

Supplementary Information for

**Super-Resolution Atlas of SARS-CoV-2 Infection Reveals Protease-
Dependent Organelle Maturation, dsRNA Landscapes, and Intracellular
Structural Proteins**

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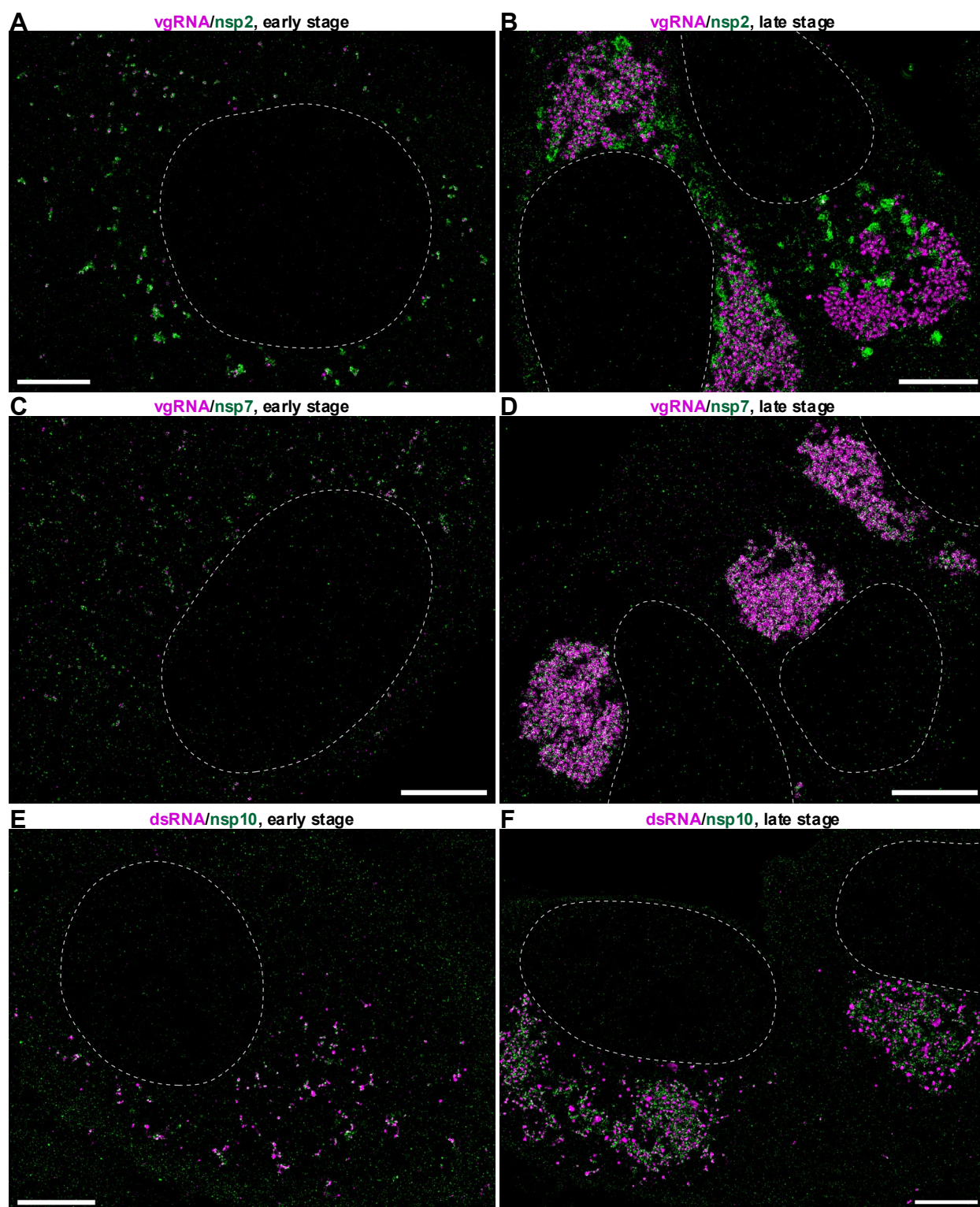
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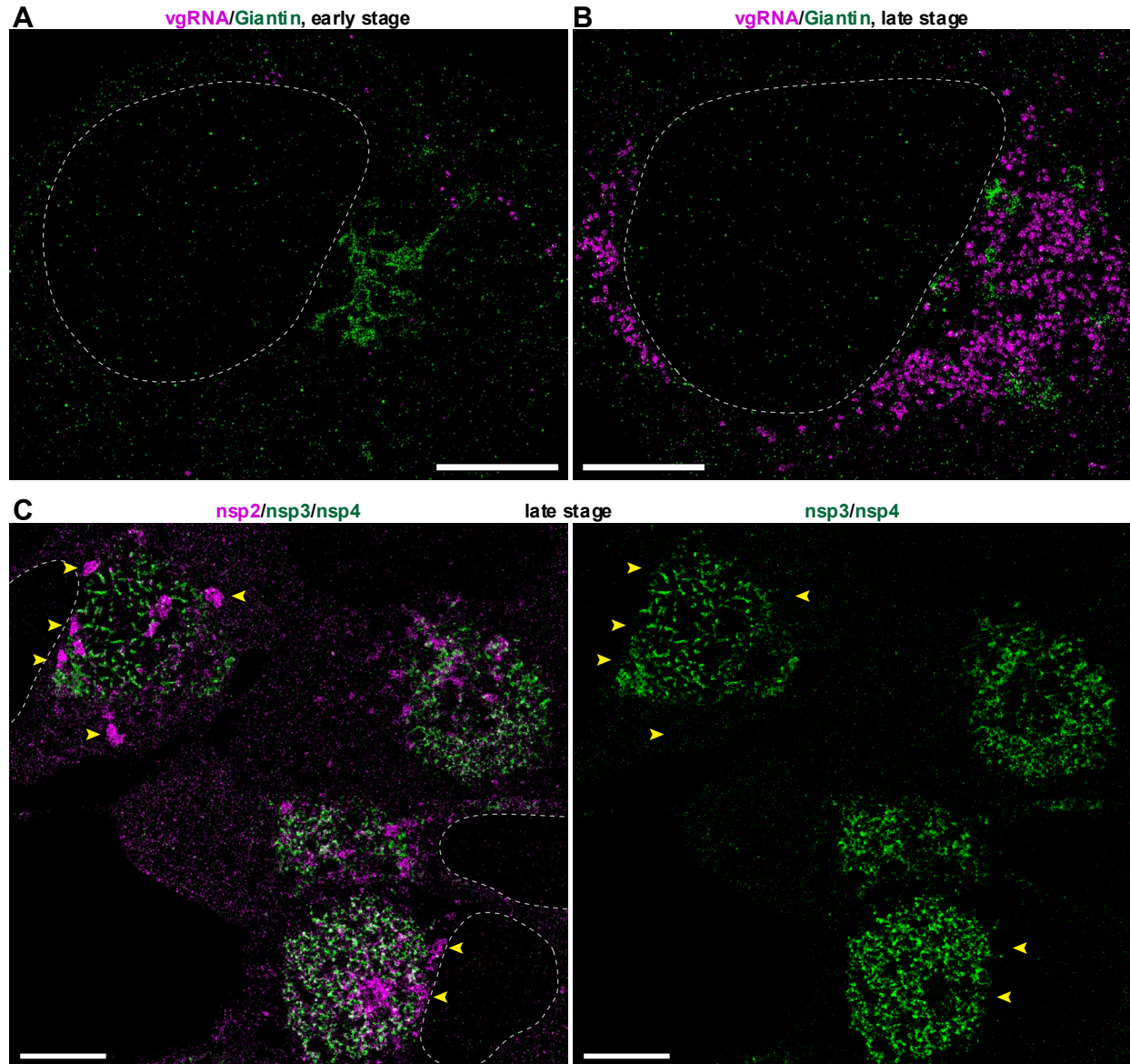
Supplementary Fig. 1: Morphological classification of infection stages in asynchronous cell populations.

