CHAPTER 6

General discussion
DISCUSSION

The reticulovesicular replication network

The distantly related nidoviruses SARS-CoV and EAV both modify the endoplasmic reticulum (ER) into a reticulovesicular network (RVN) with which their RNA synthesis is thought to be associated. By using electron tomography (ET; chapters 2 and 5) to study the replication structures of two distantly related nidoviruses, we discovered that the previously documented double-membrane vesicles (DMVs) induced by infection with both viruses are continuous with each other and the ER via narrow membrane connections, often just 8 nm in diameter. Although these narrow connections at first glance may seem rather insignificant, their discovery refined our view of nidovirus-host interactions during replication. An ER-origin of replication vesicles was previously suggested for both coronaviruses [94,100,101] and arteriviruses [65,97], however, the fact that the DMVs of both virus families are connected and thus form a membraneous network was not known. The discovery of nidovirus-induced RVNs not only supported the notion that these viruses use the ER as membrane donor for RVN formation, but also implied that nidovirus replication maintains a close physical relationship with the ER throughout infection. In particular, this was demonstrated by two studies using drugs that affect the morphology and function of the ER, brefeldin A (BFA; chapter 3) and the translation inhibitors puromycin (PUR) and cycloheximide (CHX; chapter 4). BFA treatment, which triggered dilation of the ER, resulted in a faster transformation of DMVs into vesicle packets and affected DMV formation and RTC function [240]. The blockage of DMV formation by PUR treatment would seem a likely result of protein translation inhibition; however, the rapid reduction of RNA synthesis by preformed complexes cannot be solely attributed to translation inhibition because this drastic effect was not observed after translation inhibition with CHX, a difference that might be explained by the fact that PUR induces much more pronounced changes of ER morphology (Chapter 4).

Coronavirus DMVs were first described in ultrastructural studies of cells infected with mouse hepatitis virus (MHV) [102,262]. Next to DMVs, diverse membrane clusters were observed, which were named tubular bodies and reticular inclusions [102]. Recently, iEM studies have shown that the tubular bodies contain the structural envelope (E) protein, which likely induces the tubular morphology when it accumulates in the cell [235]. Also upon SARS-CoV-infection, a second membrane structure was observed in close proximity of the first detectable DMVs from 2 h p.i. on (Chapter 2). We chose to name this structure “convoluted membranes” (CM) by analogy to similar structures induced by flavivirus infection, which were proposed to be the site of viral genome translation and polyprotein processing [39,170]. ET and iEM on SARS-CoV-infected cells showed that SARS-CoV CMs are composed of membranes that connect the ER and DMV outer membranes and contain the majority of the nsp species for which iEM could be performed (Chapter 2). Similar results have recently been published for MHV
using iEM and showed that several nsps as well as the structural nucleocapsid (N) protein are present on DMVs as well as CMs, but not on other MHV-induced structures like the tubular bodies [235]. In contrast to our observations for SARS-CoV, these authors did not observe continuities between DMVs and CM, which is likely explained by the use of conventional 2-D TEM rather than ET. For SARS-CoV, these membrane connections were only discovered when using ET, while in 2-D TEM they were not apparent. Additionally, it was claimed that the CMs appeared only after DMV formation and are therefore unlikely to be a precursor structure of DMVs [235]. However, in our ET studies, from the earliest moment of detection onwards, the DMVs induced by both SARS-CoV (Chapter 2) and MHV (Knoops et al., unpublished data; Fig. 1) were accompanied by the reticular CM structures. Theoretically, these contradicting results may be explained by differences between the host cells used in these two studies (HeLa-CEACAM versus Vero E6), although no clear differences were observed in any aspect of RVN structure or development when comparing different cell lines infected with MHV (Knoops et al., unpublished data). It is also possible, and perhaps more likely, that early in infection the small CM structures remained invisible to Ulasli and co-workers in their 2-D analysis and were only detected at later time points when they had become larger. Besides the structures described by Ulasli et al., we observed vesicle packets (VPs) in MHV-infected Vero E6 cells (Knoops, unpublished data; Fig. 1C), the same type of structures that became very pronounced late in SARS-CoV infection (Chapter 2).

