

The architecture of membrane compartments involved in hepatitis C virus genome replication revealed by cryo-electron tomography

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Chapter 2 contains methods from collaborative work, developed and executed by members of the Astbury Biostructure Electron Microscopy (EM facility, University of Leeds) and Electron biology Imaging centre (eBIC, Diamond Light Source, Harwell)

Chapter 4 contains data from collaborative work with the EM facility and eBIC. Mr Martin Fuller (EM facility) prepared ultrathin sections by resin-embedding and collected TEM images. Dr Tom O Sullivan (EM facility) developed the CEMOVIS workflow, including data collection. Dr Yehuda Halfon acquired cryo-ET images from lamellae (EM facility). Dr James Gilchrist (eBIC) facilitated lamellae generation at eBIC.

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Dedication

This study is dedicated to my beloved parents, who have been the source of inspiration, guidance, and strength. Their love and encouragement have shaped my personal growth.

It is also a tribute to a loving memory of a heart-warming conversation with my grandfather when I was a young child. He inspired me to pursue a career in science, saying, *“If you aspire to be a doctor, aim for a doctorate in science. That way, you'll contribute significantly to the well-being of a society”*

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Abstract

Hepatitis C virus (HCV) affects an estimated 71 million people worldwide and is a major cause of chronic liver disease. HCV infection induces rearrangements of host cytoplasmic membranes, resulting in the formation of a network of membranous compartments known as replication organelles (ROs). These ROs provide platform for virus genome replication. Despite the presence of single membrane vesicles (SMVs), double membrane vesicles (DMVs), and multimembrane vesicles (MMVs) observed in HCV-infected cells or those harbouring a subgenomic replicon (SGR), the specific RO housing the assembly of the replication complex has remained elusive. While DMVs were predominantly observed and proposed as potential sites for HCV genome replication, attempts to pinpoint the precise location of the non-structural (NS) proteins within these ROs using immunolabelling methods for electron microscopy yielded inconclusive results

To unfold the organisation of NS proteins within the ROs, two novel *in situ* cryo-electron tomography (cryo-ET) workflows were established. This study aimed to provide a deeper understanding of ROs utilising Huh7 cells harbouring HCV JFH-1 subgenomic replicon (SGR), where NS5A is fused with eGFP. The current knowledge of ROs was predominantly based on the correlative light and electron microscopy (CLEM) techniques employing chemical fixation and resin-embedded methods. To improve on this, cryo-preserved cell specimens using cryo-focused ion beam (cryo-FIB) milling and ultramicrotomy (CEMOVIS) were employed. For the first time, *in situ* cryo-ET on lamellae (prepared by cryo-FIB) and ultrathin sections (prepared by ultramicrotomy) from replicon cells revealed insights into the content of these ROs.

Cryo-electron tomograms reveal that NS proteins assemble within a subset of DMVs, SMVs, and MMVs, challenging the notion that HCV utilises a specific organelle exclusively for hosting the replication complex. Instead, the intricate network of vesicles may serve as an immune evasion strategy, consistent with the literature. The identified DMVs fell into three distinct categories: the first showing clustered densities suggestive of replicase assembly, the second exhibiting thread-like densities, indicating a potential role in safeguarding intermediate dsRNA filaments from host immune surveillance, and the third appearing empty. In addition to studying DMVs, the structural characteristics of SMVs, MMVs, and multi-vesicular bodies (MVBs) were thoroughly examined. Notably, this investigation marks the first instance of directly observing the arrangement of NS proteins within an MMV. The cryo-ET methodologies established in this study provide a foundation for deciphering the architecture of the HCV replication complex in the near future, leveraging advanced computational tools.

Abbreviations

2D: Two dimensional
3D: Three dimensional
aa: Amino acids
Å: Angstrom
A, amp: Ampere
Apo: Apolipoprotein
APEX: Ascorbate peroxidase
ACDP: Advisory committee on dangerous pathogen (ACDP)
AF: Alexa Fluor
AR: Aspect ratio
Amp^r: Ampicillin resistance
ATG: Autophagy related gene (or protein)
AH: Amphipathic helix
APEX: A modified ascorbate peroxidase derived from soybean
bp: Base pair
BSL-2: Biosafety level 2
BCA: Bicinchoninic acid
BSA: Bovine serum albumin
CDC: Centre for Disease Control and Prevention centre
CD81: Cluster of differentiation 81
CLDN1: Claudin-1
Chol: Cholesterol
Cer: Ceramides
CsA: Cyclosporin A
Cyphs: Cyclophilins
CIP: Alkaline phosphatase calf intestinal
cDNA: Complementary DNA
CO₂: Carbon dioxide
Cryo-EM: Cryo-electron microscopy
Cryo-FM: Cryo-fluorescence microscopy
Cryo-CLEM: Cryogenic- correlative light and electron microscopy
Cryo-CLET: Cryogenic- correlative light and electron tomography
Cryo-FIB: Cryogenic- Focussed ion beam milling
CEMOVIS: Cryo-electron microscopy of vitreous sections
Cy5: Sulfo-Cyanine5 maleimide
Cys: Cysteine
CLIP tag: Orthogonal tag further engineered from SNAP

dH₂O: Deionised water
 DAA: Directly acting antiviral
 DAPI: 4',6-diamidino-2-phenylindole
 DAB: Diaminobenzidine
 DEPC: Diethyl pyrocarbonate
 DMEM: Dulbecco's Modified Eagle Medium
 DMV: Double membrane vesicle
 DMInV: Double membrane Inner vesicle
 DMSO: Dimethyl sulfoxide
 DNA: Deoxyribonucleic acid
 DNase: Deoxyribonuclease
 dNTP: Deoxyribose nucleotide triphosphate
 DTT: 1,4-Dithiothreitol
 e⁻: electron
 EFTEM : energy-filtered TEM
E.coli: Escherichia coli
 eGFP: Enhanced green fluorescence protein
 EDTA: Ethylenediaminetetraacetic acid
 ER: Endoplasmic reticulum
 EM Facility: The Astbury Biostructure Electron Microscopy facility at University of Leeds
 ET: Electron tomography
 EM: Electron microscopy
 FM: Fluorescence microscopy
 FPs: Fluorescent proteins
 Feo: Fusion of neomycin phosphotransferase and Firefly luciferase
 FACS: Fluorescence-activated cell sorting
 FBS: Foetal bovine serum
 FAPP2: glycosphingolipid transporter four-phosphate adaptor protein 2
 Flippers: Fluorescent indicator and peroxidase for precipitation with EM resolution
 FIAsH: Fluorescein arsenical hairpin binder-ethanedithiol
 GLB: Glasglow lysis buffer
 GFP: Green Fluorescence Protein
 Gt (gt): Genotype
 G418: Geneticin sulphate
 GNN or GND: Polymerase deficient SGR
 hr: hour

h.p.e: hour post electroporation
HCV: hepatitis C virus
H2B: Histone2B
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEK: Human embryonic kidney
HCC: Hepatocellular carcinoma
HIV: Human immunodeficiency virus
HSPG: Heparan sulphate proteoglycan
HPF: High pressure freezing
His: Histidine
Halo tag: Self-labelling protein derived from haloalkane dehalogenase enzyme DhaA
ICTV: The International Committee on Taxonomy of Viruses
InV: Inner vesicle
IRES: Internal ribosome entry site
IF: Immunofluorescence
IL: Interleukin
IRF-3: regulatory factors 3
IFN- α : Interferon alpha
IPTG: Isopropyl β -D-1-thiogalactopyranoside
JFH-1: strain derived from a patient with Japanese fulminant hepatitis
Kb: Kilobase
kD: Kilo Daltons
KCl: Pottassium chloride
LVPs: Lipoviral particles
LB: Lysogeny broth
LD: Lipid droplet
LDLs: Low-density lipoproteins
LM: light microscopy
LN₂: Liquid nitrogen
mRNA: Messenger RNA
MMV: Multimembrane vesicle
MVB: Multi vesicle body
MES: 2-Morpholinoethanesulphonic acid sodium salt
MiniSOG: Mini Singlet oxygen generator
MT: Microtubule
MAVS: Mitochondrial antiviral signalling protein
mCherry: Red fluorescent protein
MiniSOG: Mini singlet oxygen generator

MSP: N-terminal major sperm domain
 MgCl₂: Magnesium Chloride
 MW: Membranous web
 MOPS: 0.2 M 3-(N-morpholino) propane sulphonic acid
 NS: Non-structural
 NEAA: Non-essential amino acids
 NMR: nuclear magnetic resonance
 NEB: new England biolabs
 NaCl: Sodium chloride
 Nt: nucleotide
 Nu: nucleus
 NTP: Nucleoside triphosphates
 NF-κB : Nuclear factor κB
 NPC1L1: Niemann-Pick C1-like 1
 NaCl: Sodium Chloride
 NTRs: Non-translated regions
 Ni-NTA: Nickel-nitrilotriacetic acid
 OD₆₀₀: Optical density at 600 nm
 ORF: Open reading frame
 OCLN: Occludin
 ORF: Open reading frame
 OSBP: Oxysterol-binding protein
 PCR: Polymerase chain reaction
 PBS: Phosphate buffered Saline
 PF: Plunge freezing
 PFA: Paraformaldehyde
 PDI: Protein disulfide-isomerase
 PVDF: Polyvinylidene membrane
 PIPES: Piperazine-N.N'-b 2-ethanesulfonic acid
 PAGE: Polyacrylamide gel electrophoresis
 PI-4: Phosphatidylinositol 4
 PI4P: Phosphatidylinositol 4- phosphate
 PI4KIIIα: Phosphatidylinositol 4 kinase III α
 PE: Phosphatidylethanolamine
 PC: Phosphatidylcholine
 PI: Phosphatidylinositol
 PA: Phosphatic acid
 pLDDT: Predicted Local Distances Difference Test

pmol: Picomoles
 PAE: Predicted aligned error
 pTM score: Predicted template modelling score
 p56: hypo-phosphorylated
 p58: hyper-phosphorylated
 QD: Quantum Dots
 QQ: Quantile-Quantile
 RNA: Ribonucleic acid
 RG: Reporter genes
 RNase: Ribonuclease
 RIG-I: Retinoic acid-inducible gene I
 RASs: Resistant associated substitutions
 RLU: relative light units
 RLuc: Renilla Luciferase
 RPM: Revolutions per minute
 RT: Room temperature
 Rab: Ras-associated binding
 RACK1: Receptor for activated C kinase 1
 ReAsH: Fluorescent, membrane-permeable biarsenical compound
 SDS: Sodium dodecyl sulphate
 siRNA: Small interfering RNA
 SM: Sphingomyelin
 SF2: Superfamily 2
 SGR: Subgenomic replicon
 SGR NS3-NS5B or WT: SGR JFH-1 Feo NS3-NS5B
 SGR JFH-1 NS5A-eGFP: SGR JFH-1 Feo NS3-NS5B (with NS5A tagged with eGFP)
 SGR JFH-1 NS5A-mCherry: SGR JFH-1 Feo NS3-NS5B (with NS5A tagged with mCherry)
 SGR JFH-1 NS5A-eGFP-CoilE: SGR JFH-1 Feo NS3-NS5B (with NS5A tagged with eGFP and CoilE)
 SGR JFH-1 NS5A-eGFP-MiniE: SGR JFH-1 Feo NS3-NS5B (with NS5A tagged with eGFP and MiniE)
 SGR JFH-1 NS3-MiniE NS5A-eGFP: SGR JFH-1 Feo NS3-NS5B (with NS3 tagged with MiniE and NS5A tagged with eGFP)
 SR-BI: Scavenger receptor, class B type 1
 ss RNA: single stranded RNA
 SEM: Scanning electron microscopy
 SVR: Sustained virological response

SMV: Single membrane vesicle
SNAP tag: Modified form of DNA repair enzyme, human O⁶ alkylguanine DNA-alkyltransferase (AGT)
SMInV: Single membrane inner vesicle
STA: Subtomogram averaging
TGN-46: Trans Golgi network-46
TEM: Transmission electron microscopy
TAE buffer: Tris acetate buffer
TBS: Tris buffered Saline - Urea
TBS-T: Tris buffered Saline-tween 20
TCEP: Tris 2-carboxyethyl phosphine
TOMM20: Mitochondrial import receptor subunit
TLR3: Toll-like receptor 3
TRIF : Toll-interleukin-1 receptor domain-containing adaptor-inducing beta interferon
TfR1: Transferrin receptor protein1
VAPs: Vesicle-associated membrane proteins (VAMP)-associated proteins
VAP-A: Vesicle-associated membrane protein-associated protein A
ViCs: Vesicles in Cluster
VLDLs: Very low-density lipoproteins
VIPER: Versatile Interacting peptides MiniE-MiniR or CoiE-CoiR
UA: Uranyl acetate
UTR: Untranslated regions
UPR: Unfolded protein response
V: Volts
WHO: World health organisation
WT: Wild-type
Zn: Zinc

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Chapter 1| Introduction

1.1 Hepatitis C virus (HCV)

1.1.1 HCV transmission and epidemiology

Currently, an estimated 58 million individuals worldwide are chronically infected with Hepatitis C virus (HCV), and there are around 1.5 million new infections reported annually. Without intervention, acute infections can progress to liver cirrhosis and hepatocellular carcinoma (Garcia M, 2001; WHO, 2023). HCV spreads primarily through parenteral routes, with intravenous drug administration and blood transfusions being the most common methods. Additional potential transmission routes include body piercing, tattooing, intranasal cocaine use, and accidental injuries involving contaminated needles (Conry-Cantilena et al., 1996).

The virus is classified in the Hepacivirus genus within the *Flaviviridae* family. Through sequencing and phylogenetic analysis of HCV isolates from diverse geographical locations, it has been determined that the virus can be classified into eight genotypes (gt) and 93 subtypes (Figure 1.1). These genotypes and subtypes exhibit differences at the nucleotide level of ~ 30% and 15% respectively (Schnell et al., 2018; Hedskog et al., 2019). At present, the International Committee on Taxonomy of Viruses (ICTV) has officially recognised and updated the classification to include 93 subtypes (Smith et al., 2023).

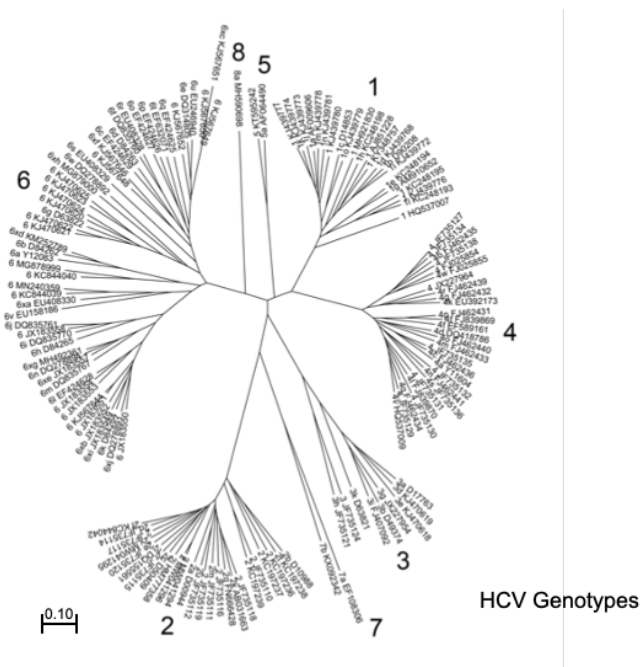


Figure 1.1: Phylogenetic classification of family of *Flaviviridae* and HCV genotypes.

Adapted from the official International Committee on Taxonomy of viruses (Smith et al., 2023).

The global prevalence of HCV exhibits variations, with the most widespread infections attributed to genotype 1 (46%) and genotype 3 (30%), with respect to geographic distributions of genotypes. Specifically, one-third of genotype 1 cases are concentrated in East Asia, while genotype 3 cases are predominantly found in South Asia (Messina et al., 2015). Genotypes 2, 4, and 6 collectively account for 9.1%, 8.3%, and 5.4% of HCV cases worldwide, respectively. Genotype 2 is predominantly prevalent in East Asia, whereas the highest number of genotype 4 cases is observed in North Africa and the Middle East. Genotype 5 represents the smallest proportion of cases globally, accounting for less than 1% of all HCV cases, with the majority found in Southern and Eastern sub-Saharan Africa (Messina et al., 2015). The World Health Organization's (WHO) commitment to eliminating HCV cases (Dhiman and Premkumar, 2020) has led to comprehensive investigations of HCV prevalence in individual countries. Progress reports for elimination from countries such as China (Zhao et al., 2022), Mexico (De la Torre Rosas et al., 2021), Europe (Pawlotsky, 2023), and UK (Burki, 2023) have been reported in recent years.

1.1.2 Morphology of HCV virion

The morphology of HCV particles found in HCV-infected blood donors was initially determined by an indirect immunogold electron microscopy (EM) study. Polyclonal and monoclonal antibodies against putative HCV envelope proteins were tested on two plasma samples with high HCV RNA titres. By sucrose density gradient centrifugation, spherical virus-like particles were found to be of 55 to 65 nm in diameter with spike like projections in 1.16 g/ml fractions (Kaito et al., 1994). The viral core, housing the viral genome, was measured at 30-35 nm, while the viral spikes on the virion membrane, formed by E1 and E2 glycoprotein heterodimers, were found to be 6 nm. (Kaito et al., 2006). HCV JFH-1 virions from cell culture imaged by negative stain transmission electron microscopy (TEM) and cryo-EM had a diameter of 50 nm (Yu et al., 2007). The overall morphology of the virion is not icosahedral due to its association with lipoproteins, low-density lipoproteins (LDLs) and very low-density lipoproteins (VLDLs) (Yu et al., 2007; Gastaminza et al., 2010; Catanese et al., 2013; Vercauteren et al., 2014). Cryo-EM micrographs of HCV virus, HCV- lipoviral particle (LVPs) and a schematic model of HCV-LVP is shown in Figure 1.2.

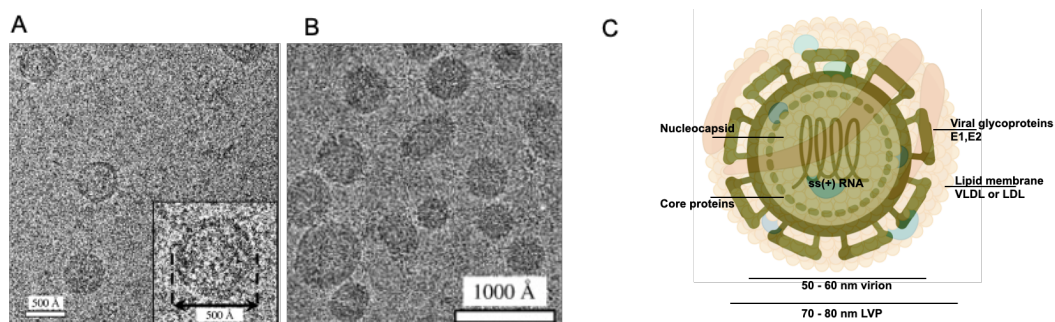


Figure 1.2: Morphology of HCV virus and virus like particle.

[A] Cryo-EM micrograph of JFH-1 HCV virions produced in cell culture and purified by iodixanol density gradient centrifugation. The diameter of the virion was established as ~50 nm with a smooth surface morphology and with a multilayer outer structural organisation (inset). [B] Cryo-EM micrograph of HCV-LPs illustrating the heterogeneity in size. [A,B]: micrographs reproduced from (Yu et al., 2007). [C] Schematic representation of HCV-LP illustrating the overall morphology of HCV-LP.

1.1.3 Models to study virus replication

The progress in HCV research can be attributed to the development of several model systems which are frequently used for investigating various aspects of the HCV lifecycle. The *in-vitro* models employ established cell lines and primary hepatocytes, *ex-vivo* models implement liver slices, and *in-vivo* models rely on using transgenic and humanised mice, Tupaia (tree-shrew) and chimpanzee (Morozov and Lagaye, 2018). To study virus replication, infectious clones, and subgenomic replicons (SGR) and HCV cell-cultured-derived virions (HCVcc, typically based on the JFH-1 clone or chimaeras consisting of JFH-1 replicase genes) are utilised.

The HCV genome was identified in 1989 from infected chimpanzee (Choo et al., 1989). Later, a full-length functional clone of HCV complementary DNA (cDNA) was constructed. The RNA transcripts from the clones were found to be infectious and caused disease in chimpanzees after direct intrahepatic inoculation (Kolykhalov et al., 1997). Similarly, consensus cDNA clones of H77 (gt1a) were constructed in a cassette vector with fixed 5' and 3' termini that led to infectious RNA transcripts (Kolykhalov et al., 1997). However, it was the generation of SGR cloned from HCV genomes that accelerated our understanding of the molecular biology of HCV. A monocistronic or a bicistronic SGR can be generated depending on the research objective. A monocistronic SGR is under the control of one internal ribosome entry site (IRES), whereas a bicistronic SGR is composed of two IRES (Khan et al., 2020). Examples of bicistronic SGRs are illustrated in Figure 1.3.

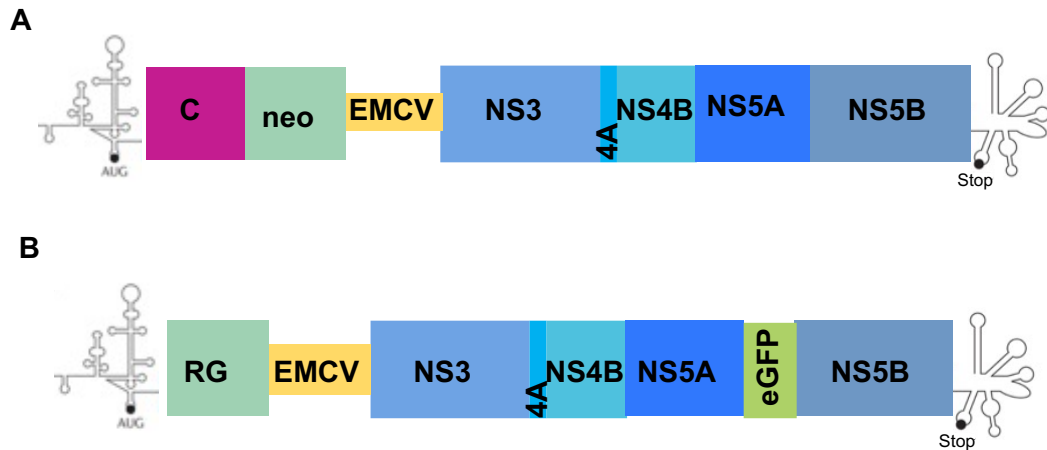


Figure 1.3: Schematic examples of bicistronic SGRs comprised with two IRES.

[A] Classical bicistronic SGR, HCV IRES at 5'UTR drives the translation of first 16 codons of core, followed by a neomycin (*neo*) resistance reporter gene (RG) carrying *neo*; EMCV IRES drives the translation of NS3-5B coding region [B] Variation of the bicistronic SGR from A, HCV IRES drives the translation of RG, and NS5A is fused with a fluorescent tag reporter.

A cell culture system was established in Huh7 cells through the transfection of a HCV SGR derived from gt1a, which included a neomycin resistance gene (*neo*) (Lohmann et al., 1999; Bartenschlager, 2002). This became a fundamental workhorse in HCV research and paved a way to understand replication of HCV in cell culture model systems. The JFH-1 genome and constructed SGRs demonstrated efficient replication in Huh7 cells and the ability to generate infectious particles upon transfection with JFH-1 transcripts (Wakita et al., 2005). However, the infection efficiency of JFH-1 derived from Huh7 was observed to be low and suggested a role of cellular environment as a major determinant for HCV replication.

Cell lines from population of Huh7 and Huh7-clones, which supported adapted and nonadapted SGR replication, were treated with interferon-alpha (IFN- α) (cured) to eliminate HCV RNA (Blight et al., 2002). This facilitated establishment of the cured cell lines Huh7.5 (Blight et al., 2002), characterized by a missense mutation in the retinoic acid-inducible gene I (RIG-I or DDX58). In general, a great proportion of the treated cells demonstrated the ability to support HCV replication, enabling the transient detection of both SGR and full-length genome replication through various assays (Blight et al., 2002). RIG-I gene participates in intracellular antiviral defence (Sumpter et al., 2005). However, cellular factors other than RIG-I were suggested to be responsible for controlling HCV replication (Feigelstock et al., 2010).

1.1.4 Genome arrangement

HCV possesses a 9.6 kb enveloped positive single-stranded genome. This genome is composed of a single open reading frame (ORF), situated between two untranslated regions (UTR), namely the 5'UTR and 3'UTR (Choo et al., 1989; Kato et al., 1990). The 5'UTR and 3'UTR are both highly structured and conserved among genotypes as well as subtypes (Bukh et al., 1992; Blight and Rice, 1997). UTRs are crucial for viral replication and proliferation. Translation is initiated by IRES at the 5'UTR in endoplasmic reticulum (ER) (Lindenbach et al., 2005). The translated polyprotein is proteolytically processed into ten proteins by host and viral proteases (Moradpour et al., 2007). The genome organisation, polyprotein processing, protein topology on a membrane with their functions are illustrated in Figure 1.4.

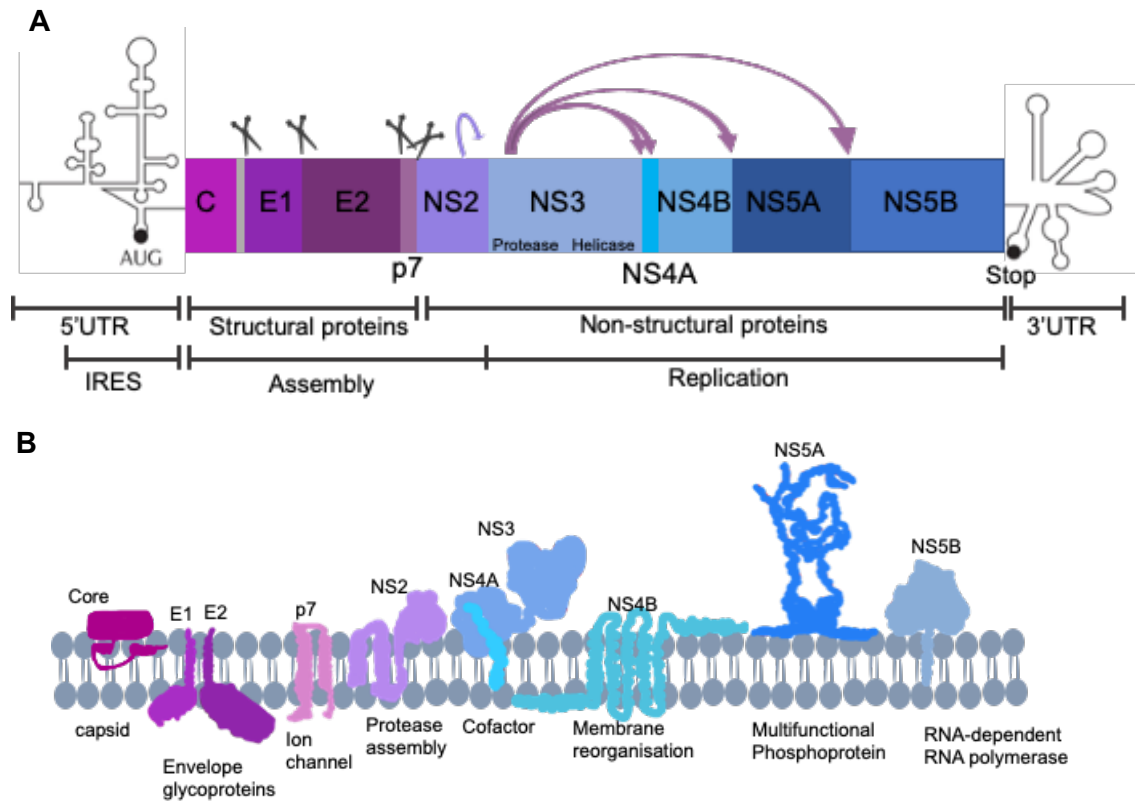


Figure 1.4: HCV genome organisation and membrane topology of proteins after polyprotein processing.

[A] The polyprotein, typically consisting of 3011 amino acid residues, is encoded by a single ORF flanked by highly conserved 5'UTR and 3'UTR. The 5'UTR contains the IRES, and the start and stop codon are indicated with a black dot located in the UTRs. Host cell and viral proteases cleave the polyprotein into ten mature proteins, categorized as structural and non-structural proteins. The first third of the polyprotein includes four structural proteins—core, envelope glycoproteins E1 and E2, and the p7 viral cation channel—that mainly contribute to viral particle formation. The remaining six NS proteins—NS2, NS3, NS4A, NS4B, NS5A, and NS5B—are primarily involved in viral RNA replication and play roles in HCV particle assembly and release. Cellular signal peptidases and viral proteases cleave the polyprotein, indicated by scissors and arrows, respectively. [B] Membrane topologies and functions of cleaved proteins. Each protein is tethered to the membrane by either amphipathic α helices (core, NS5A, NS3) or transmembrane segments. NS3 is bound to the membrane via a small α -helix and via the NS4A protein intercalated with the NS3 protease domain. The membrane topologies and functions of cleaved proteins are diverse. While NS5A is depicted as a dimer, most, if not all, proteins form homo or heterodimers (e.g., core and E1E2) or oligomeric complexes (e.g., p7). Adapted from (Bartenschlager et al., 2013).

1.2 HCV lifecycle

1.2.1 Binding, entry, and post-entry events

The process of HCV replication is intricate, requiring coordinated interactions among various host receptors and viral proteins. Many researchers have identified several host factors involved in the processes of viral entry. These processes includes the viral attachment to the surface of the hepatocyte, receptor mediated endocytosis of the viral particle and endosomal fusion to release HCV genome into cytosol (Colpitts et al., 2020)

The HCV virus particles, after circulating in the bloodstream as either free particle or enclosed by host LDLs, enter the target cell by interacting with a series of cell surface receptor proteins. Initially, HCV LVPs engage with heparin sulfate glycosaminoglycans (HSPG), followed by interactions with the LDL receptor (LDLR) and scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002; Catanese et al., 2010). In addition to viral attachment, these host factors have been shown to contribute to post-binding and internalisation (Colpitts et al., 2020).

Cell surface factors SR-B1, CD81 (cluster of differentiation 81) and CLDN1 (Claudin1) act as viral receptors and coordinate the viral internalisation in temporally regulated process. Expression levels of SR-B1 have been shown to play a key role in virus internalisation rates (Grove et al., 2007). It has been suggested that the SR-B1 protein that is involved in cholesterol uptake binds to E2, resulting in a modified LVP with enhanced interactions between E2 and CD81 (Catanese et al., 2010). However, it remains undetermined whether SR-B1 and CD81 form a complex facilitating the entry steps. While experimental and mathematical approaches indicate that both CD81 and SR-B1 are essential for efficient entry, a minority of viral particles appear capable of entry without SR-B1 (Kalemera et al., 2019).

The complex formation between viral protein E2 and CD81 receptor (Roberto et al., 2000) facilitates the activation of signalling pathways resulting in activation of tyrosine kinase receptors such as epidermal growth factor receptor (EGFR) (Lupberger et al., 2011), Rho and Ras GTPases (Brazzoli et al., 2008; Zona et al., 2013) Activation of EGFR subsequently activates HRas which promotes actin rearrangements, thereby inducing a lateral diffusion of CD81 to interact with tight junction proteins CLDN1 and Occludin (OCLN) (Lupberger et al., 2011). EGFR-CD81 interaction facilitates the translocation of the virus to the tight junction for endocytosis (Mailly and Baumert, 2020). Additionally, EGFR has been proposed to help recruit clathrin coated vesicles for endocytosis (Baktash et al., 2018). Interactions with entry factors promote internalisation via clathrin-mediated endocytosis, and dynamin-dependent endocytosis (Hsu et al.,

2003; Tscherne et al., 2006; Blanchard et al., 2006; Farquhar et al., 2012) as well as alternate endocytic pathways (Matsuda et al., 2014). Other host factors identified that contribute to HCV internalisation include, Niemann-Pick C1-like 1 (NPC1L1) as an entry factor in cell culture and a role in cholesterol uptake, (Sainz et al., 2012), transferrin receptor 1 in particle uptake (TfR1) (Martin and Uprichard, 2013) and Abl tyrosine kinase, acting during clathrin-mediated endocytosis (Min et al., 2017).

After viral internalisation, HCV particles have been shown to transport with GFP-Rab5a positive endosomes, along with actin stress fibers (Coller et al., 2009). Endocytic vesicles eventually mature into acidic endosomes, thus promoting low pH-dependent HCV fusion. The low pH is critical in driving the conformational rearrangement of the glycoproteins to release the fusion peptide. CD81, plays a dual role in entry and to render E1E2 competent for fusion with acidic compartments (Fénéant et al., 2014).

HCV glycoproteins E1 and E2 form a non-covalent heterodimer that mediates fusion. Two distinct studies have highlighted the crucial involvement of E1 and E2 transmembrane domains. Additionally, these studies have demonstrated the significance of different regions of the E2 ectodomain in viral entry and assembly, respectively (Ciczora et al., 2007; Albecka et al., 2011). HCV fusion machinery is unique as E1 protein forms trimers (feature of typical fusion protein) but the formation is co-dependent of expression of E2. On the other hand, E2 harbours a central immunoglobulin (Ig) fold, common among class II fusion protein, the crystal structure revealed a compact globular fold that is inconsistent with class II fusion fold. The molecular mechanisms on the intra-intermolecular interactions of E1 and E2 that drives fusion remains to be elucidated (Colpitts et al., 2020). The acidic environment of the endosomes induces conformational changes in the envelope proteins, facilitating the fusion of the viral and endosomal membranes (Blanchard et al., 2006). A schematic model of HCV entry steps is shown in Figure 1.5.