Although all samples were fixed at 24 hours post-infection (hpi), the viral life cycle in A549-ACE2 cells is asynchronous, resulting in a population containing cells at different functional phases of replication. Cells were stratified based on the morphology of replication organelles (ROs) observed via super-resolution microscopy.

(A, C, E) "Early morphological stage" is characterized by isolated, individual DMVs (typically $\lesssim$ 200 nm in diameter) distributed in the cytoplasm.

(B, D, F) "Late morphological stage" is defined by the presence of large, merged vesicle packets and dense perinuclear accumulations of viral content. This morphological transition is consistent across different replicase components and RNA species, as shown by side-by-side comparisons of vgRNA (magenta) and nsp2 (green) **(A, B)**; vgRNA (magenta) and nsp7 (green) **(C, D)**; and dsRNA (magenta) and nsp10 (green) **(E, F)**.

The white dashed curves indicate the approximate position of the nucleus edge. Scale bars: 5 μ m.



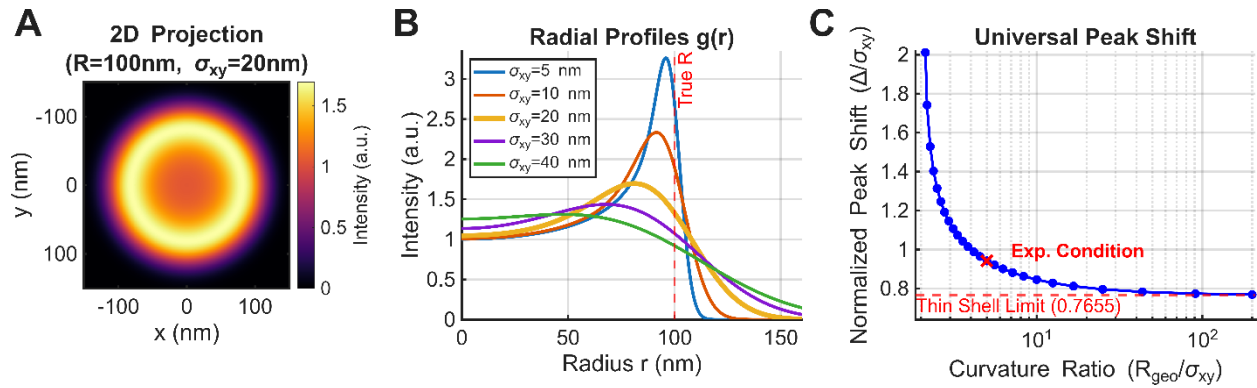
Supplementary Fig. 2: The Golgi marker Giantin remains spatially distinct from replication organelles, and nsp2 segregates from nsp3/nsp4 during Golgi remodeling.

(A) SR image of vgRNA (magenta) and Giantin (green) in the early morphological stage indicates that the Golgi apparatus retains its typical compact, perinuclear ribbon-like structure and is spatially distinct from the isolated vgRNA puncta–early ROs.

(B) In the late morphological stage, the Golgi apparatus undergoes fragmentation and dispersal. The Giantin signal remains separate from the large, merged vgRNA clusters, indicating that Giantin is not relocated to or recruited into the viral replication organelles.

(C) SR image of nsp2 (magenta) costained with a mixture of anti-nsp3 and nsp4 antibodies (green) in the late infection state. Nsp2 accumulates in dispersed structures consistent with remodeled Golgi bodies (yellow arrowheads) which are distinctly devoid of nsp3 and nsp4 signals.

The white dashed curves approximate the position of the nuclear edge. Scale bars, 5 μm .