**Made from the ER**

Both arteriviruses [65,106] and coronaviruses [85,87,182] encode three non-structural proteins (nsps) that contain hydrophobic domains and presumably anchor the RTC to cellular membranes. In support of the conclusion that the nidovirus-induced RVN is derived from rough ER (rER), ribosomes were found to abundantly decorate its outer membranes (Chapters 2 and 5) and co-localization was observed of the main protein of the cellular Sec61-translocon channel co-localized and SARS-CoV nsps (Chapter 3). Ribosomes dock to the translocon in ER membranes while being engaged in the co-translational insertion of trans-membrane (TM) and secretory proteins. While translation is on-going, the nascent polypeptide chain leaves the ribosomal exit tunnel and is scanned for signal and/or TM sequences by the signal recognition particle (SRP). Upon SRP binding, the entire complex (i.e. ribosome, nascent chain, and SRP) is targeted to the SRP-receptor in the ER membrane, where translation continues with the nascent chain entering the translocon channel [59]. However, when a TM sequence is specific for the SRP-independent pathway, the nascent protein is first completed in the cytosol where it is kept in an unfolded conformation by cytosolic chaperones [273]. In mammalian cells, protein translocation is almost exclusively co-translational and it remains unclear how the unfolded proteins are targeted to the ER, although insertion into the membrane seems to be facilitated by the Sec61/Sec62/Sec63 complex [274]. For now, it is unclear whether
Figure 1. EM images of the membrane modifications that are induced by Mouse Hepatitis Virus. Vero E6 cells containing the CEACAM1-receptor were infected with MHV-A59, cryo-fixed by plunge-freezing and processed for TEM analysis as described earlier (Chapters 2 and 3). (A) At 8 h p.i., clusters of DMVs were visible in the cytoplasm and were often accompanied by clusters of convoluted membranes (CM). The arrows in the insets show the connections of DMVs with CM structures that were readily visible in our TEM-images. (B) In a time-course experiment, the first MHV-induced DMVs were detected at 4 h p.i. and, also at this early time-point, were connected with CM membranes (arrows). (C) Besides DMVs, also vesicle packets (VP) were occasionally found at 8 h p.i. in which multiple inner-vesicles shared the same outer membrane.
membrane insertion of the nidovirus transmembrane replicase subunits occurs co- or post-translationally, although the efficient integration of MHV nsp3 in microsomes suggested the involvement of an SRP-dependent ER-translocation mechanism [85]. If the SRP pathway indeed directs the nascent replicase polyprotein towards ER membranes, this would be the first step in which its template, the viral genome, would become physically associated with the ER membrane via translating ribosomes [275]. Multiple ribosomes would translate ORF1a or ORF1ab, simultaneously producing polyproteins and thus securing the membrane asso-

![Figure 2. Models for the formation of DMVs from ER membranes.](image)

(A) Double-budding mechanism. After association of the replicase polyproteins with ER-membranes, the ORF1A-encoded membrane domains induce a negative curvature towards the ER-lumen, thus creating an invagination into the ER. Membrane-pairing occurs by luminal interaction of replicase proteins situated on the membranes of the invagination and the opposing ER-membrane, and results in a wrapping event towards a double-membrane vesicle. In this model, one membrane-fission event at the orifice of the invagination would be necessary to explain the morphology of the connected DMV as was observed after infection with either EAV or SARS-CoV. (B) Single-budding mechanism. Similar to the double-budding mechanism, with the exception that membrane-pairing would not occur with the membrane opposing the invagination, but with the membranes surrounding the orifice. In this model, the neck-like connection of the DMV with the ER can be explained without any fusion result, although fusion would be a necessity for complete closure of the DMV. (C) Protrusion model. After association of the replicase with ER-membranes, the ORF1A-encoded membrane domains first induce membrane-pairing which afterwards results in membrane-bending. Like in the double-budding mechanism, one membrane-fission event at the orifice of the invagination would be necessary to explain the morphology of the connected DMV. Especially in this model, it is unclear what influences the direction of membrane-bending. (Figure adapted and adjusted from [97])
ciation of the genome with the ER via the ribosome-translocon interaction, potentially until the genome switches from acting as template for translation to being a template for minus-strand RNA synthesis. The mechanism of membrane-association by poliribosome formation was discovered during translation of the poliovirus genome, supporting the theory that this mechanism indeed is used in some RNA virus systems [276,277]. After SARS-CoV infection, we found that Sec61α resides almost exclusively within the RVN and therefore likely plays a major role in the insertion of replicase TM domains into the ER membrane (Chapter 3). However, upon translation of coronavirus subgenomic mRNAs, also various structural and accessory transmembrane proteins need to be inserted into the ER-membrane, a step likely utilizing the same translocation-machinery. Thus, by rearranging the host’s ER into a system which associates the RTC with the translation/translocation machinery, nidoviruses may have created the perfect system to couple RNA synthesis and the multi-step pathway leading to virus assembly.

