

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

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The submitted manuscript is a nicely structured study of the localisation of SARS-CoV-2 proteins using super-resolution fluorescence microscopy. Overall, some interesting results are presented that are entirely consistent with existing models and published data. As a very thorough characterisation of the location of viral proteins, the work is beneficial to and will be of interest to the field. However, additional data would be required to fully support some conclusions and there are several places where rather speculative statements are made without acknowledgement of alternative interpretations of the data. This should be addressed before publication could be considered. In addition, better recognition of existing models and data should be given to accurately place the work within the published knowledge.

The most noteworthy results presented in the manuscript are 1) the positioning of the viral non-structural proteins from the cytoplasmic face of the DMV to the DMV interior and their relationship with the DMV molecular pore, 2) the role of nsp5 protease activity in DMV biogenesis and 3) the presence of dsRNA linkers between DMVs and the identification of dsRNA bodies lacking the 3-4 pore.

In the first observation, the data shows that the N-terminus of nsp3 sits on the cytoplasmic face of the DMV, nsp4 sits closer to the DMV centre and nsp5-16 are localised further to the interior than nsp4. Interestingly, nsp5-16 are also positioned in line with nsp3 and 4. This data is consistent with the N-terminus of nsp3's position at the crown of the DMV molecular pore, nsp4 forming part of the pore transmembrane section and the remaining nsps making up the replication transcription complex (RTC) situated at the base of the pore within the interior of the DMV. The data presented is a thorough characterisation. And finding antibodies that recognise all these viral proteins, especially when they are assembled into the RTC, is no small achievement and an extremely valuable resource if shared with the wider research community!

The authors present their finding of nsp5-16 locating within the DMV as a novel model. However, this does not set the findings as well as it might within the existing knowledge. Nsp5 and nsp3 have long been shown to colocalise (10.1128/JVI.02501-05) and nsp3, 5 and 8 have been shown using immunoEM to localise to CMs, but also to the interior of DMVs (10.1371/journal.pbio.0060226). Furthermore, since the characterisation of the DMV molecular pore, a model whereby the RTC is incorporated into the DMV during formation has been proposed and is a favoured model among those who have historically studied coronavirus replication organelles (10.1146/annurev-biochem-052521-115736). In addition, evidence has already been published in an analogous study using alphacoronavirus 229E that nsp7, 8 and 9 are localised to the interior of the DMV with nsp4 further out and nsp3 on the exterior (10.1126/sciadv.adq958). Evidence of density sitting under the DMV pores seen using cryoET has also led to the model that the RTC is associated with and sits under the pore in the DMV lumen (10.1126/science.abd3629, 10.1126/sciadv.adq958). Therefore, the work presented here supports and is completely consistent with existing data and models and should recognise this better.

The second finding presented is the requirement for nsp5 protease function in formation of DMVs. When nsp5 function was inhibited using nirmatrelvir, DMV formation was limited, and instead concentric rings of likely paired membranes were formed (EM would be needed to confirm this result). However, preventing cleavage of the nsp3-4 junction would also give a comparable phenotype so confirmation that 3-4 cleavage is occurring would be required to confidently draw this conclusion. In addition, this section leads to some internal inconsistency with the conclusions. The authors propose that nsp5 cleavage

of the polyprotein occurs after biogenesis of the DMV, once it has sealed (lines 208-210). However, the data presented in this section (assuming 3-4 is cleaved) demonstrates that inhibition of nsp5 protease function prevents/limits DMV formation. DMV formation by the nsp3-4 self-cleaving expression cassette appears to be very rapid as formation intermediates are not readily visualised (consistent with observations in virus infected cells). Therefore, it seems likely that nsp5 cleavage of the polyprotein occurs before, during and after DMV formation. How much role nsp5 plays in truly “gating” DMV formation is not clear. Additional scenarios would need to be assessed to provide a detailed understanding of the step-wise process. And this would also require inclusion of electron microscopy imaging. However, once 3-4 cleavage is confirmed, the results presented here are an interesting and useful development in our understanding of the process of DMV formation.

The third finding presented is the existence of dsRNA connectors between DMVs as well as the observation of dsRNA aggregations lacking association with nsp3-4. These connectors are novel and, to my knowledge, have not been reported before. The use of both high-resolution imaging and 3D imaging may have helped in the identification of these structures. Although earlier super-resolution imaging of other CoV infected cells has not identified these connectors and it would be beneficial to discuss this difference. The authors propose that the connectors may represent the presence of dsRNA in the membrane connections often seen between DMVs in SARS-CoV-2 infected cells. However, these are connections of the DMV outer membrane so it is not immediately obvious how dsRNA would cross into this space. The DMV pore would deliver RNA to the cytoplasm and there is no indication yet of a pore from the DMV interior to the intra-membrane space. It has been shown that dsRNA present in cells infected with SARS-CoV-2 (and other CoVs) is located within a membrane protected environment (10.3390/v13122540), so non-membrane associated dsRNA free in the cytoplasm seems not to be a likely model (although linkers were not detected in this study). The presence of dsRNA aggregates not associated with nsp3-4 is also a new finding. The authors propose that these are membrane-less aggregates of dsRNA that might act to seed new DMVs. However, no data is presented showing whether these are membrane associated or not so this conclusion cannot be drawn. And absence of nsp3-4 does not mean there is no membrane. Previous data about the location of dsRNA in CoV infected cells indicates that it is associated with membranes via either fluorescence or electron microscopy (10.1371/journal.pbio.0060226; 10.3390/v13122540). Furthermore, the suggestion that these aggregates function to seed new DMVs is too speculative in my opinion. Directionality cannot be inferred from the data presented. It is equally possible that excess dsRNA is shuttled out of DMVs into a storage compartment. And FISH experiments were not able to show that this dsRNA is viral RNA as there was no overlap of the signals. This may be a technical issue where the denaturation steps during FISH labelling were not sufficient to allow subsequent FISH probe annealing. However, it remains a possibility that these dsRNA accumulations represent another RNA source. Overall, an interesting observation but more data is needed to really suggest function for these accumulations.

Other findings:

Nsp2 localisation to Golgi later in infection. There are no controls here to show whether nsp2 and are still colocalised or whether also colocalises with giantin. Have other golgi markers been tested? Is giantin relocalised to ROs?

Study of the localisation of structural proteins is also included, although this doesn't fit so naturally with the key findings of the manuscript and few conclusions can be drawn. M localises to large ring-like structures, potentially representing assembled particles in transit to the surface. Higher magnification images showing the M ring (and association with S in a ring) would make it more convincing that these are assembled virus particles. Some of these larger vesicles are associated with LAMP1, which may be consistent with a lysosome linked exit strategy. However, this is only one option and there is still conflicting evidence about exit route. The majority of M doesn't colocalise with LAMP1 in the data presented here, even in the large vesicles. And markers for other potential exit routes have not been tested here. So confidently drawing conclusions is rather difficult.

Next, N localises to the outside of DMVs earlier in infection and is more distributed later in infection. N has been indicated to sit on the outside of the pore to encapsidate viral RNA as it is released from the pore with evidence of RNPs close to the exterior of the pore (10.1146/annurev-biochem-052521-115736). Viral RNAs then disperse into the cytoplasm, as would be predicted as the viral RNA moves to assembly sites, or to act as mRNA for new polyprotein production. In addition, N likely exists free from RNA as it is a multifunctional protein.

Finally S location is characterised using S2 and S1 antibodies. S2 was shown to localise to ER tubules and the nuclear envelope but not associated with S1. It's not clear what would have happened to the S1 from these S2 subunits. S2 antibodies have been used in the imaging of other coronaviruses without nuclear envelope localisation being reported. This might be discussed. Perhaps inclusion of another S2 antibody might provide additional confidence.

Methodology used is appropriate. Some findings would be solidified by the inclusion of electron microscopy or correlative light and electron microscopy, although this is really outside the scope of the work. Methods details are sufficient, with the exception of infection timings. From the methods, it appears that samples were fixed at 24 hpi while results discuss “early” and “late” infection. These are very subjective terms and actual timepoints should be given.

Reviewer #2

(Remarks to the Author)

The manuscript by Andronov et al describes a super-resolution spatial atlas of SARS-CoV-2 infection in human cells, focusing on the nanoscale organization of viral components. Using advanced SMLM techniques, they created a multi-color super-resolution atlas of viral RNAs and non-structural proteins (NSPs) to resolve the spatial context of polyprotein processing and replication organelle (RO) maturation, which were previously unclear. Key findings include a protease-gated

maturation model for double-membrane vesicles (DMVs), where the nsp5 protease acts inside the DMV lumen to complete polyprotein cleavage, and the discovery of dsRNA connectors linking DMVs to pore-free dsRNA granules. Furthermore, the study investigates the effect of the antiviral drug nirmatrelvir, demonstrating that it induces multi-layered polyprotein bodies by halting DMV maturation, which may explain the viral rebound observed after drug cessation.

Some specific questions:

1. Some dsRNA puncta have no/little viral proteins associated. Are these different maturation stages of the DMV? As a follow up, can you pseudo-time the DMV maturation based on different signals?
2. Is there any filtering applied to selected DMVs for particle averaging? Since the distances are not normalized how do you select DMVs imaged at equatorial plane?
3. None of the proteins seem to penetrate the DMV. Why do they all localize to the membrane?
4. SARS-CoV-2 also enters via endocytosis. Could the colocalization of M and LAMP-1 come from internalized viruses?
5. Please mention/indicate the time points used in the different experiments rather than early/late infection.
6. The missing colocalization between S1 and S2 is interesting and would suggest some intracellular shedding of S1 which would be a disadvantage for the virus as this could lead to inactivation of S. Please discuss.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all my comments and queries. The manuscript now places the work more accurately within the existing published work and references where there could be alternative interpretations of the data presented while still highlighting the significance of the findings. Overall, this is an impressive study that adds very valuable information to our understanding of coronavirus replication and was a thoroughly enjoyable read!