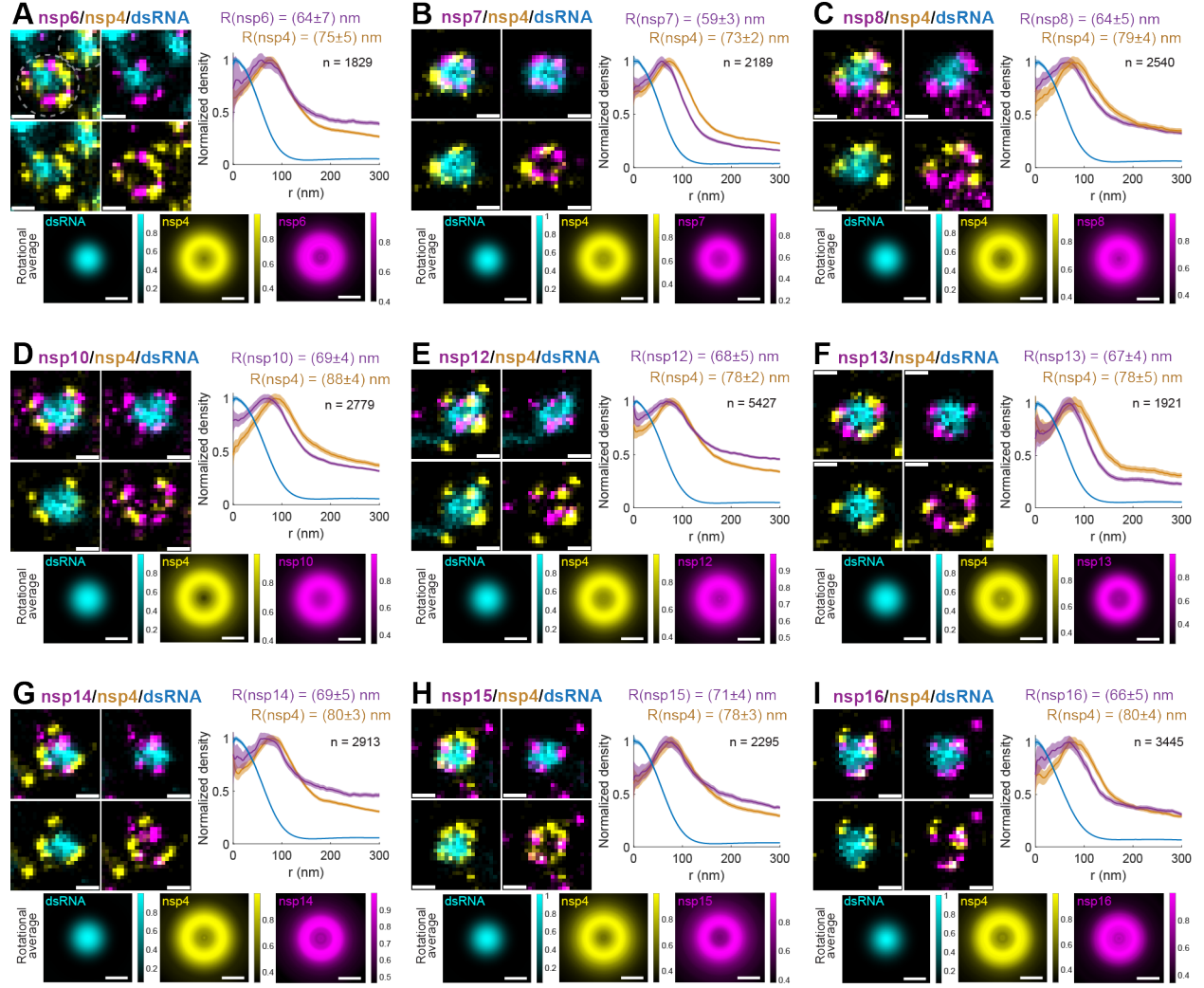


Supplementary Fig. 3. Simulation of projection effects and systematic peak shift in DMV radius measurements.

(A) Simulated 2D projection of a double-membrane vesicle (DMV) modeled as a spherical shell with geometric radius $R_{geo} = 100$ nm and lateral localization precision $\sigma_{xy} = 20$ nm. The image represents the convolution of the ideal geometric projection (Abel transform) with a Gaussian Point Spread Function (PSF).