After membrane insertion, nidovirus replicase transmembrane-domains are thought to not only constitute a membrane anchor for the RTC but also to induce membrane pairing at the luminal interface by “zipping” the two opposing ER membranes together, thus minimizing the luminal space between the membranes of DMVs and CM [65,106]. The transmembrane domains expressed from ORF1a and the expression level of the nsps that contain them may have a profound effect on the membranes in which they are inserted and are believed to induce curvature. For EAV, it was shown that the first two transmembrane replicase subunits, nsp2 and nsp3, are capable of inducing characteristic double membranes and DMVs when expressed together in the absence of any other nsps or viral RNA synthesis [65]. Two models have been proposed to explain the formation of arterivirus DMVs from ER membranes [97]. According to the “double-budding mechanism” model, the replicase proteins would first induce a negative curvature in the ER membrane and, after membrane fission, this would give rise to a vesicle within the ER lumen (Fig. 2A). Subsequently, luminal replicase TM domains in the ER and the membrane of this vesicle would interact to induce positive curvature and induce a second budding event, this time towards the cytosol, which after a second membrane fission event would result in a detached vesicle with a double membrane. In summary, in this model two opposing budding events would follow each other, while the switch from inducing negative to positive curvature would be driven by luminal interactions of replicase subunits. Similar to the double budding mechanism proposed by Pedersen et al., membrane pairing could also occur by oligomerization of proteins positioned in the membrane region surrounding the orifice (Fig. 2B). In this model, the neck-like connections between DMVs and the ER composed of two membranes, similar to what was observed in electron tomography, can be explained without any membrane fusion event, although membrane fusion at the orifice would still be a necessity for complete closure of the DMV. The alternative “protrusion-and-detachment” model operates in a similar way; however, the interaction of replicase subunits in opposing membranes, resulting in the formation of tightly-paired
membranes, would now precede the membrane-bending and -fission events that ultimately would create a sealed DMV (Fig. 2C). In this model, it remains unclear what determines the beginning and direction of curvature. The tomograms that were obtained from both SARS-CoV- and EAV-infected cells mainly showed paired-membrane structures like those proposed in the “protrusion-and-detachment” model. Vesicles within the ER that do not show the paired-membrane morphology, as proposed in the “double-budding mechanism”, were only observed in the SARS-CoV VPs, which were detected only late in infection and might result from dilating ER and dissociation of membrane protein interactions. The detachment of DMVs from the ER was not observed for the nidoviruses studied in this thesis (chapter 2 and 5) and thus suggests that the last fission event, which is part of both models, does not occur. Alternatively, an initial fission event, creating a fully detached DMV, could be followed by fusion of DMV outer membranes to generate the RVN. Still, it remains unclear how the transmembrane subunits of the replicase induce membrane pairing and curvature, and at which stage cellular proteins might be involved. It was recently found [106] that a cluster of four conserved cysteine residues in a luminal domain of EAV nsp3 is essential for DMV formation and that their replacement is lethal to the virus when evaluated in a reverse genetics system. Although experimental evidence is still lacking, it is tempting to speculate that these cysteine residues are responsible for membrane pairing by e.g. forming disulfide bridges or a tertiary structure element like a zinc finger domain. Interestingly, a mutant nsp2-nsp3 polyprotein containing an engineered N-glycosylation site in this cysteine-containing loop appeared to retain the ability to pair and curve membranes but hardly induced closed DMV structures, a defect likely due to steric hindrance by the sugar group. When this mutation was tested by reverse genetics, EAV replication was not detected until this nsp3 mutant pseudo-reverted and lost the signal for N-glycosylation [106].