Reviewer #2

(Remarks to the Author)

The authors have addressed my points and clarified my concerns.

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Response to Reviewer #1 (Remarks to the Author):

For convenience, we show the referee comments in *italics*, and our replies in [blue](#). Textual changes made in the manuscript are shown in [red](#). Please note that Supplemental Figures S1, S2, S3, S7, S8, and S10 are all new in response to the reviewers.

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[We thank the reviewer for their careful evaluation of our manuscript, for the feedback and for the constructive suggestions, which have significantly strengthened this work. In the following, we address each item in turn.](#)

The authors present their finding of nsp5-16 locating within the DMV as a novel model. However, this does not set the findings as well as it might within the existing knowledge. Nsp5 and nsp3 have long been shown to colocalise (10.1128/JVI.02501-05) and nsp3, 5 and 8 have been shown using immunoEM to localise to CMs, but also to the interior of DMVs (10.1371/journal.pbio.0060226). Furthermore, since the characterisation of the DMV molecular pore, a model whereby the RTC is incorporated into the DMV during formation has been proposed and is a favoured model among those who have historically studied coronavirus replication organelles (10.1146/annurev-biochem-052521-115736).

We thank the reviewer for highlighting these important foundational and recent studies. Regarding the placement of our findings within existing knowledge, we completely agree that the localization of the RTC and viral RNA within the DMV lumen is supported by early immuno-EM work (e.g., Knoops et al., 2008) and recent super-resolution studies (Chen et al., 2024). We have updated the text to more prominently acknowledge this consensus.

Regarding the timeline of RTC incorporation and polyprotein cleavage, a review of the broader literature indicates that this sequence of events is currently treated as an emerging hypothesis rather than a firmly established consensus. As the reviewer rightly points out, excellent recent reviews like de Beijer et al. (2024) have begun to theorize this, but notably, they refer to it strictly as a “possible scenario” to explain how large NSPs reach the interior. Similarly, Chen et al. (2024) note it as “one possibility”, and the very recent work by Perry et al. (2025) proposes this sequence exclusively through computational modeling without experimental validation. In the broader literature, polyprotein cleavage is routinely depicted as occurring entirely in the cytoplasm. Therefore, while the field has recently begun to hypothesize this ‘gated maturation’ sequence out of structural necessity (because the pore is too small for protein transit), our study provides the much-needed nanoscale spatial evidence to confirm this possible scenario experimentally.

We have revised the Introduction, Results and Discussion to better acknowledge these recent hypotheses and foundational immuno-EM studies, citing all the literature recommended by the reviewer and positioning our work as an experimental confirmation of this emerging model.

In the Introduction:

While foundational immune-EM studies have long placed replication-transcription complexes (RTCs) within the DMV lumen{Knoops, 2008 #7031}, the mechanism by which these large proteins enter the sealed DMV has remained highly elusive{de Beijer, 2024 #7931}. It has been recently hypothesized that RTCs might be incorporated during DMV formation{de Beijer, 2024 #7931;Chen, 2024 #7856;Perry, 2025 #7907}, yet studies still conflict on the precise architecture, debating whether specific components reside inside

the lumen{Andronov, 2024 #7854;Chen, 2024 #7856} or outside on the cytoplasmic face{Yang, 2024 #7855}.

In the “Nsp5 and replicase NSPs reside intraluminally and align with DMV pores for protease-gated DMV maturation” section of the Results:

In related coronaviruses, nsp5 has long been shown to colocalize with nsp3{Snijder, 2006 #7057}, to localize at convoluted membranes{Knoops, 2008 #7031}, and to be protected by membranous structures{van Hemert, 2008 #7861;Knoops, 2008 #7031}.

In the “Nanoscale localization of NSPs sheds light on DMV formation and maturation” section of the Discussion:

While schematic models routinely depict the processing of pp1a/pp1ab by PLpro and 3CLpro occurring entirely in the cytoplasm{Hartenian, 2020 #7032;Malone, 2022 #7862;Steiner, 2024 #7880;Marano, 2024 #7886}, much evidence places vgRNA replication and the mature RTCs inside the DMVs{Andronov, 2024 #7854;Doyle, 2021 #7683;Chen, 2024 #7856;Snijder, 2020 #7078}. Because the central channel of the DMV pore (width < 20 Å{Huang, 2024 #7853}) is too narrow for large, folded proteins to traverse, it has recently been hypothesized that the large coronaviral NSPs must be enclosed at the time of DMV formation{de Beijer, 2024 #7931;Chen, 2024 #7856}.

In addition, evidence has already been published in an analogous study using alphacoronavirus 229E that nsp7, 8 and 9 are localised to the interior of the DMV with nsp4 further out and nsp3 on the exterior (10.1126/sciadv.adq9580). Evidence of density sitting under the DMV pores seen using cryoET has also led to the model that the RTC is associated with and sits under the pore in the DMV lumen (10.1126/science.abd3629, 10.1126/sciadv.adq9580). Therefore, the work presented here supports and is completely consistent with existing data and models and should recognise this better.

We thank the reviewer for highlighting these excellent structural and imaging studies. We agree that our data supports and complements the recent super-resolution mapping performed in the alphacoronavirus HCoV-229E by Chen et al., as well as existing cryo-ET models (such as Wolff et al.).

Moreover, our study significantly advances beyond the prior super-resolution studies (Chen et al, 2024) in several key ways. First, we provide a comprehensive map of the highly pathogenic betacoronavirus SARS-CoV-2, rather than the alphacoronavirus 229E. Second, our multiplexed dSTORM approach achieves higher spatial resolution (~15–20 nm) compared to the STED/SIM techniques used by Chen et al., allowing us to map nearly all NSPs with respect to both dsRNA and nsp4. Finally, we perform not only radial but also angular pair-correlation analysis. This allows us to map the precise localization of NSPs

circumferentially with respect to the nsp3/nsp4-labeled pore complex, providing a more detailed and comprehensive mapping of the DMV architecture.

To ensure these distinctions and connections are clear, we have expanded our text in both the Results and Discussion sections to better integrate and compare our work with these specific studies.

In the “Nsp5 and replicase NSPs reside intraluminally and align with DMV pores for protease-gated DMV maturation” section of the Results:

Previous studies, including our earlier work{Andronov, 2024 #7854} and recent analogous super-resolution mapping in the alphacoronavirus HCoV-229E{Chen, 2024 #7856}, have localized the RdRp catalytic subunit nsp12 and the accessory domains nsp7, nsp8, and nsp9 to the DMV lumen. Furthermore, cryo-electron tomography (cryo-ET) studies have observed unassigned luminal densities positioned directly beneath the DMV pore{Wolff, 2020 #7069}. However, high-resolution spatial data providing the molecular identities for the remaining non-structural proteins have been lacking.

In the “Nanoscale localization of NSPs sheds light on DMV formation and maturation” section of the Discussion:

By leveraging the high spatial resolution of dSTORM (~15–20 nm), our study expands upon recent STED/SIM observations in the alphacoronavirus HCoV-229E{Chen, 2024 #7856} to encompass the entire SARS-CoV-2 replicase. Furthermore, by incorporating not only radial but also angular pair-correlation analysis with respect to the pore complex, our data provide direct molecular confirmation for the unassigned RTC densities observed beneath the pore in cryo-ET data{Wolff, 2020 #7069}, offering a comprehensive, detailed map of the mature replication organelle.

The second finding presented is the requirement for nsp5 protease function in formation of DMVs. When nsp5 function was inhibited using nirmatrelvir, DMV formation was limited, and instead concentric rings of likely paired membranes were formed (EM would be needed to confirm this result). However, preventing cleavage of the nsp3-4 junction would also give a comparable phenotype so confirmation that 3-4 cleavage is occurring would be required to confidently draw this conclusion. In addition, this section leads to some internal inconsistency with the conclusions. The authors propose that nsp5 cleavage of the polyprotein occurs after biogenesis of the DMV, once it has sealed (lines 208-210). However, the data presented in this section (assuming 3-4 is cleaved) demonstrates that inhibition of nsp5 protease function prevents/limits DMV formation. DMV formation by the nsp3-4 self-cleaving expression cassette appears to be very rapid as formation intermediates are not readily visualised (consistent with observations in virus infected cells). Therefore, it seems likely that nsp5 cleavage of the polyprotein occurs before, during and after DMV formation.

How much role nsp5 plays in truly “gating” DMV formation is not clear. Additional scenarios would need to be assessed to provide a detailed understanding of the step-wise process. And this would also require inclusion of electron microscopy imaging. However, once 3-4 cleavage is confirmed, the results presented here are an interesting and useful development in our understanding of the process of DMV formation.

We thank the reviewer for this highly insightful critique. The reviewer raises an excellent point: differentiating between an uncleaved nsp3–nsp4 junction and an uncleaved nsp4–nsp16 block is critical to validating our models of DMV and MLB formation.