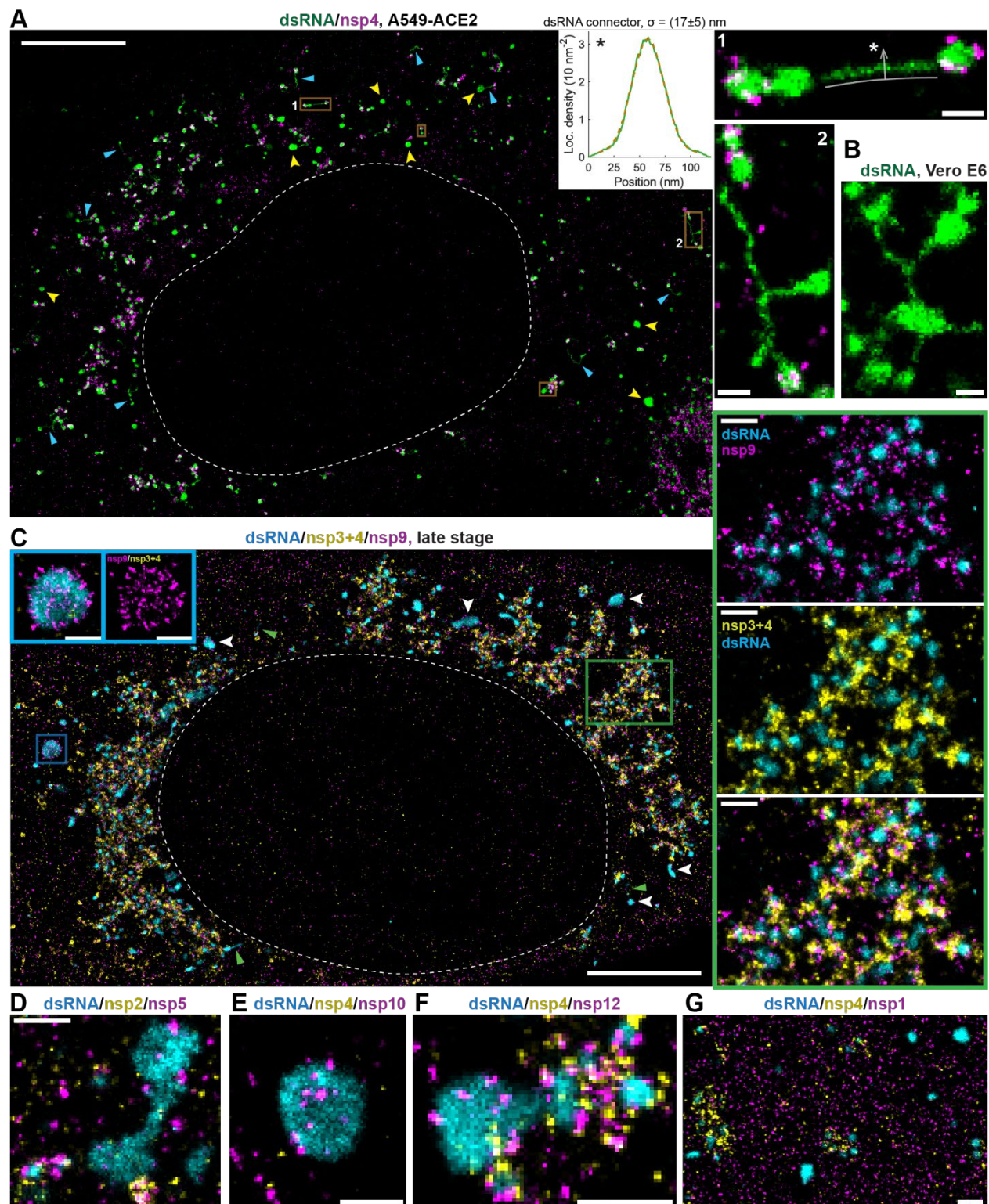
(B) Theoretical radial intensity profiles $g(r)$ calculated for a fixed geometric radius ($R_{geo} = 100$ nm) and varying localization precisions ($\sigma_{xy} = 5\text{--}40$ nm). The profile calculated for $\sigma_{xy} = 20$ nm (bold yellow curve) strongly resembles the experimental profiles for many DMV-associated proteins, with the exception of an additional nearly constant background that is trivial and was neglected in this theoretical development. The vertical dashed line indicates the true geometric radius. Note that as the blur width (σ_{xy}) increases relative to the shell radius, the position of the intensity maximum (r_{peak}) shifts systematically inward.

(C) Universal master curve quantifying the systematic peak shift $\Delta = R_{geo} - r_{peak}$. The shift, normalized by the localization precision (Δ/σ_{xy}), is plotted as a function of the dimensionless curvature ratio (R_{geo}/σ_{xy}). In the thin-shell limit ($R_{geo} \gg \sigma_{xy}$), the shift approaches a constant value of $\approx 0.77\sigma_{xy}$ (dashed red line). For the experimental conditions (indicated by the red cross; $R_{geo} \approx 100$ nm, $\sigma_{xy} \approx 20$ nm), curvature effects slightly increase this shift to approximately $0.9\sigma_{xy}$.



Supplementary Fig. 4: Nanoscale spatial organization of non-structural proteins within DMVs based on three-color SR imaging.

(A–I) SR imaging and quantitative radial analysis maps the distribution of nsp6–nsp8 (A–C), nsp10 (D), and nsp12–nsp16 (E–I) multiplexed with nsp4 and dsRNA. Each panel displays a representative SR image of an individual DMV, the corresponding radial density distribution function $g(r)$, and a rotationally averaged projection of the radial distribution. Dashed circles in A delineate two distinct DMVs. Peak radial positions (R) are indicated above the plots as the mean $\pm$ 95% confidence interval (CI), derived from Gaussian fitting of the peak region of $g(r)$ following bootstrap resampling. The sample size (n) denotes the total number of DMVs analyzed per target combination. Analyses were performed on DMVs across 22 (A), 16 (B–D, H), 35 (E), 12 (F, G), and 18 (I) distinct early-stage cells. Scale bars: 100 nm.



Supplementary Fig. 5: Subcellular organization of dsRNA connectors and granules in SARS-CoV-2-infected cells.

(A) 2D dSTORM imaging of dsRNA and nsp4 reveals diverse dsRNA localization patterns. Magnified regions (right) corresponding to the yellow boxed areas in the main image highlight dsRNA connectors bridging DMVs. The inset displays a cross-sectional localization density profile of dsRNA, calculated orthogonal to the white curve in region 1, in the direction of the arrow (with longitudinal density averaged). The width of the fitted Gaussian function (σ , dashed line) is presented as the mean $\pm$ SD ($n = 10$ connectors from the represented cell).

(B) Intercluster dsRNA connectors are similarly evident in Vero E6 cells labeled exclusively for dsRNA.

(C–G) Three-color 2D SR images display large dsRNA granules and aggregates.