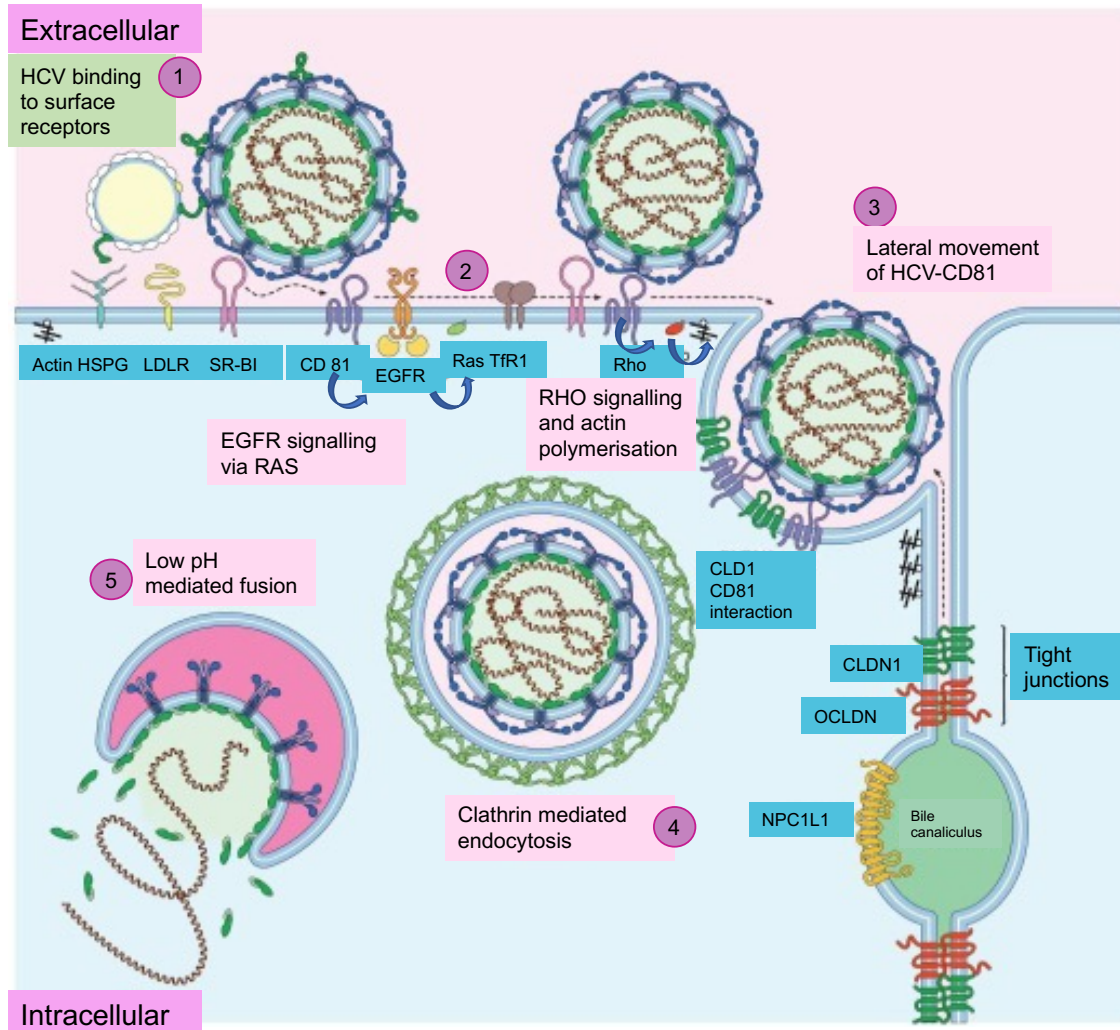


Figure 1.5: HCV entry into the cell.

Step 1: HCV LPs attach to a series of cell surface receptors: HSPG, LDLR and SR-B1 before interacting with CD81. Step 2: Interaction of CD81 with viral envelope protein E2 mediates EGFR signalling through Rho GTPases. Step 3: Lateral movement of the viral particle to sites of cell-to-cell contact. Step 4: Interaction of CD81 with Claudin1 (CLDN1) facilitates HCV internalisation by clathrin-mediated endocytosis. Step 5: The acidic and low pH of endosomal compartment induces HCV membrane fusion and release of the genome into the cytoplasm. Adapted from (Lindenbach and Rice, 2013).

1.2.2 Polyprotein translation

Translation of HCV polyprotein begins with the attachment of the viral RNA to the host ribosome. The IRES located within the 5'UTR facilitates cap-independent translation initiation, enabling the ribosome to bind directly to the viral RNA a rough endoplasmic reticulum (Lancaster et al., 2006) .There are various methods through which the initiation of the translation process can take place, and is reviewed extensively (Niepmann and Gerresheim, 2020) .

In one potential pathway, the IRES can directly engage the 40S ribosomal subunit, forming a binary complex. Subsequently, this complex interacts with eIF3 (eukaryotic initiation factor 3), resulting in the assembly of the 40S-IRES-eIF3 unit (Niepmann and Gerresheim, 2020). Alternatively, the IRES can associate with an already assembled 40S-eIF3 complex. Following these initial steps, the ternary complex (40S-IRES-eIF3) incorporates eIF2, which is loaded with tRNAⁱMET and GTP, along with eIF5 and eIF1a. Once the HCV AUG start codon is identified, the release of decharged eIF2-GDP is facilitated by eIF5 (Niepmann and Gerresheim, 2020). The 60S subunit then joins the 40S subunit, assisted by eIF5b. Subsequently, eIF5B, eIF1A, and eIF3 disengage from the ribosome, preparing it for the commencement of the first initiation and elongation steps. (Niepmann and Gerresheim, 2020). There are multiple other pathways discussed for 80S-IRES formation in healthy and stress related conditions, and when there are limited initiation factors available.

Recently, detailed investigations on how IRES captures ribosomes was analysed by cryo-EM structures and showing that HCV IRES binds to both an actively translating 80S ribosome, as well as the 40S subunit (Yokoyama et al., 2019). As a result, a proposed model of HCV translation hijacked by HCV IRES was derived and is shown in Figure 1.6.

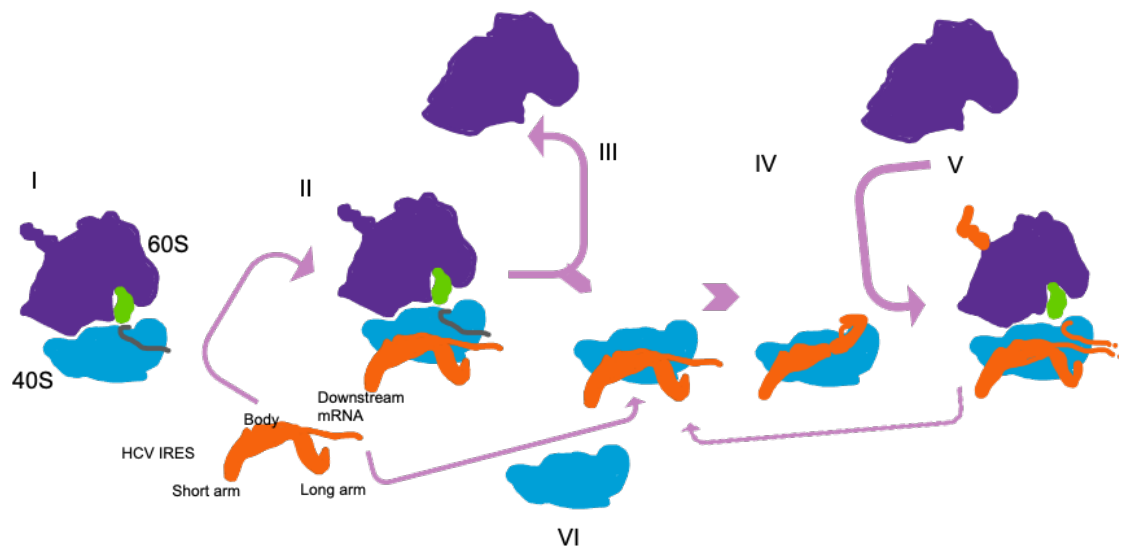


Figure 1.6: Hijacking of translation machinery by HCV IRES.

(I) A distinct mRNA downstream of the HCV IRES is translated by a ribosome. (II) The translating 80S ribosome is seized by the HCV IRES. (III) Even after translation termination, the HCV IRES remains attached to the 40S subunit. (IV) Using its elongated arm, the HCV IRES positions its downstream mRNA onto the 40S subunit. (V) The HCV IRES holds onto the captured 80S ribosome, which continues to translate its own downstream mRNA (VI) The HCV IRES also targets a free 40S subunit. Adapted from (Yokoyama et al., 2019).

1.2.3 Genome replication: Biogenesis of the membranous compartments induced by HCV

Polyprotein translation and genome replication are two inter-connected processes which are mediated by HCV RNA regulatory elements and cellular proteins (Isken et al., 2007; Romero-López and Berzal-Herranz, 2009; Niepmann et al., 2018). For the HCV replication, a negative strand RNA is synthesised by the NS5B RNA-dependent RNA polymerase which may further act as a template to synthesise the positive strand of the viral RNA (Murakami et al., 2006). The architecture of how non-structural proteins assemble to form the replicase machinery is unknown and is the focus of this study.

Positive-sense RNA viruses induce membrane rearrangements in the host cell forming specialised compartments for genome replication. HCV infection induces production of numerous vesicles derived primarily from ER and was referred to as the membranous web (MW) (Romero-Brey and Lohmann, 2016). This MW is composed of single, double, multi-membrane vesicles, and has been observed in the cytoplasm of HCV infected

hepatocytes by EM (Egger et al., 2002; Gosert et al., 2003). In recent years, these specialised compartments (or vesicles) have been defined as replication organelles (RO) as they serve as a platform for the assembly of the replicase machinery (Wolff et al., 2020a). On the basis of these structures, positive strand viruses are classified into two groups (Romero-Brey and Lohmann, 2016; Wolff et al., 2020a). One class primarily forms spherules via the formation of invaginations in specific host organelles, such as the ER, mitochondria or endolysosomes (Wolff et al., 2020a). The other class generates vesiculotubular membrane clusters comprising of different structural elements, among which double membrane vesicles (DMVs) are found to be a recurrent motif (Wolff, 2020).

Ultrastructural analyses by EM on HCV induced structures either by replicons or an infectious virus have reported accumulation of a variety of membrane vesicles, among which double membrane vesicles (DMVs) are the most predominant (Romero-Brey et al., 2012). DMVs are reported typically in the range of 100-300 nm in diameter and are distributed throughout the cytoplasm of cells (Romero-Brey et al., 2012; Romero-Brey and Lohmann, 2016). From this study, models suggesting that DMVs could be generated either by evaginations or invaginations from the ER were reported.

Our understanding of the ROs and how HCV use these compartments for replication of their genome is limited and challenging due to the morphological variations in these membranes and complexity of host-cellular protein interactions. Therefore, this study aims to investigate these membranous compartments by employing *in-situ* sample preparation techniques and cryo-electron tomography (cryo-ET).

1.2.3.1 Linkage between membrane alterations in host cells and HCV RNA synthesis

Among the several types of vesicles observed during HCV infection, the functional link between DMVs as replication sites was proposed based on the fact that double stranded RNA (dsRNA), presumptively an RNA intermediate, was localised to the lumen of these vesicles by immunogold labelling EM (Ferraris et al., 2010). Also, purified DMVs contain HCV RNA (Ferraris et al., 2010) and retain the ability to synthesise RNA *in vitro* (Paul et al., 2013). The production of DMVs increases during an infection and correlates with the kinetics of HCV replication as determined by immunofluorescence (Romero-Brey et al., 2012). The accumulation of DMVs was also observed around lipid droplets (LDs) (Romero-Brey et al., 2012), which play an important role in the assembly of virions by association with both the core and NS5A, suggesting the closed spatial organisation of replication and assembly site (Miyazawa et al., 2007; J.Y. Lee et al., 2019).

The key argument against DMVs as *bona fide* replication sites is based on the apparent absence of a pore or an opening. Electron tomography (ET) of HPF-FS resin embedded sections has shown that only 8 % of DMVs have been observed with an opening towards the cytosol (Romero-Brey et al., 2012), posing the question whether only a minority of the DMVs are actively participating in replication at a given time or if this was due to the resolution limit of the methods used so far to visualise them (Romero-Brey and Lohmann, 2016). Alternatively, DMVs could transiently open to facilitate the transfer of nucleotides and NS proteins, swiftly closing thereafter to protect them from detection by the host immune system. It is also possible that the five non-structural proteins (NS3-5B) that form the replication complex could reside on the surface (Paul et al., 2014a). Larger and more complex multi-membrane vesicles (MMV) are also observed by EM as the infection progresses, however the role of MMVs during an infection is not clear (Ferraris et al., 2010; Romero-Brey et al., 2012).

1.2.3.2 Role of viral proteins and host pathways in the formation of DMVs

DMVs are observed following HCV infection but also in cells harbouring SGR, suggesting that structural proteins are not crucial for replication and formation of the DMVs (Lohmann et al., 1999; Ferraris et al., 2010; Romero-Brey et al., 2012). The intracellular membrane rearrangements were compared on cells transfected with SGR encoding NS2-5B and NS3-5B polyprotein. In both cases, DMVs resembled those observed in HCV infected cells. However, lower amounts of DMVs were noted when transfected with NS2-5B replicon compared to NS3-5B replicon. Additionally, cells expressing polyprotein NS3-5B (in the absence of UTRs) also induced formation of DMVs, suggesting these structures are induced by viral proteins independent of HCV RNA replication. The model (NS3-5B polyprotein fragment, with and without UTRs) was also used to demonstrate that highly potent NS5A inhibitor (Daclatasvir) disrupt MW formation independent of RNA replication (Berger et al., 2014). Polyprotein expression of NS3-5A, lacking NS5B (RdRp) also generated MW mainly composed of elongated DMVs, classified as double membrane tubules (DMTs), suggesting that NS5B is not a mandatory building block of MW but still influences their morphology (Romero-Brey et al., 2012).

The potential of individual HCV proteins to induce membrane alterations was also characterised. NS3/4A forms a stable membrane-associated complex and are physiologically relevant, therefore NS3/4A complex was studied together and observed to induced large amounts of smooth single membrane vesicles (SMVs)(Romero-Brey et al., 2012). NS4B generated the MW with compact clusters of vesicles about 85 nm. NS5A led to the formation of DMV and MMV, although with low abundance and different sizes as compared with the polyprotein NS3-5B expression (Romero-Brey et al., 2012).

NS5B induced enlargements of the ER with invaginations present occasionally. This suggested all the NS proteins involved in the replicase machinery could individually induce the membrane alterations, with NS5A inducing the most membrane curvature (Romero-Brey et al., 2012). Therefore, the replicon system (NS3-5B) was chosen for the current study to induce membrane alterations similar to HCV infected cells. The differences in the formation of vesicles from polyprotein expression and individual NS proteins is shown in Figure 1.7.

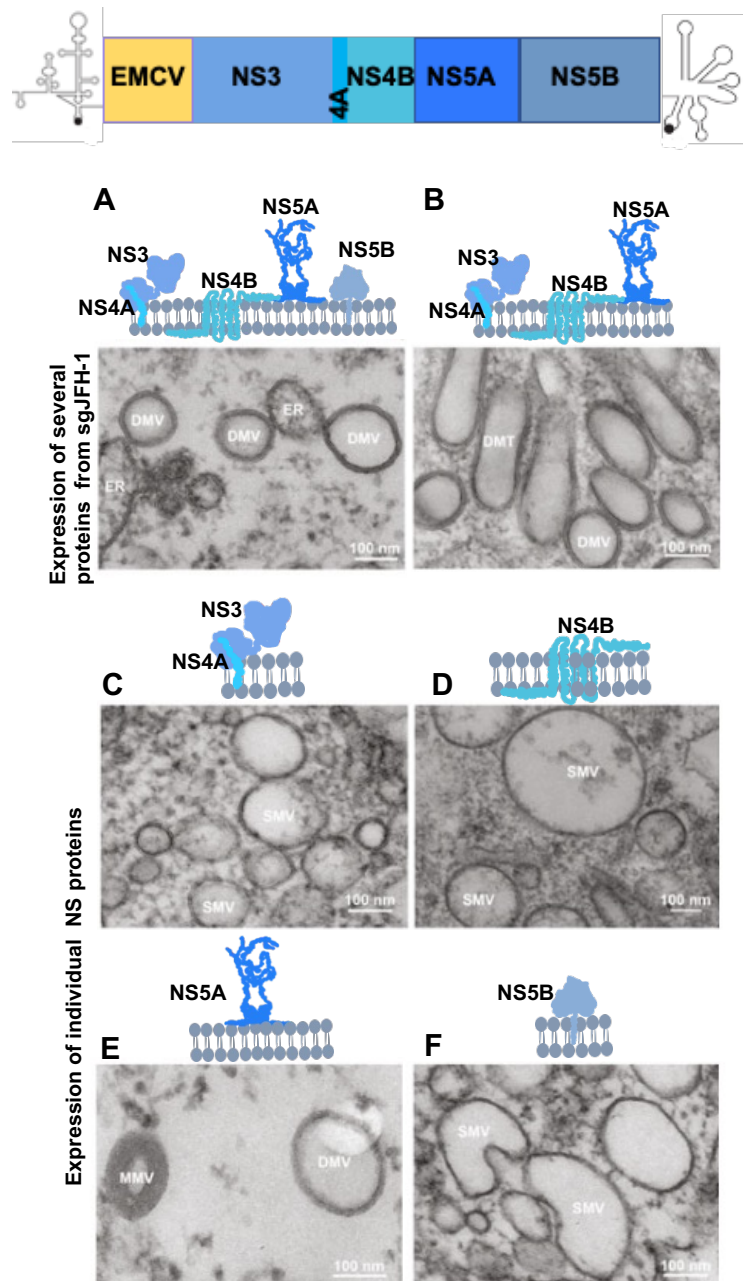


Figure 1.7: Analysis of the membrane alternations induced by polyprotein and individual NS.

[A] Membrane alterations induced by SGR polyprotein NS3-5B [B] Membrane alterations induced by SGR polyprotein NS3-5A. Expression of polyprotein lacking NS5B induced smaller DMVs and elongated DMTs with a variable diameter of 166 ± 92 nm compared to the expression of NS3-5B. [C] Expression of NS3/4A complex induced swollen ER sheets and variable diameter of 203 ± 93 nm. [D] Expression of single NS4B was found to generate larger SMVs (325 ± 168 nm). [E] Expression of NS5A leads to formation of DMVs and MMVs, identical to polyprotein expression in cells harbouring SGR or infected with HCV. [F] NS5B led to enlarged ER sacs with an average diameter of 370 nm. Adapted from (Romero-Brey and Lohmann, 2016).

Overall, biogenesis of DMVs is a complex process and requires multiple steps with numerous interactions between host and viral proteins. This includes the initiation of both positive and negative curvature, membrane pairing, and various membrane fission events, depending on the specific topology of the DMV (Wolff et al., 2020a). All viruses that induce DMV formation by remodelling the ER appear to hijack membranes of the secretory pathway and autophagy pathways (Wolff et al., 2020a). The induction of autophagy is well studied and is hypothesised to provide the membrane structures for the formation of HCV factories (Chan and Egan, 2005; Ke and Chen, 2011; Shinohara et al., 2013; Mohl et al., 2016).

The process of autophagy induced by HCV is orchestrated by direct interactions with the viral proteins (primarily NS5A or NS4B) (Guévin et al., 2010; Su et al., 2011; Aweya et al., 2013) which alter the maturation of autophagosomes and prevent fusion with lysosomes (Sir et al., 2008). The role and importance of ATG5 (autophagy related 5) has been reported in DMV biogenesis in HCV replication. Limiting the formation of ATG12 conjugates during infection has resulted in production of smaller and fewer DMVs (Fahmy and Labonté, 2017). In recent years, NS5A was shown to induce autophagy and DMV formation with the help of RACK1 (receptor for activated C kinase1) (J.S. Lee et al., 2019). Rack1 was shown to promote autophagy by enhancing the ATG14L-Beclin1-Vps34-Vps15 complex under starvation conditions. This complex is referred to as vesicle nucleation complex and is crucial for autophagy induction (Zhao et al., 2015). Coimmunoprecipitation experiments suggested that distinct components of the vesicle nucleation complex interact with HCV NS5A directly or indirectly (J.S. Lee et al., 2019). The morphological similarities in HCV DMV and autophagosomes suggests that they may share common biogenesis pathways.

1.2.3.3 Structure and functional role of non-structural proteins involved in the replicase assembly

1.2.3.3.1 Structure and function of NS3 and the cofactor NS4A

The NS3 is a bifunctional protein composed of 631 aa (JFH-1 is 67 kDa, GenBank: BAB32872.1) with serine protease and helicase activities that are essential for HCV replication (Jin and Peterson, 1995). NS4A is a 54 amino acid (8 kDa) protein and acts as a cofactor for NS3 protease activity (Lin et al., 1997). NS3 is cleaved from the polyprotein at the N-terminus by the viral NS2/NS3 autoprotease (Grakoui et al., 1993). NS3 along with its cofactor NS4A is involved in cleavage of the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions (Bartenschlager et al., 1993; Failla et al., 1995).

Multiple mutational and structural studies indicated that helicase and protease domains of NS3 are linked covalently, and thus interact and regulate each other. Structural analysis of an engineered single chain (sc) NS3-4A protein (gt1) demonstrated that the C-terminus of the helicase resides in the protease active site, supporting a *cis*-cleavage at the NS3/4A junction (Yao et al., 1999). This study also implies the potential occurrence of domain rearrangement between the protease and helicase. Such rearrangement would enable the protease to execute trans cleavages at junctions of other NS proteins (Yao et al., 1999).

The N-terminal domain of NS3 encodes for a serine protease domain (Grakoui et al., 1993). This domain adopts a chymotrypsin-like fold, which is a common structural motif in many serine proteases. This fold is composed of central beta-sheets surrounded by alpha-helices creating a well-defined catalytic cleft where substrate binding and catalysis occur (Kim et al., 1996). The protease's enzymatic activity relies on a catalytic triad composed of histidine, aspartate, and serine (Gu and Rice, 2013).

The C-terminal domain of NS3 has helicase and NTPase activity responsible for binding and unwinding of the viral RNA in a 3'-5' direction during replication (Kim et al., 1998; Murayama et al., 2007). The NS3 helicase domain belongs to the superfamily 2 (SF2) of helicases, containing the two RecA-like domains. Crystallographic structures of three distinct conformational states revealed that nucleic acids bind to the surface formed by the rec-A domains which can swing toward each other upon nucleotide binding defining the structural basis of an inchworm/ratchet motion (Gu and Rice, 2010).

JFH-1 NS3 shares 79 to 81% sequence identity with gt1 isolates. Crystallographic structural analysis of JFH-1 NS3-4A with gt1b NS3-4A observed both structures to be

composed of four globular domains arranged in a tetrahedral fashion (Zhou et al., 2018). Domains 1 (D1) and 2 (D2) constitute an ATPase core, which is capped by domain (D3) completing the RNA binding cleft. The protease domain in complex with NS4A is packed along the back of these domains (Zhou et al., 2018). The key difference in the two genotypes is in the conformational shift in position of D2 (and not amino acid composition), caused by a twist in the conserved B14/15 hairpin loop (Zhou et al., 2018). The hairpin loop has been shown to play an important role in SF2 helicase enzymes in RNA unwinding and ATP hydrolysis (König et al., 2013). The crystal structure of NS3 with NS4A cofactor is shown in Figure 1.8.

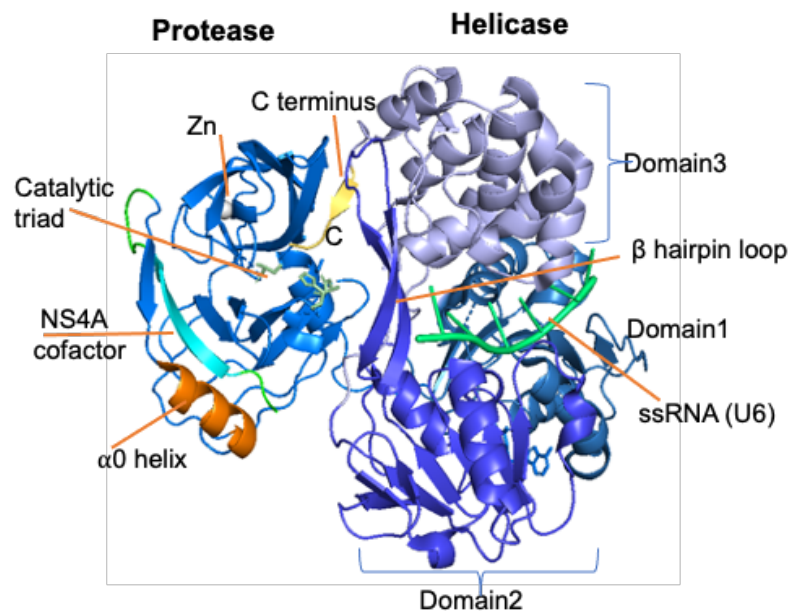


Figure 1.8: Crystal structure of NS3 from gt1b (PDB: 3O8R) in complex with ssRNA (U6).

The protease domain is coloured in azure blue, with NS4A cofactor highlighted in cyan, α_0 helix in orange and the catalytic triad in olive green. The Helicase Domain 1, 2, 3 are coloured as true blue, violet and lavender respectively. The ssRNA is shown in green and the C-terminal end residue are shown in yellow. Adapted from (Gu and Rice, 2013).

NS3/4A plays an important role in confounding the host innate immune response to viral infection by impeding the activation of the transcription factors interferon regulatory factors 3 (IRF-3) and nuclear factor kB (NF-kB). Upon viral RNA detection in host, the NS3/4A protease activity disrupts the Toll-like receptor 3 (TLR3) or retinoic acid-inducible gene (RIG-I) pathways by cleaving the important adaptor proteins mitochondrial antiviral signalling protein (MAVS) and Toll-interleukin-1 receptor domain-containing adaptor-inducing beta interferon (TRIF). Additionally, a conserved NS3 surface patch orchestrates NS2 stimulation, NS5A hyperphosphorylation and HCV genome replication (Isken et al., 2015). NS3 interacts with NS5B mediated by the protease domain of the NS3 (Zhang et al., 2005).

1.2.3.3.2 Structure and function of transmembrane protein NS4B

NS4B is a hydrophobic protein composed of 217 aa (27 kDa). NS4B was shown to localise in the ER as punctate foci by subcellular fractionation, immunofluorescence analyses and double label-confocal microscopy when it was expressed individually or in the context of the entire HCV polyprotein (Hügler et al., 2001). NS4B is liberated by NS3-4A serine protease (Tanji et al., 1994). The topology of NS4B is believed to have an N terminal part composed of two amphipathic helices, a central part with four transmembrane domains and a C-terminal part with two helices (Gouttenoire et al., 2010). A predicted model of NS4B by ColabFold is shown in the Figure 1.9. Predicted local difference test (pLDDT) shows the confidence score per residue. The sequence coverage and predicted aligned error (PAE) is shown in appendix Figure 10.

NS4B plays a key role in the formation of replication complexes and is shown by EM to induce formation of membranous web (MW) by providing a scaffold for HCV replication. NS4B alone can induce the MW on its own via oligomerisation (Egger et al., 2002; Paul et al., 2013). NS4B has a predicted amphipathic helix (AH), found to be conserved among HCV isolates. The N-terminal AH in NS4B mediates membrane association and has a role in HCV replication. Five site directed mutations within the hydrophobic face of the AH resulted in inhibited membrane association. These mutations were also unable to sustain replication under G418 selection pressure (Elazar et al., 2004). Complete disruption of NS4B AH resulted in abolished RNA replication while a partial disruption resulted in intermediate level of replication (Elazar et al., 2004). NS4B C-terminal mutants were also found to influence the intracellular mobility and phosphorylation of NS5A (Jones et al., 2009).

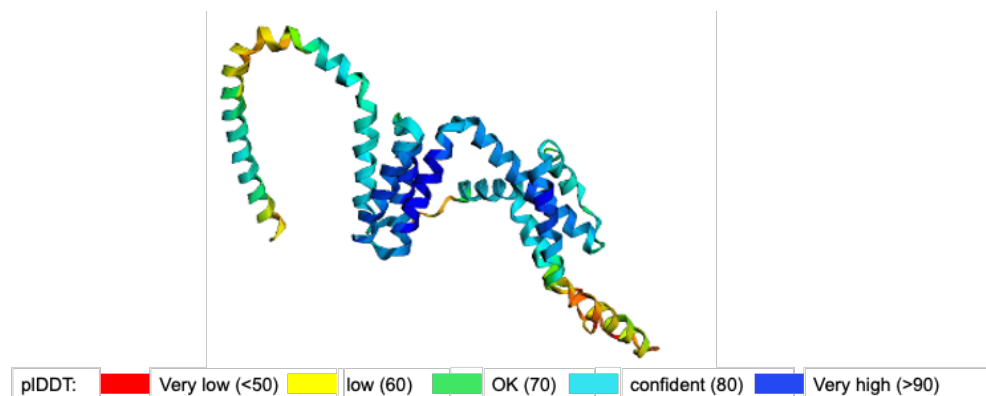


Figure 1.9: Structure prediction of NS4B by ColabFold

Coloured by the per-residue confidence score from 1-100 pLDDT (see colour key).
The structure is composed primarily of helices.

1.2.3.3.3 Structure and function of multifunctional protein NS5A

NS5A protein is composed of 466 amino acids (49 KDa) (Kaneko et al., 1994). NS5A is released from the polyprotein by NS3 protease (Tanji et al., 1994). The structure of NS5A is characterised by an N-terminal amphipathic alpha helix which is conserved across genotypes and plays an important role to target NS5A to intracellular membranes (Elazar et al., 2003; Penin et al., 2004). Bioinformatics-assisted modelling predicted domain organisation model of NS5A also constitutes three structural domains (I, II and III) separated by low complexity sequences (LCSI and LCSII), and a Zn binding motif in Domain I (Tellinghuisen et al., 2004). This was also corroborated by proteolysis experiments. Domain I has been crystallised and found to form two distinct non-overlapping homodimer configurations (Tellinghuisen et al., 2005; Love et al., 2009; Lambert et al., 2014). The differences in NS5A dimer configuration have been documented (Figure 1.10B), indicating the possible existence of an RNA binding groove between the monomers (Tellinghuisen et al., 2005), as opposed to the absence of such a groove (Love et al., 2009). This suggests that NS5A may possess distinct biological properties. Moreover, by introducing mutations at the dimer interfaces, NS5A self-interaction was disrupted, RNA replication and virion assembly was inhibited with some genotypic differences (Shanmugam et al., 2018). The reduced NS5A self-interaction was also associated with altered NS5A-CypA interaction, NS5A hyperphosphorylation and NS5A sub-cellular localisation, indicating the importance of NS5A-self interactions (Shanmugam et al., 2018).

Four conserved cysteine residues were predicted to form a zinc binding motif (C39,C57,C59, and C80) and includes a structural motif (CX17CXCX20C) that is well

conserved among Hepacivirus and Pestivirus (Tellinghuisen et al., 2004). Additionally, four conserved cysteine residues were identified in domain I responsible for NS5A-NS5A (dimer) interactions (Lim et al., 2012). Domains II and III are less conserved and are found to be natively unfolded (Liang et al., 2007; Hanouille, Verdegem, et al., 2009). Domain II is important for RNA replication and may also contribute to the RNA binding properties of the protein. Domain III is essential for the assembly of the virion.

When the polyprotein NS3-NS5A is expressed in tissue culture cells, two alternatively phosphorylated forms have been detected by protein electrophoresis (Koch and Bartenschlager, 1999; Neddermann et al., 1999). A hypo phosphorylated p56 (or basal phosphorylation) characterised by one or more residues phosphorylated at the C-terminal or central part of the protein (Tanji et al., 1995). A hyperphosphorylated p58 has been shown to be phosphorylated at one or more serine residues (Ross-Thriepland et al., 2015b; Goonawardane et al., 2017) within the surface exposed LCSII connecting domains II and III (Neddermann et al., 1999), and depend on the presence of NS4A (Kaneko et al., 1994). This differential regulation has been attributed to regulation of RNA replication and assembly of the virion (Appel et al., 2005; Masaki et al., 2008). The sequence and structures of NS5A domain I are illustrated in Figure 1.10.

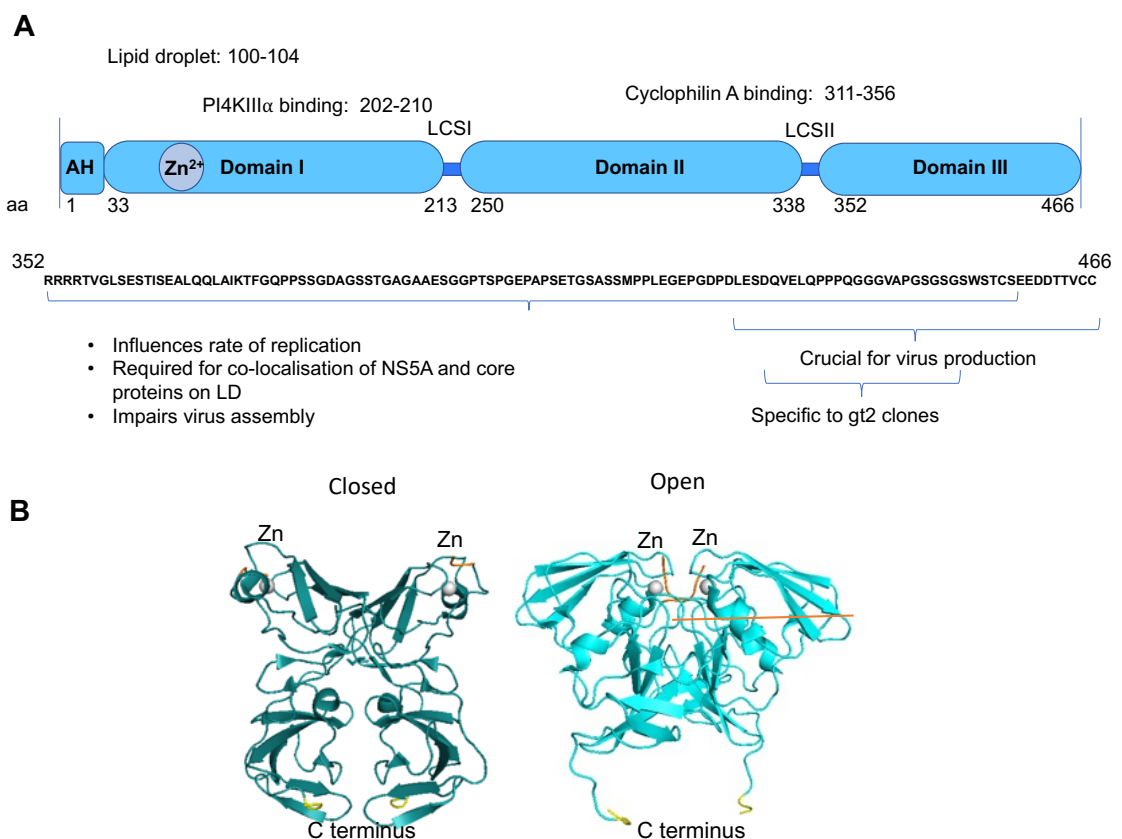


Figure 1.10: Sequence and structure of NS5A.