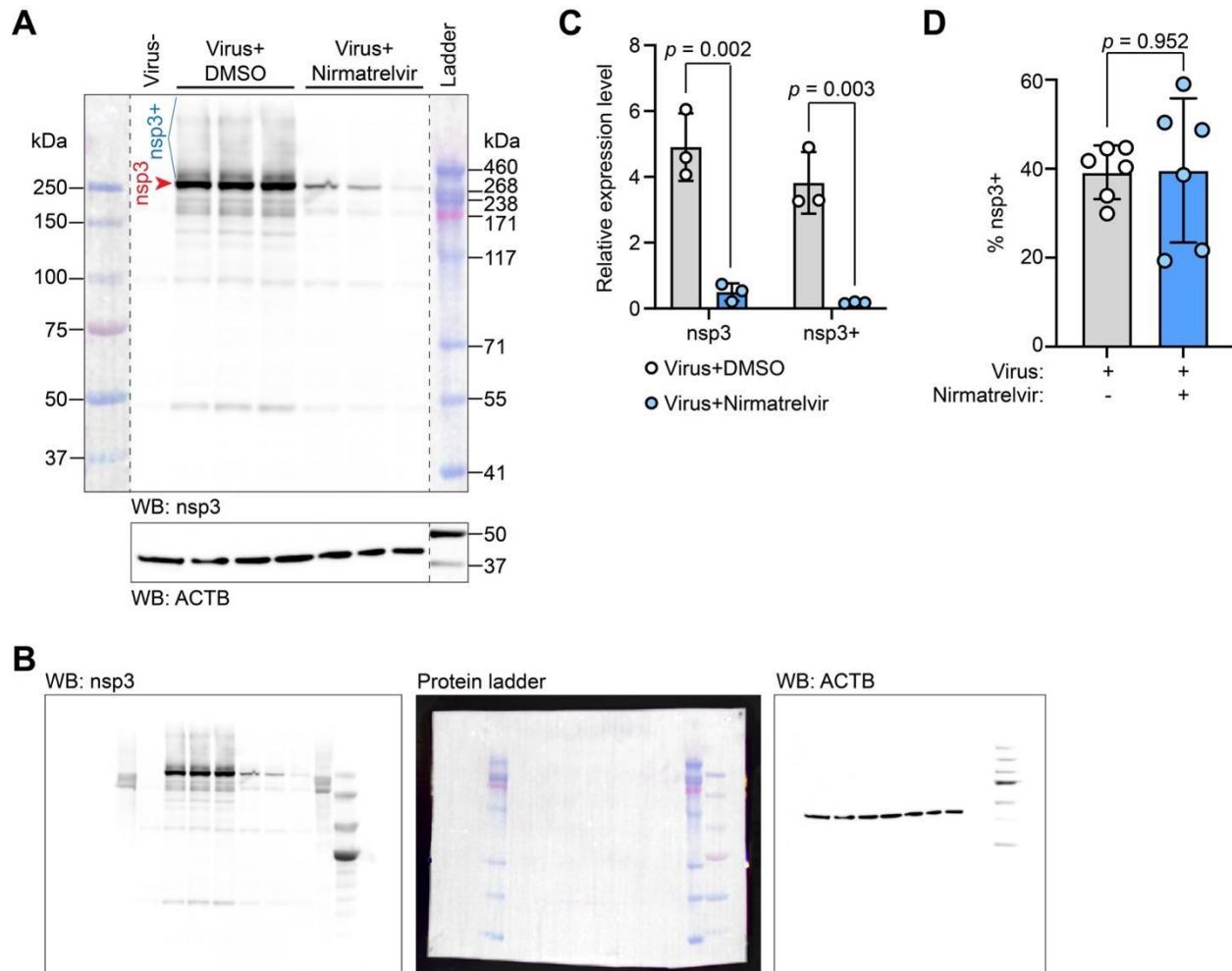
Confirmation of nsp3–nsp4 cleavage:

To directly address this concern, we performed Western blot studies on lysates from SARS-CoV-2-infected cells treated with nirmatrelvir or DMSO, probed with an anti-nsp3 antibody (Supplementary Fig. 10). If the nsp3–nsp4 junction was successfully cleaved, we would expect the appearance of a nsp3 band of 217.3 kDa. If the nsp3–nsp4 junction remained uncleaved, we would expect the appearance of high-molecular-weight bands above the nsp3 band, which correspond to an nsp3–nsp4 fusion (273.5 kDa) and potentially up to nsp3-nsp11/nsp16 polyproteins. To validate our method for detecting such large proteins, we used an extended overnight membrane transfer and two high-range protein ladders. The successful transfer of the 460 kDa ladder marker confirmed that our Western blot could effectively detect proteins in the expected size range of nsp3–nsp4 fusion and larger (Supplementary Fig. 10A-B).

The nsp3 protein band (Supplementary Fig. 10A, red arrow) was successfully detected in both Virus+DMSO and Virus+Nirmatrelvir groups, and its specificity was confirmed by its absence in the Virus- control group. The expression level of nsp3 protein was decreased by almost ten-fold after nirmatrelvir treatment (Supplementary Fig. 10C), suggesting potent inhibition of the virus by nirmatrelvir. We also detected signals above the nsp3 protein band (“nsp3+”, blue bracket) in the Virus+DMSO groups (Supplementary Fig. 10A), indicating that uncleaved nsp3 precursors are present in SARS-CoV-2 infected cells. We quantified the intensity of the nsp3 band (Supplementary Fig. 10A, red arrow) and the intensity of nsp+ bands, to calculate the percentage of nsp3+ proteins relative to total nsp3 proteins. We found that the expression level of nsp3+ proteins was also significantly decreased after nirmatrelvir treatment (Supplementary Fig. 10C). Nevertheless, the percentage of nsp3+ protein did not significantly change between Virus+DMSO and Virus+Nirmatrelvir groups (Supplementary Fig. 10D), indicating that nirmatrelvir treatment has no significant influence on the cleavage between nsp3-nsp4.

Mechanistically, the nsp3–nsp4 junction is cleaved by the viral papain-like protease (PL^{pro}, located within nsp3) rather than M^{pro}. Our results confirm that nsp3 is released from nsp4 regardless of whether nsp5 is inhibited by nirmatrelvir or not.

We attempted to perform Western blot studies by staining nsp4. However, despite our nsp4 antibody working well for SR imaging, it did not show confident staining on Western blot. We further tested commercial nsp4 antibodies (Novus Biologicals, NBP3-15991-100ul; Cat no. DA239), which failed to produce a specific signal in Western blot assays.



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.

Refined mechanism of DMV biogenesis and MLB formation:

With nsp3–nsp4 cleavage confirmed, we now construct a more refined model of DMV encapsulation and MLB formation that aligns with both our observations and established literature:

1. Encapsulation: It has recently been shown that mature, individually expressed NSPs cannot penetrate the narrow DMV pores to enter pre-formed DMVs (10.1083/jcb.202306101). Therefore, our observation that nsp5–nsp16 reside within the mature DMV lumen (Fig. 3I) implies they are co-enclosed during biogenesis. Because these proteins are translated as a polyprotein at the ER, they remain physically tethered to the invaginating membrane (via the transmembrane domains of nsp4 and nsp6) and are encapsulated as the membrane curves and seals.

2. MLB formation (nirmatrelvir): Our Western blot confirms that under nirmatrelvir treatment, nsp3 and nsp4 separate. This allows their ectodomains to interact across the ER lumen, which is known to drive membrane zippering. However, because M^{pro} is inhibited, the downstream transmembrane domains of nsp4 and nsp6 remain covalently tethered within the massive nsp4–16 polyprotein block. Previous studies have demonstrated that when the nsp4–nsp5–nsp6 polyprotein cannot be efficiently cleaved, normal DMVs fail to form (10.1128/mbio.01658-17). We therefore propose that this uncleaved nsp4–16 block restricts membrane flexibility. While the cleaved nsp3 and nsp4 initiate zippering, the steric constraint of the uncleaved downstream block prevents the membranes from curving into spherical vesicles. Consequently, the membranes form the stacked, relatively flat zippered sheets (MLBs) we observe in our super-resolution data.

We agree with the reviewer that polyprotein cleavage is likely an ongoing, dynamic process. M^{pro} cleavage is not strictly a single "gate" that opens only after closure; rather, the successful cleavage of the nsp4–6 region by M^{pro} is likely a continuous structural prerequisite that allows the membranes to achieve the curvature necessary for DMV closure. We have revised the manuscript to reflect this more grounded model, and we acknowledge that confirming the precise membrane topology of these intermediate MLBs is a vital next step for correlative light and electron microscopy (CLEM), which is a relatively challenging technical experiment outside the scope of this paper.

In the “Nsp5 and replicase NSPs reside intraluminally and align with DMV pores for protease-gated DMV maturation” section of the Results:

Because nsp5 is the main coronaviral protease, indispensable for viral polyprotein processing and for viral replication, its intraluminal localization provides direct spatial evidence for an emerging model of polyprotein processing coupled to DMV formation. We propose that the nsp5–nsp16 polyprotein block is co-enclosed during DMV biogenesis. In this scenario, partially uncleaved pp1a/pp1ab precursors are engulfed as the membrane seals, allowing nsp5 to subsequently complete the polyprotein processing within the protected DMV lumen.

In the “Protease inhibition by nirmatrelvir generates multilayer polyprotein bodies that persist and precede rebound” section of the Results:

Considering that preventing cleavage of the nsp3–nsp4 junction could also lead to a comparable phenotype of limited DMV formation observed under nirmatrelvir treatment, we next performed Western blot studies to determine whether nsp3–nsp4 cleavage occurs under nirmatrelvir, which is critical to validating our models of DMV and MLB formation. We probed nsp3 using an anti-nsp3 antibody in Western blot analysis of lysates from SARS-CoV-2-infected cells treated with nirmatrelvir or DMSO. Specific detection of nsp3 was confirmed, by the absence of its signal in the uninfected control group (Supplementary Fig. 10A-B, Virus-). The expression level of nsp3 was significantly decreased after nirmatrelvir treatment (Supplementary Fig. 10C), while the proportion of uncleaved nsp3 (nsp3+) did not significantly change between DMSO and nirmatrelvir treated groups (Supplementary Fig. 10D), indicating that nirmatrelvir treatment has no significant influence on the cleavage between nsp3 and other proteins, including nsp4. Since the nsp3–nsp4 junction is cleaved by the viral papain-like protease (PL^{pro}, located within nsp3) rather than M^{pro}, this confirms that nsp3 is released from nsp4 even when nsp5 is fully inhibited by nirmatrelvir.

