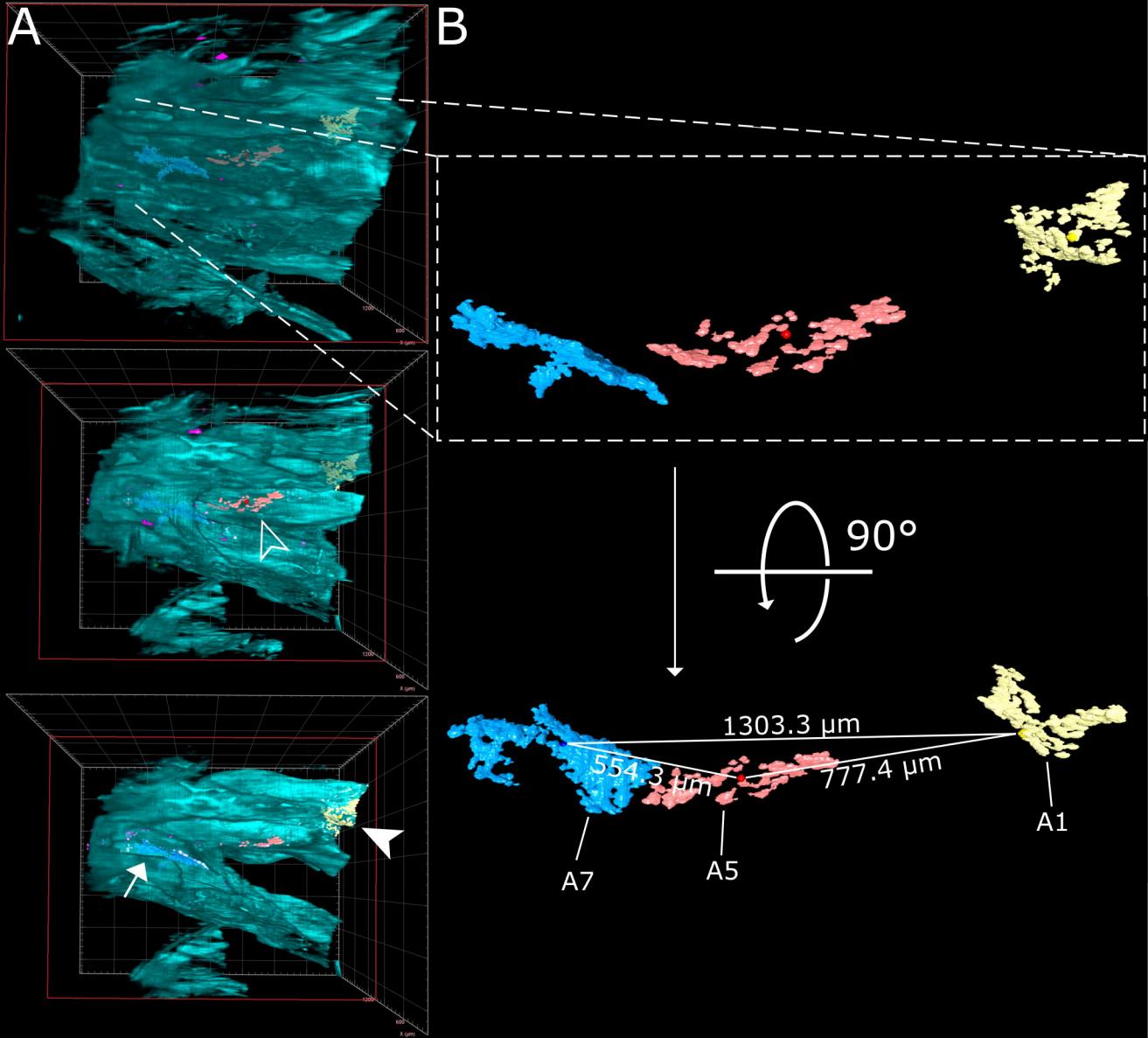
Z: 1032 - 2450 μm

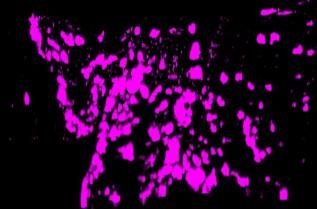
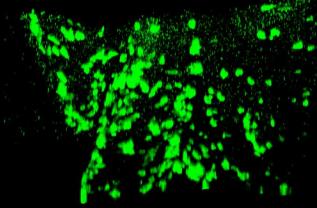
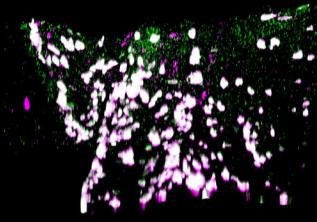
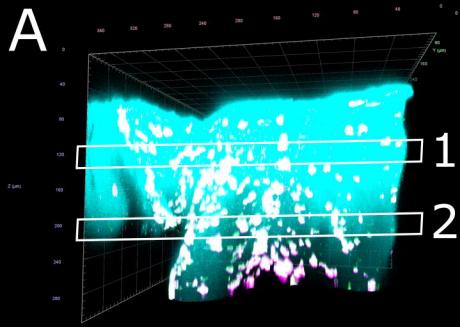
Z: 2622 - 3168 μm