Particularly in the case of coronaviruses, the CM structures that extensively labeled for the replicase proteins did not have the characteristic vesicular morphology and comparison of their 2D projection-pattern with in silico-generated templates of different cubic-membrane organizations revealed a 3-D gyroid-based cubic membrane architecture [103,278]. The formation of these geometrical and crystalloid membrane conformations is normally induced by a high concentration of transmembrane proteins with the ability to self-interact [279]. Unfortunately, a surrogate system to study these membrane modifications, e.g. expressing only the trans-membrane nsps of coronaviruses, has not yet been developed. Such a system might provide valuable clues about the mechanism and chronology of CM and DMV formation. Moreover, reconstitution experiments involving isolated nidovirus trans-membrane nsps and “empty” proteoliposomes, as well as insertion of nsps into translocon-containing microsomes during in vitro translation could provide useful information about the exact type of curvature that these proteins can induce [280] and the question whether they need host factors to do so.
Recently, it was suggested that depletion of LC3, which is a marker protein for autophagosomes, inhibits MHV replication to a certain extent \( (\textit{i.e.} \) a 4-fold reduction of virus-driven protein expression was measured) [263]. Autophagy was previously implicated in coronavirus replication because some light microscopy studies reported that LC3 co-localizes with SARS-CoV [96,226] and MHV replicase proteins [263], although other studies could not confirm these observations [100,281]. This may in part be explained by the use of transiently expressed GFP-LC3 fusion proteins, which were found to not co-localize with nsps and seemed to be excluded from the virus-induced membrane structures, whereas endogenous LC3 appears to co-localize partly with coronavirus replicase proteins [263]. An in-depth colocalization analysis, including iEM, is clearly required to determine if and where LC3 is indeed present in RVN membranes. The light microscopy study of Reggiori et al [263] showed a certain level of LC3 co-localization with MHV nsp2 and nsp3, which were suggested to reflect the localization of DMVs. However, since CM, much more than DMVs, are the structures that contain the largest amounts of replicase proteins, it is likely that any co-localization between LC3 and coronavirus replicase will be concentrated in the CMs. It was also suggested that Atg5 knockdown, which results in autophagy-deficient cells, impaired DMV formation after MHV-infection [105], but this result was contradicted in a follow-up study showing that Atg5 knockdown does not have an effect on DMV formation and that autophagy is not required for replication [226]. This was again confirmed by Reggiori and co-workers, who showed that also the essential autophagy protein Atg7 is dispensible for MHV replication [263]. In the same paper, it was also claimed that the MHV-induced RVN, besides LC3, contains EDEM1 and OS-9, which both are part of the ER-associated protein degradation (ERAD) machinery [263]. These proteins are normally located in the ER-lumen and target misfolded proteins for ubiquitination and subsequent degradation by cytosolic proteasomes, a process also involving the sec61-translocon for “dislocation” across the ER membrane [282]. In contrast to their proposed role in the Sec61-mediated dislocation pathway however, it was suggested in a structural study that EDEM1-containing smooth-membrane vesicles might pinch off from the ER and would subsequently be exported into the autophagy-lysosomal pathway [283]. Whereas the authors of the latter paper suggested that this is an alternative way to remove misfolded proteins from the ER, Cali and co-workers [284], who introduced the term “EDEMosomes” for these small vesicles, interpreted the observed phenomena as a way to downregulate the amounts of ERAD chaperones such as EDEM1 and OS-9 themselves, in order to prevent unnecessary degradation of newly synthesized proteins in the ER. Subsequently, it was claimed that these EDEMosomes form the scaffold for the formation of MHV-induced replication-associated membranes [263], although it remains entirely unclear how this would be achieved. In particular, it is difficult to envision how 200 to 300-nm double-membrane structures that are decorated with ribosomes [198] would arise from 150-nm smooth single-membrane vesicles that are stuffed with protein cargo. In-depth ultrastructural and biochemical studies will be needed to test the viability of the EDEMosome hypothesis.
For a complete understanding of the cellular pathways that nidoviruses use for induction of their replication membranes, it will be important to identify the specific host proteins and mechanisms involved. Recently, it was shown that both flavi- and enterovirus RTCs are able to recruit phospholipid-modifying enzymes, e.g. phosphatidylinositol 4-kinases (PI4K), and change the lipid composition of their replication membranes which seems essential for viral replication [285,286]. Furthermore, the enterovirus polymerase seems to specifically bind phosphatidylinositol-4-phosphate lipids which might be a key feature in the membrane association of the RTC and subsequent viral RNA synthesis [286]. It will be highly informative to understand whether also nidoviruses influence the lipid environment of the RVN, which viral and host proteins might be involved in this process, and what their exact role in RVN formation might be.