In the “Nanoscale localization of NSPs sheds light on DMV formation and maturation” section of the Discussion:

We propose that polyprotein processing is dynamically coupled to DMV biogenesis. In this model, nsp3–nsp4 interactions initiate membrane bending while nsp4 remains spatially coupled to the partially uncleaved nsp5–nsp11/16 block within pp1a/pp1ab (Fig. 7B). This coupling during DMV biogenesis is likely essential for luminal delivery, as it has been shown that when mature replicase NSPs are expressed individually—outside the context of the polyprotein—they completely fail to translocate into pre-formed DMVs{Yang, 2024 #7855}. Recent computational modeling independently supports a similar sequence in which pore formation precedes nsp5-mediated cleavage and traps the replicase inside the DMV{Perry, 2025 #7907}. Once initial cleavages relieve steric constraints to permit membrane closure, a subsequent trigger (e.g. the increased concentration of NSPs, which might favor the nsp5 dimerization necessary to increase its activity{Ferreira, 2022 #7892}) would stimulate 3CL^{pro} to finish DMV maturation.

In Fig. 7: Summary of findings from the super-resolution spatial Atlas of SARS-CoV-2 infection, (B) Proposed model of DMV formation, polyprotein processing, and disruption by nirmatrelvir:

(1) The process begins with vgRNA translation into polyproteins pp1a/pp1ab on the endoplasmic reticulum (ER) membrane. The polyprotein is anchored by the transmembrane domains of nsp3, nsp4 and nsp6. Nsp1 is cleaved by the PLpro domain of nsp3 shortly after translation, while Mpro (nsp5) possibly auto-cleaves and begins processing the C-terminal domains (nsp5–11/16).

(2) This early cleavage allows the cis-interaction of the nsp3 and nsp4 ectodomains, leading to membrane bending, while nsp5–11/16 stays attached to the nsp4 C-terminus via non-covalent and remaining covalent bonds.

(3) This is followed by pore formation, which partitions nsp2 and nsp3 to the cytosolic face of the pore, while the maturing nsp5–11/16 proteins are compartmentalized on the opposite side of the growing double membrane.

(4) The DMV membrane closes, encapsulating vgRNA; the binding of vgRNA to the nsp7–nsp11/16 complexes ensures vgRNA localization within the DMV interior.

(5) Within the closed DMV, optimal local concentrations of nsp5 complete the cleavage of pp1a/pp1ab to produce a mature RO.

In the Future perspectives:

Methodologically, as shown previously{Dahlberg, 2020 #7447}, correlative fluorescence–cryo-EM (CLEM) will be essential to add molecular specificity to high-resolution volumes. This approach is required to structurally confirm whether the dsRNA connectors are enveloped by the membranes of host ER tubules, and to validate the zippered membrane architecture of the nirmatrelvir-induced MLBs.

The third finding presented is the existence of dsRNA connectors between DMVs as well as the observation of dsRNA aggregations lacking association with nsp3-4. These connectors are novel and, to my knowledge, have not been reported before. The use of both high-resolution imaging and 3D imaging may have helped in the identification of these structures. Although earlier super-resolution imaging of other CoV infected cells has not identified these connectors and it would be beneficial to discuss this difference. The authors propose that the connectors may represent the presence of dsRNA in the membrane connections often seen between DMVs in SARS-CoV-2 infected cells. However, these are connections of the

DMV outer membrane so it is not immediately obvious how dsRNA would cross into this space. The DMV pore would deliver RNA to the cytoplasm and there is no indication yet of a pore from the DMV interior to the intra-membrane space. It has been shown that dsRNA present in cells infected with SARS-CoV-2 (and other CoVs) is located within a membrane protected environment (10.3390/v13122540), so non-membrane associated dsRNA free in the cytoplasm seems not to be a likely model (although linkers were not detected in this study). The presence of dsRNA aggregates not associated with nsp3-4 is also a new finding. The authors propose that these are membrane-less aggregates of dsRNA that might act to seed new DMVs. However, no data is presented showing whether these are membrane associated or not so this conclusion cannot be drawn. And absence of nsp3-4 does not mean there is no membrane. Previous data about the location of dsRNA in CoV infected cells indicates that it is associated with membranes via either fluorescence or electron microscopy (10.1371/journal.pbio.0060226; 10.3390/v13122540). Furthermore, the suggestion that these aggregates function to seed new DMVs is too speculative in my opinion. Directionality cannot be inferred from the data presented. It is equally possible that excess dsRNA is shuttled out of DMVs into a storage compartment. And FISH experiments were not able to show that this dsRNA is viral RNA as there was no overlap of the signals. This may be a technical issue where the denaturation steps during FISH labelling were not sufficient to allow subsequent FISH probe annealing. However, it remains a possibility that these dsRNA accumulations represent another RNA source. Overall, an interesting observation but more data is needed to really suggest function for these accumulations.

We are highly grateful to the reviewer for this thorough and constructive critique. The reviewer raises excellent points regarding membrane topology, directionality, and technical caveats that have significantly improved our interpretation of these novel dsRNA structures.

Regarding why previous studies missed the connectors: We attribute the novel identification of these structures to the higher resolution of our dSTORM approach combined with optimized labeling. Because these connectors are very thin (~15–20 nm) and faint compared to the massive dsRNA density within the DMV core, they are easily obscured by background or might be missed by thresholding in lower-resolution techniques (like STED/SIM), especially if the labeling density was suboptimal.

Regarding membrane protection and the "membrane-less" claim: The reviewer is completely correct to point out that viral dsRNA is typically shielded in membrane-protected environments, and that the absence of the nsp3/nsp4 pore complex does not equate to the absence of a lipid bilayer. We have removed the term "membrane-less" from the manuscript (referring to the massive granules now as "nsp3/nsp4-lacking" or "non-canonical"). Regarding the dsRNA connectors, because they have not been observed in previous correlative or DL studies, their exact membrane topology remains entirely unknown. To reconcile our observation of these connectors with the established protection of dsRNA, we hypothesize in the text that they might be enveloped within the lumen of host-derived ER

tubules known to interconnect DMVs, though we note this requires future structural confirmation.

Regarding the "seeding" hypothesis vs. storage: We fully concede that suggesting these aggregates definitively act to "seed" new DMVs was too speculative, as we cannot strictly infer directionality from fixed-cell data. We have integrated the reviewer's alternative hypothesis: these large, nsp3/nsp4-lacking dsRNA accumulations could equally represent specialized compartments for the shuttling, storage, or eventual degradation of excess viral RNA.

Regarding the RNA source and FISH limitations: The reviewer makes an excellent point about the technical limitations of RNA FISH, which we completely agree with: standard denaturation steps are frequently insufficient to allow FISH probes to anneal to highly stable, protected viral dsRNA duplexes. Because of this, proving the origin of the RNA via hybridization is challenging. However, we argue against a cellular/host RNA source based on three converging observations: (1) we do not observe comparable bright dsRNA clusters in non-infected cells, making a massive, infection-induced burst of endogenous dsRNA highly unlikely; (2) the granules are specifically decorated with viral replicase components (nsp5, 8, 9, 13, 16); (3) as shown in our data (Fig. 4F), these granules are occasionally physically tethered directly to canonical DMVs (which are known viral replication sites) via the dsRNA connectors.

The manuscript has been updated accordingly. In the abstract:

[...]Second, we identify dsRNA connectors bridging DMVs and **non-canonical, nsp3/4-lacking** dsRNA granules decorated with replicase components[...]

In the Discussion, section "Cytoplasmic dsRNA granules and inter-DMV connectors route replication intermediates":

We also observe thin, linear dsRNA 'connectors' that bridge neighboring DMVs and sometimes tether DMV clusters to these non-canonical dsRNA granules (Fig. 4C–G, Supplementary Fig. 4A, Supplementary Fig. 5A–C). To our knowledge, these inter-organellar bridges have not been previously described for coronavirus infection. The visualization of these faint conduits was likely enabled by the high spatial resolution and optimized labeling density of our dSTORM approach, as such thin structures (~15–20 nm) can easily be obscured by background or might be missed by thresholding compared to the extremely bright dsRNA cores in lower-resolution studies. The exact membrane topology of these connectors and granules is currently unknown. Since the massive dsRNA granules lack the canonical nsp3/nsp4 pore complex, their exact biogenesis is unclear; however, the absence of nsp3/nsp4 does not preclude the presence of a protective lipid bilayer. Considering that viral dsRNA is typically shielded from host sensors in membrane-protected environments, one compelling hypothesis is that these cytoplasmic connectors are not 'naked', but rather

enveloped within the lumen of the host-derived ER tubules known to interconnect DMVs{Wolff, 2020 #7069}.

Functionally, while these interconnected networks might serve as conduits for RNA traffic to seed new replication sites, their directionality cannot be inferred from our fixed-cell data. Alternatively, rather than active replication centers, these non-canonical dsRNA accumulations may represent specialized compartments for the shuttling, storage, or sequestration of excess viral dsRNA away from the primary replication sites. Recognizing that standard FISH denaturation steps are often insufficient to melt highly stable, protected dsRNA duplexes for probe annealing, definitively proving the origin of the RNA within these dense granules via hybridization is technically challenging. However, a viral origin is highly probable because: (i) comparable massive dsRNA accumulations are absent in non-infected cells, making an infection-induced burst of host dsRNA unlikely; (ii) they are specifically decorated with multiple viral replicase proteins; and (iii) they are occasionally physically linked to canonical, replication-competent DMVs via the dsRNA connectors (Fig. 4F). Defining the precise membrane topology and molecular composition of the connectors, alongside the material properties of these viroplasm-like granules, remains a key direction for future work.

In the Future perspectives:

[...] Equally critical is defining the precise membrane topology of the novel inter-DMV connectors and nsp3/nsp4-lacking dsRNA granules identified here. Future studies must determine whether these massive accumulations represent active hubs for seeding new DMVs or specialized compartments for viral RNA storage and sequestration.

Methodologically, as shown previously{Dahlberg, 2020 #7447}, correlative fluorescence–cryo-EM (CLEM) will be essential to add molecular specificity to high-resolution volumes. This approach is required to structurally confirm whether the dsRNA connectors are enveloped by the membranes of host ER tubules, and to validate the zippered membrane architecture of the nirmatrelvir-induced MLBs.