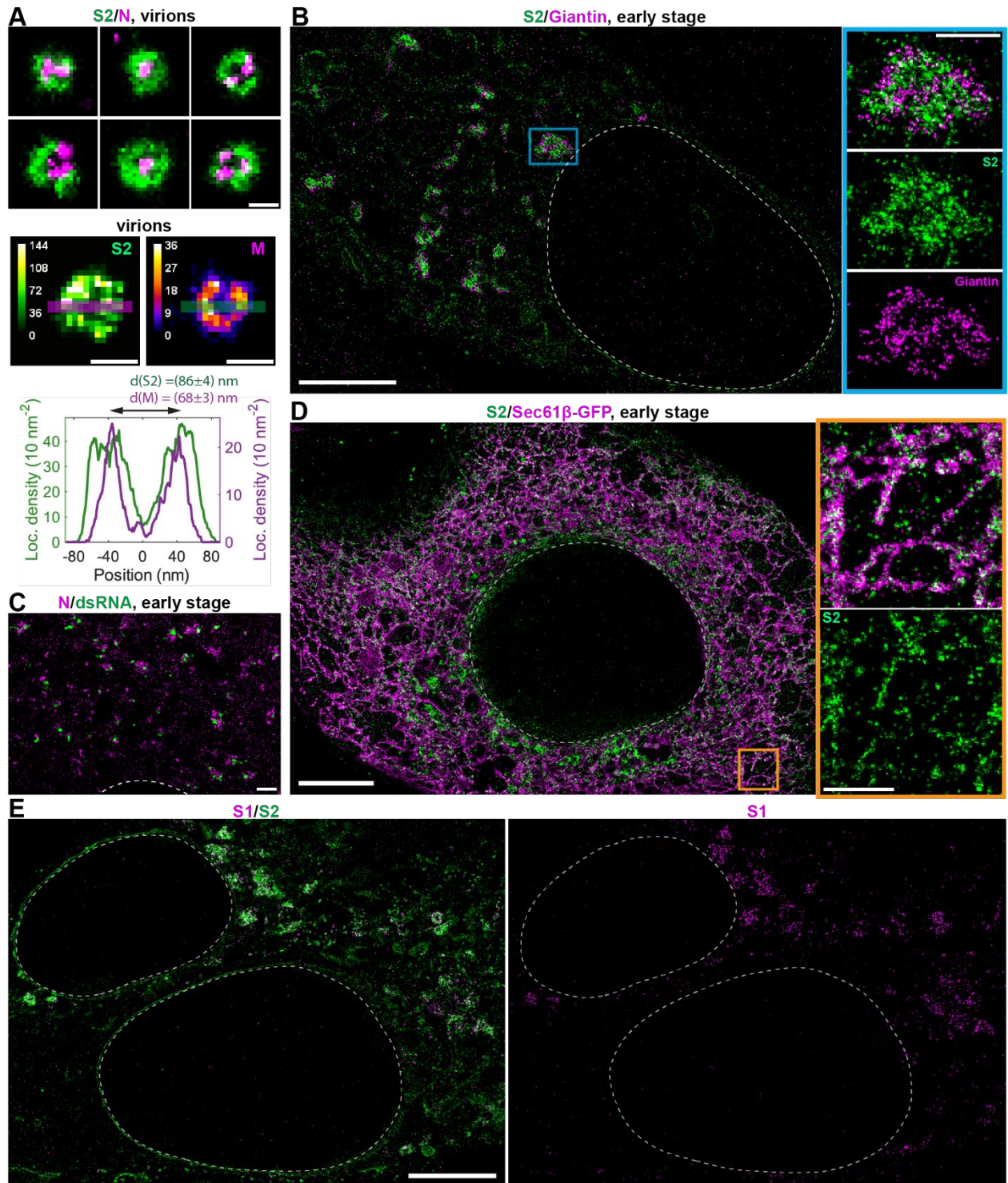
(C) In late-stage infection, cells contain massive, round and elongated dsRNA structures. These aggregates lack DMV pore proteins (nsp3+4, labeled with a mixture of anti-nsp3 and anti-nsp4 primary antibodies) but recruit nsp9. Magnified insets (blue frame) detail a single large dsRNA aggregate devoid of pores. Conversely, the right panels (green frame) illustrate the typical perinuclear accumulation of dsRNA, nsp9 and nsp3+4 characteristic of late-stage DMVs and vesicle packets⁵. In panels **A** and **C**, arrowheads denote additional large dsRNA aggregates, while triangles highlight fiber-like dsRNA connectors.

(D) An irregularly shaped dsRNA aggregate is decorated with discrete nsp5 puncta but lacks nsp2.

(E, F) Large dsRNA structures largely devoid of nsp4 are nevertheless decorated with nsp10 (**E**) and nsp12 (**F**).

(G) Voluminous, nsp4-deficient dsRNA granules occupy cytoplasmic zones entirely lacking nsp1.

Images in **A** and **C–G** were acquired in A549-ACE2 cells. White dashed curves approximate the position of the nuclear envelope. Scale bars: 5 μm (main images in **A, C**), 500 nm (**D–G** and magnified boxes in **C**), and 200 nm (**B** and magnified boxes in **A**).



Supplementary Fig. 6: Subcellular localization and nanoscale organization of structural proteins in SARS-CoV-2-infected cells.

(A) SR imaging of SARS-CoV-2 virions reveals the concentric organization of the S2 subunit around the N protein-labeled core (top). Both S2 and the membrane (M) protein form ring-like structures within the virions (middle). Cross-sectional localization density profiles, calculated along the indicated lines in the middle panels, demonstrate that M rings have a distinctly smaller diameter than S2 rings. The peak-to-peak distance between the two density lobes (d) was calculated by fitting the profiles with a sum of two Gaussians ($n = 24$ and 27 virions for M and S2, respectively). Values of d are represented as the mean $\pm$ 95% CI. Images of S2 and N were acquired from virions deposited on coverslips as previously described⁵, whereas M images were acquired from virions located at the cell periphery.