**Place of nidovirus RNA synthesis: Concealed**

Optimization of the cryo-fixation protocols for electron microscopy analysis greatly improved the preservation of the morphology of nidovirus-induced membrane rearrangements, in particular the DMV interior (Chapters 2 and 5). Upon cryo-fixation, the interior of both SARS-CoV- and EAV-induced DMVs appeared to be packed with a granular substance after plunge-freezing and, for EAV, contained an electron-dense core with surrounding halo after high-pressure freezing (HPF). Similar internal structures were observed after HPF of cells infected with members of the picornavirus family, e.g. poliovirus [49]. As observed for EAV (Chapter 5), the interior of DMVs induced by Foot-and-mouth disease virus (FMDV) contained electron-dense cores upon HPF, which were not seen after conventional chemical fixation [121]. Also for flaviviruses, it was shown that the ultrastructure of the replication vesicles differs between samples prepared by chemical versus cryo fixation [38]. Whereas chemically fixed Dengue virus-infected cells contained spherular ER invaginations, resembling those proposed in the first step of the “double budding” model, cryo-fixation revealed a DMV morphology in which the inner and outer membranes are tightly apposed.

Compared with conventional chemical fixation, which resulted in empty DMVs [100,104], the cryo-EM protocols are less prone to fixation artifacts, thus significantly improving ultrastructure and resolution. Next, these improved protocols were adapted for iEM and made it easier to distinguish between the different components of the RVN on the basis of their morphology (Chapters 2, 3, and 5). A clear difference in the localization and amount of labeling of RVN components was found when iEM was performed with antibodies directed against nsps and dsRNA. Large amounts of replicase proteins that would not contain TM domains after their proteolytic release, like the SARS-CoV main protease (nsp5) and primase (nsp8), and the EAV polymerase (nsp9) and endoribonuclease (nsp11), were found associated with the double membranes of the RVN. These findings suggest these membranes to be the scaffold for the enzymatically active replicase proteins, however, the dsRNA, which is thought to
represent the key nucleic-acid intermediate of viral RNA synthesis, was mainly found inside the apparently sealed DMVs and co-localized only with a small fraction of the nsps (chapter 2 and 5).

This apparent paradox could be explained if only a fraction of the replicase proteins inside the infected cell is actively engaged in RNA synthesis, as postulated for other RNA viruses [47]. Still, it remains unclear where exactly RNA synthesis occurs. Membranes that were isolated from infected cells and retained active RTCs, were used to investigate the synthesis of genomic RNA and subgenomic mRNAs in vitro [188,247]. In these studies, it was shown that RTC activity was insensitive to nuclease and protease digestion, and only became sensitive after detergent treatment. These experiments strongly suggested that RTC activity is somehow protected by membranes, although no information was obtained about the exact nature of these membrane compartments. When dsRNA accessibility was investigated in situ by nuclease treatment and antibody labeling of semi-permeabilized EAV-infected cells, a similar result was obtained supporting the observation obtained by tomography that the majority of dsRNA is enclosed within membranes (chapter 5).

The in vitro and in situ data make it tempting to speculate that DMVs are indeed the place of RNA synthesis. However, ET showed that the vast majority of DMVs did not have clear openings connecting their interior with the cytosol (Chapters 2, 3 and 5), in contrast to what was observed when ET was employed for other +RNA viruses [36,38,41]. If the nidovirus-induced vesicles are indeed sealed, this poses the question of how import of nucleotides into the vesicles and export of RNA products out of the vesicles is organized. In the in vitro system, only the genomic RNA remained membrane-associated whereas the subgenomic mRNAs were released [188,247]. Attempts have been made to detect newly made viral RNA products by iEM [97,104], but these remained largely inconclusive due to RVN fragility and preparation artifacts, labeling efficiency and the possibility that RNA products may be transported to another location within the labeling timeframe.