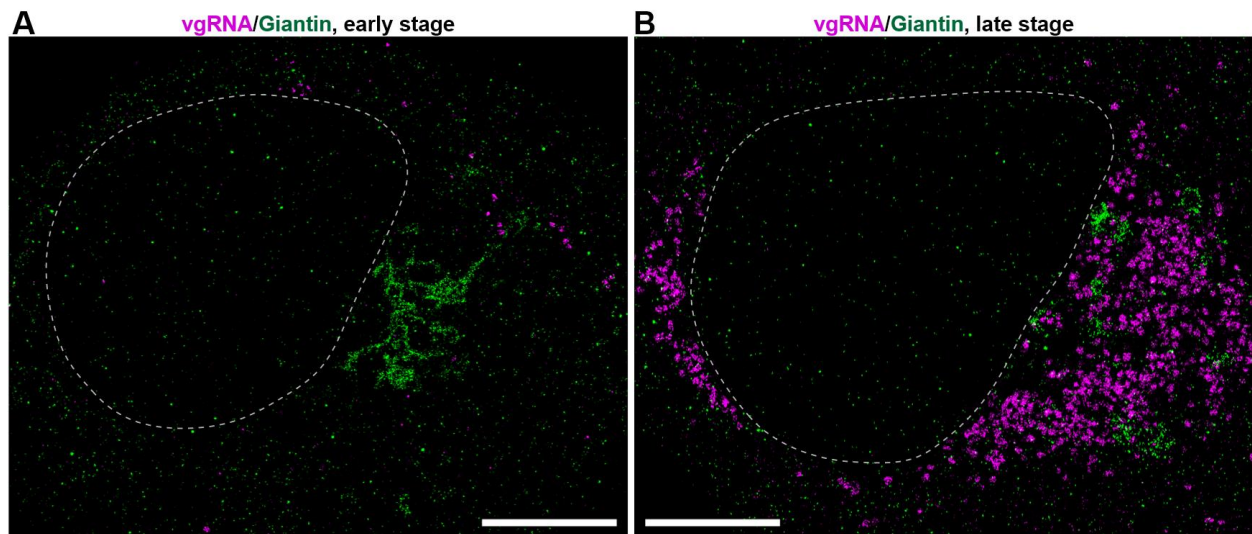
Future work should move beyond static snapshots to live-cell, single-molecule tracking. Resolving these dynamic trajectories will clarify the exact spatiotemporal sequence of polyprotein cleavage during DMV biogenesis, establish the directionality of RNA transport [...]

Other findings:

Nsp2 localisation to Golgi later in infection. There are no controls here to show whether nsp2 and 3 are still colocalised or whether 3 also colocalises with giantin. Have other golgi markers been tested? Is giantin relocalised to ROs?

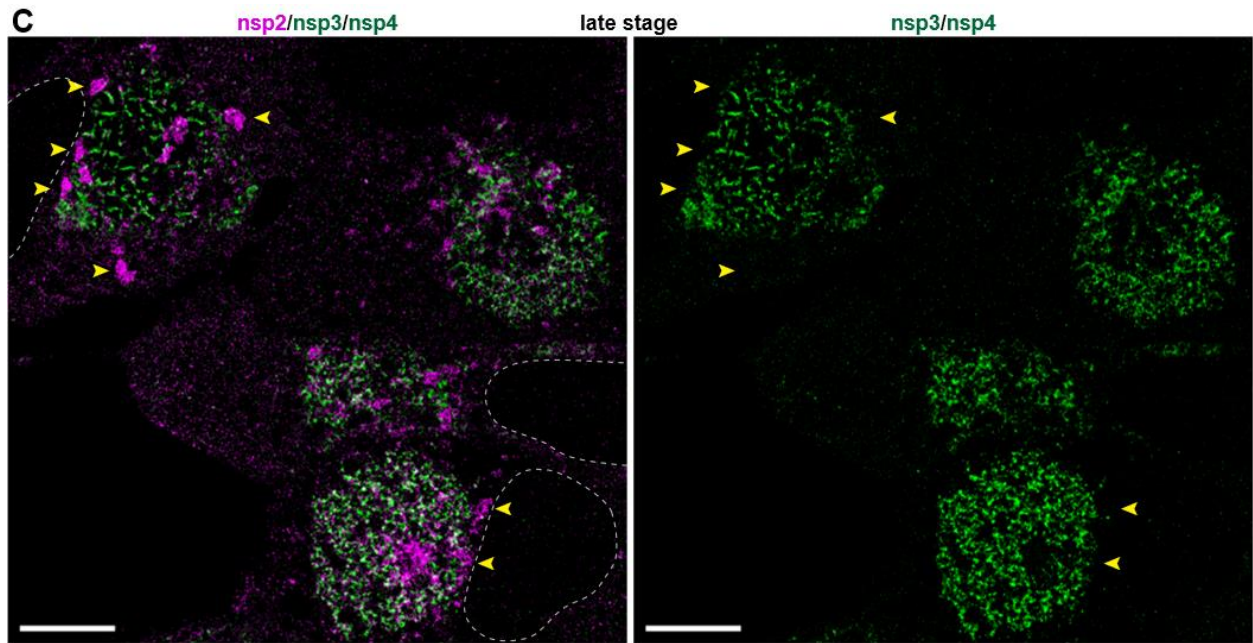
We thank the reviewer for these important questions regarding the spatial segregation of viral components and the integrity of the Golgi apparatus.

To directly address whether the Golgi matrix protein Giantin is recruited to replication organelles (ROs), we performed co-imaging of Giantin with vgRNA (which we established as a robust RO/DMV marker in our previous work, 10.1038/s41467-024-48991-x). As shown in the new Supplementary Fig. 2A-B (below), while the Golgi apparatus undergoes significant remodeling (fragmentation) during late-stage infection, the Giantin signal remains spatially distinct from the vgRNA clusters (ROs). This suggests that Giantin is not recruited to ROs.



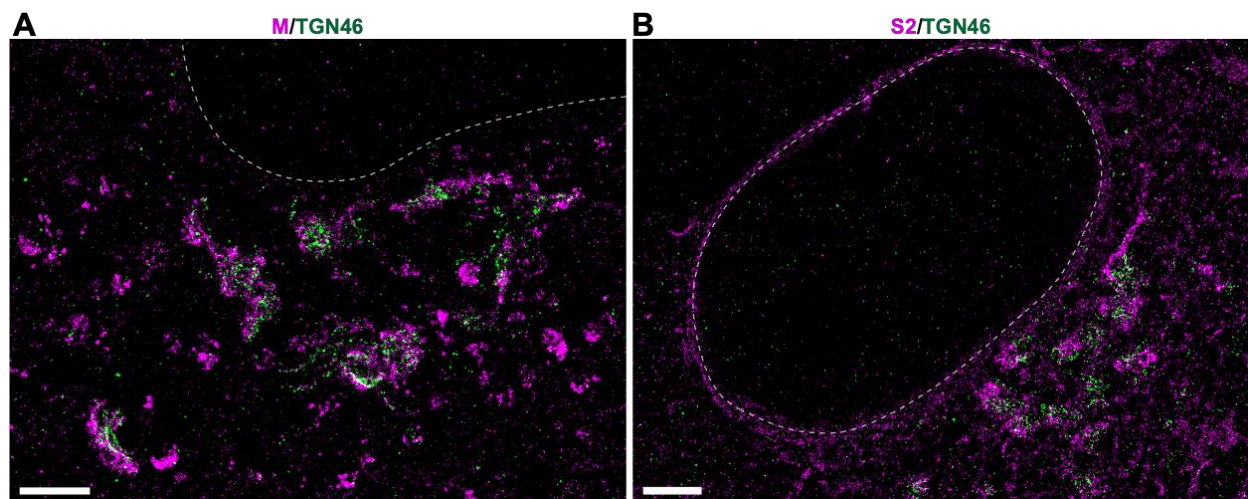
Supplementary Fig. 2: The Golgi marker Giantin remains spatially distinct from replication organelles, and nsp2 segregates from nsp3/nsp4 during Golgi remodeling. **(A)** SR image of vgRNA (magenta) and Giantin (green) in the early morphological stage indicates that the Golgi apparatus retains its typical 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. The white dashed curves approximate the position of the nuclear edge. Scale bars, 5 μ m.

To further investigate nsp2 relocalization in late infection, we co-stained nsp2 with a mixture of antibodies against nsp3 and nsp4. As shown in the new Supplementary Fig. 2C (below) the nsp2 signal (magenta & yellow arrowheads) in late-stage infection is spatially segregated from the nsp3/nsp4-positive RO clusters (green). This provides direct experimental evidence that nsp2 dissociates from the core replication complex and, unlike nsp3/nsp4, relocalizes to the fragmented Golgi (an example image of nsp2 with the Giantin-labeled Golgi is available in Fig. 2D).



Supplementary Fig. 2: (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.

As suggested by the reviewer, we extended our analysis to the trans-Golgi network (TGN) using the marker TGN46. We observed that TGN46, like Giantin, displays a fragmented phenotype in late infection. Furthermore, we found that the Membrane (M) and Spike (S2) proteins are often colocalized with or adjacent to TGN46, in line with their trafficking via the TGN, as shown in the new Supplementary Fig. 7 (below)



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 μ m.

The Results section of the manuscript has been updated to include in the section “Ribosome-bound nsp1 is excluded from DMVs, and nsp2 shifts from DMV/CMs to fragmented Golgi during infection”:

While the Golgi apparatus retains its typical interconnected ribbon morphology in early infection, it undergoes significant fragmentation in the late stage, consistent with previous reports of SARS-CoV-2–driven remodeling{Cortese, 2020 #7609;Zhang, 2025 #7878} (Supplementary Fig. 2A, B). We confirmed that the Golgi matrix protein Giantin remains spatially distinct from ROs throughout this process (Supplementary Fig. 2A, B). Strikingly, however, nsp2 relocates to these fragmented Golgi structures (Fig. 2D). This specific Golgi association is, to our knowledge, novel and suggests evolving host-interaction roles for nsp2 during organelle remodeling.