[A] Amino acid sequence and domain organisation of NS5A. Sequence regions associated with functional activity are shown, (Appel et al., 2005; Hughes et al., 2009)

[B] Two crystal dimeric structures of NS5A Domain I without amphipathic helix, proposed RNA binding groove is denoted by orange line. PDB structures, left: 3FMQ, right: 1ZH1.

1.2.3.3.4 Structure and function of polymerase protein NS5B

NS5B is the RNA dependent RNA polymerase (RdRp) and belongs to a class of membrane proteins called tail-anchored proteins (Schmidt-Mende et al., 2001). The characteristic features of these proteins include (1) posttranslational membrane targeting via a hydrophobic C-terminal insertion sequence that traverses the phospholipid bilayer, which is mapped to the C-terminal part of NS5B (21 aa) (Ivashkina et al., 2002) and is essential for RNA replication in cell culture (Ivashkina et al., 2002). However, it is not important for *in-vitro* RNA synthesis when expressed and purified in heterologous system (Lohmann et al., 1997). In NS5B, the transmembrane helix and the catalytic domain are separated by a linker, highlighting the other key characteristics of these tail-anchored proteins (2) an integral membrane association and (3) cytosolic orientation of the functional protein domain (Ivashkina et al., 2002).

The crystal structure of NS5B of gt2a has the same homologous organisation as observed in gt1b and other polymerases, encompassing a catalytic domain organised as right hand disposition of fingers, palm (subdomains), and thumb domains (Biswal et al., 2005). *In-vitro* RNA synthesis by NS5B can be classified into four distinct steps namely, RNA binding, initiation, elongation, and termination (Simister et al., 2009). Two forms of the NS5B structures have been reported, a closed state encircled by the catalytic domains on one side, and a linker and beta-flap (or β -hairpin) on the other. These closed structures imply two functional roles (1) the closed structure represents and allows the initiation state of the polymerase and allow space for a single stranded template and priming nucleotides (Simister et al., 2009). However, once the RNA synthesis moves from initiation to elongation stage, the linker must be removed for a major structural rearrangement towards open state to allow egress of the primer-template duplex. Such 'open' conformations have been reported with a gt 2a NS5B capable of binding dsRNA (Biswal et al., 2005), NS5B complexed with RNA primer-template (Mosley et al., 2012), and various crystal structures of stalled polymerase during the initiation and elongation of RNA synthesis (Appleby et al., 2015). (2) The switch from initiation to elongation changes the position of the elongating polymerase in relation to the membrane due to the distortion of the linker from the catalytic cleft (Bartenschlager et al., 2013; Romero-Brey and Lohmann, 2016). The crystal structures

lack the linker sequences and thus the topology of NS5B in a physiological environment in a replication complex remains to be elucidated. *De novo* priming by NS5B for negative strand synthesis occurs at the 3' UTR and requires two nucleotide binding sites within the enzyme to synthesise a dinucleotide primer (Ferrari et al., 2008). High concentrations of GTP have been shown to stimulate *de novo* initiation by binding to an allosteric site (Bressanelli et al., 2002). The NS5B sequence and structural organisation is shown in Figure 1.11

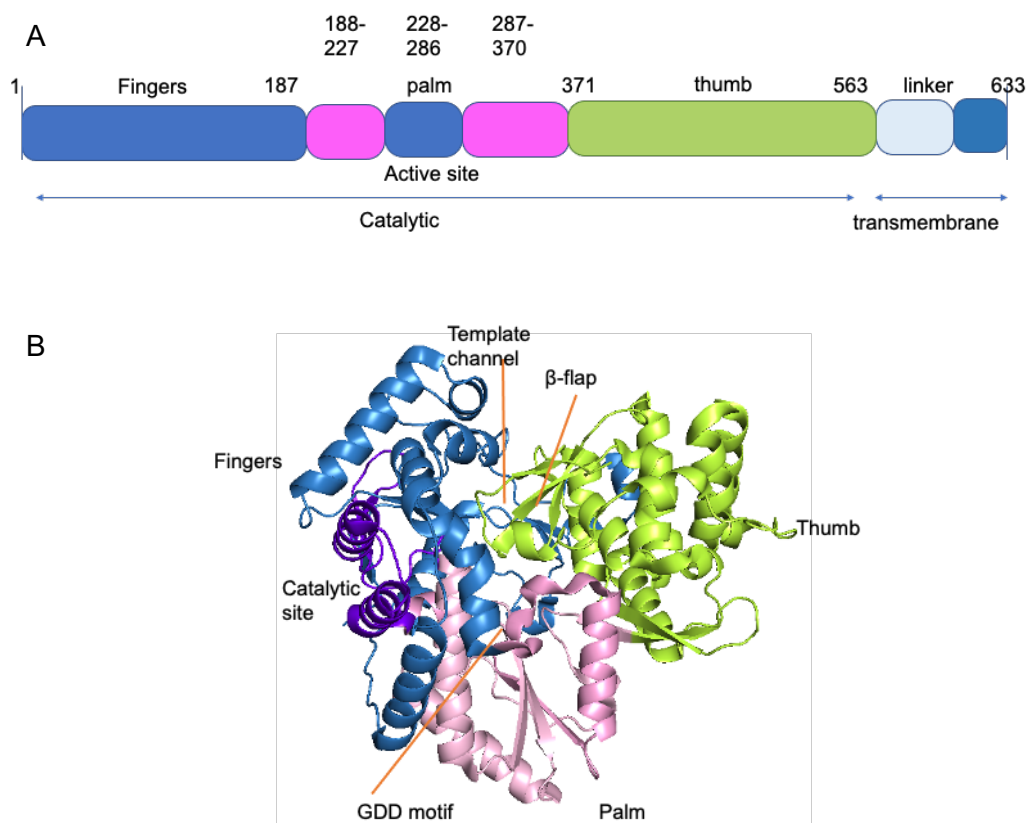


Figure 1.11: Sequence and structure of NS5B from JFH-1 (PDB:315K).

Domains are colour coded as shown in the sequence, Fingers, Thumb, palm are depicted as blue, green and pink. The catalytic site is highlighted in purple and specific regions are indicated by orange line.

1.2.3.4 Role of lipids and host cellular proteins in the biogenesis of membranous compartments

The membrane lipid composition in the host cell determines the membrane curvature providing membrane flexibility and fluidity to form distinct shapes observed in HCV induced membrane compartments (Bley et al., 2020). Lipids differentially regulate distinct curvatures, such as phosphatidylinositol 4- phosphate (PI4P) and lysophospholipids (LysoPL) are inverted cone-shaped lipids, inducing positive curvature.

Cholesterol (Chol), ceramides (Cer), unsaturated fatty acids (UFAs) and phosphatidylethanolamine (PE) are cone-shaped lipids and induced a negative curvature. Phosphatidylcholine (PC), Phosphatidylinositol (PI) and phosphatic acid (PA), and sphingomyelin (SM) are cylindrical shape lipids and thus induce flat membrane (Bley et al., 2020). The formation of DMV requires modifications of its lipid profile to expand the size, increase positive membrane curvature and recruitment of additional accessory factors (Paul et al., 2014a). Given that HCV derives membranous compartments primary from ER, the role of an ER resident enzyme -phosphatidylinositol 4 kinase III α (PI4KIII α), and its product phosphatidylinositol 4- phosphate (PI4P) has been studied extensively.

In mammalian cells, PI4-kinases constitutes two types with two isoforms each (PI4KIII α and PI4KIII β), which are responsible for synthesis of different PI4 pool (Balla, 2006). PI4KIII α is localised in the ER, the plasma membrane (Balla et al., 2005), and parts of Golgi (Bianco et al., 2012), whereas PI4P localises mainly in the Golgi and the inner leaflet of the plasma membrane. PI4P recruits multiple host factors involved in vesicle budding and lipid biosynthesis and therefore provide 'signatures' to distinct membrane compartments (Hammond et al., 2012). Together they are important in maintaining the functionality and integrity of HCV membrane structures (Berger et al., 2009; Li et al., 2009; Borawski et al., 2009). PI4P accumulates in HCV replication cells due to recruitment and activation of PI4KIII α by NS5A and NS5B (Reiss et al., 2011; Berger et al., 2011) and disruption of PI4KIII α by either pharmacological inhibition or knockdown of expression has resulted in reduced DMV diameter (Reiss et al., 2013). Additionally, the interaction of PI4KIII α with NS5A has been mapped to seven amino acids at the C-terminus of Domain I, which are important for viral replication and PI4KIII α activation (Reiss et al., 2013; Harak et al., 2014). PI4KIII α affect HCV replication not only through its impact on PI4 but also by influencing the regulation of NS5A phosphorylation by either facilitating the synthesis of NS5A-p56 or supressing p58 synthesis (Reiss et al., 2013).

PI4 and cholesterol play critical roles in replication organelle formation. Addition of PI4 to membranes has shown to modulate membrane curvature (Furse et al., 2012). In addition, PI4 helps to recruit lipid transfer proteins to add other lipids such as sphingolipids and cholesterol (Holthuis and Menon, 2014). DMVs are found to be enriched in cholesterol by 9-fold, and the removal of cholesterol reduces the DMV size and HCV genome stability (Paul et al., 2013). Additionally, by using fluorescent marker for Chol (TopFluor cholesterol), NS5A-positive structures were found to be enriched with oxysterol-binding protein (OSBP) (Wang et al., 2014). The cholesterol transport protein OSBP and glycosphingolipid transporter four-phosphate adaptor protein 2 (FAPP2) are vital for HCV replication (Wang et al., 2014; Khan et al., 2014). When PI4KIII α was

silenced, the colocalisation of OSBP and NS5A was lost, indicating that OSBP was recruited via PI4P (Wang et al., 2014).

Given the formation of numerous vesicles, vesicle-associated membrane proteins (VAMP)-associated proteins (VAPs) which are expressed ubiquitously (WEIR et al., 1998; Nishimura et al., 1999) and interact with wide variety of cellular proteins (Skehel et al., 1995) and viral proteins (Hamamoto et al., 2005; Goonawardane et al., 2017) have been investigated. Cellular host factors VAP-A and VAP-B are involved in ER homeostasis and vesicular trafficking (Gao et al., 2004; Evans et al., 2004; Hamamoto et al., 2005). By encoding an affinity tag to NS4B in a functional JFH-1 SGR, NS4B-containing membranes were isolated from stable replicon cells by density gradient centrifugation and HA-affinity capture. By biochemical and electron microscopy analysis, purified double membrane vesicles (DMVs) that contained NS3 and NS5A were also found to be enriched with VAP-A, VAP-B and cholesterol, highlighting the role in the HCV replication process (Paul et al., 2013). NS5A has been shown to interact with VAP-A and VAP-B proteins in a phosphorylation dependent manner emphasising the role of NS5A in the formation of membranous replication factories (Gao et al., 2004; Evans et al., 2004; Hamamoto et al., 2005). NS5A binds to OSBP which is mediated by the adaptor proteins VAP-A and VAP-B (Tu et al., 1999; Hamamoto et al., 2005). OSBP shuttles between ER and Golgi, delivering cholesterol from ER to Golgi and PI4P from Golgi to ER, and is assisted by VAP-A and VAP-B (Mesmin et al., 2013).

Given the role in vesicle transport, the involvement of Ras-associated binding (Rabs) proteins in HCV MWs has also been studied. Rabs belong to Ras superfamily of small GTPases that continuously cycle between cytosol and different membranes. Rabs are associated with distinct membrane bound compartments highlighting their importance in regulation of vesicle trafficking processes, including vesicle formation, motility and tethering and fusion to the acceptor membrane and signalling to other organelles (Corbeel and Freson, 2008). Rab 2, 5, 7 have been shown important for HCV RNA replication (Manna et al., 2010; Romero-Brey and Lohmann, 2016). Rab5 is found on early endosomes (EEs) and Rab7 locates on late endosomes (LEs). Rab5 regulates endocytosis and EE fusion and Rab7 directs the transport from ILEs to lysosomes (Mizuno-Yamasaki et al., 2012; Romero-Brey and Lohmann, 2016). NS4B-C-terminal domain has been suggested in the recruitment of Rab5 to the NS4B foci, contributing to a role in the formation of replication sites (Stone et al., 2007; Berger et al., 2009; Aligo et al., 2009). Rab18 was believed to promote physical interaction between LDs and ER (Ozeki et al., 2005), and were later shown to facilitate direct association of replicase components with LD via direct interaction of NS5A and active GTP-bound Rab18

(Salloum et al., 2013). Therefore, Rab18 might act as a joining force for physical coupling of HCV replication and virion production (Dansako et al., 2014).

The role of cyclophilins (CyPs, ubiquitous molecular chaperones) in HCV replication was identified by inhibition of replication by cyclosporin A (CsA). CsA treatment on cells expressing gt1b replicon showed decreased expression of viral proteins NS5A and NS5B (Watashi et al., 2003). Compared to gt1b, JFH-1 (gt2) showed less suppression of replication, with the difference of CypB responsible to stimulate RNA binding activity of NS5B in gt1b but not in gt2a (Naoto et al., 2006). Direct interaction of CypA and CypB with NS5A domain II and domain II have been identified and characterised by NMR (Hanouille, Badillo, et al., 2009). A common binding site within NS5A for CypA and NS5B suggest a potential role in the regulation of replication (Rosnoblet et al., 2012). Cyclosporine was observed to block *de novo* synthesis of membranous web but had no little effect on the established replication organelles. However, this block was lifted with mutations in NS5A, suggesting a role for CypA dependent modifications in the biogenesis of HCV replication (Madan et al., 2014). The role of several host cellular proteins have been studied demonstrating the importance of these factors in modulating HCV replication through affecting MW biogenesis (Li et al., 2021).

1.2.4 Viral assembly and release

The newly synthesised RNA can be either used as template for production of proteins. or packaged into virions (Zayas et al., 2016). Cytosolic lipid droplets (cLDs) are lipid storage organelles derived from the ER, and are composed of a hydrophobic core with a phospholipid monolayer (Gross and Silver, 2014). A key role in virus production is the recruitment of HCV proteins to the sites of cLDs (Boulant et al., 2006; Miyanari et al., 2007). For the formation of a viral particle, the genome encapsidation (by the core protein) as well as membranous envelope is acquired by budding into the ER, in proximity to cLDs where HCV proteins are first assembled (Gastaminza et al., 2008). It was shown recently by correlative light and electron microscopy (CLEM) that during assembly, structural proteins (core, E2) of HCV re-localise to the replicase and together induce the wrapping of cLDs by ER membranes. Membrane wrapped cLDs are in close proximity to viral replication organelles, allowing viral proteins to spatially coordinate replication and assembly (J.Y. Lee et al., 2019).

Multiple interactions between the HCV proteins facilitate the assembly and egression of HCV particles. After the proteolytic cleavage of the polyprotein precursor, the early phase of the viral particle formation is initiated by the interaction of NS5A with the core protein (Miyanari et al., 2007). This interaction is enhanced by a triglyceride-synthesizing enzyme

acyl CoA: diacylglycerol acyltransferase 1 (DGAT1) (Camus et al., 2013). NS5A is an RNA-binding phosphoprotein that is involved in both replication and assembly (Tellinghuisen et al., 2008; Masaki et al., 2008). There are two distinct regions in the domain III of NS5A, composed of basic amino acids and a separate cluster of serines, which are involved in the recruitment of replication complex to the core and the transfer of HCV RNA to the core respectively (Zayas et al., 2016). The core is composed of three domains: DI is involved in the interaction with the viral RNA and formation of homooligomers, DII targets the protein to the cLD, and DIII serves as a signal sequence that directs the nascent polyprotein to the ER membrane (Zayas et al., 2016). The complete processing of the core by signal peptide peptidase is vital for its association with cLDs, as disruption of processing is shown to reduce HCV titres (Targett-Adams et al., 2008).

The two structural proteins NS2 and p7 are involved in the recruitment of non- structural proteins and core-containing cLDs to the assembly complex. Together they are required for the correct organisation and recruitment of proteins to the assembly complex (Jones et al., 2007; Jirasko et al., 2010; Popescu et al., 2011; Gentzsch et al., 2013). Although NS2 catalytic activity is not required for virus production and is dispensable in the assembly process (Jirasko et al., 2010), the transmembrane domains of NS2 play a significant role in the recruitment of E1/E2 and NS3/4A complexes to sites of core associated cLDs (Phan et al., 2009; Counihan et al., 2011). Likewise, p7 is not involved in replication but deletion of p7 in HCV does not produce infectious particles and signifies the importance of p7 in assembly of the virion (Steinmann et al., 2007; Jones et al., 2007). The non-structural protein complex NS3/4A may be required for packaging of HCV genomes in a helicase dependent way. It has been reported that the helicase domain of NS3 is required for HCV particle production at a step after the association of core and NS5A but before the assembly of progeny virus (Yi et al., 2007; Ma et al., 2008). The schematic of virus assembly is shown in Figure 1.12

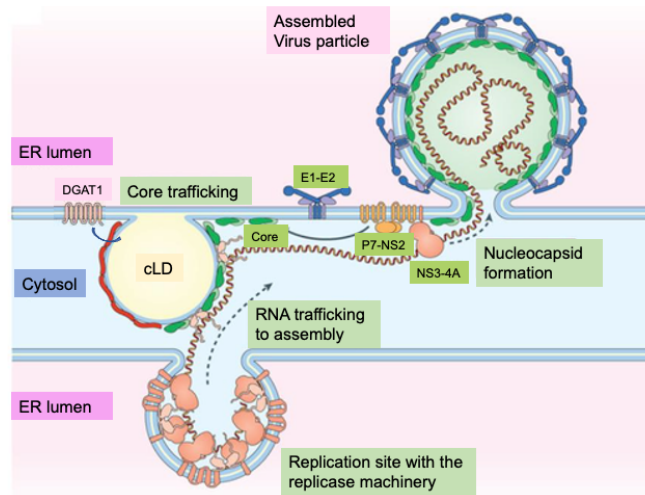


Figure 1.12: Schematic illustration of the assembly of viral particles in proximity to a replication organelle and cLDs.

Viral RNA is transported from the replication complex to the LD, where core and NS5A facilitates the assembly process. The interaction of NS2-p7 and NS3-4A recruits the core protein to the assembly site. Finally, the virions are assembled by recruitment of E1 and E2 and budding into the ER. Adapted from (Lindenbach and Rice, 2013).

The newly packaged virions then bud from the assembly complex using the endosomal sorting complex required for transport pathway (ESCRT) (Corless et al., 2010; Ariumi et al., 2011; Tamai et al., 2012). The virions then travel through the trans-Golgi network (TGN) and endosome pathways where the E1/E2 envelope proteins are glycosylated in a process of maturation (Gastaminza et al., 2008; Vieyres et al., 2010; Mankouri et al., 2016). This process is shown to disrupt the TGN architecture in an NS2 dependent manner which results in redistribution of the TGN to the HCV core on lipid droplet regions (Mankouri et al., 2016).

To finalise maturation, the virions are transported to the Golgi compartment. Recently, it has been shown that HCV can induce the fragmentation of the Golgi which raises the questions of whether the destruction of HCV-induced Golgi is a possible trigger for liver necrosis (Hansen et al., 2017). The virions are released at the plasma membrane by trafficking through the endosome network in a VAMP-1 (Vesicle-associated membrane protein-associated protein) dependent manner (Coller et al., 2012). The endosomal protein Rab18 (Ras-related protein) positive LDs colocalised with NS5A and appeared to promote the physical association of NS5A and other replicase components with LDs (Zayas et al., 2016), suggesting possible role of endosomal network in the release of the viral particle.

HCV particles isolated from serum and cell culture mediums constitute infectious particles with different buoyant densities. As the particles are pleiomorphic in nature, the size, buoyant density and infectivity might differ which makes them difficult to study (Morozov and Lagaye, 2018). Although, it was well established that the secretion of the HCV particles after the virion maturation uses the very-low-density lipoproteins (VLDL) pathways and depends on both the VLDL and the apolipoprotein (apoB) (Morozov and Lagaye, 2018). More recent evidence suggests that HCV release may be independent of the VLDL pathways (Mankouri et al., 2016). Accordingly, it was also shown that the VLDL pathway requirements of apoB and microsomal transfer protein are dispensable for HCV particle assembly (Jiang and Luo, 2009; Benga et al., 2010). The diverse buoyant density of infectious particles presents as a major problem in understanding the classical “assembly-maturation-release” process (Morozov and Lagaye, 2018).

1.3 Correlative light and electron microscopy (CLEM)

integrated with cryo- electron tomography (cryo-ET)

Correlative light and electron microscopy (CLEM) combines electron microscopy's high-resolution imaging with the capacity to spatially localise specific proteins of fluorescence light microscopy, enabling targeted imaging of specific macromolecules at molecular detail and in cellular context. CLEM has become instrumental for virologists studying host-virus interactions (Verkade and Collinson, 2019). While optical microscopy reveals viruses as small dots due to their size, electron microscopy offers the resolution necessary for understanding the virus life cycle's ultrastructure (Bykov et al., 2016). Technological advancements since the 1960s in sample preparation, electron microscopes, and detectors have facilitated 3D tomography approaches for morphological analysis of viruses (Hillier, 1950). However, challenges persist, including artefacts from sample preparation and imaging techniques. Various CLEM approaches exist for visualizing viral infections, each with its own set of advantages and disadvantages (Bykov et al., 2016), as shown in Figure 1.13 and Figure 1.14.

This study focuses on cryo-CLEM, allowing near-native state observations with optimal preservation of fluorescence and specimen ultrastructure. Challenges include sample handling, the need for specialized cryo-light microscopy and cryo-EM setups, and low contrast in the resulting EM images (Bykov et al., 2016). Cryogenic temperatures pose difficulties for fluorescence imaging due to sample sensitivity, fluorophore blinking, vitreous ice refraction, and cryo-stage instabilities (Kaufmann et al., 2014). Despite challenges, recent studies suggest potential solutions, and ongoing method development aims to overcome these obstacles (Bykov et al., 2016).

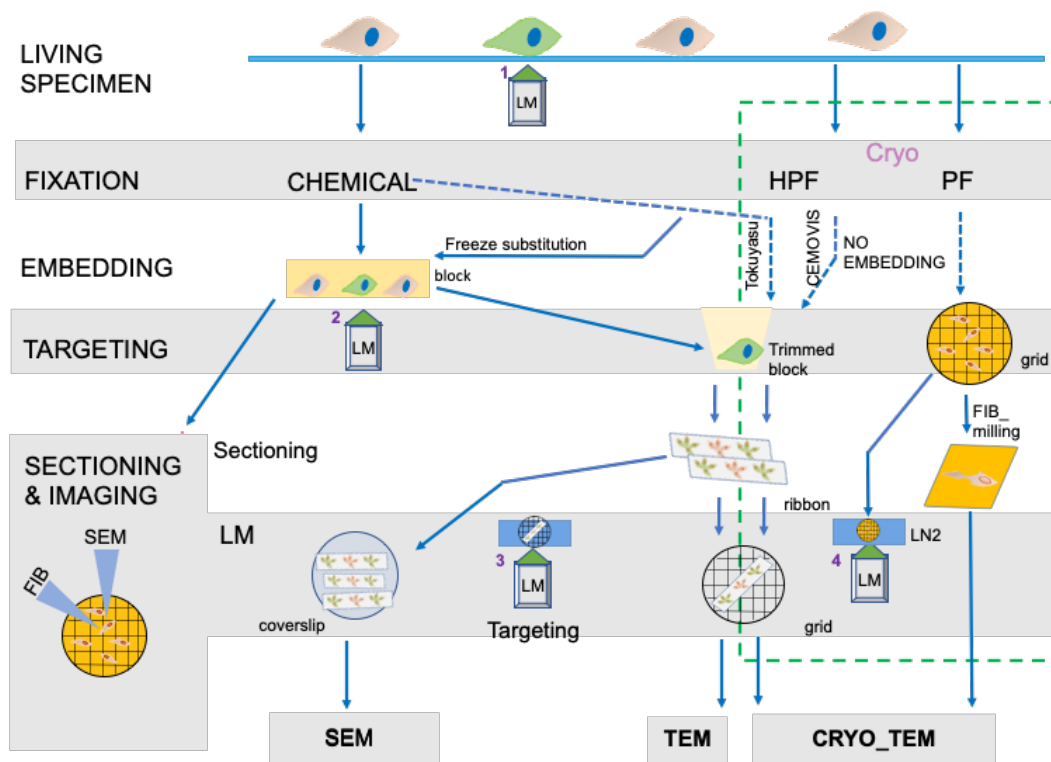


Figure 1.13: EM sample preparation workflow with various strategies for light microscopy (LM) incorporation.

Figure depicting the most common EM sample preparation techniques, workflow from living specimen to the final micrograph registration step. Various strategies of LM incorporation are shown: 1-LM on living or prefixed specimen, 2-LM on entire block of embedded specimen, 3-LM on sections at room temperature, and 4-cryo-LM on vitreous sections or whole vitrified cells. Workflow number 4 is used in this study. Various methods of CLEM workflow are depicted. First, the sample is fixed either chemically at room temperature or with High-Pressure freezing (HPF) or plunge-freezing (PF). HPF and PF fixed samples can be further processed at cryo-conditions (CEMOVIS, FIB-milling, or direct imaging). Chemically fixed cells can be embedded in gelatin, preserved in cryoprotectant (sucrose), plunge frozen in liquid nitrogen, cryosectioned and then immunolabeled at RT using the Tokuyasu method to preserve cellular ultrastructure. HPF-fixed cells can in turn be embedded using freeze-substitution protocol for sectioning at room temperature. The block is trimmed to remove the unwanted material and focus on the region of interest aided by reference marks or light microscopy. Embedded specimens are sectioned using an ultra-microtome, mounted on EM grids or coverslips and imaged with TEM (transmission electron microscopy) or SEM (scanning electron microscopy). LM data are used to target the acquisition. Alternatively sectioning and imaging of the embedded specimen can be done simultaneously using FIB/SEM or SBSEM instrument. Adapted from (Bykov et al., 2016)

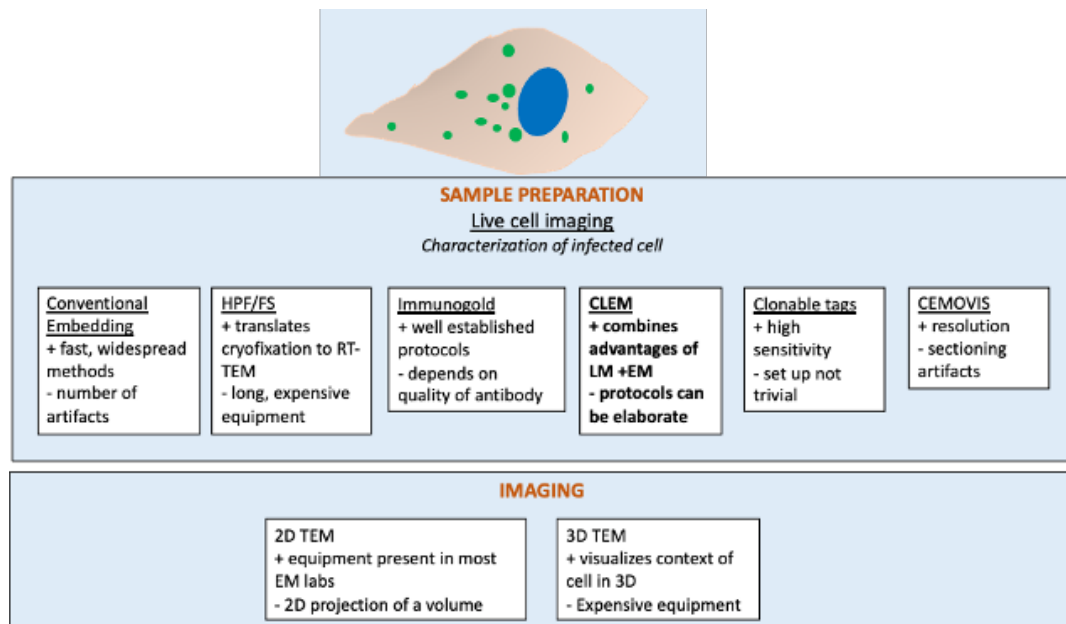


Figure 1.14: Schematic summary of the advantages and limitations of different approaches for sample preparation and imaging.

Adapted from (Sachse et al., 2019).

1.3.1 Principles of cryo-ET: tool for investigating host cellular architecture after viral infection.

In biological sciences, transmission electron microscopes (TEM) offer an unparalleled approach for understanding the physical structure of biomolecules and their biological function. The concept of electron tomography, a process of ‘imaging by sections’ proposed by Radon in 1917 (Radon, 1917), which eventually was integrated with TEM in the 1960s, paved a way to determine the 3D structure of a biological object from a set of 2D projections (De Rosier and Klug, 1968; Herman, 1972; Ercius et al., 2015). The general approach of cryo-electron tomography involves collecting a series of 2D cryo-EM images, known as projections, which are typically acquired by tilting the stage from ± 60 degrees. This set of projections are referred to as a tilt-series. The reconstruction workflow then involves computational alignment of the tilt-series to generate a 3D reconstructed volume called a tomogram (Cope et al., 2011).

The slab geometry of the specimen holder creates a limitation on the range of tilt-angles that can be collected (typically ± 60 degree) and this missing angular range is known as the ‘missing wedge’ (Hagen et al., 2017). Tilt-series can be acquired in three main ways, namely unidirectional (-60 to $+60$), bi-directional (0 to -60 and 0 to $+60$) and dose-symmetric (alternating between $+$ and $-$ tilts with tilt angle increments, usually starting

from zero). To minimise the missing wedge, dual axis tomography can be employed (Arslan et al., 2006). When the goal of a tilt-scheme is to ensure high-resolution information is maintained, three main factors are considered: the angular increments between tilt-images, the total angular range covered and the order in which the tilt projections are collected (Wan and Briggs, 2016). The various tilt-schemes are illustrated in the Figure 1.15A. For cryo-preserved samples, the dose symmetric scheme is optimal as the high-resolution information is concentrated in the lower tilts where the sample is thinnest yielding a maximum information transfer (Hagen et al., 2017).

Tilting the specimen introduces lateral stage displacement and increased specimen drifts and therefore tracking and autofocus is performed before every image for efficient collection of high-quality information. Autofocus and tracking corrects for the stage movements parallel and perpendicular to the electron beam respectively. Autofocus ensures reproducible defocus across a tilt-series whereas tracking helps to maintain the field of view constant in each tilt image. A range of commercially available packages account for most of these factors and allows automated tomographic data collections (Wan and Briggs, 2016).

The total electron dose that can be applied to a biological sample without causing radiation damage to record a series of images is very limited. Therefore, a dose fractionation is generally applied over the 30-120 images upto 100 electrons per $\text{\AA}^2$ (Cope et al., 2011; Wan and Briggs, 2016). Moreover, the fractionated dose over the tilt-series results in low contrast and low signal to noise ratio (SNR) and is observed more as the thickness of the sample increases with high-tilts (Roman I Koning et al., 2018).

To reconstruct a 3D volume in a tomogram from the acquired tilt-series, pre-processing of raw images is required. The data are collected in movie mode on a direct electron detector allowing multiple single shot acquisitions producing higher frame rates (La Grange et al., 2015). The high frame rate of the particles during cryo-ET imaging experience two types of motion. 1) Global mechanical motion is introduced by subtle changes in the stage movements between beam exposures at different tilt-angles. 2) The electron beam induced particle motion that results in small movements in between frames collected at each tilt angle (Shen et al., 2024; Pyle and Zanetti, 2021). The high frame rate of the direct detectors allows this beam induced particle motion to be captured in movies. Thus the frame alignment is important for correction of any beam-induced motion to produce motion-corrected images with high-resolution information restored (Brilot et al., 2012; Fernandez et al., 2018). For tilt-series processing of frame alignments, open-source software like motioncor2 can effectively be employed (Zheng et al., 2017).

Additionally, any shifts and rotations for compensation of the stage shifts during the collection are determined and corrected by cross-correlation of two images in the commonly used IMOD pipeline for reconstructing tomogram (Kremer et al., 1996).

Several different methods are available to reconstruct a tomogram (Wan and Briggs, 2016). However, two of the most common methods are backprojection and iterative reconstruction. Backprojection methods are real space analogs to Fourier synthesis approaches (Bracewell and Riddle, 1967). The Fourier synthesis is based on the central slice theorem, where Fourier transform of each projection image is calculated and placed in a plane of 3D Fourier space. When all the tilts are included, a reverse Fourier transformation is applied to generate a real-space 3D reconstruction. Most commonly a weighted back projection (WBP) is applied which accurately reproduces the densities within the biological specimen by reweighting overrepresented frequencies (Koning et al., 2018).

Finally, tomograms derived from cellular architecture are crowded and difficult to interpret. Therefore, tools for denoising, segmentation and modelling are employed to understand the information observed in the tomograms (Heymann, 2021). A schematic of tilt-series collection to segmentation is illustrated in Figure 1.15B.