iEM is a popular technique to locate the products of viral RNA synthesis products in situ on cell sections, however, a few disadvantages inherent to the technique must be taken into account. In iEM, a specific first antibody is used to detect the target which is then visualized using a second antibody containing a gold particle. This indirect labeling method is not only prone to background labeling, in particular if antigens have a low labeling intensity, but it also has a limited lateral resolution of ~30 nm. Therefore, it will reveal the localization of the target with an uncertainty that might e.g. make the difference between being inside, outside or on the membrane of DMVs [287]. Furthermore, antibodies will only bind to the surface that became exposed by sectioning of the specimen and, thus, projection-images can show gold particles superimposed on structures which in fact do not contain the target.

One approach to locate products of viral RNA synthesis is the use of an antibody highly specific for dsRNA [177,179], which in turn can be detected with immuno-gold particles (Chapter 2, 3 and 5). However, apart from the question whether the labeled RNA product was
part of an active complex at the time of fixation, it is as yet unclear whether all dsRNA detected upon +RNA virus infection indeed represents viral RNA, and therefore other methods should be used to confidently localize the active RTC. Detection of virus-specific sequences with complementary oligonucleotide probes, combined with immuno-gold detection, may be an option but these probes should be designed carefully to discriminate between the different cellular and viral RNAs that are present in the infected cell. For detection of active RTCs, the viral negative-stranded RNA would preferably have to be targeted, which has a higher chance of being part of a complex that is actively engaged in viral RNA synthesis. On the other hand, negative-stranded RNAs are underrepresented and can hybridize to an excess of complementary positive-stranded RNA molecules, thus complicating the detection of these molecules in iEM. To improve the specificity of iEM detection of negative-stranded RNA sequences, padlock probes could be used which are detected by the rolling-circle amplification method. Briefly, padlock probes are oligonucleotides containing two target-complementary ends which become circularized by DNA ligation after hybridizing to a target DNA [288] or RNA sequence [289]. Besides the high labeling-specificity, a major advantage of this method is that the rolling circle amplification increases the signal of the probe [290,291]. Interestingly, methods are also being developed that will allow detection of proteins in complex with DNA/RNA via padlock-probes [292] and might thus be explored in the future to locate nsps on template RNAs.

Ultrastructural studies have been combined with metabolic labeling of viral RNA synthesis using labeled uridine [51,189] or uridine-triphosphate (UTP) [97,104] and are possibly the most straightforward approaches to localize the active site of RTC. Labeled uridine crosses the cell-membrane efficiently and is metabolized to uridine triphosphate (UTP) which can be incorporated in newly synthesized RNA, with actinomycin D treatment ensuring that most of the RNA made is indeed viral RNA. Labeled UTP itself cannot cross the plasma membrane and needs to be transfected or micro-injected in order to reach the viral RTC. Many different forms of labeled uridine and UTP are available and Bienz and co-workers were among the first to use radiolabeled (tritiated) uridine in ultrastructural studies to localize the active poliovirus RTC by autoradiography [51]. Although this method is highly specific, the large silver-grain size identifies the radioactive spot with a low lateral resolution and the relatively long exposure times (weeks to several months) may be considered cumbersome. Nowadays, halogenated uridine or UTP are used more often and, if incorporated in newly made RNA, can subsequently be detected with an anti-bromo-UTP (BrUTP) monoclonal antibody by iEM. Apart from the iEM complications described above, the BrUTP labeling times needed for the BrUTP to efficiently enter the cell after transfection and to be incorporated into a detectable amount of RNA products likely exceeds the timeframe needed for RNA products to be transported to another location. Furthermore, the variety of labeling efficiency might prevent a synchronized labeling of different cells in a culture and thus microinjection of infected cells would probably be a more useful, alternative approach. Ideally, labeling times
of 5 to 15 minutes should be achieved, which are more realistic given the estimated rate of viral RNA synthesis. However, they also make the detection of the incorporated halogenated-uridine more difficult. Electron spectroscopic imaging (ESI) in combination with ET might be used as a more sensitive alternative to conventional iEM detection with an antibody. During transmission EM, electrons interact with the sample by means of inelastic and/or elastic scattering. Inelastically scattered electrons decrease image quality, however, by using ESI one can specifically detect inelastically scattered electrons that suffered a specific energy loss due to their interaction with the specimen [257]. The energy loss is characteristic for the element that induced it and therefore, by filtering out electrons with an energy loss in a specific range, it is possible to map different atoms in the specimen. ESI is a well-established technique in materials sciences, but its application to biological samples is relatively novel. Especially in combination with ET, the sensitivity of electron spectroscopic imaging has the potential to detect halogenated-uridine labeled viral RNAs inside thin sections.