To confirm that this Golgi-localized nsp2 population undergoes spatial segregation from the main replication complex, we co-imaged nsp2 with the established DMV markers nsp3 and nsp4. As expected, in late-stage infection, nsp2 is distinctly segregated from the nsp3/nsp4-positive RO regions (Supplementary Fig. 2C), further supporting its unique role away from the sites of RNA synthesis.

In the section “M protein maps the secretory–lysosomal route of SARS-CoV-2 assembly”:

Super-resolution imaging reveals that the cis/medial-Golgi marker Giantin forms a peripheral rim around these M clusters, suggesting accumulation within the cisternae. To map the exit route, we co-imaged the trans-Golgi network (TGN) marker TGN46. Unlike the encapsulation seen with Giantin, M aggregates appear closely juxtaposed to or partially overlapping with TGN46-positive structures (Supplementary Fig. 7A), consistent with the protein traversing the TGN for sorting and packaging.

And in the section “Spike subunits segregate after furin cleavage and N–vgRNA RNPs form outside DMVs”:

Consistent with M protein trafficking, S2 clusters are encapsulated by Giantin but localize adjacent to TGN46-positive structures (Supplementary Fig. 7B), indicating coordinated transit through the secretory pathway.

Study of the localisation of structural proteins is also included, although this doesn't fit so naturally with the key findings of the manuscript and few conclusions can be drawn. M localises to large ring-like structures, potentially representing assembled particles in transit to the surface. Higher magnification images showing the M ring (and association with S in a ring) would make it more convincing that these are assembled virus particles.

Given that structural proteins are essential for viral assembly and egress, determining their nanoscale spatial organization is critical for building a truly comprehensive atlas of the SARS-CoV-2 infection cycle. We have now included a brief transition in the Results section to clarify the rationale for shifting from NSPs to structural proteins:

Following the synthesis and spatial routing of vgRNA at the replication organelles, the viral life cycle shifts to packaging and egress. While this phase is governed by four main structural proteins—spike (S), membrane (M), envelope (E), and nucleocapsid (N){Jahirul Islam, 2023 #7890;Chung, 2024 #7888}—we focused our investigation on the nanoscale localization of the S, M, and N proteins to complete our spatial atlas.

One interesting discovery is that, in the peripheral cytoplasm, M proteins form virion-sized particles which aggregate into large hollow clusters (Fig. 5A, red square), referred to as M rings. We hypothesized that these virion-sized particles formed by M proteins represent assembled virions. We appreciate the reviewer's suggestion to test this hypothesis by examining the association of these "M rings" with S proteins at higher magnification.

Indeed, co-labeling of the M protein and the Spike S1 subunit revealed the co-assembly of both proteins into virion-sized particles within these hollow M clusters (Fig. 5E, top panel). While the dense packing of these particles within the clusters limits our ability to perfectly resolve individual virion morphologies using super-resolution microscopy, the co-assembly of M and S1 strongly supports our hypothesis. We have revised our conclusion to state that these nanoscale particles represent either fully assembled virions or late-stage intermediates undergoing assembly, in the "M protein maps the secretory–lysosomal route of SARS-CoV-2 assembly" section of the Results:

To assess whether these M-positive aggregates correspond to assembled virus particles, we colabeled the M protein alongside the spike S1 subunit. This revealed the co-assembly of both proteins into virion-sized particles that pack into large, hollow clusters (Fig. 5E). Our findings suggest that these nanoscale particles represent either fully assembled virions or late-stage intermediates undergoing assembly.

Some of these larger vesicles are associated with LAMP1, which may be consistent with a lysosome linked exit strategy. However, this is only one option and there is still conflicting evidence about exit route. The majority of M doesn't colocalise with LAMP1 in the data

presented here, even in the large vesicles. And markers for other potential exit routes have not been tested here. So confidently drawing conclusions is rather difficult.

We agree with the reviewer that it is premature to definitively conclude that the LAMP1-associated hollow M clusters represent a lysosome-linked egress pathway. These structures could represent either lysosomal egress or the assembly of virions within lysosomes. Furthermore, as the reviewer notes, there is conflicting evidence in the literature regarding lysosomal egress (PMID: 33157038, 34440816, 40540531). For example, a transmission electron microscopy (TEM) study of SARS-CoV-2 virus in Vero cells reveals small secretory vesicles are the principal means of SARS-CoV-2 egress (PMID: 34440816). Without investigating additional egress markers, we cannot definitively assign these LAMP1-associated clusters to the egress pathway. Therefore, we have removed the claim that large hollow M clusters are explicitly consistent with pre-egress virion assemblies, adjusting the text in the “M protein maps the secretory–lysosomal route of SARS-CoV-2 assembly” section of Results as follows: (iii) peripheral cytoplasm M proteins form virion-sized particles that aggregate into large hollow clusters (Fig. 5A, red square);

and in the “Structural protein localization patterns map virion assembly and egress” section of Discussion:

SR imaging of the spike protein suggests that these structures likely represent active sites of massive virion production, storage or transport.

Next, N localises to the outside of DMVs earlier in infection and is more distributed later in infection. N has been indicated to sit on the outside of the pore to encapsidate viral RNA as it is released from the pore with evidence of RNPs close to the exterior of the pore (10.1146/annurev-biochem-052521-115736). Viral RNAs then disperse into the cytoplasm, as would be predicted as the viral RNA moves to assembly sites, or to act as mRNA for new polyprotein production. In addition, N likely exists free from RNA as it is a multifunctional protein.

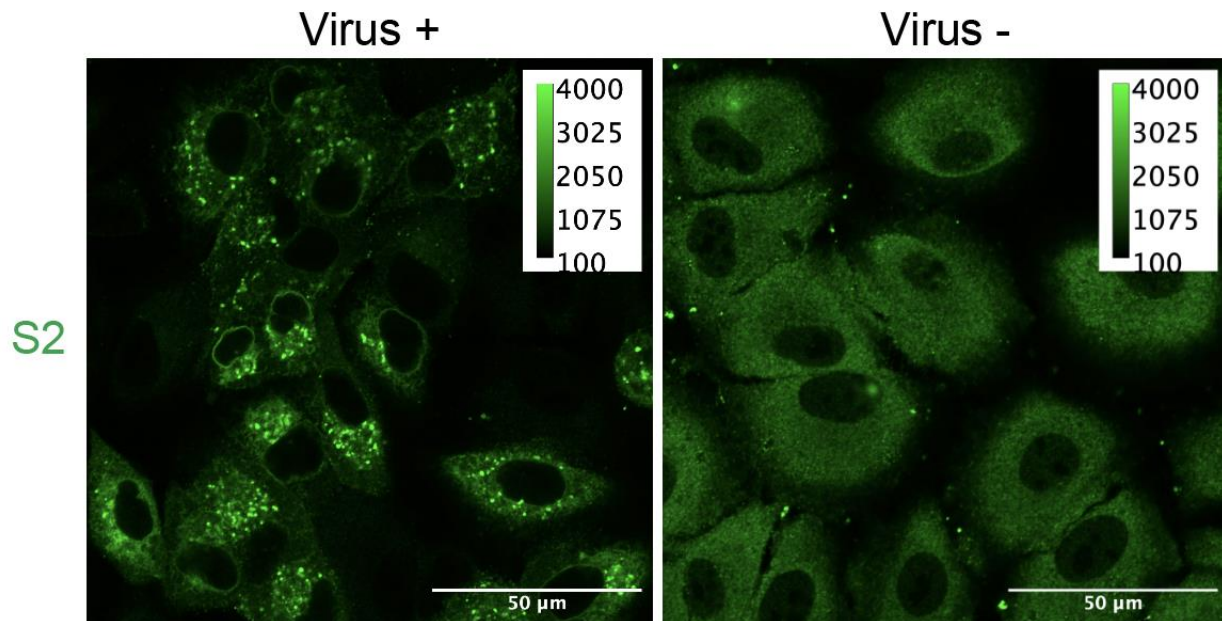
We thank the reviewer for directing us to this review paper, which summarizes cryo-ET observations of N proteins associated with the cytoplasmic face of DMV pores (<https://scholarlypublications.universiteitleiden.nl/handle/1887/3421526>). This structural model is entirely consistent with our observation that N proteins tend to accumulate immediately outside of ROs in early infection, suggesting that N proteins might participate in the encapsulation of newly synthesized viral genomic RNA as it exits the pore. We also agree with the reviewer’s interpretation of the distributed N signal in late infection. We have revised the manuscript to better integrate these mechanistic models:

In early infection, N proteins tend to accumulate in the cytosol outside and around ROs (Supplementary Fig. 6C). This observation is consistent with previous reports{Scherer, 2022 #7643;Andronov, 2024 #7854;de Beijer, 2024 #7931;Wolff, 2022 #7932} and potentially

reflects the encapsulation of newly synthesized vgRNA as it exits the DMV pore. In late infection, N proteins distribute more evenly in the cytoplasm (Fig. 5G) as vgRNA moves to assembly sites and disperses into the cytoplasm. We observed that a subset of cytoplasmic N proteins colocalized with vgRNA puncta, while a fraction remained unassociated (Fig. 5G, yellow square, and 5H). This suggests that N proteins not only assemble with vgRNA into ribonucleoprotein particles (RNPs), but may also establish a pool that performs distinct functions in the cytoplasm{McBride, 2014 #7135}.

Finally S location is characterised using S2 and S1 antibodies. S2 was shown to localise to ER tubules and the nuclear envelope but not associated with S1. It's not clear what would have happened to the S1 from these S2 subunits. S2 antibodies have been used in the imaging of other coronaviruses without nuclear envelope localisation being reported. This might be discussed. Perhaps inclusion of another S2 antibody might provide additional confidence.

To confirm this localization, we compared the anti-S2 antibody signal in infected versus uninfected cells. While the antibody specifically decorates the nuclear envelope and ER in infected cells, it produces only a diffuse, non-specific background signal in uninfected cells (see new Supplementary Fig. 8 below). This robustly excludes the possibility that the nuclear envelope/ER localization of S2 is an artifact of non-specific antibody binding.