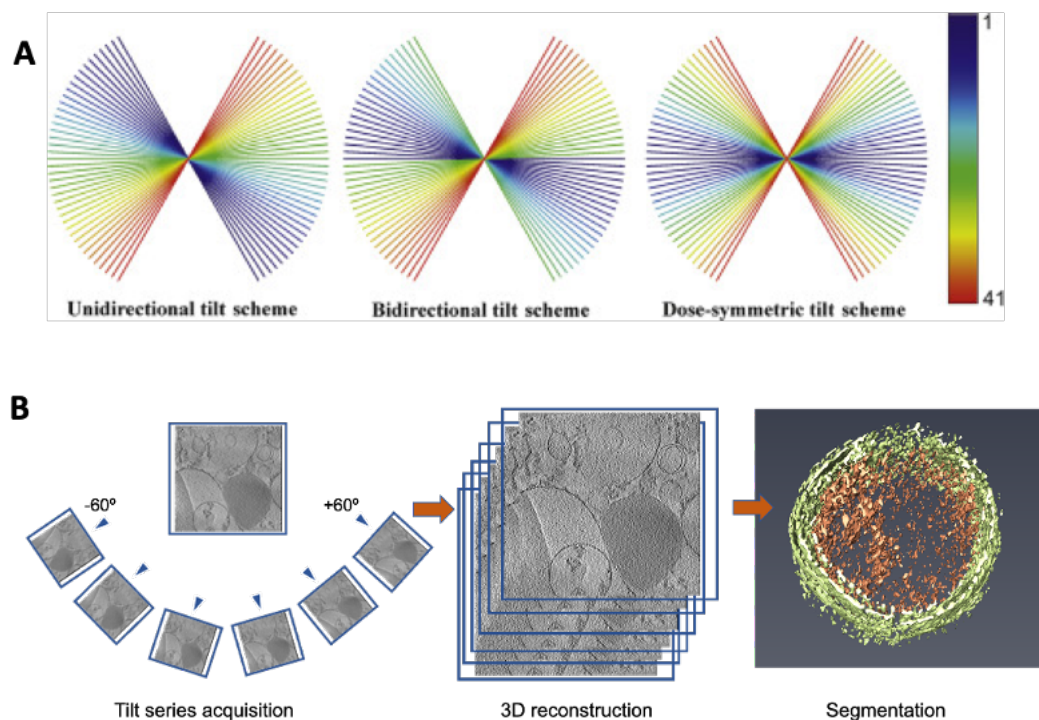


Figure 1.15: Summary of tilt-schemes and cryo-ET workflow.

[A] Schematic showing the tilt-schemes, from left to right- unidirectional (-60 to +60), bi-directional (0 to -60 and 0 to +60) and dose-symmetric (alternating between + and -tilts with tilt angle increments), taken from (Wan and Briggs, 2016). [B] Overview of cryo-ET workflow: tilt-series acquisition, 3D reconstruction and segmentation.

1.3.2 Deducing viral structures and intracellular insights through cryo-ET in virus-infected cells

In the past two decades, advancements in cryo-electron tomography (cryo-ET) have facilitated the direct visualisation of intricate, pleiomorphic 3D structures (Quemin et al., 2020). Early milestones in studying virus-host interactions by cryo-ET have been demonstrated in case of the herpes simplex virus 1 (HSV-1) and human immunodeficiency virus (HIV). For HSV-1, cryo-ET has allowed to solve the tegumentation and envelopment in intact isolated the virions during virion egress (Grünwald et al., 2003), native 3D intermediates of membrane fusion in HSV-1 (Maurer et al., 2008), HSV-1 transport and the assembly of viral particle in axons of primary neurons (Ibiricu et al., 2011) and structure of HSV-1 virus capsids within cell nuclei (Vijayakrishnan et al., 2020). For HIV, the infection process and disassembly of HIV-1 virions in the cytoplasm of the infected cells was visualised using live cell-microscopy and cryo-ET (Jun et al., 2011). More recently, the functional role of matrix domain (MA) in mature HIV virion was elucidated where MA rearranges between two distinct hexameric lattices upon maturation (Qu et al., 2021).

In recent years, cryo-ET has provided novel insights into the replication compartments of various viruses. For instance, it revealed distinctive features of viral replication compartments (spherules) in Flock house Nodavirus. These spherule invaginations derived from the outer mitochondrial membranes were packed with densities indicating RNA fibrils which correlated with RNA replication template length (Ertel et al., 2017). In the case of SARS-CoV-2, *in situ* cryo-ET from cryo-focused ion beam (cryo-FIB, details in chapter 4) generated lamellae elucidated the budding mechanism, showing the structures of virions and virus-induced double-membrane vesicles (DMVs) (Klein et al., 2020). Another study focused on the architecture of chikungunya virus (CHIKV) replication organelles (spherules) formed at the host plasma membrane. It revealed that the spherules contained a single copy of the viral genome, with the viral protein nsp1 serving as the base for the assembly of the replication complex at the neck of the spherule (Laurent et al., 2022). Moreover, the molecular architecture of the CHIKV replication complex was further elucidated to subnanometer resolution through a combination of cryo-single particle EM and cryo-ET (Tan et al., 2023).

1.4 Aims and objectives

Over the past decade, the ultrastructure of the HCV membranous web has been extensively studied using EM. While samples fixed with chemicals, followed by resin embedding and sectioning had effectively preserved membranes, they limited the investigation of proteins or replicase complex assembly. This study aimed to employ recent technological advancements in preparing cryogenic cell specimens to gain insights into the assembly of the HCV replicase machinery within the membranous compartments induced by the SGR in Huh7 cells.

In Chapter 3, stable cell lines harbouring SGR JFH-1 Feo NS3-5B (Feo: fusion of Firefly Luciferase and neomycin phosphotransferase genes) were generated and validated. The subcellular distribution of cellular markers (VAP-A, PDI, TGN-46) was examined in cells with and without SGR. The chapter's aim was to determine the suitable fluorophore (eGFP vs. mCherry) fused to NS5A for developing a cryo-correlative light and electron microscopy (cryo-CLEM) workflow.

Chapter 4 aimed to explore two cryogenic sample preparation approaches and established methodologies for *in-situ* investigation of membranous compartments induced by SGR JFH-1 Feo NS3-5B (NS5A-eGFP). The investigation primarily covers the proposed replication site, double-membrane vesicles (DMVs), followed by other compartments such as single-membrane vesicles (SMVs), multi-membrane vesicles (MMVs), and regions around lipid droplets (LDs) and endoplasmic reticulum (ER).

Chapter 5 aimed to employ alternative tools for CLEM approaches for studying *in situ* HCV membrane rearrangements. Specifically, the VIPER labelling system was explored to determine if other non-structural proteins in the replicase machinery can be tagged and integrated into the cryo-CLEM workflow. Various insertion sites in the NS3 protein were investigated for this purpose.

Chapter 2| Materials and Methods

2.1 General materials

2.1.1 Bacterial strains

Chemically competent *Escherichia coli* (*E.coli*) DH5 α were used for cloning from Life Technologies.

Bacterial strains: *E. coli* BL21 (DE3) RIL cells (Thermo Fisher Scientific) were used for protein expression and were kind gift from the Zeqiraj lab (University of Leeds). Lysogeny broth (LB) agar or culture medium was supplemented with ampicillin at 100 μ g/ml and Chloramphenicol at 50 μ g/ml for cell propagation

2.1.2 Mammalian Cells

Huh7 cells are a human hepatocyte-derived cancer cell line established in 1982, which support replication of HCV subgenomic replicons (Nakabayashi et al., 1982). Huh7.5 cells are a sub-population of cells that were established by prolonged treatment with IFN- α which allowed increased permissiveness of HCV replication (Blight et al, 2002). HEK-293T (human embryonic kidney) cells were used for transfection for western blot analysis.

Cell growth was maintained in the Dulbecco's modified Eagle's medium (DMEM, Sigma), supplemented with 10% foetal bovine serum (FBS, Gibco), 50 Units/ml penicillin, 50 μ g/ml streptomycin, 1% nonessential amino acids (NEAA, Lonza) and 2.8% HEPES (Lonza) in a humidified incubator at 37 °C with 5% CO₂. DMEM with the supplemented factors was referred as complete DMEM. For cell maintenance, cells were passaged upon reaching confluence. Cells were washed twice with 1 X PBS and detached from the culture flasks by incubating with trypsin (T4049, Sigma) at 37 °C.

2.1.3 Antibodies: Primary

Target	Species	Source	Comments	Dilution
α NS5A	Sheep	(Macdonald et al., 2003)	Polyclonal serum	Variable
α NS3	Mouse	Dr. Thomas Pietschmann	Monoclonal	1:200
PDI	Rabbit	Cell signalling Technology	Monoclonal	1:100
VAP-A	Goat	Santa Cruz Biotechnology	Polyclonal	1:100
TGN-46	Sheep	Dr Vas Poonambalam	Polyclonal	1:50
β -actin	Mouse	Merck	Monoclonal	1:10,000

Table 2.1: List of primary antibodies

2.1.4 Antibodies: Secondary

Target	Species	DYE	Source	Dilution
α Goat	Donkey	IRDYE 800	LI-COR	1:10,000
α Mouse	Donkey	IRDYE 680	LI-COR	1:10,000
α Rabbit	Goat	Alexa Fluor 488	Invitrogen	1:500
α Goat	Donkey	Alexa Fluor 488	Invitrogen	1:500
α Sheep	Donkey	Alexa Fluor 488	Invitrogen	1:500
α Rabbit	Chicken	Alexa Fluor 594	Invitrogen	1:500
α Goat	Donkey	Alexa Fluor 594	Invitrogen	1:500
α Sheep	Donkey	Alexa Fluor 594	Invitrogen	1:500
α Mouse	Goat	Alexa Fluor 594	Invitrogen	1:500
α Mouse	Goat	Alexa Fluor 647	Invitrogen	1:500

Table 2.2: List of secondary antibodies

2.1.5 Fluorescent probes

Probe	Target	Source	Dilution
BODIPY 558/568 C12	Stain Lipid droplets	Invitrogen	1:1000
DAPI (Prolong Gold Antifade reagent with DAPI)	Stain Nucleus	Invitrogen	10 μ l
Sulfo-Cyanine5 Maleimide	Conjugate with MiniR protein	Lumiprobe	Tested variable molar ratios

Table 2.3: List of fluorescent dyes

2.1.6 SGR and expression constructs

HCV genome sequences for JFH-1 (genotype 2a) used for analysis were obtained from Genbank accession numbers BAB32872.1. Full length JFH-1 SGR (Wakita et al., 2005) was used to generate JFH-1 pSGR Feo NS3-5B (Wyles et al., 2009; Ross-Thriepland et al., 2015b) using insertion site (P418, gt2) in NS5A-domain III for GFP (or mCherry) as reported (Moradpour et al., 2004). The pCMV10- NS3-5B plasmid was provided by Dr. Dan Jones at the University of Glasgow (Jones et al., 2009).

2.2 General Methods

2.2.1 Transformation of plasmids in chemically competent bacteria

For transformation, plasmid DNA (50-100 ng/μl) was incubated with *E.coli* DH5α cells at a volume ratio of 1:10 on ice for 30 minutes. The cells were subjected to heat shock at 42 °C for 1 minute and then incubated on ice for 5 minutes. Cells were recovered in 1:10 ml volume ratio in LB broth (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) in a shaking incubator for 2-3 hours at 37°C and 180 rpm. Cells were harvested by centrifugation at 3500 x g for 3 minutes. The cell pellet was resuspended in LB and plated onto LB agar plates with appropriate antibiotic and were grown at 37 °C, or 30 °C until colony formation.

Antibiotic	Final concentration
Ampicillin	100 μg/ml
Kanamycin	50 μg/ml
Chloramphenicol	50 μg/ml

Table 2.4: List of antibiotics used for transformation in bacteria

2.2.2 Preparation of plasmids from bacterial cultures

Single colonies were picked and grown in LB-antibiotic medium at 37°C and 180 rpm overnight (16-18 hours). Cells were harvested at 4,000 x g for 10 minutes. Plasmid DNA was purified using Monarch Miniprep kit (New England BioLabs (NEB)) or GeneJET Maxiprep kit (Thermo Fisher Scientific) according to manufacturer's protocol.

2.2.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was employed to amplify sequences for cloning. Oligonucleotides were purchased from Integrated DNA Technologies, were resuspended in deionised water (dH₂O) to a final concentration of 100 μM and stored at -20 °C. PCR reactions were amplified with Phusion High-Fidelity DNA polymerase (NEB), or GoTaq master mix (Promega) for colony PCR. PCR reactions were customised based on the objective. In cases where poor PCR yields were observed, 3% dimethyl sulfoxide (DMSO) was included in the steps.

2.2.4 Construct verification: Colony PCR and sequencing

Single colonies from transformation plates were picked for colony PCR with the appropriate primers to detect the inserted fragment. GoTaq master mix (Promega) was used for PCR amplification in a final volume of 25 μl. The general PCR conditions are shown in Table 2.5. The annealing and extension times were adjusted according to the

primers calculated melting temperature (T_m). Correctly identified colonies were sequenced by sanger sequencing at Genewiz (Azenta Lifesciences).

Stock concentration		Final concentration
2 X GoTaq master mix	12.5 μ l	1X
Forward Primers (10 μ M)	1 μ l	0.4 μ M
Reverse Primer (10 μ M)	1 μ l	0.4 μ M
Colony	single	
dH ₂ O	10.5	
Total volume	25 μ l	

PCR Reaction	Temperature	Time	cycles
Initial Denaturation	95 °C	30 seconds	1 X
Denaturation	95 °C	30 seconds	35 X
Annealing	55 °C	30 seconds	
Extension	68 °C	45 seconds	
Final Extension	68 °C	5 minutes	1 X

Table 2.5: Colony PCR setup and parameters

2.2.5 Restriction digestion

Restriction digestion enzymes were supplied by NEB or Promega. Reactions were carried out in 20 μ l volume in recommended buffers at indicated temperatures. At least 1 Unit of enzyme per μ g of DNA was incubated for a minimum of 1 hour.

2.2.6 DNA agarose gels

Variable percentage of DNA agarose gels were prepared using 1 X in TAE buffer (40 mM Tris-HCl, 0.11% (v/v) acetic acid, 1 mM ethylenediaminetetraacetic acid [EDTA]) with SYBR safe stain at a 1 in 10 dilution (10,000U, Invitrogen). DNA samples were loaded after combining with 1 X purple loading dye (NEB) and run in 1 X TAE buffer at 80 Volts (V). Hyper ladder I marker (Bioline) was used for reference.

2.2.7 DNA fragment excision from agarose gels

DNA fragments were visualised under blue light illumination with orange filter. The fragments of interest were excised using clean blades and extracted using Monarch Gel Extraction kit (NEB) according to manufacturer's protocol.

2.2.8 DNA Ligation

Following gel extraction, both DNA insert and linearised vector, were incubated with T4 ligase (NEB) in a 10 µl reaction and incubated at 16 °C overnight. The ligation product was transformed into competent DH5α cells. Alternatively, Instant Sticky End Ligase mix (NEB) was added and mixed thoroughly using a pipette at RT and immediately placed on ice.

2.2.9 Linearisation and purification of DNA for *in-vitro* transcription

Plasmid DNA (~5 µg) was digested using *Xba*I (NEB) restriction enzyme with cut smart buffer in 50 µl reaction volume at 37 °C for 1 hour. Following digestion, 50 µl of dH₂O was added to the digested product to bring the final working volume up to 100 µl. Equal volume of phenyl chloroform was added to bring the total volume to 200 µl. The sample was centrifuged in a benchtop centrifuge for 10 minutes at 16000 x g. The aqueous phase was carefully extracted into a RNAase free Eppendorf tube. An equal volume of 100% chloroform was added and vortexed thoroughly. The mixture was centrifuged for 10 minutes at maximum speed and the aqueous phase was retrieved. Depending on the volume of the separated aqueous phase, 1/10 volume 3M sodium acetate was added with 2.5 volumes of 100% ethanol. The samples were stored at -20 °C overnight to precipitate the DNA. Thereafter, the samples were centrifuged at 16000 x g for 30-40 minutes to pellet the DNA. The supernatant was removed, and the pellet was washed with an equal volume of 70% ethanol and centrifuged again at 16000 x g for 30 minutes. Excess ethanol was removed and dried by placing the tube in a heat block set at 42 °C. Finally, the linearised DNA was resuspended in 12 µl nuclease-free water. The concentration was measured using a nanodrop.

Alternatively, Monarch PCR and DNA clean-up kit (NEB) was used to purify linearised DNA after digestion using manufacturer's instructions and eluted in 12 µl nuclease-free water.

2.2.10 *In-vitro* transcription of RNA and purification

The purified linearised DNA sample was treated with RNA secure (Invitrogen) to inactivate all RNase activity. To an RNase-free reaction tube, 1 µl of RNA secure and 7 µl DNA (~1 µg) was added. The reaction was incubated at 65 °C for 10 minutes and then cooled on ice, followed by the addition of 10 µl of T7 2 X buffer and 2 µl of T7 enzyme mix to the reaction tube from the RiboMax Express T7 kit (Promega). The sample was gently mixed and incubated at 37 °C for 2 hours. Following incubation, 2.5 µl DNase was added to the reaction tube, mixed and incubated at 37 °C for 20 minutes.

Alternatively, HiScribe™ T7 RNA synthesis kit (NEB) was used. To an RNase-free reaction tube, 1 µg of template DNA was mixed with 10 µl of nucleoside triphosphates (NTP) buffer mix and 2 µl of T7 polymerase mix, and the reaction was adjusted to a final volume of 50 µl with nuclease free water. The sample was gently mixed and incubated at 37 °C for 2 hours. Following incubation, 2 µl DNase was added to the reaction tube, mixed and incubated at 37 °C for 15 minutes.

The RNA Clean & Concentrator kit (Zymo Research) was used to purify transcribed RNA as per the manufacturer's instructions. The concentration was determined using a nanodrop.

2.2.11 RNA agarose gel

A 1% agarose gel was used to test the integrity of the purified RNA, where 0.3 g agarose was mixed with 3 ml of 10 X MOPs buffer (0.2 M 3-(N-morpholino) propane sulphonic acid (MOPS), 20 mM sodium acetate, 10 mM EDTA) and 25.6 ml of dH₂O. The suspension was heated until dissolved, and then cooled to 60 °C. Once cool, 1.4 ml formaldehyde from a 40% stock, and 3 µl SYBR safe stain (Invitrogen) was added and allowed to set. RNA samples were resolved by mixing 1 µg of RNA with 1 X RNA loading dye (NEB), and heating at 65 °C for 10 minutes before loading into the gels. RNA gels were run in 1 X MOPs buffer at 80 V for 30 minutes.

2.2.12 DNA, RNA, protein quantification

Nucleic acid samples were quantified using a nanodrop UV-Vis spectrophotometer using preprogramed applications for dsDNA, ssDNA, and RNA. Alternatively, for protein concentration, Bicinchoninic acid assay (BCA) Protein Assay kit (Pierce, Thermo Fisher Scientific) was followed as per manufacturers' instructions, with the BSA standards prepared in the appropriate buffer.

2.2.13 RNA Electroporation

Huh7 or Huh7.5 cells were washed twice in ice-cold 1 X PBS, and then resuspended in diethyl pyrocarbonate (DEPC)-PBS. Using a square-wave protocol 2 µg of RNA was electroporated into 0.2×10^7 cells at 260 V for 25 ms. Cells were resuspended in complete DMEM to a final concentration of 1.7×10^5 cells/ml and seeded accordingly.

2.2.14 Luciferase assay

To monitor the replication of HCV SGRs over a period of 72 hours, 300 µl media of 1.7×10^5 cells/ml electroporated cells were seeded in 24-well plates. Cells were lysed at 4-, 24-, 72-, and 96- hour post electroporation (h.p.e) using 100 µl of 1 X Firefly Luciferase buffer (Biotium), by incubating overnight at -20 °C. In a white 96-well plate (Greiner), 50 µl of lysed samples and 30 µl of Luciferase reagent (Biotium) were mixed. Samples were set up in triplicate for the accuracy of the experiment. The FLUOstar Optima luminometer (BMG Labtech) was used to measure the levels of luciferase activity. Relative luminescence units (RLU) were recorded and compared.

2.2.15 Generation of stable cell lines

For generating stable cell lines by selecting colonies of cells resistant to antibiotic pressure, 0.2×10^7 Huh7 and Huh7.5 cells were electroporated with 2 µg of RNA transcripts and resuspended in complete DMEM. For the generation of SGR NS5A-eGFP and NS5A-mCherry stable cell lines, colony-forming assays were conducted. For this, duplicates of 10^5 , 10^4 , 10^3 Huh7 cells electroporated with RNA transcripts were combined with non-electroporated (non-transcripts) cells to a final seeding density of 1.8×10^5 cells. These cells were seeded in 6-well plates and incubated at 37°C, 5% CO₂ for 48 hours. The spent media was discarded, and the cells were washed with 1 X PBS. Polyclonal cell colonies were selected using a final concentration of geneticin sulphate (G418) of 700 µg/ml (Santa Cruz Biotechnology). Upon iterative rounds of G418 selection, the antibiotic concentration was reduced, and the cells were maintained at 400 µg/ml of G418.

For generating a stable cell line for SGR NS5A-eGFP-MiniE, 0.2×10^7 Huh7.5 electroporated cells were seeded in T75 flasks and incubated at 37°C, 5% CO₂ for 48 hours. The spent media was discarded, and the cells were washed with 1 X PBS. Cells were selected using a final concentration of G418 at 250 µg/ml and maintained at the same concentration.

2.3 Fluorescence microscopy

2.3.1 HCl treatment on coverslips

To prepare coverslips for seeding cells suitable for confocal fluorescence microscopy, 22 mm coverslips (VWR) were rinsed with dH₂O, washed in 1M HCl for 30 minutes followed by three washes with dH₂O. The coverslips were then incubated with 100% ethanol for 30 minutes, dried, placed in a petri dish and autoclaved.

2.3.2 Seeding cells on coverslips

Huh7 cells harbouring SGRs were maintained in complete DMEM in a humidified incubator at 37 °C with 5% CO₂. Cells were washed with 1 X PBS and detached using 1 X trypsin. Subsequently, cells were transferred into a falcon tube and harvested by centrifugation at 1200 X g for 3 minutes. Cells resuspended to 5 X 10⁴ in complete DMEM, and 300 µl of the cell suspension was seeded per coverslip in a 6-well plate. Alternatively, all harvested cells were resuspended in 2 ml complete DMEM and subjected to fluorescence-activated cell sorting (FACS). FACS was conducted by Dr. Ruth Hughes or Dr. Sally Boxall (Bioimaging Facility, University of Leeds). During the FACS sorting, cells were placed in 37 °C and 5% CO₂ incubator to keep them healthy. The cells were collected by gating the brightest fluorescence which were indicative of an actively replicating cell. After the cells were sorted by FACS, 5 X 10⁴ cells resuspended in 300 µl of complete DMEM were seeded per coverslip. Cells were gently pipetted on to the coverslips, ensuring full coverage of the surface area, and incubated for 20 minutes at RT. Complete DMEM (~ 2.2-2.5 ml) was added to the side of the well and incubated at 37 °C and 5% CO₂ for 19-22 hours before fixation.

2.3.3 Immunofluorescence Protocol

Culture media was gently aspirated and discarded after 19-22 hours of incubation. Cells were washed 3 x 5 minutes with 1 X PBS. Cells were fixed with 4% formaldehyde for 20 minutes at RT, followed by 3 x 5-minute washes with 1 X PBS. Cells were permeabilised using 0.2 % Triton X-100 for 10 minutes, washed 3 x 5 minutes with 1 X PBS, and subsequently blocked with 3% BSA-PBS for 30 minutes. To stain the cells with primary antibody, 100 µl of primary antibody diluted in 3% BSA-PBS and 0.02% sodium-azide, was pipetted onto parafilm and the coverslips were placed cell-side down onto the antibody. Following incubation, coverslips were washed 3 x 5 minutes with 1 X PBS. Coverslips were then incubated with the secondary antibody (or BODIPY dye) diluted in 1% BSA-PBS at RT for 1 hour, followed by 3 x 5-minute washes with 1 X PBS. Prolong Gold Antifade reagent containing 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen) was used to mount the coverslips onto glass microscope slides and cured at RT for 2.5 hours before long term storage at 4 °C. In cases, where this staining method did not yield expected results from the control samples, alternate staining methodology were tested and are detailed in Chapter 3.

2.3.4 Confocal microscopy

Z- stack images were acquired on Zeiss LSM880 upright confocal microscope. Plan Achromat objectives with 40X and 63X magnification (oil immersion) with numerical aperture of 1.4 was utilised. Post images were processed using Fiji (Schindelin et al., 2012).

NS5A and NS3 foci analysis in Chapters 3 and 5 were processed as follows. Z-stack images acquired were projected using the Z-stack function in Fiji. The images were converted into 8 bit, and a 'Gaussian blur' (sigma-2) plugin was applied. 'Otsu thresholding' was applied, where the parameters were kept the same between two images to be compared. To separate connected NS5A foci, the 'Watershed' function was applied. Area of a focus was calculated using the 'Analyse Particles' function. For colocalisation analysis, the 'Colocalisation Finder' plugin was used (Zheng et al., 2017). Data were exported and various analysis were performed using statistical tools in GraphPad Prism.

2.3.5 Image acquisition using the Zeiss Axio Vert A1 microscope

Images were acquired using the Zeiss Axio Vert A1 microscope in brightfield and fluorescence with 10X or 20X objectives. The VIS-LED wavelength is 400-700 nm.

2.3.6 Image acquisition using the EVOS microscope

Images were acquired using the EVOS microscope (Thermo Fisher Scientific) in brightfield and using a GFP filter with 4x, 10x and 20x objectives. The GFP-AMEP4651 cube has 470/22 Excitation and 510/42 Emission Filter.

2.4 Electron microscopy

2.4.1 Sample preparation using resin embedding and sectioning

Huh7 cells (control) and Huh7 cells harbouring SGR NS5A-eGFP were trypsinised and harvested at 1200 x g for 3 minutes. The pelleted cells were fixed in 2.5% glutaraldehyde (25% EM Grade, Agar Scientific) in 0.1M sodium phosphate buffer for a minimum of 2.5 hours. The subsequent sample preparation steps were performed by Mr Martin Fuller (Astbury Biostructure Laboratory, Electron Microscopy Facility, University of Leeds). The cell pellet was washed twice for 30 minutes in 0.1M sodium phosphate buffer. The cell pellet was post-fixed with 1% osmium tetroxide in 0.1M phosphate buffer for 1 hour and washed twice for 30 minutes with 0.1M phosphate buffer. The sample was dehydrated by incubating for 20 minutes with 20%, 40%, 60%, 80%, and 2X 100% ethanol. The cell pellet was then immersed in propylene oxide twice for 20 minutes. Freshly prepared resin mix composed of 50% propylene oxide and 50% Araldite epoxy resin was added and allowed to infiltrate the pellet for several hours to overnight (Luft, 1961). A resin mix

composed of 25% propylene oxide and 75% Araldite epoxy resin was then added and allowed to infiltrate the sample for several hours. Subsequently, fresh Araldite epoxy resin was added for 3-8 hours. The sample was transferred to embedding moulds with fresh Araldite epoxy resin and allowed to polymerise overnight at 60 °C. Ultrathin sections (80 -100 nm) were picked up on 3.05 mm grids and stained with saturated uranyl acetate for 2 hours, followed by Reynolds Lead citrate for 15 minutes. In general, for material in suspension the sample is handled in small Eppendorf tubes which are spun down after each stage to change the solution, then re-suspended using a whirly mixer. The sample is then polymerised in the Eppendorf tube at 60 °C.

2.4.2 Image acquisition using the FEI Tecnai T12 TEM

Cellular EM images were acquired using the FEI Tecnai T12 TEM at three different magnifications from ultrathin sections prepared by resin embedding and sectioning. Data acquired by Mr Martin Fuller.

Magnification	Pixel size
2900x	3.8 nm x 3.8 nm
4800x	2.3 nm x 2.3 nm
11,000x	0.99 nm x 0.99 nm

Table 2.6: Data acquisition parameters on TEM-T12

2.4.3 Rendering EM grids hydrophilic

Quantifoil Finder 200 mesh gold grids R1.2/1.3 (Gilder G200F1) and C-flat 200 mesh gold1.2/1.3 (Electron Microscopy Sciences) were used for cryo-ET. For EM workflow development and optimisation, Square Pattern 200 mesh gold EM grids (Agar Scientific) were formvar and carbon coated by Mr. Martin Fuller.

Grids were placed on a parafilm-wrapped glass slide and glow discharged in the Quorum GloQube (Quorum Technologies) to render grids hydrophilic and negatively charged. A negative polarity cycle of 20 mA current was applied for a duration of 30-60 seconds.

2.4.4 Final workflow for cell culture on EM grids

Quantifoil and/or C-flat gold EM grids (Electron Microscopy Sciences) were glow discharged and transferred to the centre of a glass bottom cell culture dish (Nunc TM, 150680). Grids were functionalised with 7 µl of fibronectin (Sigma-Aldrich) at 25 µg/ml concentration at 37 °C for 1 hour in a dry bead bath. Meanwhile, fluorescence activated cell sorting (FACS) was performed to harvest 5 X 10³ and 10 X 10³ cells in 500 µl of complete DMEM. The cells were harvested by centrifugation at 1200 X g for 3 minutes in a benchtop centrifuge. Approximately 450 µl volume was discarded and cells were resuspended in 50 µl complete DMEM and seeded on to the EM grids at various cell

densities. Cells were incubated on the EM grids for 20 minutes at RT. For optimum cell growth, 2 ml of complete DMEM was added per dish and incubated at 37 °C, 5% CO₂ for 20- 24 hours.

2.4.5 Sample vitrification by plunge freezing

Cells cultured on EM grids were plunge frozen using Leica EM GP Automatic plunge freezer (Leica Microsystems) in liquid ethane cooled to -180 °C, and stored in liquid nitrogen. Before plunge-freezing, 3 µl of 10 nm Colloidal Gold Labelled Protein A (Boster Bio) gold fiducial marker, or 1 X PBS was applied to the side of the grid harbouring the cells and 0.5 µl was applied to the back of the grid in the humidifier chamber. The chamber was set to 90% humidity at 8 °C, and blot time was varied between 5 seconds to 8 seconds for each sample. Grids were blotted using No.1 Whatman paper. Grids were clipped in C-rings and placed into autogrids dedicated for screening and data collection using the FEI Titan Krios TEM (Thermo Fisher Scientific), and storage in liquid nitrogen.

2.4.6 Sample thinning by cryo-focussed ion milling (FIB-SEM)

Lamellae were prepared using a focused gallium ion beam on a dual-beam focused ion beam-scanning electron microscope (FIB-SEM) at the stage temperature of -180 °C. Cells positioned in the centre of grid squares were selected for milling. An organo-metallic platinum protective layer was applied onto the cells before milling. Rough milling was performed gradually in three steps at a stage angle of 12° using a gallium beam. Final fine milling was performed at the end of the milling session to yield lamellae with a desired nominal thickness. Lamellae generation was carried out by Dr. James Gilchrist (Electron BioImaging Centre (eBIC), at Diamond Light Source) using the Scios CryoFIB-SEM (Thermo Fisher Scientific). Dr Gilchrist also provided guidance on the generation of lamellae using the Aquilos CryoFIB-SEM (Thermo Fisher Scientific) using automated and user-friendly guides.

Instrument	Dataset	Platinum coating (time applied for)	Final beam current	Final lamellae thickness	Progress monitored by SEM
Scios	1	5-6 seconds	37 pA	200 nm	2.2-10 kV with ETD and T2 detectors
Aquilos	2	7-10 seconds	50 pA	160 nm	2-5 kV with ETD detectors and Auto TEM

Table 2.7: Acquisition parameters for lamellae generation

2.4.7 Sample vitrification by high-pressure freezing

Dr Tom Sullivan established the workflow for freezing SGR NS5A-eGFP cells using the high-pressure freezing approach (Astbury Biostructure Laboratory, Electron Microscopy Facility, University of Leeds). SGR NS5A-eGFP cells at $\sim 1 \times 10^6$ per mL were resuspended in 100 μ l of cryoprotectant (20% w/v Dextran 40,000 in DMEM). The sample was applied to 100 μ m deep wells of an Au type-A specimen carrier, ensuring the wells were slightly overfilled to avoid air bubbles, and covered with the flat-side of a lipid-coated Au type-B carrier. Excess liquid was removed with filter paper and the sample was high-pressure frozen at ~ 2100 bar and -190 °C with a Leica EM ICE High-Pressure Freezer (Leica Microsystems).

2.4.8 Sample thinning by Cryo- ultramicrotomy

Dr Tom Sullivan established the workflow for preparing ultrathin sections using cryo-ultramicrotomy (Astbury Biostructure Laboratory, Electron Microscopy Facility, University of Leeds). High-pressure frozen samples were trimmed at 45° and trim 20° angles, and cryo-immuno sectioned at 35° with a Leica UC7 Ultramicrotome equipped with an FC7 cryogenic chamber cooled to -160 °C (Leica Microsystems). Images were acquired with a stereomicroscope within the FC7 chamber and correlated with fluorescent images acquired on the Leica Thunder Imager (Leica Microsystems) to accurately determine the region to target for thinning. A $150 \times 100 \times 40$ μ m trapezoid block of tissue was shaped, from which 90 to 40 nm ribbons were cut. Using an electrostatic gun and micromanipulators, the ribbons were adhered to Quantifoil R2/2, Cu 300 mesh grids (EMS) which had been glow discharged for 60 seconds at 30 mA using an easy glow (Cressington glow discharger).

2.4.9 Image acquisition using the Leica THUNDER Imager: cryogenic fluorescence microscopy

High-pressure frozen, cryo-preserved carriers and cryo-EM grids with SGR NS5A-eGFP cells were screened and imaged with a Leica THUNDER Imager EM Cryo CLEM (Leica Microsystems) with a HC PL APO 50x/0.9 NA cryo objective, Orca Flash 4.0 V2 sCMOS camera (Hamamatsu Photonics) and a Solar Light Engine (Lumencor). Z-stacks images were acquired with the frame size of 2048 x 2048 pixels, with 30% intensity and an exposure time of 0.2 seconds using the LASX software (Leica Microsystems). Images were processed using Fiji (Schindelin et al., 2012).

Filter	Excitation	Emission	Dichroic
GFP	470/40	525/50	495
DAPI	365/50	460/50	400

Table 2.8: Filter properties on Leica THUNDER

2.4.10 Image correlation of cryo-EM and cryo-FM maps

The Maps software (Thermo Fisher Scientific) was used to correlate between fluorescent images and electron micrographs. Low magnification (atlas map: 125 X, pixel size 819.2 Å, spot size: 7, illuminated area: 1100 µm) electron micrographs were correlated based on broken grid squares, and distinctive patterns designed into the centre of Quantifoil grids. At medium magnification (overview: 580 X, pixel size 224.4 Å, spot size: 6, illuminated area: 313 µm), specific grid squares were aligned. At high magnifications (Search map: 8700 X, pixel size 28.8 Å, spot size: 10, illuminated area: 12 µm), holes in the carbon support film acted as fiducial makers to aid correlation. The correlation between fluorescent images and electron micrographs after tomogram collection was performed using the 'Martin Correlate' Matlab script (Schorb and Briggs, 2014).