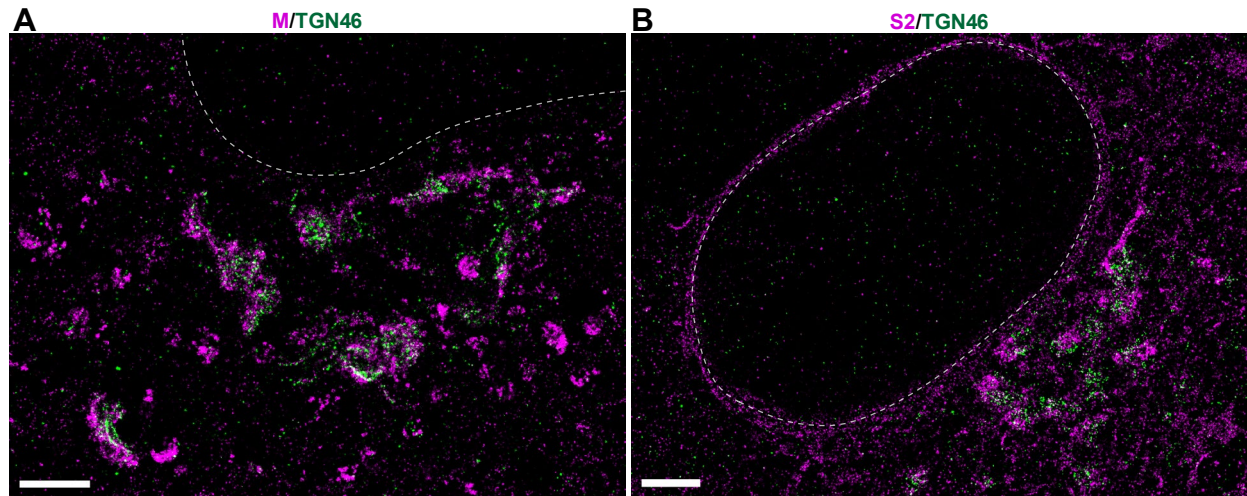
(B) S2 localizes within Golgi bodies as indicated by the Giantin-labeled rim.

(C) The N protein is distributed within the cytosol in close proximity to dsRNA-labeled ROs.

(D) A fraction of S2 localizes to ER tubules (labeled with an anti-GFP antibody). Magnified regions (right) correspond to the orange boxed area in the whole-cell image.

(E) Unlike S2, S1 subunit does not localize to either ER tubules or the nuclear envelope.

Images were acquired in A549-ACE2 cells (**A–C**, **E**) and Vero E6-Sec61 β -GFP cells (**D**). White dashed curves indicate the approximate position of the nuclear envelope. Scale bars: 100 nm (**A**), 1 μ m (**C** and magnified images in **B**, **D**), and 5 μ m (**E**, whole-cell images in **B**, **D**)

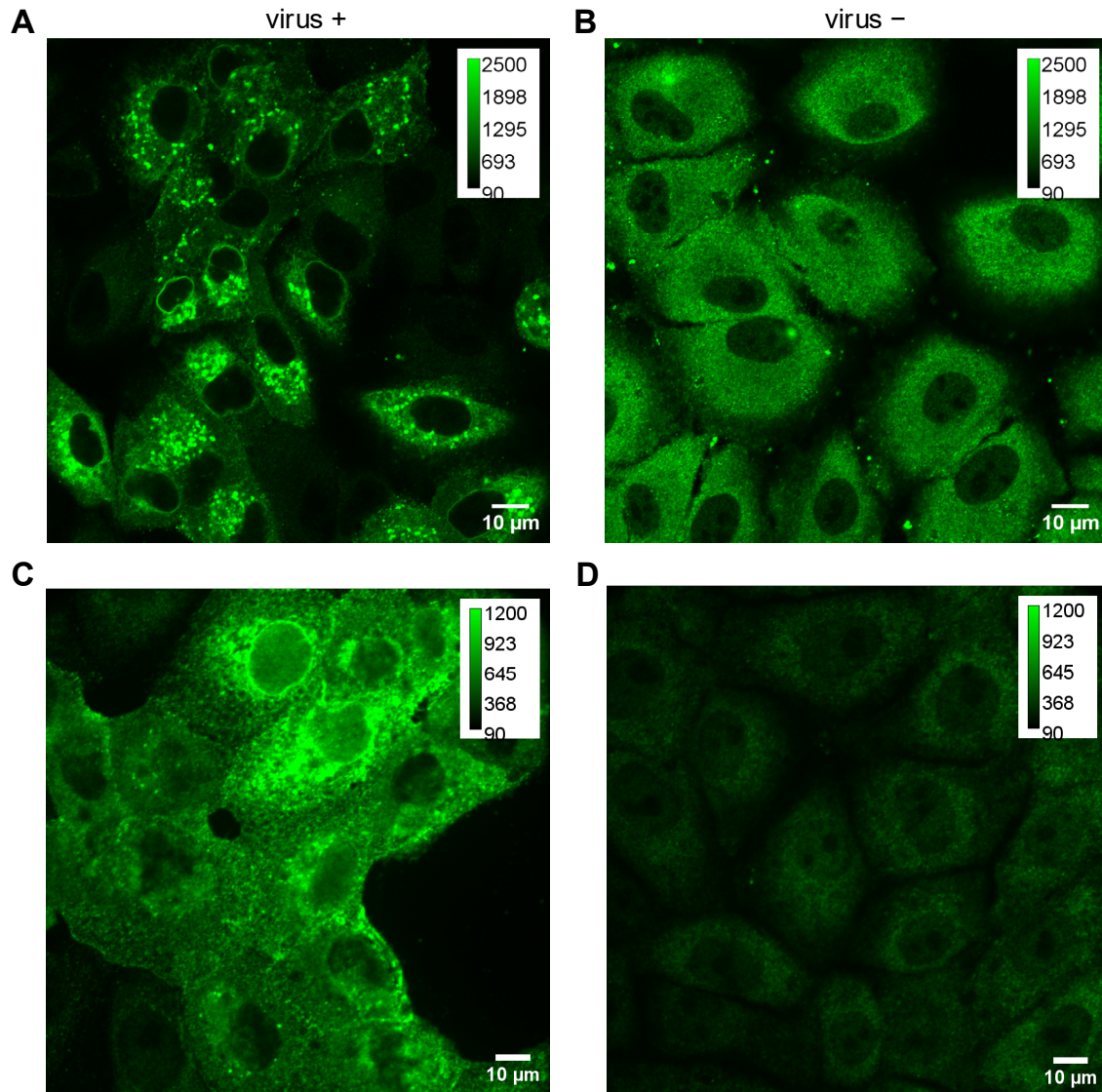


Supplementary Fig. 7: Structural proteins M and S2 localize to the trans-Golgi network.

(A) Membrane protein (M) forms dense aggregates that are spatially juxtaposed to and partially overlap with TGN46 structures.