Supplementary Fig. 8: Validation of anti-S2 antibody specificity. Immunofluorescence staining of SARS-CoV-2-infected and uninfected A549-ACE2 cells using S2 antibody confirms the specificity of the S2 antibody detection. Of note, the non-specific background signal in the uninfected control samples is frequently higher than the signal observed in uninfected bystander cells within the

infected samples. This is likely due to the depletion of the primary antibody by the highly abundant S2 antigen present in the infected cells.

Regarding the reviewer's excellent question about the fate of the missing S1 subunits, a review of the literature provides a clear biological mechanism. The SARS-CoV-2 spike protein is virtually entirely processed at the S1/S2 site during biosynthesis in the host cell, predominantly by endogenous furins (DOI: 10.1016/j.cell.2020.02.058). While this cleavage is strictly necessary to bias the spike protein into an open conformation for ACE2 binding (DOI: 10.1038/s41586-020-2772-0), it intrinsically destabilizes the assembled protein structure.

This instability leads precisely to the premature, intracellular shedding of the S1 subunit that our imaging captures. Because our study utilized the original USA-WA1/2020 isolate—which lacks the later evolutionary D614G mutation that emerged specifically to stabilize the S1/S2 interaction and limit this shedding (DOI: 10.1038/s41467-020-19808-4)—this pronounced, pre-adaptation shedding phenotype is distinctly visible in our spatial atlas. (We also discuss this evolutionary dynamic in our response to Reviewer #2, Point 6).

To clarify this mechanism for the readers, we have expanded our Discussion in the “Structural protein localization patterns map virion assembly and egress” section to address this evolutionary and structural dynamic:

Furthermore, the lack of colocalization between S1 and S2 subunits on ER tubules and the nuclear envelope suggests that a fraction of the spike protein is cleaved and dissociates within the host cell, potentially through furin-mediated processing{Walls, 2020 #7053}. The extent of this S1/S2 processing represents a critical evolutionary trade-off for the virus. On the one hand, cleavage at the S1/S2 boundary is advantageous and necessary for propagation, as it biases the spike protein toward an open conformation that promotes ACE2 receptor binding{Benton, 2020 #7942} and enhances membrane fusion and virulence{Takeda, 2022 #7941}. On the other hand, this cleavage intrinsically destabilizes the S protein{Benton, 2020 #7942}, leading to the premature intracellular shedding of S1 that we observe.

Since S1 is strictly required for viral entry, its shedding inactivates the spike protein and reduces overall infectivity, a functional cost that has been directly demonstrated in pseudotyping studies{Walls, 2020 #7053}. Consequently, viral evolution continuously fine-tunes this process to maximize the open conformation while limiting detrimental S1 shedding—a structural balance effectively achieved by the globally dominant D614G mutation, which stabilizes the spike to prevent shedding while efficiently binding ACE2{Zhang, 2020 #7943}. Because our study utilized the original USA-WA1/2020 isolate, which lacks this stabilizing mutation, our spatial atlas provides a direct visualization of this pronounced, pre-adaptation shedding phenotype.

Methodology used is appropriate. Some findings would be solidified by the inclusion of electron microscopy or correlative light and electron microscopy, although this is really outside the scope of the work. Methods details are sufficient, with the exception of infection timings. From the methods, it appears that samples were fixed at 24 hpi while results discuss “early” and “late” infection. These are very subjective terms and actual timepoints should be given.

We thank the reviewer for pointing out the ambiguity regarding infection timings. We clarify that while all samples were fixed at 24 hpi, the infection in this cell line is asynchronous, resulting in a heterogeneous population of cells at somewhat different points in the viral life cycle. Therefore, we classified cells based on the observed morphology of the fluorescence signal of dsRNA/vgRNA/nsp3/nsp4 (as we previously established in 10.1038/s41467-024-48991-x) instead of the timing. In the revised manuscript, we have added a clarification in the Results defining these categories, and a section in the Methods ("Classification of Infection Stages"):

Although all samples were fixed at 24 hours post-infection (hpi), the viral life cycle is asynchronous. To distinguish functional phases of replication, we classified cells into an 'early morphological stage' (characterized by isolated DMVs, diameter $\lesssim 200$ nm) and a 'late morphological stage' (characterized by merged ROs and vesicle packets) (Supplementary Fig. 1), following the morphological transitions we previously characterized{Andronov, 2024 #7854}.

Classification of Infection Stages

While all samples were fixed at a single chronological timepoint (24 hpi), the infection progression in A549-ACE2 cells is asynchronous. We classified cells into 'Early' or 'Late' infection stages based on the distinct morphology of the replication organelles{Andronov, 2024 #7854}. 'Early stage' cells were defined by the presence of distinct, individual DMVs (typically $\lesssim 200$ nm in diameter) distributed in the cytoplasm, while 'Late stage' cells were identified by the presence of large, merged vesicle packets and dense perinuclear accumulations. This morphological transition is consistent across various viral targets (Supplementary Fig. 1). For S and M labeling, 'Early stage' was defined by the presence of fluorescence signal in the cytoplasm with a minimal amount of virion-like particles at the cell surface, and the 'Late stage' was defined by the presence of a high density of virus particles at the cell surface. Cells with ambiguous morphology were excluded from stage-specific analysis.

We have added new Supplementary Figure 1, which shows side-by-side examples of the specific morphologies used to assign "Early" (isolated DMVs) versus "Late" (merged ROs)

status. This classification relies purely on the reference labels (dsRNA/vgRNA/nsp3/nsp4) and does not bias the analysis of the other NSPs presented in the atlas.

Response to Reviewer #2 (Remarks to the Author):

For convenience, we show the referee comments in *italics*, and our replies in blue. Textual changes made in the manuscript are shown in red. Please note that Supplemental Figures S1, S2, S3, S7, S8, and S10 are all new in response to the reviewers.

The manuscript by Andronov et al describes a super-resolution spatial atlas of SARS-CoV-2 infection in human cells, focusing on the nanoscale organization of viral components. Using advanced SMLM techniques, they created a multi-color super-resolution atlas of viral RNAs and non-structural proteins (NSPs) to resolve the spatial context of polyprotein processing and replication organelle (RO) maturation, which were previously unclear. Key findings include a protease-gated maturation model for double-membrane vesicles (DMVs), where the nsp5 protease acts inside the DMV lumen to complete polyprotein cleavage, and the discovery of dsRNA connectors linking DMVs to pore-free dsRNA granules. Furthermore, the study investigates the effect of the antiviral drug nirmatrelvir, demonstrating that it induces multi-layered polyprotein bodies by halting DMV maturation, which may explain the viral rebound observed after drug cessation.

We thank the reviewer for the time spent reviewing our manuscript, for the positive feedback and for their highly useful suggestions.

Some specific questions:

1. Some dsRNA puncta have no/little viral proteins associated. Are these different maturation stages of the DMV? As a follow up, can you pseudo-time the DMV maturation based on different signals?

We thank the reviewer for this careful observation and the insightful suggestion regarding the pseudo-timing of DMV maturation.

Regarding the dsRNA puncta that appear to have little to no viral proteins associated, the reviewer has identified the non-canonical dsRNA granules that we characterize in detail later in the manuscript (Fig. 4F-J). Visually, these large dsRNA accumulations can appear "protein-poor" because they completely lack the prominent nsp3/nsp4 pore ring. Furthermore, while they are decorated with luminal RTC components (nsp5-16), this protein localization is sparser and often visually overpowered by the massive, highly dense dsRNA

signal. As discussed in the text, we hypothesize these may represent specialized compartments for the storage or sequestration of excess dsRNA, rather than canonical active replication sites.

Regarding the concept of pseudo-timing DMV maturation, we strongly agree with the reviewer's underlying logic: static SMLM snapshots of asynchronous infections inherently capture different stages of the organelle lifecycle. However, based on time-controlled experiments from our previous super-resolution study (Andronov et al., 2024, Nat. Commun.), we found that dsRNA is actually an early saturating marker, rather than a late-stage accumulation marker.

Specifically, we previously demonstrated that following membrane closure, DMVs rapidly produce a dsRNA core that reaches size and intensity saturation early in infection (by 6 hpi in Vero E6 cells). In contrast, the subsequent late-stage maturation (e.g., up to 24 hpi in Vero E6) is characterized by a massive intra-luminal accumulation of positive-sense vgRNA, which coincides with the physical swelling of the DMV, while the dsRNA core size remains stable or even slightly decreases.

Because the canonical DMVs investigated in the current study primarily exhibit the prominent dsRNA cores and limited vgRNA content characteristic of the early-to-intermediate active replication phase, using dsRNA size to pseudo-time them into "late" stages would be not as accurate. However, we completely agree that contextualizing these SMLM snapshots within the broader organelle lifecycle is highly valuable, and we have expanded our Discussion to explicitly integrate our current structural findings with the temporal maturation model established in our prior work:

Recognizing that SMLM yields static snapshots of an asynchronous infection, the diverse compositional states we observe reflect an ongoing organelle maturation trajectory. As we previously established using time-controlled infections{Andronov, 2024 #7854}, DMV maturation proceeds through distinct phases defined by RNA synthesis. Following the M^{pro}-gated membrane closure described here, early-stage DMVs undergo rapid negative-strand synthesis, forming the dense dsRNA cores visualized in our structural atlas. Given that dsRNA accumulation saturates early in infection, the transition to late-stage DMV progression is instead characterized by the massive production and intraluminal accumulation of positive-sense vgRNA, which drives subsequent DMV expansion{Andronov, 2024 #7854}. The canonical, pore-containing DMVs captured in our current high-resolution datasets predominantly represent this early-to-intermediate active replication phase, prior to extensive vgRNA-induced swelling.

2. Is there any filtering applied to selected DMVs for particle averaging? Since the distances are not normalized how do you select DMVs imaged at equatorial plane?

We appreciate this suggestion to clarify the processing of DMV localizations. We do not restrict our analysis to DMVs imaged precisely at the equatorial plane, nor do we apply morphological filtering beyond the basic localization criteria detailed in the Methods.