2.4.11 Cryo-electron tomography (cryo-ET)

Dr. Yehuda Halfon (Astbury Biostructure Laboratory, Electron Microscopy Facility, University of Leeds) facilitated data acquisition for cryo TEM. Cryo-ET was performed using the FEI Titan Krios TEM (Thermo Fisher Scientific), operated at 300 kV equipped with a Falcon 4i direct electron detector and a Selectris imaging filter (Thermo Fisher Scientific) in the energy-filtered TEM (EFTEM) nanoprobe mode. For dataset 1, lamellae or whole cells were mapped at a magnification of 8700 X, -50 µm defocus, pixel size 2.88 nm (28.8 Å) to localize all vesicles. Tilt series datasets were acquired at magnifications of 42,000 X (corresponding pixel sizes at the specimen level: 3.0 Å) using the Tomography 5.11 software (Thermo Fisher Scientific). A dose-symmetric acquisition

scheme with a constant electron dose of $1.552 \text{ e}^-/\text{\AA}^2$ per projection, target defocus ranging from -3 to $-5 \text{ }\mu\text{m}$, and energy filter slit at 10 eV was used covering the tilt range from 60° to -60° with 2° increments. Starting angle for lamellae for dataset 1 was 0° . Individual projections were acquired in counting mode using dose fractionation. Total dose per tilt series was $94.672 \text{ e}^-/\text{\AA}^2$. Four individual frames per projections were aligned and summed 'on-the-fly' using custom python scripts which utilised commands from MotionCor2 (Zheng et al., 2017), and IMOD software packages (Kremer et al., 1996).

For dataset 2, lamellae or whole cells were mapped at a magnification of $15,000 \times$, $-50 \text{ }\mu\text{m}$ defocus, pixel size 0.85 nm ($8.5 \text{ }\text{\AA}$) to localize all vesicles. Tilt series were acquired at magnifications of $64,000 \times$ (corresponding pixel sizes at the specimen level: $1.9 \text{ }\text{\AA}$) using the Tomography 5.13 software (Thermo Fisher Scientific). A dose-symmetric acquisition scheme with a constant electron dose of $2.94 \text{ e}^-/\text{\AA}^2$ per projection, target defocus ranging from -3 to $-5 \text{ }\mu\text{m}$ and energy filter slit at 10 eV was used covering the tilt range from 71.2° to -46.8° with 3° increments. Starting angle for lamellae for dataset 2 was 12.14° . Individual projections were acquired in counting mode using dose fractionation. Total dose per tilt series was $120.68 \text{ e}^-/\text{\AA}^2$. Eight individual frames per projections were aligned and summed 'on-the-fly' using custom python scripts which utilised commands from MotionCor2 (Zheng et al., 2017), and IMOD software packages (Kremer et al., 1996).

2.4.12 Tomogram reconstruction and volume rendering

Raw images were motion-corrected using MotionCor2 (Zheng et al., 2017). The tilt series images were aligned and reconstructed with the eTOMO interface of IMOD software package (Kremer et al., 1996). Tomograms from Dataset 1 were generated with fiducial-less alignment. All tomograms were reconstructed using weighted back-projection with bin 2. Another round of bin 2 was applied during image analysis using Binvol command. Dataset 2 tomograms were generated using the Aretomo software (Zheng et al., 2022) with a bin 4. Segmentation of selected tomograms was performed using the Amira software (Thermo Fisher Scientific) by keeping the threshold of all objects the same.

2.4.13 Statistical analysis on diameter of the vesicles

The minor and major axis was measured for each vesicle using IMOD's 'measure' function (Diameter of vesicles was measured by averaging minor and major axis. The aspect ratio was calculated by dividing major axis by minor axis. Data were exported to GraphPad Prism and analysed using non-parametric tests.

2.5 Cloning of the VIPER tag in non-structural proteins

2.5.1 Cloning of CoiLE and MiniE in NS5A protein

2.5.2 Plasmid DNA purification of CoiLE and MiniE

CoiLE and MiniE sequences were synthesised with flanking 5' and 3' BglII sites in the pUC19-amp vector and were received as lyophilised DNA from Genewiz (Azenta Lifesciences). Codons were optimised for CoiLE and MiniE avoiding any internal restriction sites (highlighted in bold orange in Table 2.9). Plasmid DNA was resuspended in filtered dH₂O and used to transform in DH5α cells and grown at 37 °C overnight. DNA from cells was harvested and purified as mentioned in section 2.2.2.

For restriction digestion, 10 µg of CoiLE-DNA and MiniE-DNA was digested with BglII (NEB) at 37 °C for 3 hours. Samples were resolved on a 2% agarose gel at 80 V and then purified using the Monarch Gel Purification kit (NEB). Purified digested fragments were eluted in 20 µl of filtered dH₂O.

CoiLE base sequence	L E I E A A F L E R E N T A
	CTG GAG ATC GAG GCC GCC TTC CTG GAG AGG GAG AAC ACA GCC
	L E T R V A E L R Q R V Q
	CTG GAG ACA AGG GTG GCC GAG CTG AGG CAG AGG GTG CAG
	R L R N R V S Q Y R T R Y
MiniE base sequence	AGG CTG AGG AAC AGG GTG AGC CAG TAC AGG ACA AGG TAC
	G P L
	GGA CCC CTG
	L E I E A A F L E R E N T
	CTG GAG ATC GAG GCC GCC TTC CTG GAG AGG GAG AAC ACA
MiniE base sequence	A L E T R V A E L R Q R V
	GCC CTG GAG ACA AGG GTG GCC GAG CTG AGG CAG AGG GTG
	Q R L R N E Y G P L
	CAG AGG CTG AGG AAC GAG TAC GGA CCC CTG

Table 2.9: CoiLE and MiniE sequences synthesised by Genewiz

Nucleoside specifically chosen to avoid forming restriction site is highlighted in bold orange.

2.5.3 Vector Plasmid DNA digestion

Restriction digestion of 2 µg of the SGR JFH-1 NS5A-eGFP vector DNA was performed with BglII at 37 °C for 3 hours. The 5' end of the vector was dephosphorylated for 15 minutes at 37 °C with 1 unit of alkaline phosphatase, calf intestinal (CIP, NEB). Samples were resolved on a 2% agarose gel at 80 V, purified using the Monarch Gel Purification kit (NEB) and eluted in 20 µl of filtered dH₂O. Ligation of SGR JFH-1 NS5A-eGFP with CoilE and MiniE was performed as detailed in section (2.2.8).

2.5.4 Cloning of MiniE in NS3 protein in SGR

MiniE tag was introduced at 7 novel insertion sites in the NS3 protein using the SGR JFH-1 NS5A-eGFP construct by overlap PCR. A schematic of this approach is shown in Figure 2. 11 and the primers are listed in Table 2.10. The insertion site for MiniE with linker sequences are shown in appendix Table 9. PCR reactions were individually customised based on the sizes of the fragment. General PCR set up and parameters are shown in Table 2.11.

MiniE fragment was derived from the construct MiniE-puC AW, which was used as template with Primers C and D. SGR JFH-1 NS5A-eGFP was used as a template for Primers A, B, E, F (Figure 2.1).

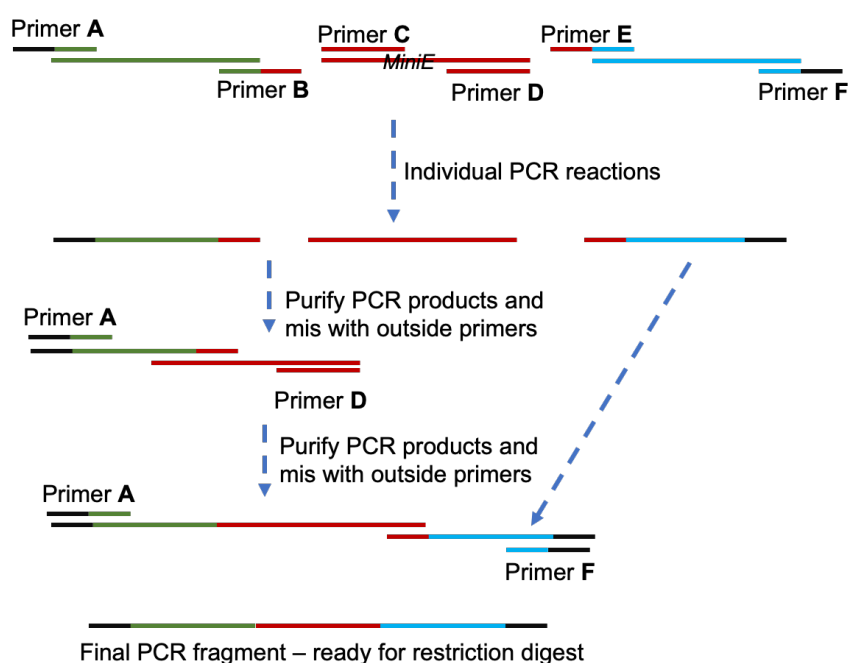


Figure 2.1: Schematic illustration of the overlap PCR strategy employed for designing SGR NS3-MiniE NS5A-eGFP constructs.

Adapted from (Hobert, 2002)

To generate construct SGR NS5A-eGFP and NS3-MiniE	Primer A (5'-3')
NS3-MiniE_M0, D25, P2, A5, G90, C99	CATCGCCTTCTATCGCCTTCTTGACGAG
NS3_MiniE_E628	GGCCCTATTACCAATGAGGTCACCCTCA
	Primer B (5'-3')
NS3-MiniE_M0	GCCTCGATCTCCAGGAGTCCGGACATGGTA TCATCG
NS3-MiniE_D25	GCCTCGATCTCCAGGAGTCCGGAGTCACGC CCCGTCATACTCACCCT
NS3-MiniE_P2	GCGGCCTCGATCTCCAGGGGAGCCATGAAT TCGGTATCATCGTG
NS3-MiniE_A5	GGCGGCCTCGATCTCCAGTCCAGCAGTGAT GGGAGCCATG
NS3-MiniE_G90	CCTCGATCTCCAGTCCCCCAGGGGGGCTG GGCCAGCC
NS3-MiniE_C99	GGCGGCCTCGATCTCCAGACACTTGCACGG CTCCAAAGACTTGGTCCC
NS3-MiniE_E628	GGCGGCCTCGATCTCCAGTCCCTCAAGGTC AGCTTGCATGC
	Primer C (5'-3')
NS3-MiniE_M0, D25, P2, A5, G90, C99,	CTGGAGATCGAGGCCGCCTTCCTGGAGAG GGAGAA
	Primer D (5'-3')
	CAGGGGTCCGTA CTCTCGTTCTCAGCCTCTG
	Primer E (5'-3')
NS3-MiniE_M0	TGAGGAACGAGTACGGACCCCTGGGACTCT CCGGTACCGCTCCCATCA
NS3-MiniE_D25	AGTACGGACCCCTGGGACTCTCCAGGACAG AACAGGCCGGGGAAGT
NS3-MiniE_P2	GAGTACGGACCCCTGGCTCCCATCACTGCT TATGCCCAGC
NS3-MiniE_A5	GGAACGAGTACGGACCCCTGTATGCCACG AAACACGAGG
NS3-MiniE_G90	ACGAGTACGGACCCCTGACCAAGTCTTTGG AGCCGTGC

NS3-MiniE_C99	GAGTACGGACCCCTGGGAGCCGTCGACCTA TATCTGG
NS3-MiniE_E628	AACGAGTACGGACCCCTGGTCATGACCAGC ACGTGGGTCC
	Primer F (5'-3')
NS3-MiniE_M0, D25, P2, A5, G90, C99	CCTAATGTTGGGATTGATGCCATGTGCC
NS3_MiniE_E628	CCTGATCAGACTCCAGGTCCGGATC

Table 2.10: List of primers used for generating SGRs with NS3-MiniE

Stock		Final concentration
5x GC Phusion buffer	10 µl	1X
Phusion enzyme	0.5 µl	1 units/50 µl
Primers (10 µM)	2.5 µl	0.5 µM
Templates (10 ng/ µl)	1 µl	0.2 ng/ µl
DNTPs (10 mM)	1 µl	0.2 mM
dH₂O	variable	
Total volume	50 µl	

Touch down PCR	Temperature	Time
Initial Denaturation	98 °C	10 sec
Denaturation	98 °C	10 sec
Annealing	Touchdown: 72 °C (10X)- 62 °C (20X)	30 sec
Extension	72 °C	variable
Final Extension	72 °C	5 min

Table 2.11: Overlap PCR set up and reaction parameters

2.5.5 Cloning of the MiniE tag in the NS3 protein in pCMV10 vector

Due to the unavailability of compatible restriction sites between the SGR JFH-1 NS5A-eGFP and pCMV10 NS3-5B plasmids, an EcoRI site was introduced before the initiation codon, methionine, in the SGR JFH-1 NS3-MiniE NS5A-eGFP plasmids (with NS3-MiniE-M0, NS3-MiniE-D25) by overlap PCR. Primers used to introduce EcoRI site in JFH-1 NS3-MiniE NS5A-eGFP are listed in Table 2.12. The fragments NS3-MiniE-M0 and NS3-MiniE-D25, were double digested with PmeI and SpeI (NEB) and were ligated

with double digested SGR JFH-1 NS3-5B. Subsequently, the SGR NS3- MiniE-M0 and SGR NS3-MiniE-D25 were double digested with EcoRI and XbaI (NEB) and then ligated with double digested pCMV-10 NS3-5B. Ligation was performed as mentioned in section 2.2.8 An overall schematic of the cloning strategy is shown in Figure 2.2. PCR reactions were optimised accordingly, as shown in Table 2.11.

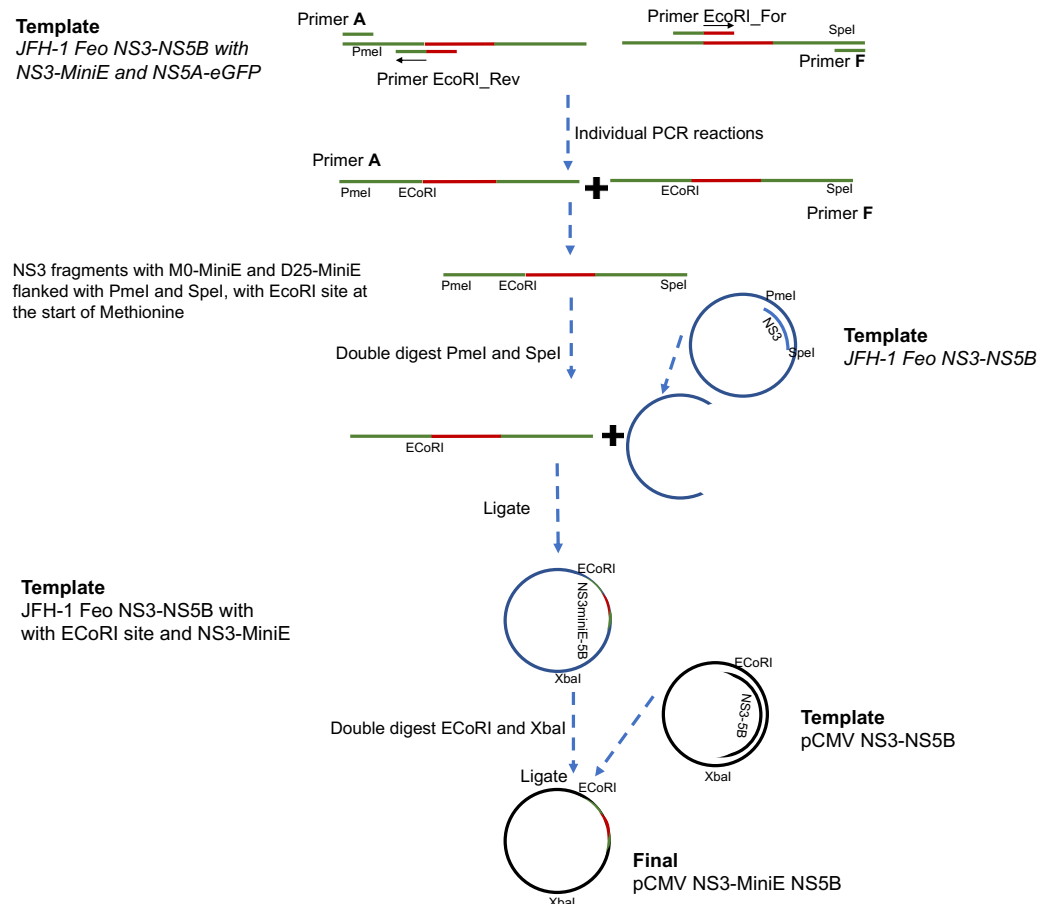


Figure 2.1: Schematic illustration of the subcloning strategy for NS3-MiniE-M0 and NS3-MiniE-D25 from SGR to pCMV-10 NS3-NS5B.

Forward	Primer	CGATGATACCGAATTCATGTCCGGACTCCTGGAGAT
EcoRI_NS3MiniE_M0		
Reverse	Primer	ATCTCCAGGAGTCCGGACATGAATTCGGTATCATCG
EcoRI_NS3MiniE_M0		
Forward	Primer	CGATGATACCGAATTCATGGCTCCC
EcoRI_NS3MiniE_D25		
Reverse	Primer	GGGAGCCATGAATTCGGTATCATCG
EcoRI_NS3MiniE_D25		

Table 2.12: List of primers to introduce EcoRI site in SGR JFH-1 NS3-MiniE NS5A-eGFP.

2.5.6 MiniR expression, purification, and conjugation with Cy5-maleimide

2.5.6.1 MiniR Expression

MiniR-pET28 expression construct was kindly provided by Prof. Kimberley Beatty (Oregon Health & Science University). The MiniR-pET28 plasmid was transformed using BL-21(DE3) RIL cells with 50 µg/ml kanamycin and 50 µg/ml Chloramphenicol. Individual clones were selected for test expression and grown in 2X yeast extract tryptone media (2X YT media for 1 litre:16 g tryptone, 5 g NaCl,10 g Bacto Yeast extract, pH 7). The expression (lysates and elutions) was analysed on 12% Bis tris gel (NuPAGE, Invitrogen) by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run in 1X MES buffer (300 mM MES salt, 50 mM Tris base, 1mM EDTA, 0.1% w/v SDS) at 180 V. The colony with the best expression was used for scaling up the expression and was stored as a glycerol stock at -80 °C. For a 500 ml culture, a 1:200 dilution of overnight culture was used and grown at 37 °C, 180 rpm. The expression was induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to an optical density (OD₆₀₀) between 0.8-1, and expressed for 6 hours at 30 °C, 180 rpm. Cells were harvested at 4,000 X g for 20 minutes. The weight of the pellet was measured and stored at -20 °C.

2.5.6.2 MiniR Purification

Cell pellet collected from MiniR protein expression was thawed on ice. Based on the cell pellet size, it was resuspended in 10 X volume of Buffer B (Table 2.13). Cells were lysed by using a 1-inch sonication probe on ice at 5% amplitude, pulsed for 1 second and then left to rest for 3 seconds on ice for 80 cycles. The lysate was clarified by centrifugation in a refrigerated centrifuge at 4 °C, at 10,000 X g for 30 minutes. Using a syringe, clarified lysate was applied to a 5ml Ni-NTA HisTrap™ fast flow column (Cytiva) (prepacked column with 90 µm agarose particle size). The resin was washed with 2 column volumes

(CV) of Buffer B, followed by 3 CV of Buffer C (Table 6) and then eluted with 1 CV Buffer E (Table 2.13). The flow-through, all washes and elutions were collected and placed on ice. All samples were resolved using 12% or 4-12% Bis tris SDS-PAGE gels (NuPAGE™, Invitrogen) in 1X MES buffer at 180 V. The SeeBlue Plus2 (Invitrogen) marker was used as a protein standard. Quick Coomassie (Neo Biotech) was used for staining protein bands at RT. Pooled elutions were concentrated using Amicon Ultra Centrifugal filters (Millipore) with a 3 kDa molecular weight cut off (M.W.CO).

Buffers	Recipe
Buffer B	6 M Urea, 100 mM NaH ₂ PO ₄ , 10 mM Tris-Cl, 10mM Imidazole, pH 8
Buffer C	6 M Urea, 100 mM NaH ₂ PO ₄ , 10 mM Tris-Cl, 20mM Imidazole, pH 6.5
Buffer E	6 M Urea, 100 mM NaH ₂ PO ₄ , 10 mM Tris-Cl, 300mM Imidazole, pH 4.5
TBS urea buffer (exchange)	2 M Urea, 100 mM NaH ₂ PO ₄ , 10 mM Tris-Cl, 10% glycerol, pH 7.4
Labelling buffer	125 mM NaH ₂ PO ₄ , 200 mM NaCl, 1.2 mM EDTA, 3.9 M ammonium sulfate, pH 7.5
5x Protein dye	300 mM Tris, 50 mM TCEP, 10 % SDS (w/v), 65% glycerol (v/v), 0.025% Ponceau red (v/v)

Table 2.13: List of buffers used for MiniR purification (Doh et al., 2020).

2.5.6.3 MiniR conjugation with Sulfo-Cyanine5-maleimide

Labelling and purification of MiniR conjugated to sulfo-cyanine5 maleimide (Cy5, Lumiprobe) was established by using an improvised protocol, developed by BSTG (BioScreening Technology Group, Faculty of Biological sciences, Leeds). Briefly, a 2 mM stock of Sulfo Cy5-maleimide was prepared in DMSO. A 150 µl of immobilized tris 2-carboxyethyl phosphine (TCEP) disulphide-reducing gel (Thermo Fisher scientific) was washed by mixing thoroughly with 3 X 300 µl of 1 X PBS containing 1 mM EDTA, and then centrifuged at 1000 X g for 1 minute. To the washed gel, 4 µl PBS containing 50 mM EDTA was added with 180 µl of MiniR at a concentration of 0.5 mg/ml, and incubated in a speed rotator for 2 hours at 4 °C. The ~180 µl supernatant containing MiniR molecules with reduced cysteines was collected in a black Eppendorf tube, to protect from light, containing 6 µl of 2 mM Sulpho Cy5-Maleimide. The labelling reaction was incubated at 4 °C for 21 hours. Following incubation, 20 mM 1,4-Dithiothreitol (DTT) was added to quench the TCEP-maleimide reaction. The mixture was cleaned to remove

excess fluorophores using a PD G25 desalting spin column (Cytvia) with 3 kDa M.W. C.O, as per manufacturers' instructions. Briefly, the PD G25 column was washed with 5 X 400 µl of TBS urea buffer (Table 2.13) and centrifuged at 800 X g for 1 minute. The upper level of the gel bed was marked and placed facing outwards in the centrifuge. A minimum of 400 µl of labelled reaction was used to elute. Absorbance was measured at A_{280} and A_{650} using a nanodrop. Samples were collected pre- and post-desalting for SDS-PAGE analysis.

2.5.7 Immunofluorescence analysis for detecting MiniE constructs with the MiniR-Cy5 probe

To detect MiniE constructs with the MiniR-Cy5 probe, control and replicon cells were seeded, fixed and blocked as detailed in section. To stain cells with MiniR-Cy5, 100 µl of MiniR-Cy5 diluted in 3% BSA-PBS was pipetted onto parafilm and coverslips were placed cell-side down onto the antibody and incubated in dark for 1 hour at RT. Following incubation, coverslips were washed three times with 3 X 5-minute washes with 1 X PBS. Prolong Gold Antifade reagent with DAPI (Thermo Fisher Scientific) was used to mount the coverslips onto glass microscope slides which were cured at RT overnight.

2.5.8 Immunofluorescence for detecting NS3 with anti-NS3 antibody

Control and replicon cells were seeded, fixed and blocked as detailed in chapter 5. To stain cells with primary anti-NS3 antibody, 200 µl of antibody diluted 1:200 in 3% BSA-PBS was pipetted directly onto the coverslips in the well and incubated at RT for 1 hour. After 3 washes with 1 X PBS, the coverslips were incubated with 500 µl of secondary antibody diluted 1:500 in 1 % BSA-PBS at RT for 1 hour and then washed 3 times with 1 X PBS. Prolong Gold Antifade reagent with DAPI (Thermo Fisher Scientific) was used to mount the coverslips onto glass microscope slides which were cured at RT overnight.

2.5.9 DNA transfection for western blotting

HEK 293T cells were seeded into a 6-well plate at a concentration of 3×10^5 per well and incubated for 24 hours at 37 °C, 5% CO₂. Cells were washed with 1 X PBS and incubated with 1 ml Opti-MEM (Gibco) at 37 °C before transfection media was added. 2 µl of Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) was diluted in 200 µl Opti-MEM and incubated at RT for 5 min. Meanwhile, 2 µg of pCMV-10-NS3-5B plasmid DNA, control or two pCMV-10 NS3-MiniE recombinant plasmids were mixed with 200 µl of Opti-MEM and incubated at RT for 5 min. The transfection reagent and DNA were mixed and incubated at RT for 20 minutes before adding to the cells in a drop-wise manner. After 6 hours, the transfection medium was removed and replaced with fresh complete DMEM. Cells were harvested at 48 hours post transfection, washed twice with

1X PBS, and lysed in 1X Glasgow Lysis Buffer (1X GLB: 10 mM PIPES, pH 7.2, 120 mM KCl, 30 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 10% Glycerol) with protease and phosphatase inhibitors at -20 °C overnight.

Inhibitors	Stock concentration	Working concentration
Leupeptin	1 mg/ml	1 µg/ml
Pepstatin A	1 mg/ml	1 µg/ml
Aprotinin	2 mg/ml	2 µg/ml
AEBSF (pefabloc)	100 mM	0.2 mM
Na ₃ VO ₄	2 mM	2 mM
NA pyrophosphate	5 mM	5 mM

Table 2.14: List of protease and phosphatase inhibitors

2.5.10 Detection of NS5A and NS3 in pCMV-10 constructs using Western blotting

Cell lysates that were lysed in 1X GLB (section 2.5.9) were thawed and clarified by centrifugation at 3000 X g in a bench top refrigerated centrifuge for 5 minutes at 4 °C. The cell supernatant was transferred into a fresh tube, and the total protein concentration was determined by BCA assay. Equal amounts of protein was prepared for control and experimental samples in SDS-loading buffer (5 X buffer: 1M Tris-HCl, pH= 6.8; 10% SDS, 10% β-mercaptoethanol; 40% glycerol; bromophenol-blue) and resolved on a prepared 12% tris- glycine SDS-PAGE gel in 1X tris-glycine buffer (diluted from 10X stock: 576 g glycine, 121.2 g Tris base and 20% SDS), and ran at 180 V. The SeeBlue Plus2 (Invitrogen) marker was used a protein standard.

The SDS gel was transferred into 1X Towbin buffer (25 mM Tris, 192 mM Glycine, 20% methanol). Polyvinylidene difluoride membrane (PVDF) was activated for 30 seconds in 100% methanol and then transferred into Towbin buffer before setting up the gel-membrane sandwich in the Trans-blot Turbo Transfer system (Bio-rad). The transfer was performed using a pre-programmed Bio-Rad mini-gel protocol which had a total run time of 40 minutes at 25 V, with the first 30 minutes being run at 1.0A and the last 10 minutes were run at 1.3A. Once the transfer was complete, the membrane blocked with blocking buffer composed of 75% Tris Buffer Saline-Tween20 buffer (TBS-T: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20), combined with 25% LI-COR Intercept^R blocking buffer (LI-COR) and incubated for 20 minutes at RT. The membrane was then washed with 5 X 5-minute TBS-T buffer. Primary antibodies (Table) were diluted in blocking buffer and applied to the membrane, and incubated overnight at 4 °C. Subsequently, the membrane was washed 5 X 5-minute TBS-T buffer, before being

incubated with secondary antibodies (Table 2.2) diluted in blocking buffer at RT for 1 hour. The membrane was washed 5 X 5-minute TBS-T buffer, dH₂O for 10 minutes and then dried between blotting membrane for a minimum of 30 minutes before visualising on LI-COR Odyssey SA Imaging system (LI-COR).

2.5.11 ColabFold modelling

For the generation of MiniE-MiniR, NS3-MiniE, NS5A-eGFP-MiniE models, Google ColabFold (Mirdita et al., 2022) available publicly was employed (<https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb>).

For modelling using ColabFold, the sequences of full-length JFH-1 protein, the protease domain and the helicase domain were obtained from Genbank (accession number: BAB32872.1), and the sequences for MiniE and MiniR taken from a previously published paper (Doh et al., 2020). The sequences were submitted to the ColabFold pipeline and models ranked by the software were visualised in Pymol.

Chapter 3| Validation of the subgenomic replicon for the establishment of a cryo-correlative light and electron microscopy method

3.1 Introduction

3.1.1 Considerations for HCV subgenomic replicon for the integration into cryo-CLEM methodology

The biosafety level of HCV according to the 'Biosafety in Microbiological and Biomedical Laboratories' manual published by the Centre for Disease Control and Prevention Centre (CDC) is considered as Biosafety level 2 (BSL-2) (Yi, 2010). However, in the UK, the advisory committee on dangerous pathogens (ACDP) in the manual for 'The Approved List of Biological Agents' classes HCV under human pathogen hazard group 3, and therefore any work carried out with infectious HCV virus must be performed at containment level 3 (CL3) (<https://www.hse.gov.uk/biosafety/information.htm>). The Astbury Biostructure Electron Microscopy facility at the University of Leeds (EM facility) is only approved to work with viruses at containment level 2 (CL2). As the goal of this project was to determine the architecture of the HCV-induced membranous compartments *in-situ* by cryo light and electron microscopy (cryo-CLEM), the use of an HCV subgenomic replicon (SGR) was considered suitable for the investigation.

HCV SGR's have contributed significantly to the discovery of pan-genotypic direct-acting antivirals (DAAs) by facilitating the treatments that deliver a sustained virologic response (SVR) (Han et al., 2019; Khan et al., 2020). The HCV genome is translated into 10 proteins that is cleaved by cellular and virus proteases. The proteins are classified as three structural (core, E1, E2) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B). The structural and NS regions are separated by a membrane bound peptide known as p7 (Jirasko et al., 2010). It was demonstrated in a proof-of-principle investigation that the minimal requirement of HCV replication and translation machinery requires the non-translated regions (NTRs), and the non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B (Lohmann et al., 1999; Bartenschlager, 2002). Therefore, HCV replicons are subgenomic RNA that are capable of autonomously replicating in hepatoma cells (Bartenschlager, 2006; Lohmann, 2009).

The first replicon system was established using a consensus GT1b isolate sequence composed of two independent gene clusters (Lohmann et al., 1999). The first cistron was composed of the HCV 5' NTR and 16 codons of the core gene fused in-frame with the neomycin phosphotransferase (neo) selection gene. The second was integrated with internal ribosome entry sites (IRES) that directed translation of non-structural viral proteins. The replicon is then cloned downstream of the T7 promoter in a plasmid and served as a template to generate bicistronic transcripts (Lohmann et al., 1999; Blight et al., 2002) (Figure 1.3 in Introduction). The autonomous replication in Huh7 cells was proved by labelling HCV RNA with H³ uridine in the presence of actinomycin D, which is

an inhibitor of DNA but not RNA-dependent RNA polymerases (Lohmann et al., 1999). When Huh7 cells were transfected with the bicistronic transcripts, the *neo* gene that confers resistance to the cytotoxic aminoglycoside antibiotic G418, then enabled the selection of “stable replicon cells” (Lohmann et al., 1999; Khan et al., 2020).

The variations of this classical replicon system were used to establish the GT1b (Ikeda et al., 2002), GT2a (Kato et al., 2003), GT3 (Saeed et al., 2012; Ward et al., 2020), GT4 (Saeed et al., 2012; Peng et al., 2013), GT5a (Wose Kinge et al., 2014), and GT6a SGRs (Yu et al., 2014; Camus et al., 2018). To facilitate direct and more efficient methods of analysis, *neo* was replaced with firefly luciferase (FLuc) or *Renilla* luciferase (RLuc) (Krieger et al., 2001; Camus et al., 2018). Feo is a fusion of neomycin phosphotransferase and FLuc reporters that allows the selection of positive clones under G418 selection and also monitors viral replication (Wakita et al., 2005). Furthermore, the insertion of GFP to NS5A protein in C-terminal domain permitted direct visualisation of events during the replication cycle (Moradpour et al., 2004). The insertion site used to introduce eGFP in NS5A was later employed to create the pSGR-JFH-1 NS5A-eGFP replicon (Wakita et al., 2005; Marie-Emmanuelle et al., 2019). Since then, NS5A has been used as a surrogate marker for replication. The majority of the ultrastructural studies investigating HCV membranous compartments have used JFH-1 virus or SGR with NS5A-eGFP (Ferraris et al., 2010; Romero-Brey et al., 2012; Ferraris et al., 2013; J.Y. Lee et al., 2019). Therefore, to perform comparative analysis, SGR JFH-1 Feo NS5A-eGFP was considered for this study. Another SGR with NS5A-mCherry was also generated (Wang et al., 2014) to identify and characterise a PI-4 kinase effector in HCV replication.

In this chapter, pSGR JFH-1 Feo NS5A-eGFP and NS5A-mCherry were used to generate stable replicon G418-selected cells. The subcellular localisation of NS5A were compared in Huh7 cells expressing SGR NS5A-eGFP and SGR NS5A-mCherry. Several host factors have been reported to play a role in the formation of the HCV replication sites, such as PI4KIII α , OSBP, VAP-A and VAP-B, cyclophilin and many others (Romero-Brey and Lohmann, 2016). Therefore, to validate NS5A-eGFP and mCherry expression, the subcellular localisation of NS5A with three cellular markers was considered.

NS5A is a multifunctional protein with an N-terminal amphipathic helix that associates with cellular membranes. During HCV replication, NS5A localises to the ER-derived membranous web (MW) (Riva et al., 2021). Using the SGR, by fractionation and immunomicroscopy, the localisation of HCV non-structural proteins in G418 selected cells were confirmed to localise exclusively to the ER, confirming the hypothesis that

HCV replication is ER-membrane associated (Mottola et al., 2002). The MW is primarily derived from the ER resulting in the formation of single-, double- and multi-membrane vesicles (SMV, DMV, MMV). The colocalisation studies from an immunofluorescence experiment using Huh7 cells infected with HCV Jc1 (intragenotypic chimera composed of sequences of the J6 and JFH-1 strains) and processed after 48 hours, revealed protein disulphide isomerase (PDI) colocalisation with the core, E2, NS3, NS4B, NS5A (Lohmann et al., 1999). Therefore, PDI was employed as one of the positive replication markers for colocalisation with NS5A.