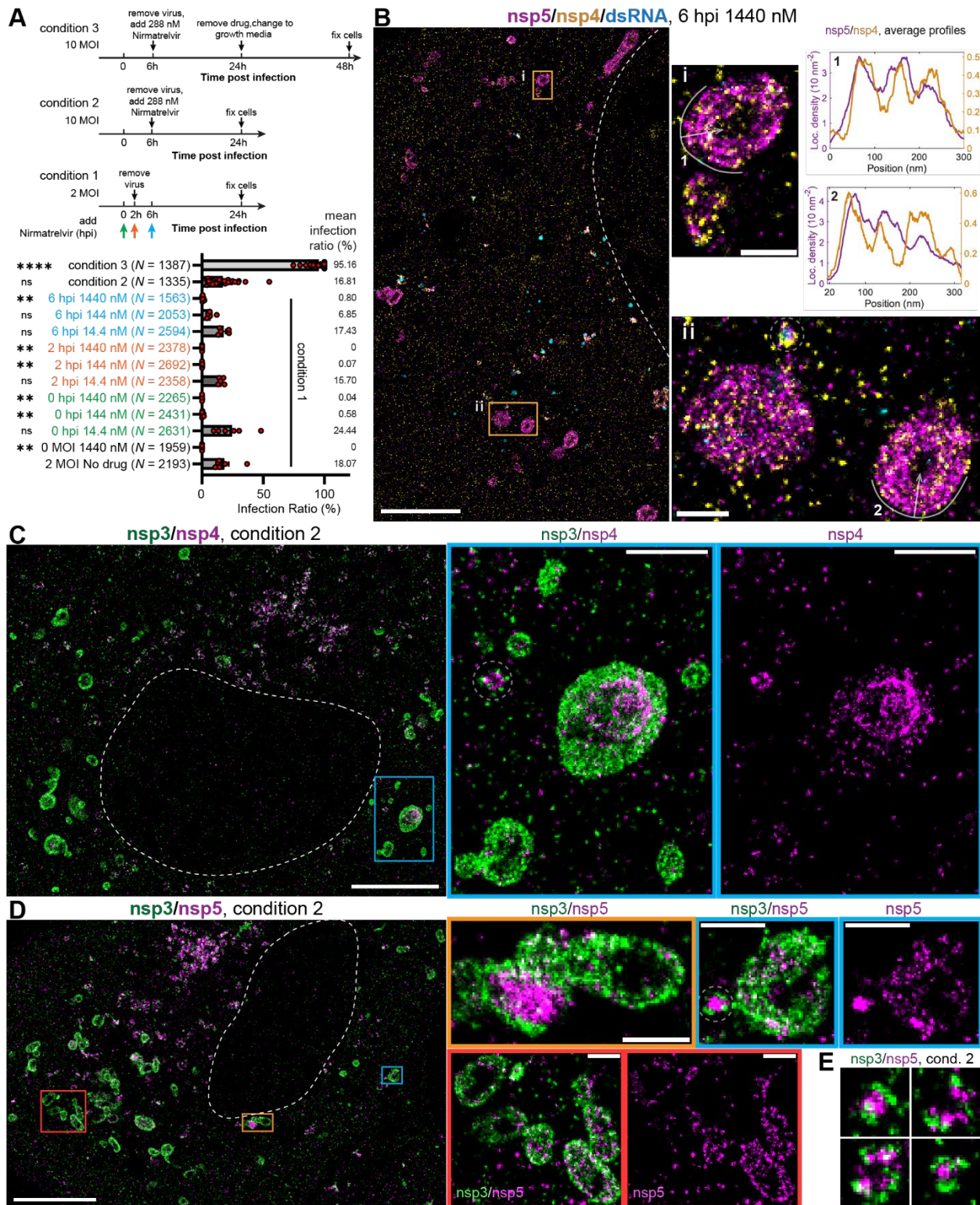
(B) Similarly, spike subunit 2 (S2) clusters are observed adjacent to TGN46.

White dashed curves indicate the approximate position of the nuclear envelope. Scale bars, 2 μm .



Supplementary Fig. 8: Validation of anti-S2 antibody specificity.

(A–D) To confirm the specificity of the anti-S2 antibody and validate the genuine localization of the S2 subunit, confocal imaging was performed on SARS-CoV-2–infected (A, C) and uninfected control (B, D) cells. Distinct, specific localization of S2 to the nuclear envelope and endoplasmic reticulum (ER) is observed exclusively in the infected cells (A, C). Of note, the non-specific background signal in the uninfected control samples (B) is frequently higher than the signal observed in uninfected bystander cells within the infected samples (A). This is likely due to the depletion of the primary antibody by the highly abundant S2 antigen present in the infected cells. Images were acquired in A549-ACE2 cells (A, B) and Vero E6 cells (C, D), representing two biologically independent experiments. Scale bars: 10 μm.