Z: 1892 - 2578 μm





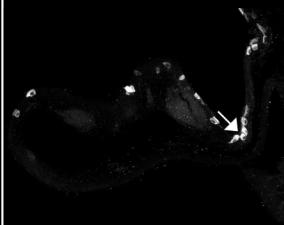


A**B**

MIP from ROI 1

SARS-CoV-2

N (#1)

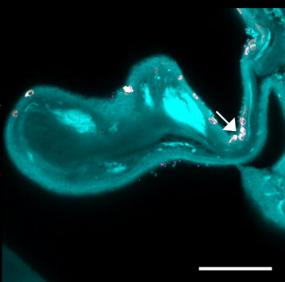


SARS-CoV-2

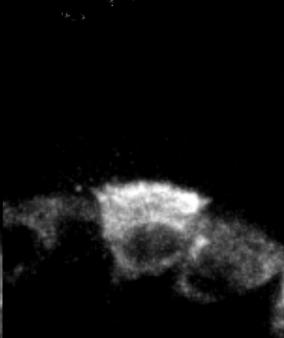
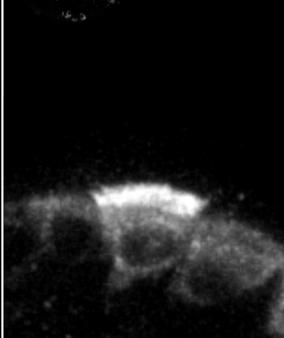
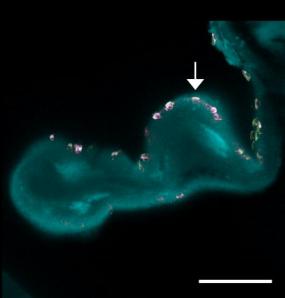
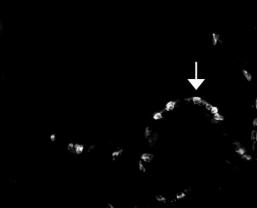
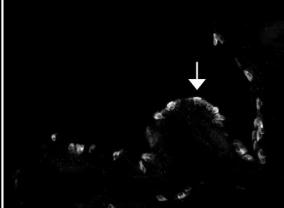
N (#2)

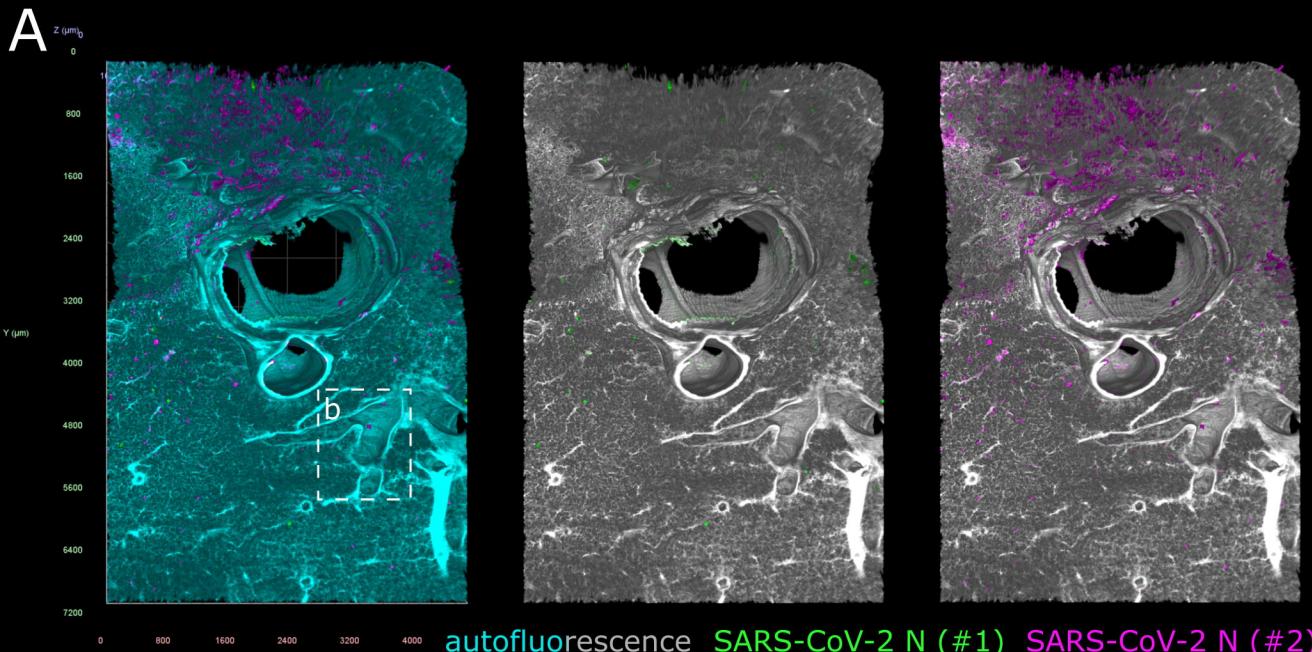


merge



MIP from ROI 2





B volumetric projections of ROI single plane