The family of VAP proteins: VAP-A, VAP-B and VAP-C, are highly conserved in mammals and orchestrate several cellular processes at the organelle-ER contact sites which have been extensively reviewed previously (Kors et al., 2022). VAP-A is a type II integral membrane protein primarily residing in the ER membrane. It contains a membrane spanning C-terminal domain, a central coiled coil domain and an N-terminal major sperm domain (MSP) which faces the cytoplasm to interact with various cellular proteins (James and Kehlenbach, 2021). *In-vitro* binding assays and *in-vivo* co-immunoprecipitation studies confirmed that NS5A binds to the C-terminal domain of VAP-A (Tu et al., 1999). Using SGR, an inverse correlation was determined between NS5A phosphorylation and VAP-A binding suggesting that hyper-phosphorylated (p58) NS5A represents a closed conformation that is unable to interact with VAP-A, whereas the hypo-phosphorylated (p56) NS5A represents an open conformation capable of strong interactions with VAP-A (Evans et al., 2004). The role of phosphorylation of a specific serine residue, S225 in NS5A in regulating interactions with cellular proteins like VAP-A for genome replication and the localisation of the replication complex was identified by immunoprecipitation, western blot, immunofluorescence and by small interfering RNA (siRNA)-mediated knockdown of VAP-A (Goonawardane et al., 2017). The colocalisation of both static and mobile NS5A-GFP with VAP-A-mCherry positive ER tubules was shown by live cell imaging in cells electroporated and infected with HCV Jc1 RNA (Eyre et al., 2014). Therefore, VAP-A was considered as another positive replication cellular marker for validating the colocalisation of VAP-A with NS5A in Huh7 cells expressing SGRs.

The trans-Golgi network protein (TGN) and associated endosomal compartments have been previously reported as a major route for trafficking and release of infectious HCV particles from Huh7 cells by siRNA-mediated silencing of TGN-associated adaptor proteins (Mankouri et al., 2016). TGN-46 staining was employed to observed differences in cells infected with JFH-1 virus or transfected with SGR, where no apparent

redistribution of TGN-46 was observed in SGR NS3-5B, and was therefore used as a control for colocalisation of SGR JFH-1 NS5A-eGFP and NS5A-mCherry (Mankouri et al., 2016).

Alongside colocalisation, differences in the brightness of fluorophores were also considered, which play an important role in imaging under cryo conditions. At 76 K (-194 °C), research using organic dyes revealed a triplet population ranging from 80% to 90%, resulting in a nearly tenfold decrease in brightness. (Giske, 2007; Kaufmann et al., 2014).

3.2 Results

3.2.1 Generation of stable cell lines: SGRs JFH-1 Feo NS3-5B (NS5A-eGFP and NS5A-mCherry)

To establish a stable cell line utilising SGR JFH-1 Feo NS3-5B (NS5A-eGFP and NS5A-mCherry), replication of the SGRs in Huh7 cells was evaluated through a Luciferase assay conducted up to 72 hours post electroporation (h.p.e). This was compared to the replication of the SGR WT JFH-1 Feo NS3-5B. The replication kinetics of SGR NS5A-eGFP were approximately $2\log_{10}$ lower at 72 hours compared to WT, while SGR NS5A-mCherry showed an approximate $3\log_{10}$ higher replication compared to the WT as illustrated in Figure 3.1. No increase in replication was observed in the replication deficient SGR-Feo DBN3 (GNN). Polyclonal cell populations were isolated under G418 selection pressure to establish a stable cell line for SGR NS5A-eGFP and NS5A-mCherry in Huh7 cells.

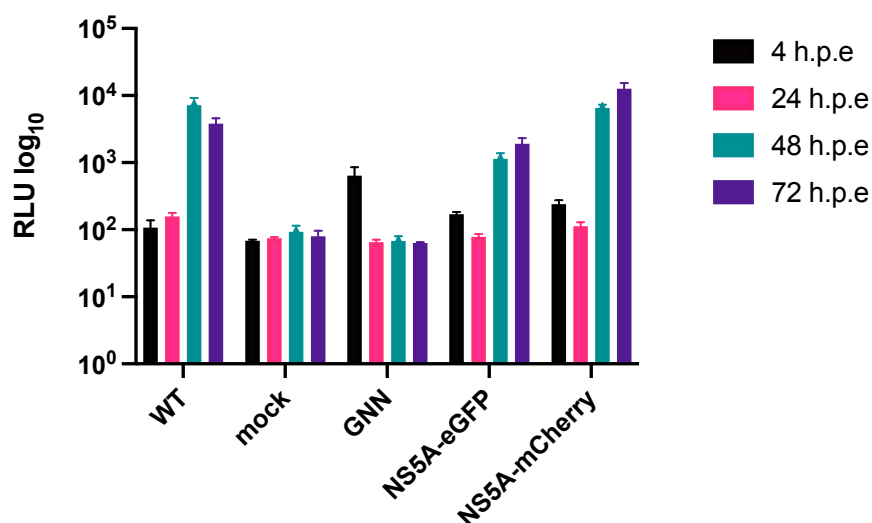


Figure 3.1: Assessment of SGR replication.

Bar graph showing the replication of SGR constructs (WT, NS5A-eGFP, NS5A-mCherry, and polymerase deficient (GNN, negative control)) measured using a Luciferase assay up to 72 h.p.e from transient electroporation of 2 µg of SGR RNA into Huh7 cells. Relative luciferase units (RLU) shown. Data represents the mean ± SD, n=1 (with three technical repeats).

3.2.2 Immunofluorescence analysis of the subcellular distribution of NS5A foci in SGR JFH-1 WT detected by Alexa Fluor 488 and 594 revealed no significant differences.

To examine and confirm the intracellular localisation of NS5A in stable cell lines (SGR NS5A-eGFP and NS5A-mCherry), the subcellular distribution of NS5A in SGR JFH-1 WT was initially assessed through immunofluorescence. Huh7 cells were electroporated with SGR WT JFH-1 and were stained with NS5A primary antibody and visualised using Alexa Fluor (AF) secondary antibodies, AF-488 and AF-594. In both instances, NS5A foci were dispersed throughout the cytoplasm as depicted in Figure 3.2A and Figure 3.2B. These observations corroborate with the previously reported NS5A distribution in cells infected with HCV JFH-1 WT (Ross-Thriepland et al., 2015a).

The distribution area of NS5A foci was examined by the 'analysing particles' function in Fiji (Schindelin et al., 2012). Descriptive statistics using GraphPad's (Prism) in-built statistical tools, revealed that the majority of the NS5A foci detected by AF-488 had larger areas within the 0.2-1 µm² range, while those detected by AF-594 had larger areas in the ranges of 1-2, 2-3, and 3-4 µm². The area of NS5A foci in both sample populations indicated the smallest foci size to be 0.02 µm² with a mean size of 1.26 µm² (Appendix Table1). As the standard deviation (Appendix Table1) between the two means differed, an unpaired t-test (Welch's test), was performed, revealing no significant differences (p value = 0.4415). The estimated mean difference in the NS5A foci (area) is presented in the Figure 3.2C.

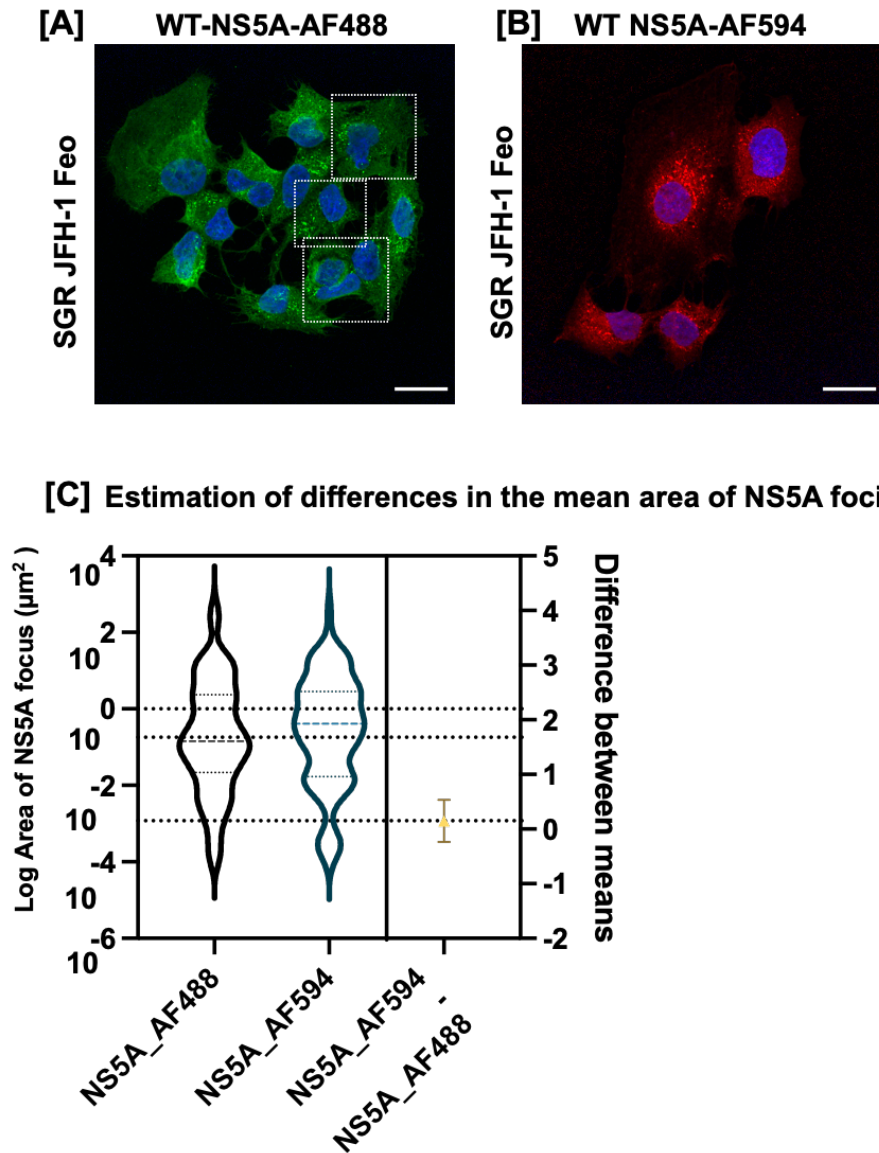


Figure 3.2: Subcellular distribution analysis of NS5A in SGR JFH-1 WT.

[A-B] Subcellular distribution of NS5A in Huh7 cells electroporated with SGR WT JFH-1. Cells were fixed and stained with anti-NS5A antibody (1:150) and DAPI (blue). [A] depicts NS5A detected by AF-488 (green), while [B] shows NS5A detected by AF-594 (red). Scale bar = 10 μm . [C] Calculated differences in the mean of the area determined of the NS5A foci by an unpaired t-test (Welch's test, GraphPad), revealing no significant differences. The left-hand side of the plot denotes all the NS5A focus points detected by AF-488 and AF-594 distributed logarithmically. The right-hand side of the plot denotes the differences in the area of NS5A mean between AF-488 and AF-594. Imaged on LSM880 confocal microscope with 63X objective (oil immersion) with N.A of 1.4.

3.2.3 Subcellular distribution of VAP-A in control Huh7 cells and Huh7 cells harbouring SGRs

The colocalisation of both static and mobile NS5A-GFP with VAP-A-mCherry positive ER tubules was shown by live cell imaging in cells electroporated and infected with HCV Jc1 RNA (Eyre et al., 2014). To evaluate the intracellular localisation of VAP-A in control Huh7 cells and subsequently validate the interaction of VAP-A with NS5A in Huh7 cells harbouring SGR NS5A-eGFP and SGR NS5A-mCherry, immunofluorescence experiments were conducted. Control Huh7 and cells harbouring SGR were stained with anti-VAP-A and then detected with fluorescent secondary antibodies. Huh7 control cells displayed a diffuse cytoplasmic distribution with an enhanced signal in the perinuclear region when cells were stained with anti-VAP-A and visualised by AF-488 nm and AF-594 nm (Figure 3.3A and Figure 3.3C). However, the pattern of VAP-A signal near the perinuclear region in Huh7 cells detected by AF-594 (Figure 3.3A) and AF-488 (Figure 3.3C) appeared to be different. Since, VAP proteins are ubiquitously expressed in the cells (Kors et al., 2022), the cellular distribution of VAP-A detected by AF-594 seemed consistent with the literature. VAP-A and VAP-B are integral ER transmembrane proteins with 63% sequence similarity (James and Kehlenbach, 2021), and undergo homo- and hetero dimerisation through their transmembrane domains and MSP domains (Nishimura et al., 1999; Kim et al., 2010). A polyclonal VAP-A primary antibody was used which could suggest the difference in the binding to the VAP-A epitopes in the cell. The difference in epitope binding could be attributed to the differences observed in the specificity of AF-594 and AF-488.

SGR NS5A-eGFP and SGR NS5A-mCherry both interact with VAP-A due to colocalisation in the cytoplasmic area. (Figure 3.3B and Figure 3.3D). As demonstrated by a change in VAP-A phenotype between Huh7 cells and Huh7 cells harbouring NS5A-mCherry was observed. The VAP-A signal was diffused throughout the cytoplasm in cells expressing SGR NS5A-mCherry and appeared to have lost the distinct perinuclear enhanced signal observed in the control cells (Figure 3.3C and Figure 3.3D). On the contrary, Huh7 cells and Huh7 cells harbouring SGR-NS5A-eGFP retained the enhanced perinuclear signal of VAP-A (Figure 3.3A and Figure 3.3B), hence colocalisation between NS5A-eGFP and VAP-A was visible. Therefore, it was considered that SGR-NS5A-mCherry may associate with cellular membrane proteins differently than SGR-NS5A-eGFP, causing changes in the network of VAP-interacting proteins, further resulting in the loss of the perinuclear signal of VAP-A.

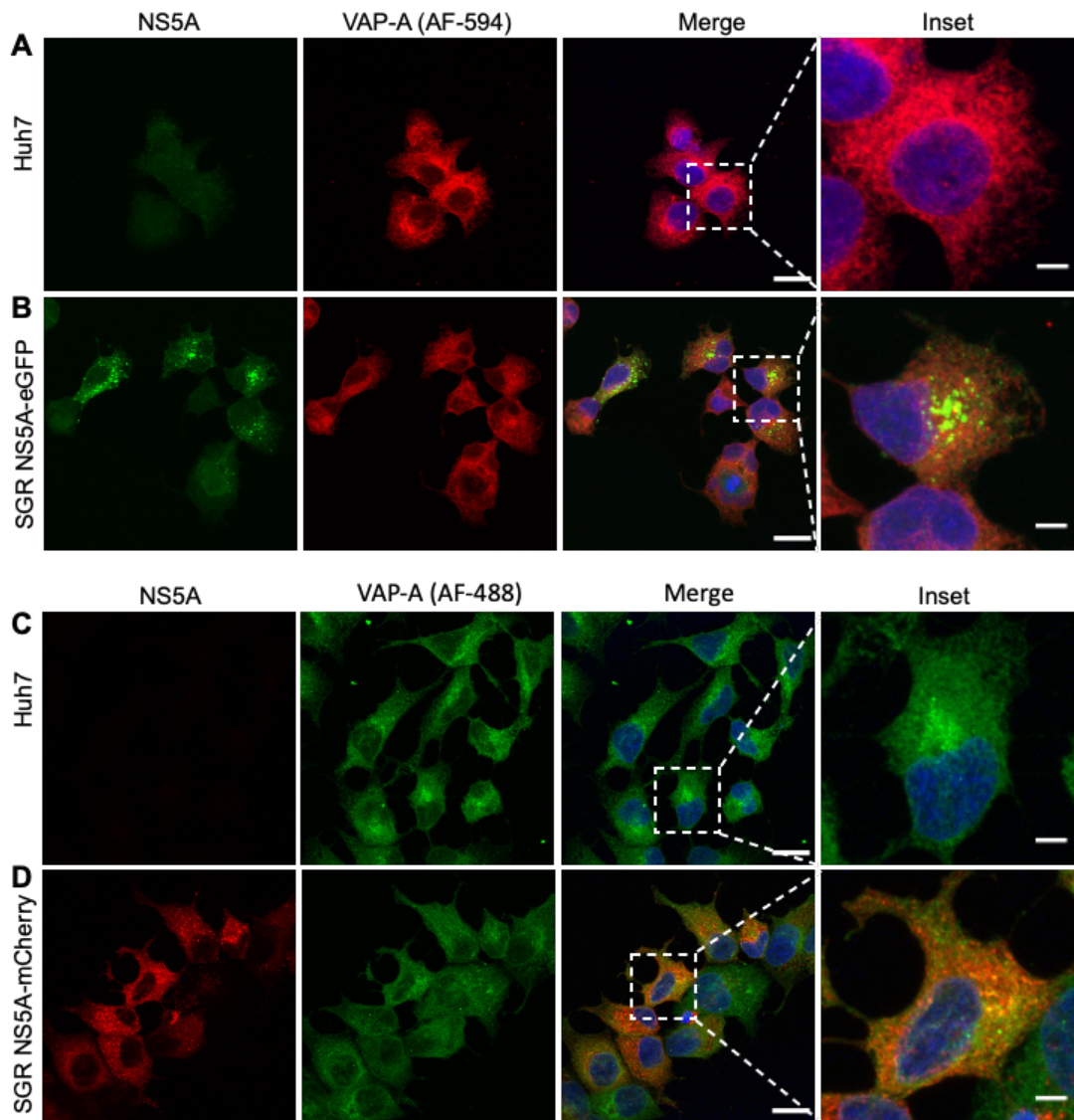


Figure 3.3: Subcellular distribution of VAP-A in control Huh7 cells and cells harbouring SGRs.

[A] Huh7 cells stained with anti-VAP-A and AF-594 (red). [B] Huh7 cells harbouring SGR NS5A-eGFP (green) stained with anti-VAP-A and AF-594 (red). [C] Huh7 cells stained with anti-VAP-A and AF-488 (green). [D] Huh7 cells harbouring SGR NS5A-mCherry (red) stained with anti-VAP-A and AF-488 (green). Colocalisation of VAP-A and NS5A are shown in respective inset. Nuclei of cells stained with DAPI (blue). Imaged on LSM880 confocal microscope with 63X objective (oil immersion) with N.A of 1.4. Scale bars = Merge: 20 μ m, Inset: 5 μ m.

To compare NS5A-foci in NS5A-eGFP and NS5A-mCherry, a similar analysis to that performed with SGR JFH-1 WT of NS5A foci (Figure 3.2) was conducted and is presented in Figure 3.4. There were no significant differences found in the mean size (area) of the NS5A foci between NS5A-eGFP and NS5A-mCherry. The minimum size of

NS5A in both the cases were $0.02 \mu\text{m}^2$, as in the case of SGR WT JFH-1 (Appendix Table 2).

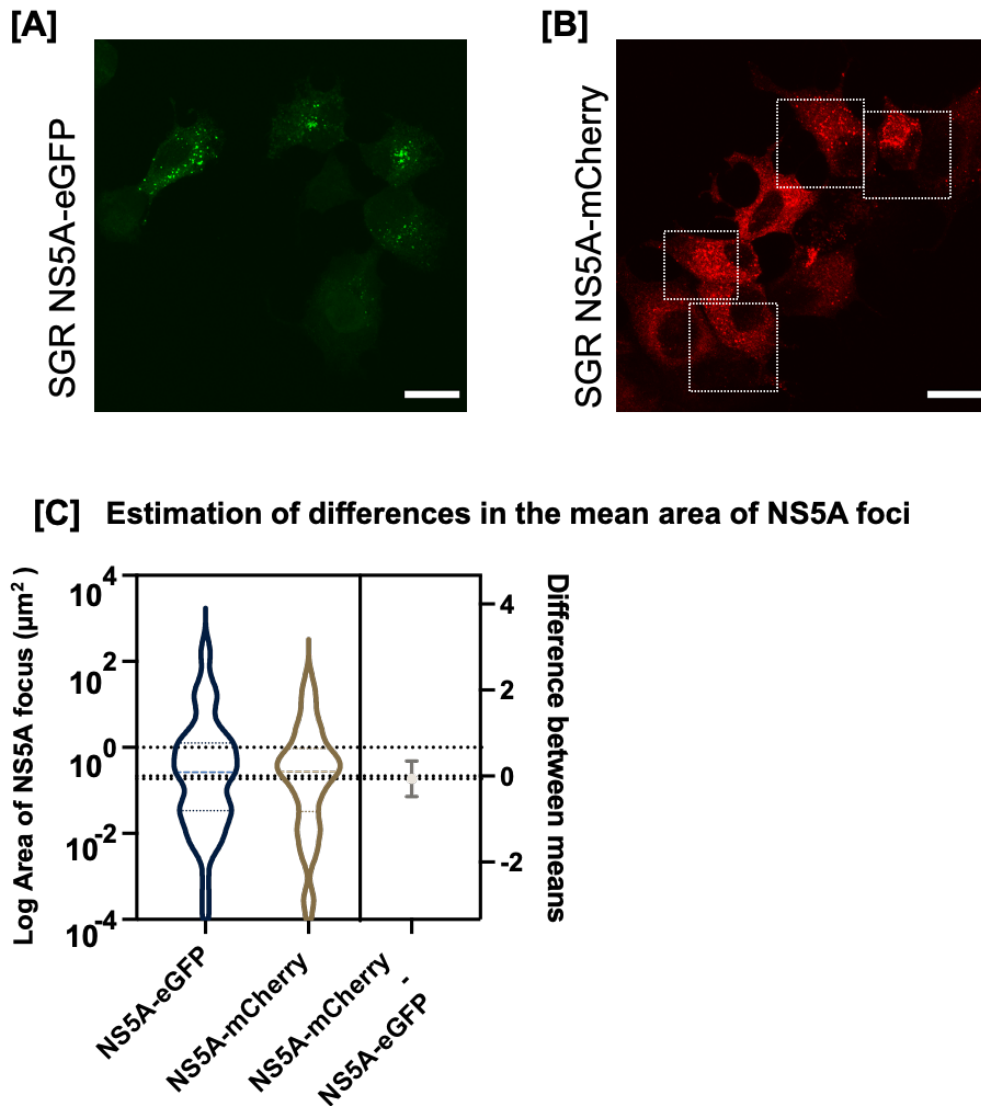


Figure 3.4: Assessment of the subcellular distribution of NS5A in SGR NS5A-eGFP and NS5A-mCherry.

[A-B] Subcellular distribution of NS5A in Huh7 cells harbouring SGR NS5A-eGFP [A] and NS5A-mCherry [B]. Cells were fixed and stained with anti-VAP-A antibody and DAPI (blue). [A] represents NS5A detected by eGFP signal (green) while [B] represent NS5A detected by mCherry signal (red). Scale bar = $20 \mu\text{m}$. [C] Calculated differences in the mean of the area determined of the NS5A foci by an unpaired t-test (Welch's test, GraphPad), revealing no significant differences. The left-hand side of the plot denotes all the NS5A focus points detected by NS5A-eGFP and NS5A-mCherry, distributed logarithmically. The right-hand side of the plot denotes the differences in the area of NS5A mean between NS5A-eGFP and NS5A-mCherry. Imaged on LSM880 confocal microscope with 63X objective (oil immersion) with N.A of 1.4

3.2.4 Subcellular distribution of PDI in control Huh7 cells and Huh7 cells harbouring SGRs

HCV viral translation and replication is closely associated with ER membranes. The effect of PDI inhibition on Huh7 cells harbouring SGR using an abscisic acid derived PDI inhibitor, origamycin, indicated suppression of HCV replication (Özcelik et al., 2018). Given the significant role of PDI in HCV replication, the subcellular distribution of NS5A, PDI, and their colocalisation was examined in Huh7 cells hosting SGR NS5A-eGFP and SGR NS5A-mCherry.

Huh7 cells harbouring SGR NS5A-eGFP and SGR NS5A-mCherry were stained with monoclonal anti- PDI and visualised with secondary antibodies to assess the association of PDI and NS5A. PDI in Huh7 control cells detected by AF-488 or AF-594 displayed a diffuse cytoplasmic distribution (Figure 3.5A and Figure 3.5C). However, PDI in Huh7 cells harbouring SGRs detected with AF-488 or AF-594 displayed mixed phenotypes of punctate and diffuse signals (Figure 3.5B and Figure 3.5D). A high level of background staining was also observed in the nucleus and was hypothesised to be due to the staining method. Due to the mixed phenotypes and no conclusive results, another staining method was investigated.

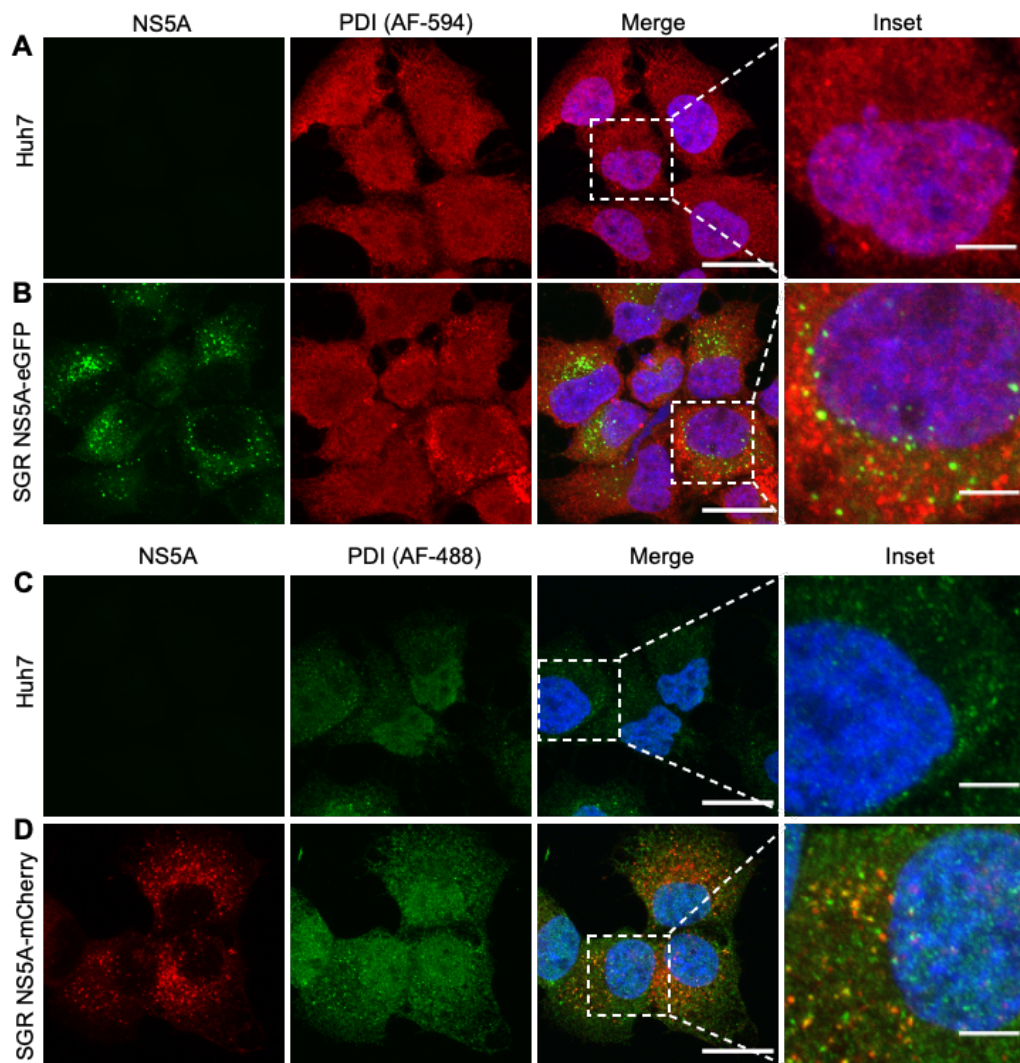


Figure 3.5: Subcellular distribution of PDI in control Huh7 cells and cells harbouring SGRs.

[A] Huh7 cells stained with anti-PDI and AF-594 (red). [B] Huh7 cells harbouring SGR NS5A-eGFP (green) stained with anti- PDI and AF-594 (red). [C] Huh7 cells stained with anti-PDI and AF-488 (green). [D] Huh7 cells harbouring SGR NS5A-mCherry (red) stained with anti-PDI and AF-488 (green). Absence of colocalisation of PDI and NS5A are shown in respective inset. Nuclei of cells stained with DAPI (blue). Imaged on LSM880 confocal microscope with 63X objective (oil immersion) with N.A of 1.4. Scale bars = Merge: 20 μ m, Inset: 5 μ m

To analyse the variations in PDI staining and distribution observed in Figure 3.5, two staining methods were further explored and compared. 1. Huh7 cells were incubated with anti-PDI antibody for 2 hours at room temperature (RT), followed by a 2hour incubation with the secondary antibody at RT (Figure 3.6A and Figure 3.6C). 2. Huh7 cells were stained with anti-PDI overnight at 4 $^{\circ}$ C, followed by incubation with the secondary antibody at RT for 1 hour (Figure 3.6B and Figure 3.6D). Primary antibody staining

performed at RT with AF-594 exhibited enhanced signal quality and reduced background in comparison to the second protocol. The manufacturer's recommended staining method with anti-PDI seemed to be more effective when cells were stained at RT, as illustrated in the merge and inset of Figure 3.6A. The results for PDI detection with AF-488 using both methods remained inconclusive.

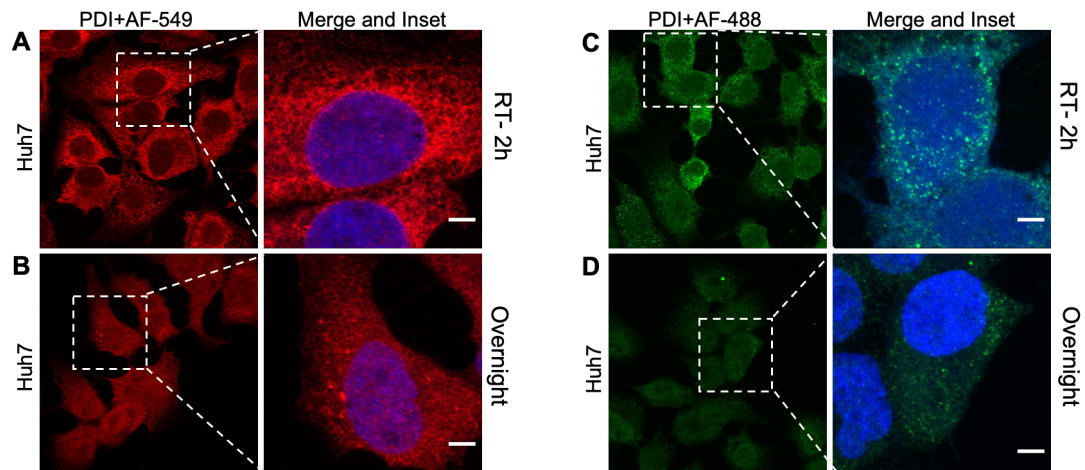


Figure 3.6: Differences in PDI staining and distribution due to incubation periods with anti-PDI antibody at different temperatures.

[A, and C] Huh7 cells were incubated with anti-PDI antibody for 2 hours at RT, and stained with secondary antibody (AF-594, red; and AF-488, green respectively) at room temperature for 2 hours. [B and D] Huh7 cells were incubated with anti-PDI antibody overnight at 4 °C, and stained with secondary antibody (AF-594, red; and AF-488, green respectively) at room temperature for 1 hour. Nuclei of cells stained with DAPI (blue). Imaged on LSM880 confocal microscope with 63X objective (oil immersion) with N.A of 1.4. Scale bar = 20 μ m.

The size of NS5A-eGFP and NS5A-mCherry foci in the cells stained with anti-PDI were similarly analysed as previously and are shown in Appendix Figure 1 and Table 3. Similarly, no significant differences were observed in NS5A foci between NS5A-eGFP and NS5A-mCherry.

3.2.5 Subcellular distribution of TGN-46 in control Huh7 cells and Huh7 cells harbouring SGRs

A partial colocalisation of NS5A with TGN-46 was observed in the cells transfected with an SGR and infected with HCV virus. However, the TGN architecture in cells infected with the JFH-1 virus was remodelled for assembly whereas the transfected cells with an SGR (NS3-5B) was observed with the intact TGN architecture (Mankouri et al., 2016). Since the SGRs (NS5A-eGFP and NS5A-mCherry) lacks structural proteins and NS2 (which was shown to be critical for the TGN rearrangements), it was considered that TGN-46 would not undergo redistribution and would be suited to study the partial colocalisation of NS5A. Therefore, TGN-46 was employed as a suitable marker for assessing its colocalisation with NS5A-eGFP and NS5A-mCherry in these SGRs.

To verify TGN-46 expression and to examine the architecture of the TGN membrane in both control Huh7 cells and cells harbouring NS5A-eGFP and NS5A-mCherry, cells were stained with polyclonal anti-TGN-46 primary antibody and, subsequently with secondary antibodies. In control Huh7 cells, a distinct perinuclear TGN stack was visualised by AF-594 (Figure 3.7A), and AF-488 (Figure 3.7C). The perinuclear TGN stack appeared mostly intact in SGR NS5A-eGFP cells (Figure 3.7B), as opposed to the NS5A-mCherry replicon cells, in which a few cells exhibited a more dispersed distribution of TGN-46 (Figure 3.7D). In both cases, partial overlap of NS5A with TGN-46 was observed, consistent with previous findings (Mankouri et al., 2016).