Because the diameter of an early DMV is typically smaller than the axial depth of field (~500 nm) of our 2D imaging system, capturing a strict equatorial slice without specialized 3D PSF engineering is not feasible. Instead, our 2D SMLM images represent the geometric projection of the entire 3D spherical shell of the DMV into a 2D plane. This projection naturally produces a ring-like density distribution that corresponds to the true 3D radial position of the labeled proteins.

We have explicitly derived the relationship between the peak position of the radial profile of the 2D projected image and the true radial position of the spherical shell target. Because the final profile is obtained by averaging a large population of DMVs distributed at random axial positions, depth-of-field effects and z-dependent PSF variations are effectively averaged away. These derivations are now described in detail in the new Methods section “Theoretical Model of DMV Projection” and in the new Supplementary Fig. 3.

The “Radial/Angular super-resolution framework validates DMV pore nsp3–nsp4 geometry” section of the Results was updated as follows:

Averaging over thousands of individual DMVs from multiple infected cells yielded a radial distribution, $g(r)$, for each NSP with a distinct peak representing its average radial position within the DMV. Although the 2D projection of these 3D spherical structures introduces central density and a systematic inward shift of absolute peak positions, relative distances between concentric layers remain robustly preserved (Supplementary Fig. 3; see Methods).

3. None of the proteins seem to penetrate the DMV. Why do they all localize to the membrane?

We thank the reviewer for this question, which allows us to clarify a crucial point about our spatial data and the biophysics of DMV formation.

First, we respectfully clarify that our quantitative analysis demonstrates that the viral replicase proteins (nsp5 through nsp16) are definitively positioned inside the DMV lumen. While the reviewer is correct that these proteins localize in close proximity to the membrane, our data proves they are situated on the inner, luminal side of the membrane rather than in the bulk cytoplasm. Because 2D SMLM projections of small, spherical vesicles can make it visually difficult to distinguish the inner versus outer face of the lipid bilayer, we utilized quantitative radial density distributions ($g(r)$) to rigorously resolve this geometry. Specifically, by using the nsp4 C-terminus as a precise reference marker for the *inner* DMV membrane, our radial profiling (Fig. 3, especially panel 3I, & Supplementary Fig. 4) demonstrates that the peak densities of nsp5–nsp16 are shifted significantly deeper (~5–25

nm for different nsp5–nsp16) into the DMV relative to the nsp4 C-terminus (Fig. 3I). This unequivocally confirms their intraluminal confinement.

Second, regarding how these massive proteins arrive inside the DMV, the reviewer's underlying intuition is completely correct: these large, folded proteins cannot simply "penetrate" or cross a pre-existing, sealed lipid bilayer, nor can they fit through the narrow ~2 nm central channel of the nsp3/nsp4 pore. Instead, as suggested in our revised Discussion and Model (Fig. 7B), these proteins do not penetrate the DMV; rather, they are engulfed by it during biogenesis.

Because the polyprotein is translated on the cytoplasmic face of the ER, the downstream NSPs (nsp5–nsp16) are physically tethered to the membrane invagination. During the maturation and closure of the DMV, the coordinated cleavages by nsp3 (PL^{pro}) and nsp5 (M^{pro}) relieve the restricting physical junctions, allowing the membrane to undergo *cis*-bending and seal. Because the cytoplasm within the invagination topologically becomes the DMV interior, the membrane effectively closes around the replication machinery, successfully trapping both partially uncleaved precursors and the non-covalently assembled RTC complex inside without requiring any proteins to cross a lipid bilayer.

4. SARS-CoV-2 also enters via endocytosis. Could the colocalization of M and LAMP-1 come from internalized viruses?

In our protocol, cells were inoculated for 2 hours before the unbound virus was washed away. Upon fixation at 24 hpi, we do not detect a significant concentration of virions in uninfected bystander cells within the same sample. Conversely, the density of virions in cells exhibiting M and S within LAMP1-positive vesicles is vastly higher. This massive accumulation strongly implies *de novo* virus production rather than the retention of the initial inoculum.

However, the reviewer raises a valid point regarding the limitations of static imaging. We cannot definitively exclude the possibility that newly released progeny virions are subsequently re-internalized by the same (or adjacent) infected cells via endocytosis. This caveat is now explicitly clarified in the manuscript:

However, given the static nature of our imaging, we cannot definitively exclude the possibility that some of these particles correspond to mature virions re-internalized via endocytosis.

5. Please mention/indicate the time points used in the different experiments rather than early/late infection.

We thank the reviewer for pointing out the ambiguity regarding infection timings. We clarify that while all samples were fixed at a single chronological timepoint (24 hpi), the infection progression in A549-ACE2 cells is asynchronous, resulting in a heterogeneous population of cells captured at different points in the viral life cycle. Therefore, we classified cells phenomenologically based on the distinct morphology of the replication organelles (using dsRNA/vgRNA/nsp3/nsp4 as markers), as we previously established in DOI:10.1038/s41467-024-48991-x

In the revised manuscript, we have added a clarification in the Results defining these categories, and a section in the Methods ("Classification of Infection Stages").

Although all samples were fixed at 24 hours post-infection (hpi), the viral life cycle is asynchronous. To distinguish functional phases of replication, we classified cells into an 'early morphological stage' (characterized by isolated DMVs, diameter ≤ 200 nm) and a 'late morphological stage' (characterized by merged ROs and vesicle packets) (Supplementary Fig. 1), following the morphological transitions we previously characterized{Andronov, 2024 #7854} (classification criteria detailed in Methods).

Classification of Infection Stages

While all samples were fixed at a single chronological timepoint (24 hpi), the infection progression in A549-ACE2 cells is asynchronous. We classified cells into 'Early' or 'Late' infection stages based on the distinct observed morphology of the replication organelles{Andronov, 2024 #7854}. 'Early stage' cells were defined by the presence of distinct, individual DMVs (typically ≤ 200 nm in diameter) distributed in the cytoplasm, while 'Late stage' cells were identified by the presence of large, merged vesicle packets and dense perinuclear accumulations. This morphological transition is consistent across various viral targets (Supplementary Fig. 1). For S and M labeling, 'Early stage' was defined by the presence of fluorescence signal in the cytoplasm with a minimal amount of virion-like particles at the cell surface, and the 'Late stage' was defined by the presence of a high density of virus particles at the cell surface. Cells with ambiguous morphology were excluded from stage-specific analysis.

We have also added Supplementary Figure 1, which shows side-by-side examples of the specific morphologies used to assign "Early" (isolated DMVs) versus "Late" (merged ROs) status. This classification relies purely on the reference labels (dsRNA/vgRNA/nsp3/nsp4) and does not bias the analysis of the other NSPs presented in the Atlas.

6. The missing colocalization between S1 and S2 is interesting and would suggest some

intracellular shedding of S1 which would be a disadvantage for the virus as this could lead to inactivation of S. Please discuss.

We agree with the reviewer that this is an interesting discovery. A review of the literature indicates that the SARS-CoV-2 Spike protein is “virtually entirely processed at the S1/S2 site during biosynthesis in HEK293T cells” presumably by furin (DOI: 10.1016/j.cell.2020.02.058). This suggests a strong biological mechanism for our observation of the missing colocalization between S1 and S2 on the ER tubules and nuclear envelope: this phenotype likely results from S1 and S2 dissociation following furin-mediated cleavage during S protein biosynthesis in host cells.

As the reviewer points out, since S1 is required for ACE2 binding to mediate viral entry, intracellular shedding of S1 is detrimental, resulting in the inactivation of the spike protein and reduced infectivity. In fact, using a murine leukemia virus (MLV) pseudotyping system, it has been demonstrated that infectivity is significantly reduced if S1 and S2 dissociate during S protein biosynthesis (DOI: 10.1016/j.cell.2020.02.058), underscoring the functional cost of S1 shedding.

However, this creates a critical evolutionary trade-off for the virus: S1/S2 cleavage is strictly necessary to bias the spike protein into an open conformation for ACE2 binding (DOI: 10.1038/s41586-020-2772-0), but it intrinsically destabilizes the protein, causing this detrimental shedding. Over the course of the pandemic, viral evolution has fine-tuned this balance to maintain cleavability while stabilizing the S1/S2 interaction to limit premature shedding, as successfully documented with the D614G mutation (DOI: 10.1038/s41467-020-19808-4). Our study uses the original USA-WA1/2020 isolate without this mutation, therefore the shedding is clearly visible.

We have expanded our Discussion in the “Structural protein localization patterns map virion assembly and egress” section to address this evolutionary and structural dynamic:

Furthermore, the lack of colocalization between S1 and S2 subunits on ER tubules and the nuclear envelope suggests that a fraction of the spike protein is cleaved and dissociates within the host cell, potentially through furin-mediated processing{Walls, 2020 #7053}. The extent of this S1/S2 processing represents a critical evolutionary trade-off for the virus. On one hand, cleavage at the S1/S2 boundary is advantageous and necessary for propagation, as it biases the spike protein toward an open conformation that promotes ACE2 receptor binding{Benton, 2020 #7942} and enhances membrane fusion and virulence{Takeda, 2022 #7941}. On the other hand, this cleavage intrinsically destabilizes the S protein{Benton, 2020 #7942}, leading to the premature intracellular shedding of S1 that we observe.

Since S1 is strictly required for viral entry, its shedding inactivates the spike protein and reduces overall infectivity, a functional cost that has been directly demonstrated in pseudotyping studies{Walls, 2020 #7053}. Consequently, viral evolution continuously fine-tunes this process to maximize the open conformation while limiting detrimental S1

shedding—a structural balance effectively achieved by the globally dominant D614G mutation, which stabilizes the spike to prevent shedding while efficiently binding ACE2[Zhang, 2020 #7943]. Because our study utilized the original USA-WA1/2020 isolate, which lacks this stabilizing mutation, our spatial atlas provides a direct visualization of this pronounced, pre-adaptation shedding phenotype.