The results obtained by the experiments described above would be valuable to address the question where exactly viral RNA synthesis takes place. However, given the fact that DMVs may contain large amounts of dsRNA, it would be straightforward to first investigate by biochemical methods whether the dsRNA indeed is viral RNA and thus represents intermediates of replication/transcription. Apart from the latter question, it is very likely that all of the UTP-labeling methods summarized above will initially detect these dsRNA structures because these structures are the first product of the viral polymerase. If short-pulse UTP labeling can be performed, fluorescence microscopy might be vital to gain information about the dynamics of the RTC. Detection of incorporated nucleotides is less prone to background signal in the permeabilized cells used for fluorescence microscopy than on cell sections for iEM. Especially, in combination with semi-permeabilization and RNA digestion (Chapter 5), it could be determined whether newly synthesized RNAs are produced inside or outside DMVs. The very specific padlocking method could then be used to determine the ratios of different viral RNAs that are present in the cytosol or within DMVs.

Furthermore, the in vitro replication/transcription system which was developed for EAV [247], might be studied by cryo-electron microscopy in more detail. As demonstrated before [293], it is possible to monitor the membrane-bound replication complex in real time by expressing a recombinant virus expressing GFP fused to one of the replicase subunits. In the same way, cells or even cell fractions that are first observed by in vivo or in vitro fluorescence microscopy could afterwards be used for electron microscopy to record high-resolution structural information [294]. This approach has recently stimulated a new development for which a (cryo-)electron microscope was equipped with an integrated laser scanning fluorescence microscope, and the detection of fluorescent signal and correlation with EM was done inside this integrated light- and electron microscope (iLEM) [295]. The development of iLEM is relatively new and in the future the technique may be combined with novel fluorescence microscopy techniques that have a superior localization resolution, e.g. STED,
PALM and STORM [296-298]. These super-resolution imaging techniques allow single particle localization of fluorescently labeled probes at a resolution of just tenths of nanometers and, also without being integrated in the iLEM, might play a significant role in the dissection of nidovirus replication in the future.

**Final remarks**

Thanks to the introduction of ET in +RNA virus replication research, detailed structural information is becoming available with respect to the replication structures that are induced in infected cells. The detailed dissection of the structural changes inside these cells allows us to begin to understand how viruses use and modify cellular components to build an infrastructure that is optimal for their replication. Overall, ultrastructural studies can establish an important framework that places biochemical data in the context of the cell and time course of infection. In this way, they will significantly contribute to our “bigger picture” of +RNA virus replication and will reveal the parallels and differences between virus orders, families, and species, which ultimately can be explained by the diversity of virus genotypes.

In this thesis, I set out to investigate the ultrastructural changes in the host cytoplasm upon infection with nidoviruses. Two prominent members of the coronavirus and arterivirus families, SARS-CoV and EAV, were chosen as models and both were found to induce similar networks of interconnected vesicles made from ER membranes. The in-depth structural dissection of these two nidovirus reticulovesicular networks revealed both common features and striking differences between these distantly related nidovirus families. Our RVN descriptions can now be used as a basis for future research, addressing e.g. the formation and function of these membrane structures and exploring the potential to use this knowledge for the development of antiviral strategies. Apart from providing answers to fundamental ultrastructural questions, the analysis of nidovirus RVN architecture also uncovered new research questions, e.g. the precise location of nidovirus replication/transcription and the mechanism involved in RNA transport from the cytosol into DMVs and/or vice versa. The lack of conventional methods to address these problems will stimulate additional technical advances, which will contribute higher-resolution information that will undoubtedly help to answer these pertinent questions.