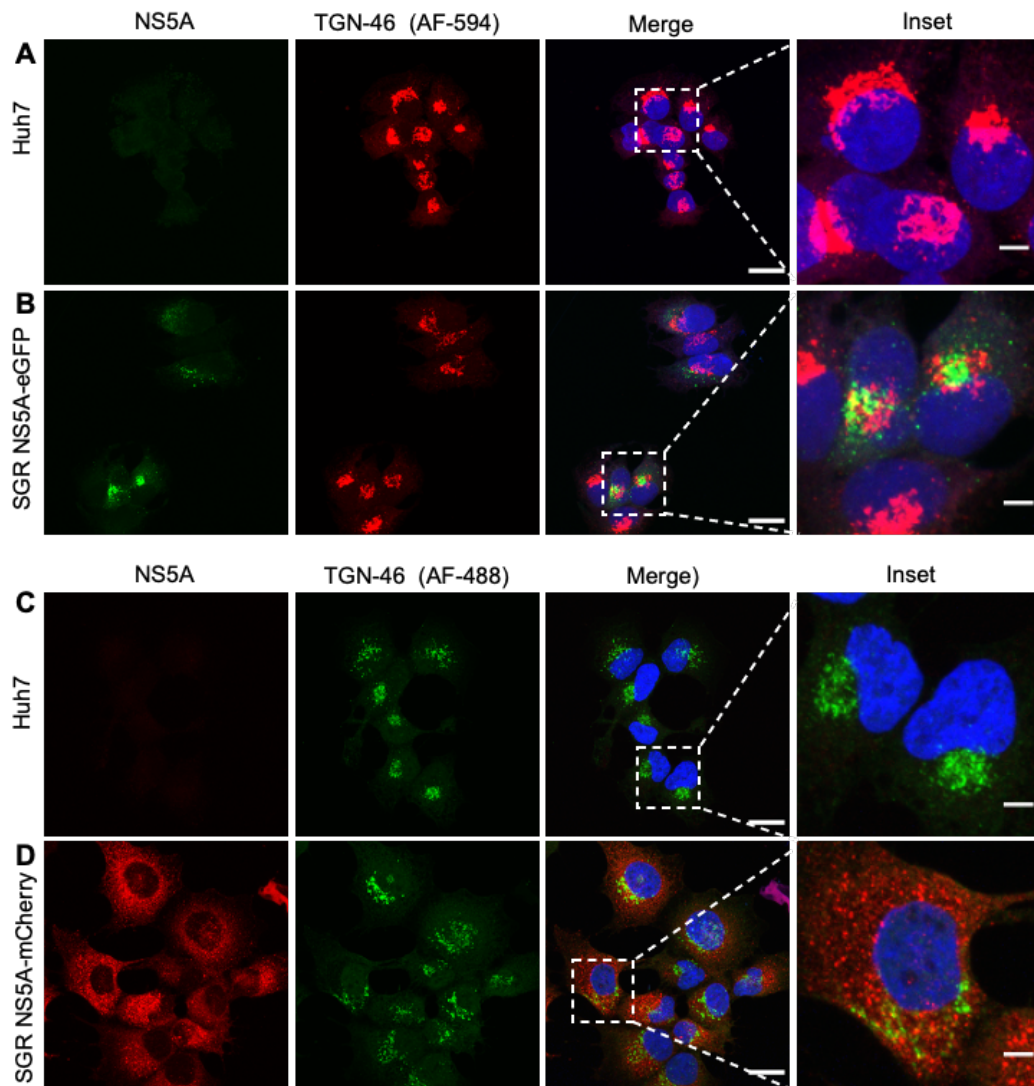


Figure 3.7: Subcellular distribution of TGN-46 in control Huh7 cells and cells harbouring SGRs.

[A] Huh7 cells stained with anti-TGN-46 and AF-594 (red). [B] Huh7 cells harbouring SGR NS5A-eGFP (green) stained with anti-TGN-46 and AF-594 (red). [C] Huh7 cells stained with anti-TGN-46 and AF-488 (green). [D] Huh7 cells harbouring SGR NS5A-mCherry (red) stained with TGN-46 and AF-488 (green). Nuclei of cells stained with DAPI (blue). Imaged on LSM880 confocal microscope with 63X objective (oil immersion) with N.A of 1.4. Scale bar = Merge: 20 μ m, Inset: 5 μ m

The size of NS5A-eGFP and NS5A-mCherry foci in the cells stained with anti-TGN-46 were similarly analysed as previously and are shown in Appendix Figure 2 and Table 4. No significant differences were observed in NS5A foci between NS5A-eGFP and NS5A-mCherry.

3.3 Discussion

To determine the best SGR for establishing a cryo-CLEM methodology, two stable JFH-1 replicon cell lines were generated - SGR NS5A-eGFP and SGR NS5A-mCherry. To compare any differences in the subcellular localisation of NS5A tagged with eGFP or mCherry, colocalisation with previously known cellular factors VAP-A, PDI and TGN-46 was examined by confocal microscopy. For detecting these cellular proteins in control Huh7 cells and cells harbouring SGR NS5A-eGFP or SGR NS5A-mCherry, secondary antibody AF-594 and AF-488 were used respectively.

Differences were observed when these cellular proteins were detected by AF-488 vs AF-594 in control Huh7 cells. Irrespective of the species and the target primary antibody, AF-488 appeared to detect mixed phenotypes. As, the conjugation method with specific linkers may vary among manufacturer's, experiments to determine whether AF-488 from different manufacturer yield better results are required. The degree of labelling may also vary, yielding varied results (Szabó et al., 2018). Alternatively, the staining methodology might have an impact on the detection and thus require further investigation.

VAPs play diverse functional roles which include organelle membrane tethering (Costello et al., 2017), regulation of cholesterol egress from the endo-lysosomal system (K. Zhao & Ridgway, 2017), interaction with autophagy proteins to modulate autophagosome biogenesis (Y. G. Zhao et al., 2018), and lipid transfer between organelles (Di Mattia et al., 2020; Ngo & Ridgway, 2009). Therefore, for the characterisation of VAP-A in control Huh7 cells and stable replicon cells, its distribution was expected throughout the cytoplasm. In Huh7 cells, VAP-A showed even cytoplasmic distribution with AF-594 detection, whereas contrastingly with AF-488, which exhibited signal enrichment around the Golgi apparatus. VAPs play a critical role in maintaining the structural and functional properties of the Golgi complex. VAPs maintain the coordinated functions of lipid transfer proteins involved in the regulation of the ER and/or Golgi membranes (Peretti et al., 2008). It remains unclear why the secondary AF antibody fused with different fluorophores detecting VAP-A in the cells show variable results. Employing VAP-A tagged with GFP (Santos et al., 2018) or mCherry (Eyre et al., 2014) may provide a better understanding of the distribution of VAP-A in Huh7 cells, thereby eliminating the photophysical properties of the AF secondary antibody and any AF-antibody conjugation led discrepancies.

Given the extensive research on membrane changes induced by HCV, which are proposed to mainly originate from the ER (Mottola et al., 2002), PDI staining was used to analyse the ER in Huh7 cells. By immunofluorescence, PDI was demonstrated to

marginally colocalise with NS5A (Pearson's correlation = 0.52) in comparison to the core (0.58), E2 (0.67) and NS3 (0.63) in HCV infected cells (Romero-Brey et al., 2012). In this study, the detection of PDI colocalisation with NS5A was hindered by high background fluorescence from the secondary antibodies. Investigating the impact of staining methods revealed that a sequential 2hour incubation with PDI followed by a 2hour incubation with secondary AF-594 at room temperature resulted in the expected ER network distribution. Recently, Castro et al. utilised a PDI subunit B antibody (P4HB; 1D3 clone, Enzo life Sciences, Exeter, UK) to demonstrate distinct cytoplasmic staining for PDI in Huh7 cells, shedding light on the role of lipin 1 and lipin 2 in the HCV replication cycle (Castro et al., 2019). The staining method and the type of PDI protein, along with its subunits, may play a significant role in immunofluorescence staining in Huh7 cells (Kozlov et al., 2010). Therefore, experiments with alternate PDI markers should be explored for characterisation of the ER in Huh7 cells.

The subcellular localisation of VAP-A, TGN-46, and PDI detected with AF-594 displayed greater consistency in control Huh7 cells. Colocalisation of NS5A-eGFP with VAP-A along with the unchanged distribution of TGN-46, aligned with previous findings for cells harbouring SGR (Eyre et al., 2014; Mankouri et al., 2016) While both NS5A-eGFP and NS5A-mCherry have been utilised in CLEM investigations (Romero-Brey et al., 2012; Grünvogel et al., 2018), NS5A-eGFP was chosen for cryo-CLEM due to its widespread use as a robust and reliable marker. The bright green signal of NS5A-eGFP allows for easy detection under a microscope with any objective lens, in contrast to the dim red signal of NS5A-mCherry. The methodology employing a combination of a primary antibody detected by the secondary antibody, AF-594 was deemed reliable for examining the intracellular localisation of cellular proteins with NS5A-eGFP. Consequently, SGR NS5A-eGFP was selected to establish a cryo-CLEM workflow as part of this study.

Chapter 4| Development of a cryo-ET workflow for in-situ investigation of HCV replication organelles

4.1 Introduction

4.1.1 Membranous web of vesicles induced by HCV infection is used by the virus as a platform for replication.

The current ultrastructural knowledge of the host membranous rearrangements derived from ER and autophagy pathways that function as specialised compartments for HCV replication has been obtained from fluorescence microscopy (FM), electron microscopy (EM) and soft X-ray microscopy (Ferraris et al., 2010; Romero-Brey et al., 2012; Paul et al., 2014b; Mohl et al., 2016; Pérez-Berná et al., 2016; J.Y. Lee et al., 2019). This intricate network of vesicles is observed during viral infection, as well as in the context of subgenomic replicons (SGR) and is commonly referred to as viral replication factories or membranous web (MW). Replication factories derived from intracellular host membranes are generated by all positive-sense single stranded RNA viruses infecting eukaryotes (Wolff et al., 2020a). In the case of HCV, MW consists of single membrane vesicles (SMVs), double membrane vesicles (DMVs), multimembrane vesicles (MMVs), multivesicular bodies (MVBs) and lipid droplets (LDs). In recent years, these vesicles are increasingly referred to as replication organelles (ROs) as they provide platform for the assembly of viral replication complex (Wolff et al., 2020b). Out of several ROs, DMVs have emerged as a recurrent motif in positive-sense RNA viruses and through EM techniques have been demonstrated as a central hub for the synthesis of vRNA (Introduction 1.2.3) (Wolff et al., 2020a).

DMVs were identified as HCV replication sites by immunogold labelling using antibodies against the non-structural (NS) proteins and the presumed replication intermediate dsRNA (Gosert et al., 2003; Ferraris et al., 2010). Correlative light and electron tomography (CLET) investigations revealed that NS5A-GFP puncta (associated with both the virus and SGR), corresponded to predominant accumulations of DMVs, surrounding LDs and in proximity to ER. SMVs and MMVs were also identified, confirming their identity as HCV replication factories (Romero-Brey et al., 2012). Additionally, the replication kinetics of double stranded RNA (dsRNA), analysed through immunofluorescence, and its colocalisation with NS5A (a replicase component) correlated with the production of DMVs. Moreover, purified DMVs were also reported to contain active viral replicases capable of *de novo* synthesis of HCV RNA (Paul et al., 2013). A summary of HCV-induced structures from virus-infected and SGR harbouring cells is shown in Table 4.1 (Romero-Brey and Lohmann, 2016).

Report	Cell type	System/Viral	Fixation method	Structure	Size
1	U-2 OS human osteosarcoma-derived	Expression of polyprotein/genotype 1a	2.5% GA + 2% OsO ₄ + 2% UA	Membranous web (MW)	85 nm
				Contiguous vesicles (CVs)	Not -provided
				Dilated ER- vesicles	Various sizes (not provided)
2	Huh 7	Subgenomic replicon / 9-13 (genotype 1b)	2.5% GA + 2% OsO ₄ + 2% UA	Membranous web (MW)	80-100 nm
3	Huh 7.5	Subgenomic replicon / JFH-1 (genotype 2a)	4% PFA and 1% GA + 1% UA	DMVs	200-500 nm
4	Huh 7.5	Infection / Jc1 (genotype 2a)	4% PFA and 1% GA + HPF	MMVs	150-200 nm
	Huh 7	Subgenomic replicon / JFH-1 (genotype 2a)	HPF-FS with acetone containing 0.2% OsO ₄ , 0.1% UA, 5% H ₂ O	DMVs MMVs	~170 nm 340 nm
	Huh 7-Lunet T7	Expression of polyprotein / JFH1 (genotype 2a)	HPF-FS with acetone containing 0.2% OsO ₄ , 0.1% UA, 5% H ₂ O		
5	Huh 7.5	Infection / JFH1 (genotype 2a)	4% PFA and 1% GA + 1% UA	Vesicles in clusters (ViCs)	100-200 nm
				Contiguous vesicles (CVs)	~100 nm
				DMVs	150-1000 nm

Table 4.1: Summary of HCV-induced replication organelles.

Adapted from (Romero-Brey and Lohmann, 2016). 1. (Egger et al., 2002). 2. (Gosert et al., 2003) 3. (Ferraris et al., 2010). 4. (Romero-Brey et al., 2012).5. (Ferraris et al., 2013). [1,2,3,5]: Cells were processed by resin-embedding, sectioning and imaged on TEM. [4]: Cells were HFP-FS, resin embedded, sectioned, and imaged by electron tomography. *GA- glutaraldehyde, PFA-paraformaldehyde, HPF-FS high pressure freezing-freeze substitution OsO₄-Osmium tetroxide, UA-uranyl acetate, DMVs- Double membrane vesicles, MMVs-Multimembrane vesicles.*

LDs served as markers in a correlative light and electron microscopy (CLEM) study, focused on investigating the ultrastructural details of the assembly of the virions in HCV infected cells. E2-NS5A double positive puncta contained ER-wrapped LDs and were suggested as the HCV assembly sites. The ER-wrapped LDs were decorated by DMVs indicating the importance of the spatiotemporal coupling of the assembly and replication sites (J.Y. Lee et al., 2019).

Direct stochastic optical reconstruction microscopy (dSTORM) was used to gain insight into the arrangement of NS proteins with respect to DMVs in infected cells. The differences in protein cluster size between NS5A and NS3 proteins involved in HCV RNA replication pointed to unique arrangement of the NS proteins within the DMV. However, how and where the replicase assembles still remains elusive (Bartlett et al., 2018).

The hypothetical model for the arrangement of replicase machinery inside the DMV is shown in Figure 4.1 (Paul et al., 2014a). While the DMVs have been suggested as the primary RO for HCV, the objective of this study is to explore all observed ROs in cells harbouring SGR with the goal of identifying and pinpointing the location of the replicase machinery.

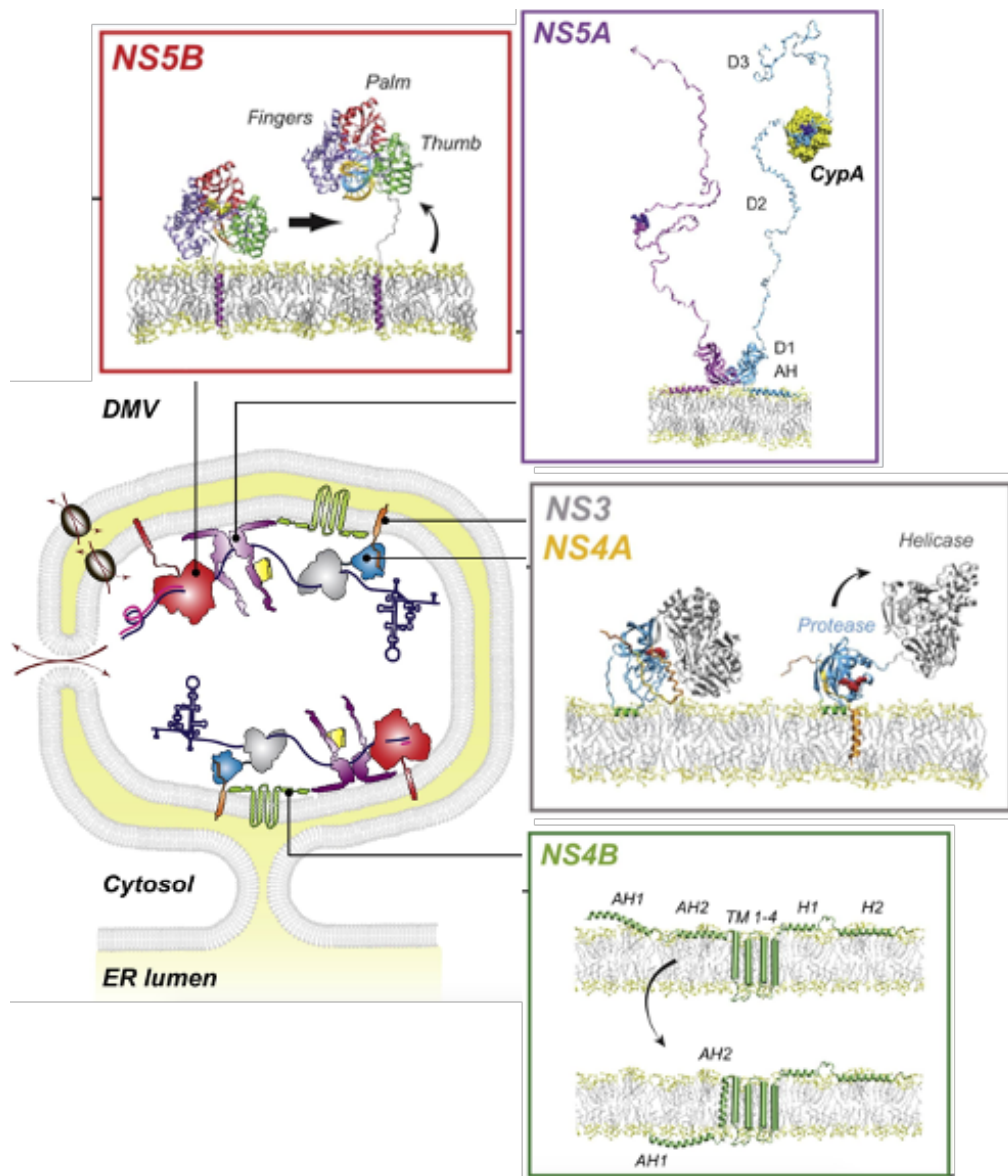


Figure 4.1: Hypothetical model of arrangement of the replicase machinery within the DMVs.

Taken from (Paul et al., 2014b).

Thus far, the approaches used to understand the cellular architecture after infection (or in cells harbouring SGR) provided the knowledge about membrane rearrangements and the role of individual NS proteins in the type of membrane rearrangements induced in the host cells. However, samples were prepared through chemical fixation combined with resin embedding and sectioning methods imposed technical limitations on directly

visualising the contents within these ROs, making it challenging to definitively identify the ROs harbouring the replication complex (RC). Therefore, the latest advancements in preparing cryogenic cell samples, which allow close-to-native imaging, were explored.

4.1.2 Cryogenic sample preparation of cells for cryo-electron tomography.

An overview of microscopy resolution limitations across the different techniques, demonstrating the restricted resolution in light microscopy and the capabilities of electron microscopy is summarised in Figure 4.2.

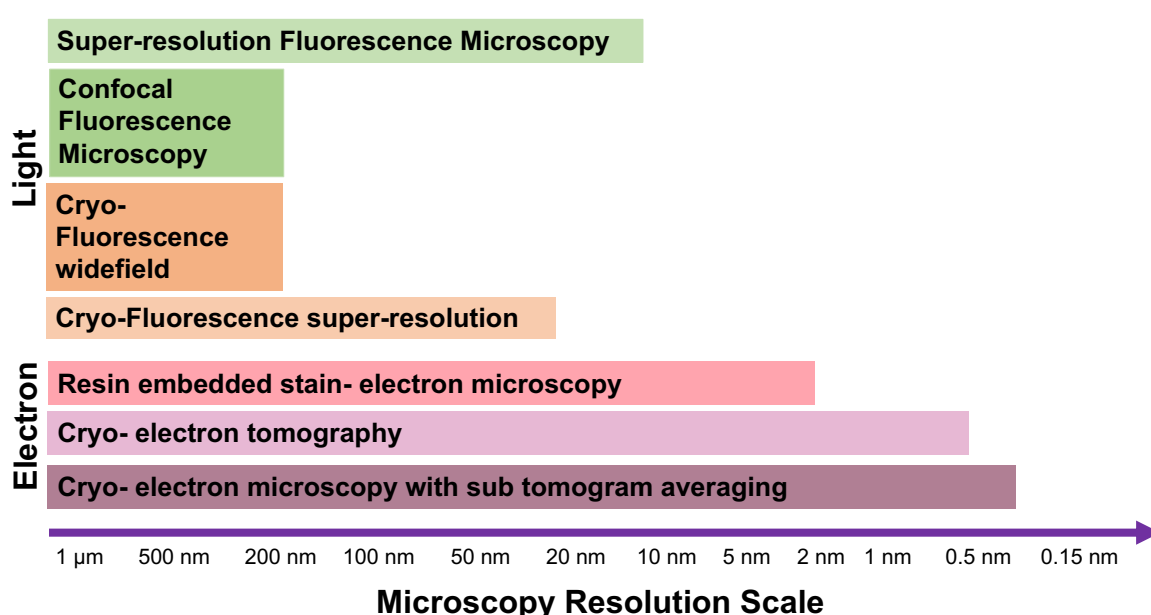


Figure 4.2: Schematic of the resolution scale of various microscopy approaches.

Tissues and eukaryotic cells are too thick for an electron beam to penetrate and therefore need to be sectioned for EM imaging (Goldsmith and Miller, 2009). A method is required that thins the cell sample (approximately 50-200 nm) after its preservation in vitreous ice (Weber et al., 2019), to enable the study of cellular compartments, protein structures and protein complexes. A cryo-preserved cell specimen suitable for cryo-ET can be prepared in two different ways. In one approach, cells can be cultured on gold (Au) EM grids, vitrified by plunge freezing in liquid ethane and thinned by cryogenic focused ion milling (cryo-FIB), typically by gallium. Gallium serves effectively as a metal ion source due to several attributes such as a low melting point, low volatility, low vapour pressure, favourable emission properties, and excellent characteristics in vacuum (Berger et al., 2023). Additionally, gallium efficiently sputters various materials due to its point source nature that allows for precise beam focusing, providing control at the nanometer scale

(Berger et al., 2023). To track the process of cell milling, a scanning electron microscope (SEM) is also installed within the same instrument and together the unit is termed a FIB-SEM. The thinned cell specimen at the end of this process is known as a lamella, which is typically in the range of 120–200 nm thick (Wagner et al., 2020).

Another approach is to high pressure freeze a suspension of cells in a carrier and subsequently section by cryo-ultramicrotomy (Chlanda and Sachse, 2014). Cryogenic electron microscopy of vitreous sections (CEMOVIS) generates ultrathin sections between 40-100 nm thick, thinner than cryo-FIB derived samples. However, these ultrathin sections contain artefacts such as compression, crevassing, chatter, and knife marks (Hutchings and Zanetti, 2018). In contrast, the lamellae derived from cryo-FIB can result in sample damage caused by gallium ions resulting in poor structural preservation. Gallium ion-induced damage is exacerbated when samples contain ice contamination and milling parameters are sub optimally adjusted. (Lucas and Grigorieff, 2023). Both these methods require user expertise; however, an automated user-friendly workflow can be employed with cryo-FIB resulting in improved throughput in sample preparation in compared to CEMOVIS. However, the limitation of cryo-FIB-SEM is the availability of the instruments and expertise which acts as a bottleneck in the overall establishment of the cryo-ET workflow.

A schematic of the two approaches is shown in Figure 4.3. A general overview of thickness scale from liver tissue, cells, lamella, and ultrathin sections is presented in Figure 4.4.

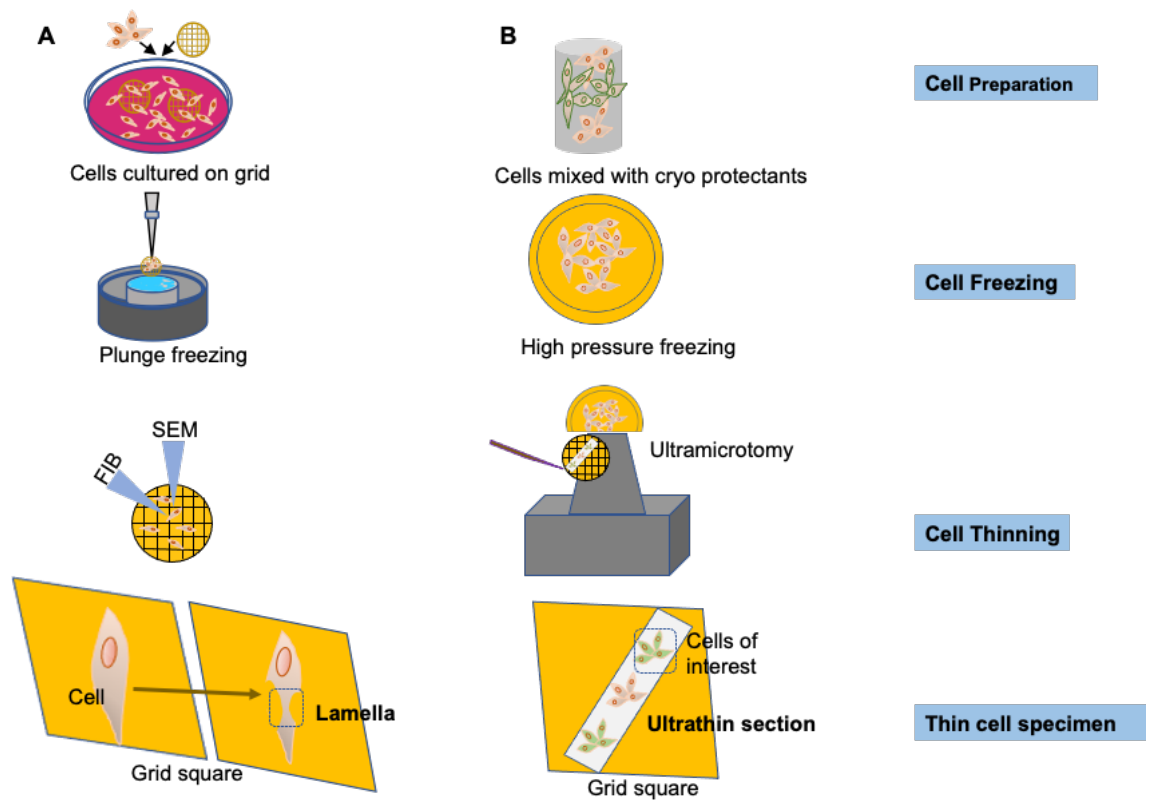


Figure 4.3: Schematic of sample preparation methods to vitrify cellular samples for cryo-ET.

[A] Workflow summarising cryo-FIB: cells are grown on gold grids, plunge frozen in liquid ethane and then milled by cryo-FIB to generate thin lamella. [B] Workflow summarising CEMOVIS: cells are high pressure frozen in a carrier and subsequently thinned by cryo-ultramicroscopy to generate ultrathin sections.

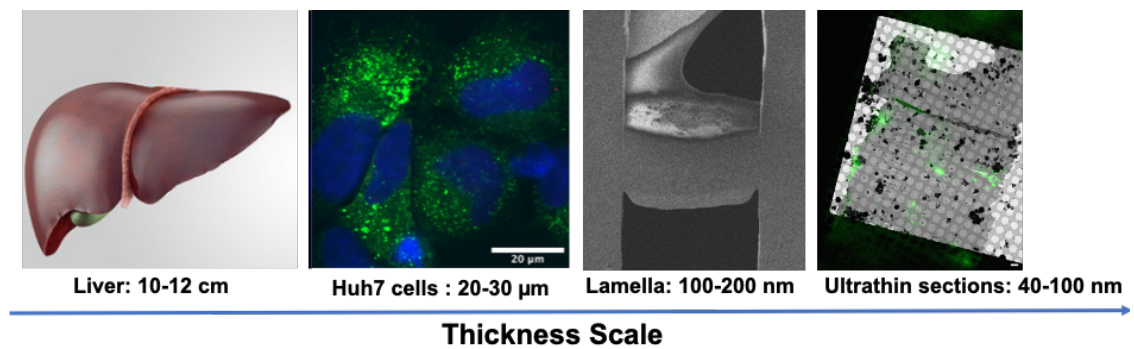


Figure 4.4: Overview of thickness scale

From left to right: liver tissue, Huh7 cells expressing HCV SGR, lamella prepared by FIB-milling, ultrathin sections prepared by ultramicrotomy and correlated with cryo-fluorescent imaging.

With the aim of elucidating the RO hosting the HCV replication complex, the objectives were: (1) To establish a cryo-CLET methodology using the replicon cells (Huh7 cells stably expressing SGR JFH-1 NS5A-eGFP). (2) To investigate the *in-situ* organisation of the ROs induced by the SGR. (3) To identify the location of the replication machinery with respect to the RO type.

4.2 Results

4.2.1 Host cellular rearrangements observed in cells stably harbouring an SGR is consistent with previous literature

Prior to implementing a cryo-ET workflow using cells harbouring SGR JFH-1 NS5A-eGFP, conventional cellular EM was employed to confirm architectural differences between control Huh7 cells and stably transfected cells expressing an HCV SGR. Mr. Martin Fuller (Astbury Biostructure Laboratory Electron Microscopy Facility) processed the control and replicon cells at RT through chemical fixation and resin embedding. Subsequently, resin embedded samples were sectioned to generate ultrathin specimens and imaged using a T12 transmission electron microscope (TEM).

The electron micrographs were acquired from Huh7 (control) and cells harbouring SGR JFH-1 NS5A-eGFP at different magnifications. Subsequently, a comparison was made between 21 cells from each condition. Differences in the cellular architecture were visually assessed around the periphery of the nucleus. Since a heterogenous replicon cell population was processed, 40-60% of cells harbouring SGR JFH-1 NS5A-eGFP were expected with membrane alterations. Out of 21 cells imaged from the replicon cell

samples, 8 were found to have modified cellular architecture compared to the control cells, which aligned with the expected ratio of success.

As the eGFP signal becomes undetectable during the embedding process in epoxy resins, a standard reference was established by utilising confocal images of cells containing SGR JFH-1 NS5A-eGFP before proceeding to cellular EM. This reference was employed to interpret the EM images. The distribution of NS5A-eGFP in a heterogenous replicon cell population indicating the various level of replication imaged by confocal microscopy is shown in Figure 4.5 A1. The differences in cellular architecture (by EM) in cells harbouring SGR JFH-1 NS5A-eGFP ranged from few speckled vesicles (Figure 4.5 B1-3) to a distinctly heavily stained large MW (Figure 4.5 C1-3). The observation of speckled cellular rearrangements in Figure 4.5 B3 could be considered equivalent to cells analysed by confocal wherein some cells exhibit low replication and therefore show only few NS5A-eGFP puncta (Figure 4.5 A2(cell 1)). Likewise, the EM image with pockets of MW around the nucleus in Figure 4.5 C1 could be designated as cells actively replicating in the cytoplasm and correspond to large, clustered gathering of puncta in confocal image (Figure 4.5 A3(cell 2)). Overall, a clear difference in the cellular architecture between the cells harbouring SGR JFH-1 NS5A-eGFP and control cells with the presence and absence of MW were observed respectively.

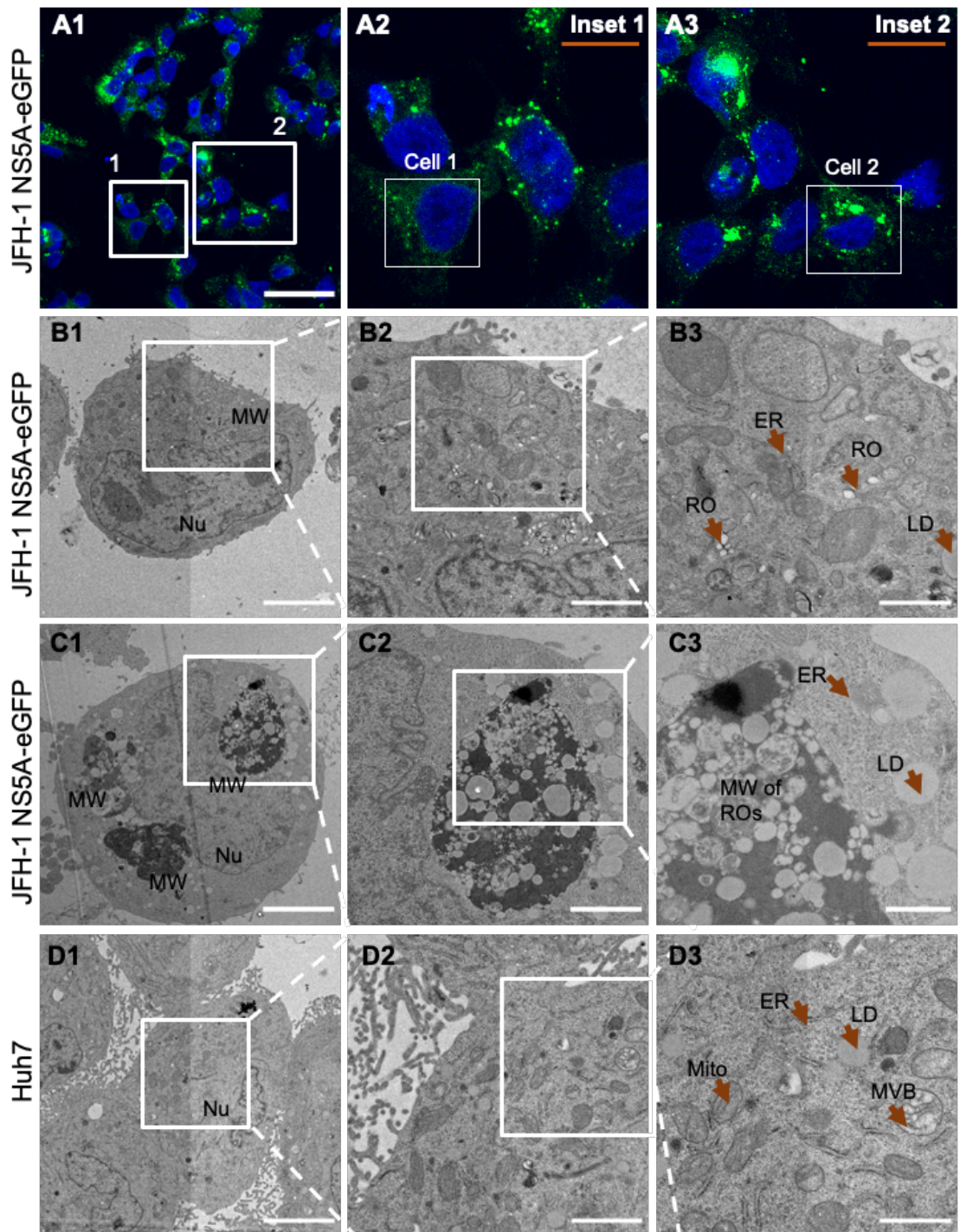


Figure 4.5: MW of ROs observed by TEM on ultrathin sections after conventional resin embedding.