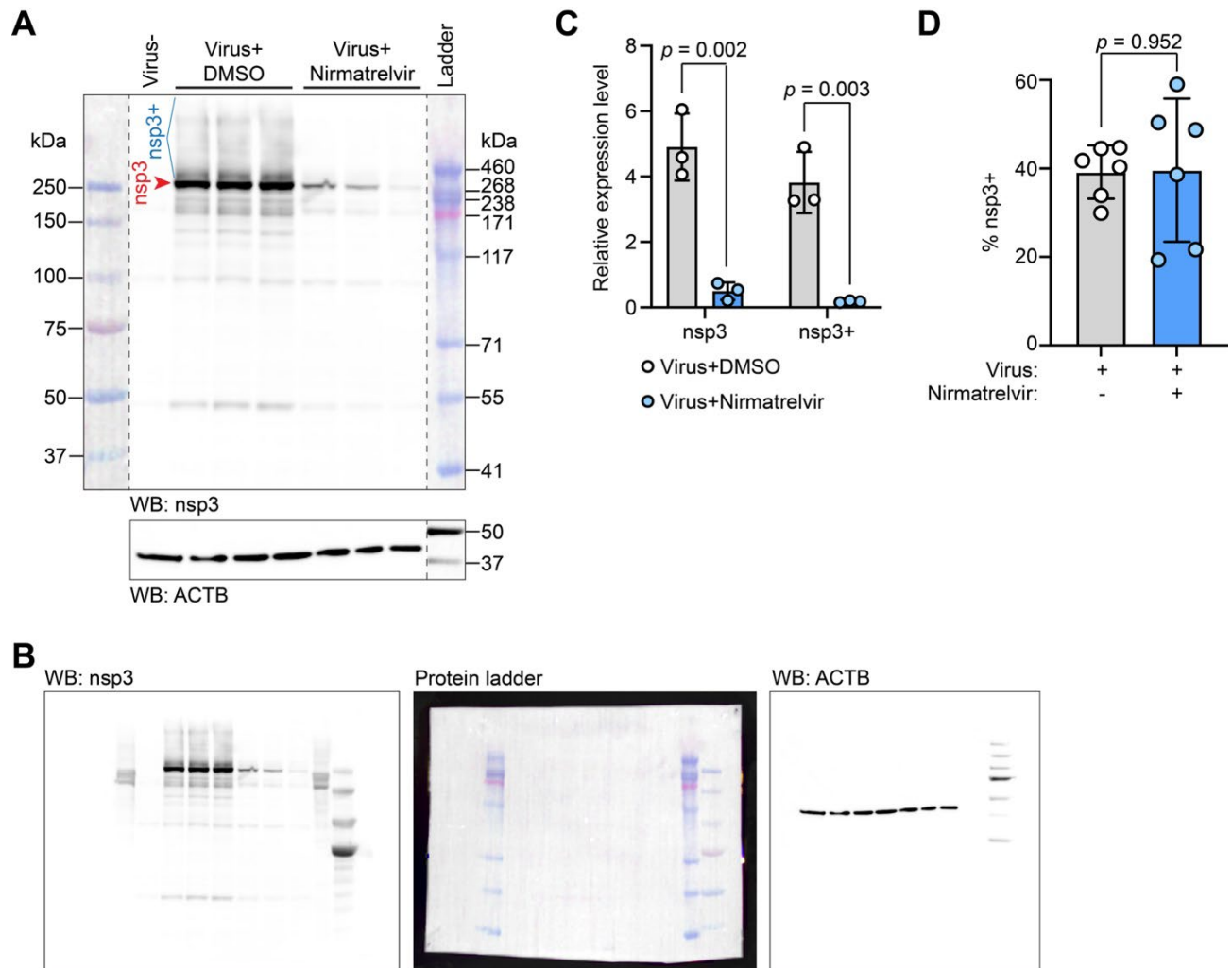
Supplementary Fig. 9: Nanoscale architecture of multilayered bodies in nirmatrelvir-treated, SARS-CoV-2-infected cells.

(A) Schematics of the experimental timelines for conditions 1–3 (top) and a corresponding scatter dot plot of the infection ratio (infected cells / total cells) across these nirmatrelvir treatments (bottom). Individual data points represent the infection ratio per field of view. Statistical comparisons against the no-drug control were performed using a one-way ANOVA with Dunnett's post hoc test. ns, not significant ($p > 0.05$); **, $p < 0.01$; ****, $p < 0.0001$. The sample size (N) denotes the total number of cells analyzed per condition.

(B) SR imaging of a cell infected at an MOI of 2, treated with 1440 nM nirmatrelvir from 6 to 24 hpi, and fixed at 24 hpi. Magnified insets (center) highlight two regions containing large multilayered bodies (MLBs). Cross-sectional localization density profiles of nsp4 and nsp5 (right), calculated orthogonal to the white curves in the central insets (with longitudinal density averaged), demonstrate that both proteins exhibit a periodic, layered organization with a spacing of approximately 80 nm.

(C–E) SR imaging of cells infected at an MOI of 10, treated with 288 nM nirmatrelvir from 6 to 24 hpi, and fixed at 24 hpi. Both nsp3/nsp4 (**C**) and nsp3/nsp5 (**D**) robustly localize to MLBs. Magnified colored boxes detail the diverse morphological variations of these MLBs. Morphologically typical DMVs are presented in **E**, with further instances delineated by dashed rings throughout panels **(B–D)**.

Experiments were performed in A549-ACE2 cells. White dashed curves indicate the approximate position of the nuclear envelope. Scale bars: 5 μm (main images in **B–D**); 1 μm (magnified boxes in **C**); 500 nm (magnified boxes in **B, D**); 200 nm (**E**).



Supplementary Fig. 10. Western blot analysis of nsp3 cleavage under nirmatrelvir treatment.

(A) Western blot analysis of nsp3 and ACTB proteins in cell lysates of A549-ACE2 cells without virus infection (Virus-), with SARS-CoV-2 virus infection and DMSO treatment (Virus+DMSO), and with SARS-CoV-2 virus infection and nirmatrelvir treatment (Virus+Nirmatrelvir). Overnight membrane transfer was conducted to guarantee large proteins were successfully transferred onto the membrane, as indicated by the 460 kDa protein ladder. Cleaved nsp3 protein bands are indicated by the red arrow. Uncleaved nsp3 protein bands (nsp3+) are indicated by the blue bracket.

(B) Raw membrane images for the Western blot results in (A). Left: nsp3 blot captured using a gel imaging system. Middle: a direct photograph of the nsp3 blot membrane, taken with a

smartphone, to clearly show protein ladders. Right: the corresponding ACTB loading control blot, captured using a gel imaging system.

(C) Quantification of the relative expression level of nsp3 and nsp3+ proteins based on the Western blot result in **(A)**. ACTB protein was used as a loading control for normalization. Three biological replicates. Two-sided, unpaired Student's t-test.

(D) Quantification of the percentage of nsp3+ proteins relative to total nsp3 proteins based on the Western blot result of **(A)**. Three biological replicates and two experimental replicates. Two-sided, unpaired Student's t-test.