[A1-A3] Cells harbouring SGR JFH-1 NS5A-eGFP imaged by confocal microscopy, demonstrating the various levels of replication in different replicon cells. This was used as a guidance to understand the difference in cellular architecture observed in EM images. [B1-3 and C1-3]. Representative EM images (low to high magnification) of cells harbouring SGR JFH-1 NS5A-eGFP. Cellular organelles, MW and RO are indicated with

a brown arrow [D1-D3] Low to high magnification EM images of control Huh7 cells with cellular organelles denoted with a brown arrow. Scale bars A1: 50 μm , A2, A3 are cropped insets of A1. Scale bars for B1, C1, D1: 5 μm . Scale bars for B2, C2, D2: 2 μm . Scale bars for B3, C3, D3: 1 μm . *MW: membranous web, RO: replication organelles, LD: Lipid droplets, MVB: multivesicular bodies, Nu: nucleus, ER: endoplasmic reticulum, Mito: Mitochondria.*

4.2.2 Workflow to establish optimum cell growth and adherence on cryo-EM grids for vitrification by plunge freezing.

To gain *in-situ* knowledge into the organisation of membrane compartments induced in cells harbouring SGR JFH-1 NS5A-eGFP, culture and vitrification of the cells are the initial major steps. For this, cells were cultured on non-toxic EM gold (Au) grids, to avoid the potential toxicity resulting of oxidised Copper (Hampton et al., 2017). Grids were rendered hydrophilic by glow-discharge using a 20 mA current for 30-60 seconds on the GloQube and then UV sterilised for 10 minutes, ensuring a clean surface for cell attachment. To identify the most efficient method for culturing cells and easy retraction of the grid for subsequent plunge freezing steps, many types of plates, the combination of plates with types of grid holders and cell culture dish were tested.

To ensure a homogenous population of cells containing HCV ROs, Huh7 cells harbouring JFH-1 NS5A-eGFP (i.e. only actively replicating cells) were selected by fluorescence activated cell sorting (FACS). Subsequently, 5×10^4 and 10×10^4 FACS-sorted cells were cultured on Au grids placed in 6-well plates, and subsequently vitrified (2.5 seconds blotting). Low magnification cryo-EM images (580X magnification on a Thermo Fisher Krios (cryo-TEM), referred to as Atlas maps) were employed to assess the results of the workflow (Figure 4.6). However, this led to an uneven distribution of cells, with either few cells adhering to the centre of the grid square or clusters of cells resulting in grid areas covered in thick ice.

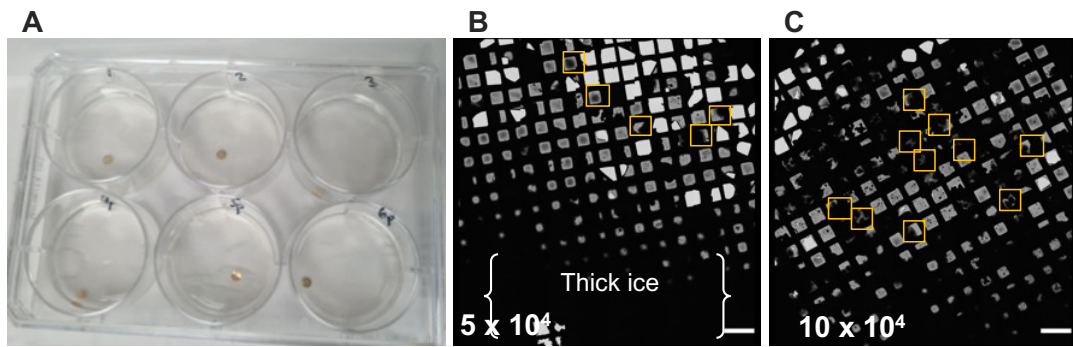


Figure 4.6: Cell culture process on Au EM grids within a 6-well plate and its examination by cryo-TEM.

[A] Initially, 5×10^4 and 10×10^4 FACS-sorted stably transfected cells in a 1 ml volume were added to a well in a 6-well plate introduced, followed by the addition of an additional 2 ml of complete DMEM for a 21 hour culture. The grids were then plunge frozen after blotting for 2.5 seconds. [B] An atlas map illustrating the screening of grids on cryo-TEM, highlighting suboptimal ice thickness and uneven cell distribution (grid square with cells are indicated by orange box). Scale bar: 100 μm

Next, 12-well plates with a homogenous cell culture suspension of 5×10^4 , 10×10^4 were tested, exploring the hypothesis that reducing the surface area could improve cell adherence and cell distribution. Bright field images from two grids from each condition (imaged in the well, using Zeiss Axio Vert A1 microscope) indicated improved cell distribution as shown in Figure 4.7 Panel A. The hypothesis of cell detachment from the grids during the extraction of the grid from the well was also tested. This presumption was tested by imaging the grid in the well, followed by extracting the grid out of the well, and then placed on a dry surface. Two examples of grids cultured with 10×10^4 cells, first imaged in the well (Panel B: G1, G2), then imaged on a dry surface (Panel B: G.1.1, G2.1), highlighting the loss of cells during this process are depicted in Figure 4.7 Panel B. Additionally, cell detachment from the grids might be caused during the process of adding fiducials and blotting steps. Thus far, 3 μl of fiducials (10 nm colloidal gold labelled protein A (GA1054)) on the grid side harbouring cells and 0.5 μl on the back side of the grid was utilised for blotting, to provide fiducial markers for downstream tilt-series alignment. This hypothesis of cell detachment during addition of fiducials was tested by re-applying 4 μl of media onto the grid placed on the dry surface, followed by manually blotting with a Whatman filter paper (Panel B: G.1.2, G2.2). The loss of cells was observed throughout the grid in subsequent steps. However, the detachment of cells was observed more during the process of grid extraction from the well, which indicated that cells are not strongly adhered well to the grid.

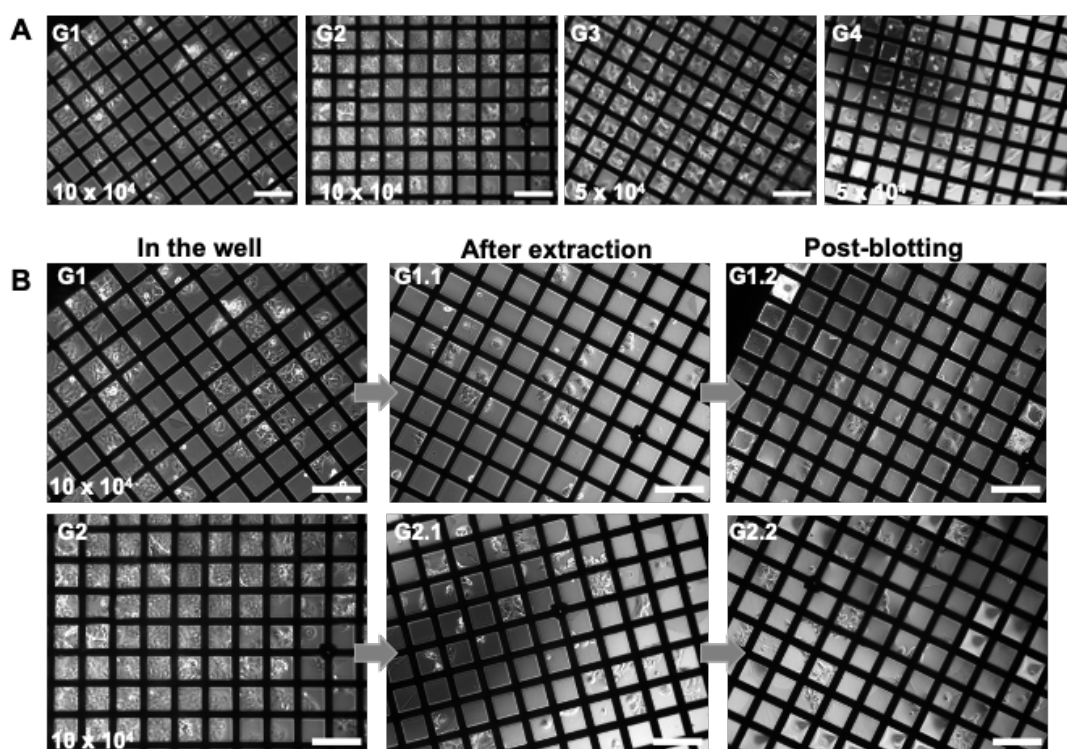


Figure 4.7: Assessment of cell distribution and adherence with FACS sorted cells harbouring SGR JFH-1 NS5A-eGFP in a 12-well plate.

[A] Bright field visualisation of cell distribution from culturing FACS-sorted 10×10^4 and 5×10^4 replicon cells on EM grids in the wells. [B] Two examples of grids cultured with 10×10^4 cells tested for cell detachment. Bright field image of grids in the well (G1, G2), extracted from the well and placed on a dry surface (G1.1, G2.1), and manually blotted on filter paper after 4 μ l of media and subsequently imaged (G1.2, G2.2). The cell detachment was observed more during the extraction of grid from the well compared to the blotting step. Images acquired on Zeiss Axio Vert A1 microscope. Scale bars: 200 μ m

Although, the combination of wells with reduced surface area and a homogenous cell population contributed to improved cell distribution to some degree, a persistent challenge remained in retrieving the grids from the well without grid deformation. Therefore, cell seeding densities in 24-well plates and 96-well plates with square and circular designed grid holders respectively were investigated (Fäßler et al., 2020). These grid holders were suggested to aid in easy retrieval of the grids from the well without causing grid warping. Differences in imaging the grids (image analysis on an EVOS microscope) when placed in square or circular grid holders are illustrated in Figure 4.8. Circular grid holders, casting shadows over the grid, were deemed impractical and subsequently discarded. Despite multiple design iterations on square grid holders, the retraction of grids remained challenging. Therefore, the grid holders were identified as tools that required further functional improvements to allow better imaging and grid handling prior to plunge freezing steps.

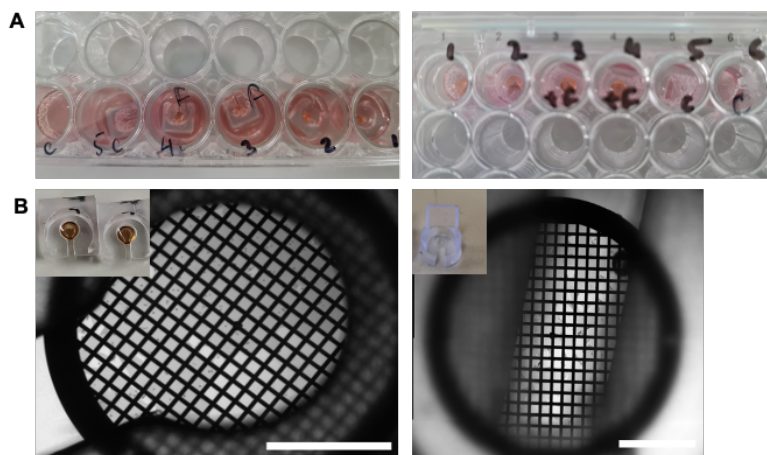


Figure 4.8: Experimental setup for evaluating grid holder functionality in facilitating easy grid retrieval from plates.

[A] Square grid holders were positioned in 24-well plates, and circular grid holders were placed in 96-well plates. [B] A bright field image comparing grids placed in square grid holders versus circular grid holders. Bright field images acquired on EVOS microscopes. Scale bars: 1 mm

Finally, a cell culture dish (Nunc™ 150680) with a total dish diameter of 40 mm, featuring a 12-mm central glass area for cell culture was tested. The 12-mm area provided a suitable surface of 1.12 cm² for cell culture. Cells were cultured on EM grids placed in the glass area. The dish proved to offer the most optimal functionality across various aspects, including surface area, imaging quality, easy retrieval of all grids without warping and grid handling prior to plunge freezing. Furthermore, the dish could be cleaned with ethanol and reused after UV treatment, which was identified as an additional advantage, contributing to a cost-efficient and sustainable workflow.

Next, cell adherence by functionalising the grids was tested (Fäßler et al., 2020; Toro-Nahuelpan et al., 2020). Cell adherence to Au EM grids coated with fibronectin at a concentration of 25 µg/ml was investigated. EM grids were coated with 7 µl of fibronectin at a concentration 25 µg/ml and incubated for 1 h at 37 °C. Cell adherence on functionalised grids was tested with a cell seeding density of 40 x 10³ cells. The difference in cell adherence on EM grids coated with and without fibronectin cultured in a glass bottom dish is illustrated in Figure 4.9 A1 and B1. The impact of manual blotting on cell adherence confirmed that cells adhered and were retained after functionalisation, thereby reducing the cell detachment, as demonstrated in Figure 4.9 A3 and B3.

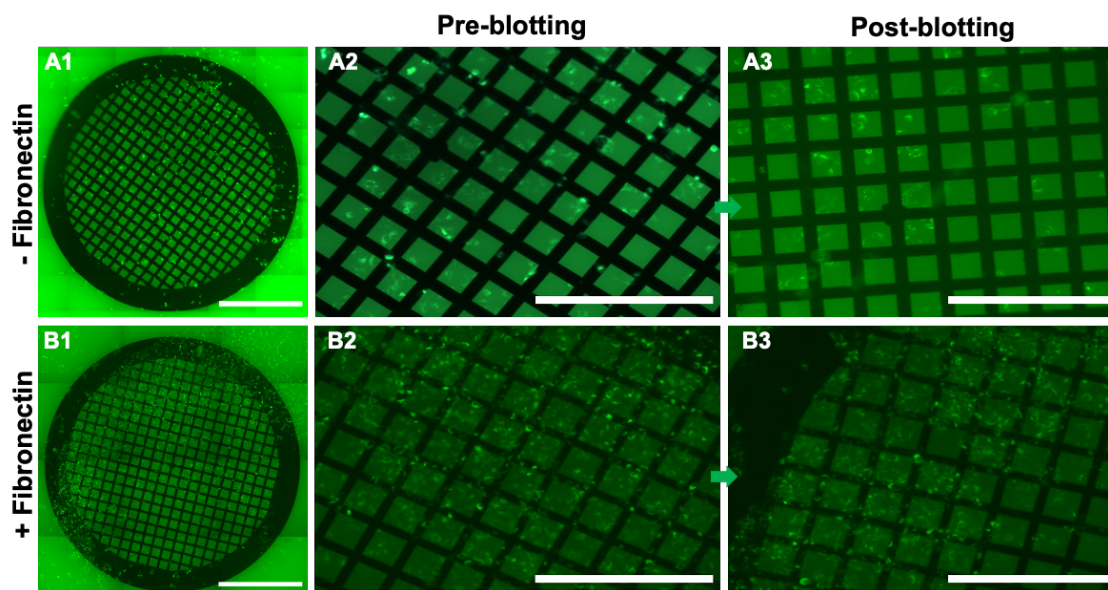


Figure 4.9: Representation of differences observed in grids cultured with 40×10^3 FACS sorted cells harbouring SGR JFH-1 NS5A-eGFP, with and without coating with $25 \mu\text{g/ml}$ fibronectin.

[A1-3]: Representative fluorescence images of a grid cultured with cells coated without fibronectin (A1), placed on dry surface to test cell detachment (A2) and blotted to test for loss during the blotting stage (A3). [B1-3]: Representative fluorescent image of a grid cultured with cells coated with fibronectin (B1), placed on dry surface to test cell detachment (B2) and blotted to test for loss during the blotting stage (B3). FM images acquired on EVOS microscopes. Scale bars A1, B1: 1 mm. A2, A3, B2, B3: 0.5 mm

After determining the most suitable dish for culturing cells on EM grids and recognising the importance of fibronectin, various cell seeding densities was tested to identify the optimum seeding density. The goal was to optimise the protocol, ensuring that cells on the grid neither became too crowded nor too sparse. Grids were seeded in triplicates with 10×10^3 , 15×10^3 , 20×10^3 , and 25×10^3 per dish. Visual assessment of grids (on EVOS microscopes) indicated 10×10^3 cells would be an optimum seeding density (Figure 4.10 A). Subsequently, these grids were plunge frozen with 2.5 seconds blot time under 90% humidity. To allow optimum blotting $3 \mu\text{l}$ of 1 x PBS was added on the cell side and $0.5 \mu\text{l}$ on the other side facing the blotting membrane. However, low-magnification EM atlas maps suggested the need for further optimisation to achieve optimal ice thickness with a seeding density 10×10^3 cells (Figure 4.10 B).

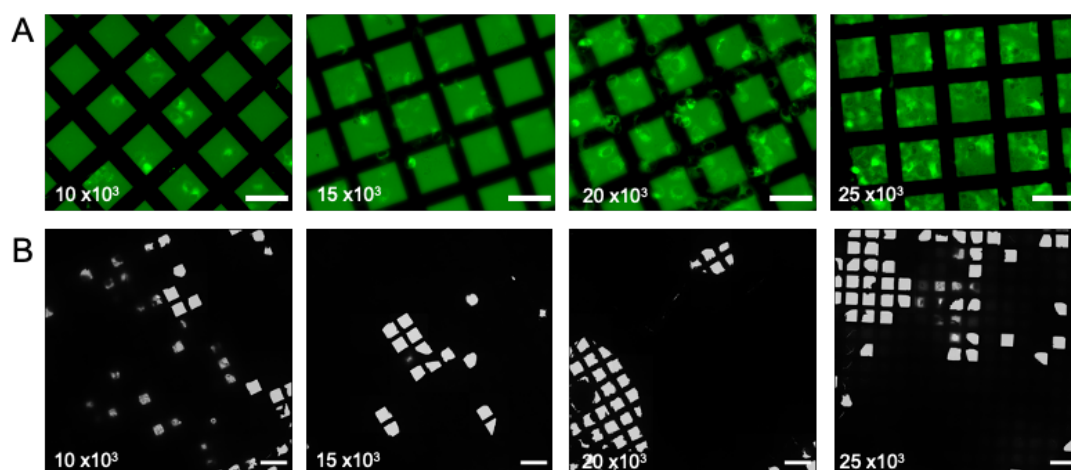


Figure 4.10: Representative images of FACS sorted cells harbouring SGR JFH-1 NS5A-eGFP cultured on EM grids in a glass bottom dish.

Representative images of FACS sorted cells harbouring SGR JFH-1 NS5A-eGFP cultured on EM grids in a glass bottom dish. [A] Differences observed by FM (EVOS microscope) of a grid area on EM grids cultured with 10×10^3 , 15×10^3 , 20×10^3 and 25×10^3 cells [B] Atlas maps illustrating the differences in the distribution of ice thickness corresponding to the grids cultured with 10×10^3 , 15×10^3 , 20×10^3 and 25×10^3 cells. Scale bars: 100 μm .

Moreover, multiple Au EM grids with the same seeding density of 10×10^3 cells and blot times ranging from 5 to 8 seconds were tested. Notably, the cell adherence and distribution were variable despite using a constant seeding density. The optimal approach identified was to employ two seeding densities and utilise a range of blot times based on the observed cell distribution (EVOS analysis). This was followed by cryo-TEM screening to determine the ideal cell spread and ice thickness.

After optimisation, the final workflow involved seeding two concentrations of homogenous FACS sorted cells harbouring SGR JFH-1 NS5A-eGFP on Au Quantifoil-finder and C-flat grids (200 mesh). The optimised conditions are presented in Table 4.2 and the overall method of culturing cells and vitrification steps are illustrated in the schematic Figure 4.11 (Method section 2.4.4). Since cell adherence was variable, an initial image analysis on an EVOS microscope allowed to make educated estimation on blotting time. In order to be compatible with Krios autoloader cassette, grids were clipped in C-clips and autogrid rings. Subsequently, grids were screened by cryo-TEM for ice thickness and number of cells. The optimum grids were taken forward for cryo-FIB milling. Generally, the success rate of grids with optimum cell distribution and ice thickness was observed to be 50% per session.

Collection method	Harvested at	Seeding volume per grid	Incubation time
FACS sorted cells in 500 μ l of complete DMEM corresponding to 5×10^3 and 10×10^3 cells	1200 x g for 3 minutes	50 μ l	20 minutes to allow cell adherence, before adding 2.5 ml of complete DMEM. Incubated for 20-24 hours at 37°C with 5% CO ₂

Table 4.2: Parameters for cell culture and vitrification

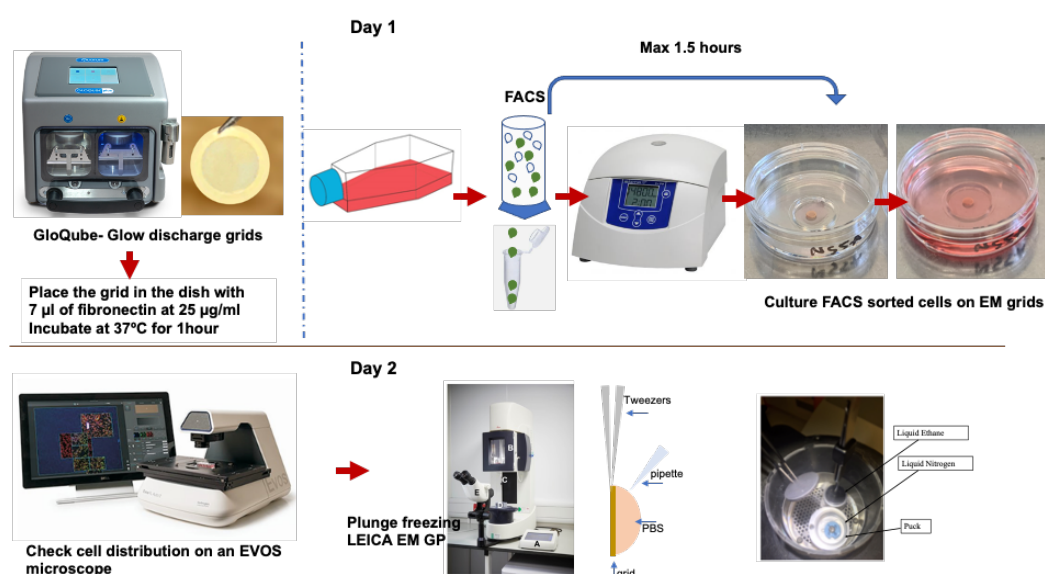


Figure 4.11: Summary of the cell culture methodology on Au EM grids and the vitrification process through plunge freezing.

Day 1 involves functionalising gold grids with 25 μ g/ml fibronectin after rendering the grid hydrophilic via glow discharge, followed by cell culture and incubation. Day 2 includes the assessment of cell distribution on an EVOS microscope. Subsequently, blotting is conducted on a LEICA EMS plunger, with 3 μ l of 1x PBS on the cell side in the blotting chamber, maintained at 90% humidity and 8°C.

Using the established methodology, two representative atlases of best grids after screening is shown in Figure 4.12. These grids were taken forward for cryo-FIB milling as there were plenty of cell targets with optimum ice thickness.



Figure 4.12: Cryo-EM Atlas maps depicting two optimal grids selected for subsequent cryo-FIB milling.

Inset G2 provides a visual guide to the preferred positioning of cells in the center of the grid square. Scale: 100 μ m

4.2.3 Preparation of lamella by cryo-FIB milling

Cell specimens thicker than 500 nm prepared by plunge freezing (or high pressure freezing) require thinning prior to imaging by cryo-ET (Roman I. Koning et al., 2018). To obtain the cellular architecture from cryo-TEM, samples need to be ideally between 100 to 200 nm thick (Sachse et al., 2019). To achieve cryogenic thin cell specimens cryo-FIB-SEM was employed to prepare thin lamellas (Lam and Villa, 2021). Specifically, two instruments were used to generate two sets of data. These instruments employed gallium ions as a source for milling and were located at the electron bioimaging centre (eBIC) at the Diamond Light Source (Harwell).

In dataset 1, there were limited targets for milling, and the cells on the grid had ice contamination from both transportation and cryo-fluorescence microscopy (cryo-FM) imaging conducted before milling. Prior to the milling process, cryo-FM images were captured for the entire grid and specific grid squares where cells were centered to guide the milling steps at the NS5A-eGFP foci position within the cell. However, the geometry of the FIB-SEM stage rendered the MAPS software (which correlates FM-EM images, commercially licensed by Thermo Fisher) was ineffective. Generally, the fluorescence guidance information proves valuable for identifying cells of interest targeted for milling and subsequent tilt-series collection (Sachse et al., 2019). In this study, the cultured cells underwent FACS sorting, with only actively replicating cells being chosen for seeding. Therefore, all the cultured cells on the grid were of interest. Considering the widespread distribution of NS5A-eGFP throughout the cytoplasm, lipid droplets were considered as

markers to identify the replication factories as previously employed (J.Y. Lee et al., 2019). The subcellular localisation of NS5A-eGFP in proximity to LDs in cells harbouring SGR JFH-1 NS5A-eGFP is shown in the appendix (Figure 3).

Additionally, search maps integrated into the tomography software (TOMO) for cryo-electron tomography (cryo-ET) collection could be utilised to locate the region of interest. Dataset 1 was collected on a Scios, and the milling steps controlled for each cell were executed by Dr. James Gilchrist until features such as lipid droplets (dark spots) were observed on the surface (Figure 4.13). Out of eight lamellae prepared from one grid, five remained intact after transportation from eBIC to Leeds, providing valuable structural information. Lamellae with a nominal thickness of 200 nm were produced using a beam current at 37 pA were generated for dataset 1. Cells located at the centre of grid squares were chosen for the milling process, which advantageously also allowed for the milling of adjacent cells, maximising the number of targets for downstream imaging.

Crystalline and amorphous ice contamination was observed during inter-device transfers (Jonker et al., 2022). Therefore, to minimise ice contamination (Tacke et al., 2021), cryo-FM imaging prior to milling was dismissed. Dataset 2 consisting of 32 lamellae prepared from a single grid using an automated and user-friendly set up on an Aquilos cryo-FIB machine was generated. These lamellae were ~160 nm thick and were produced using a beam current at 50 pA. All 32 lamellae were preserved during transportation. Key cellular features like lipid droplets and membranous rearrangements were clearly visualised on a searchmaps at a magnification of 15,000X (pixel size 0.85 nm). The advantage of automated milling was its high throughput and faster processing. However, using an automated process to generate lamellae resulted in inconsistent lamellae thicknesses, with some areas being too thick for cryo-ET imaging.

Lamellae generation involves multiple steps of rough milling to fine milling, during which several lamellae are lost. Given this, and with limited time on the instrument, 30 good lamellae were generated from 60 cell targets on a single grid, sufficient for downstream cryo-ET imaging. Example images from before and after milling on the cryo-EM grid (dataset1) is shown in Figure 4.13.

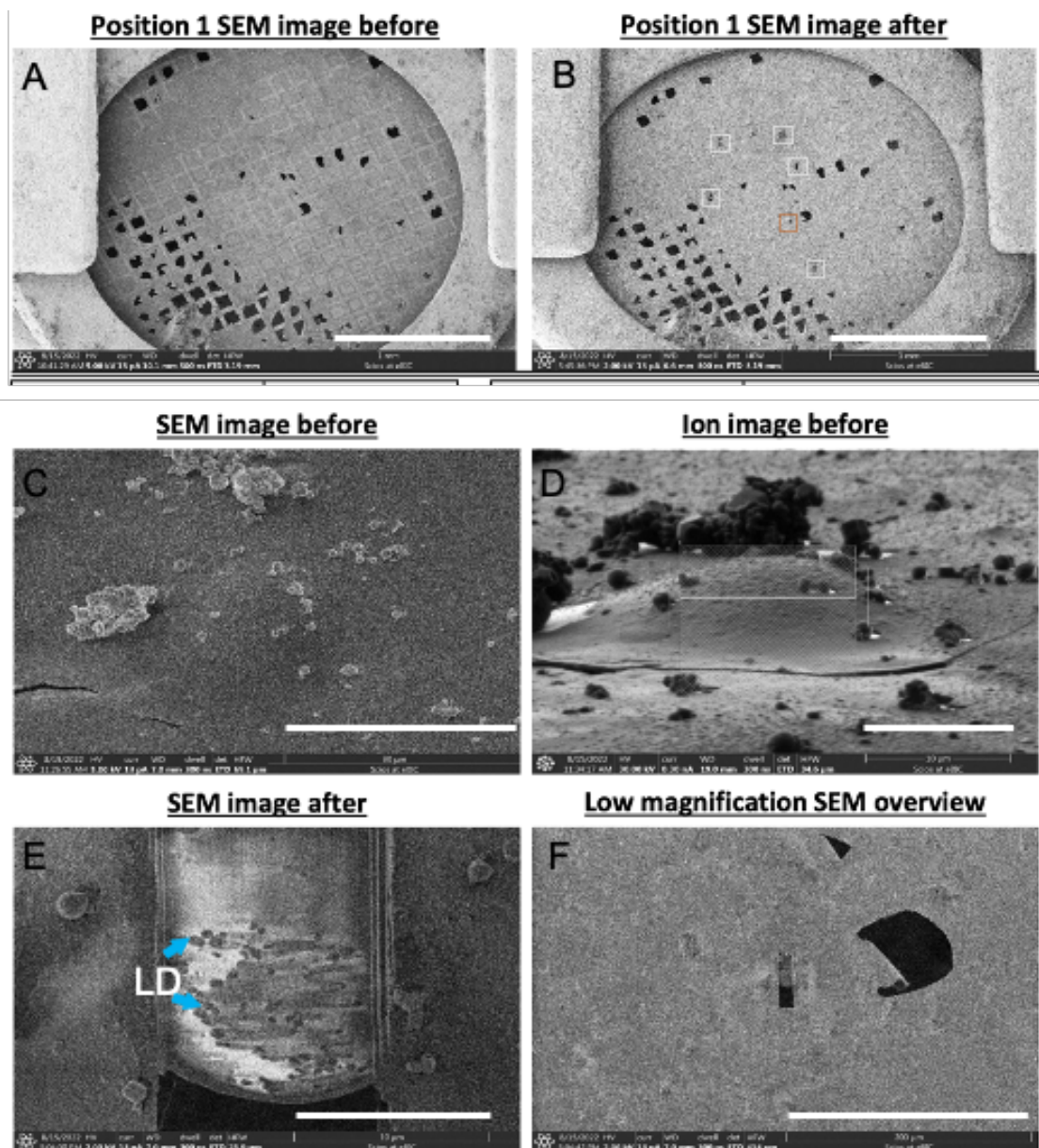


Figure 4.13: Representation of lamella generation through cryo-FIB milling.

The EM grid is depicted before milling [A] and after milling [B], featuring an organo-platinum coating. Six milled areas on the grid are highlighted in white dashed boxes, with the orange highlighted box corresponding to the lamella in [F]. A representative SEM image of a cell, ready for milling, is presented from both top view [C] and side view [D]. [E] Displays an SEM image of a lamella, emphasizing the observations of lipid droplets (LDs) on the surface. [F] A low-magnification SEM image of the same lamella shown in [E]. Scale bars- A: 1 mm, B: 1 mm, C: 30 μm , D: 10 μm , E: 10 μm , F: 200 μm .

4.2.4 Improvement on acquisition of data by cryo-ET

To visualise the molecular architecture of HCV membranous rearrangements, cryo-ET of the lamellae was employed (Eisenstein et al., 2023; Khavnekar et al., 2023). Dr. Yehuda Halfon (Astbury Biostructure Laboratory Electron Microscopy Facility, University of Leeds) facilitated data acquisition by cryo-ET. To identify regions for tilt-series acquisition, search maps with low electron dose were used as guidance. Tilt-series dataset 1 was collected at a magnification of 42,000X and pixel size of 3 Å. Whilst dataset 2 was collected at magnification of 64,000X and pixel size of 1.9 Å. A dose-symmetric tilt scheme was employed for both datasets. This tilt scheme reduces the accumulated damage from the electron beam at the angles where the specimen is at its thinnest (Berger et al., 2023). Dataset 1 consisted of 27 tilt-series which were collected from five lamellae, while dataset 2 consisted of 124 tilt-series collected from thirty-two lamellae. Individual projection frames were aligned by motion correction using dose weighting motionCor2, (Zheng et al., 2017)) and stacked into a tilt-series using an in-house python script (tomo-stacker.py, from Dr. Tom Sullivan, University of Leeds). Tilt series were reconstructed into tomograms using the eTOMO tomography pipeline from IMOD with weighted back projection (dataset 1) (Kremer et al., 1996) and with AreTomo (dataset 2) (Zheng et al., 2022).

The best way to mill lamellae is at a low angle where the beam is as parallel to the substrate as possible. This results in long lamellae, maximising the usable area for tilt-series acquisition (Lam and Villa, 2021). The lamellae angle limits the range of tilts in the cryo-ET, contributing to resolution anisotropy (Diebolder et al., 2015). Due to this pre-tilt of the lamella, the apparent sample thickness will be greater in one tilt direction on the cryo-TEM stage compared to the other direction, correlating to a greater relative tilt between the lamella and the beam (Lam and Villa, 2021). To compensate for the tilt-angle from FIB-milling, tilt series acquisition should start at the angle used as the milling angle. Lamellae generated for dataset 1 were milled at 12°, so to compensate this angle, the starting angle should have been set at 12° in the cryo-TEM. However, this step was missed and therefore, certain positions were compromised, and the correct reconstruction was not possible. Out of 27 reconstructed tomograms, 13 were suitable for analysis, while the rest appeared to be damaged by radiation, potentially due to ice contamination that resulted in uneven milling (Cheng et al., 2023). To reconstruct the tomograms, some projections had to be excluded due to failure of autofocus and tracking on the microscope. These were mostly high and low tilt increments near $\pm 60^\circ$ (in this case 72° and 48°) as well as the starting angle 0° (in this case 12°) due to failure on compensating the lamellae tilt angle. Keeping all this factors in mind, the next dataset (2)

was collected with a starting angle of 12.14° and that resulted in successful automated tomogram reconstruction by AreTomo of 124 tilt-series. In this dataset, some tomograms were discarded from analysis due to two artefacts arising from milling: ion deposition and curtaining (Lucas and Grigorieff, 2023). Two examples of lamellae from dataset 2, acquired on search map magnification of 15,000X and pixel size of 8.5 Å illustrating the different kinds of membranous rearrangements observed are shown in Figure 4.14. The top lamella contains more LDs compared to the bottom lamella which highlights more ROs (SMVs, DMVs, MVBs). Since each lamella had regions of identifiable membrane rearrangements, all possible regions were imaged by cryo-ET for unbiased analysis.

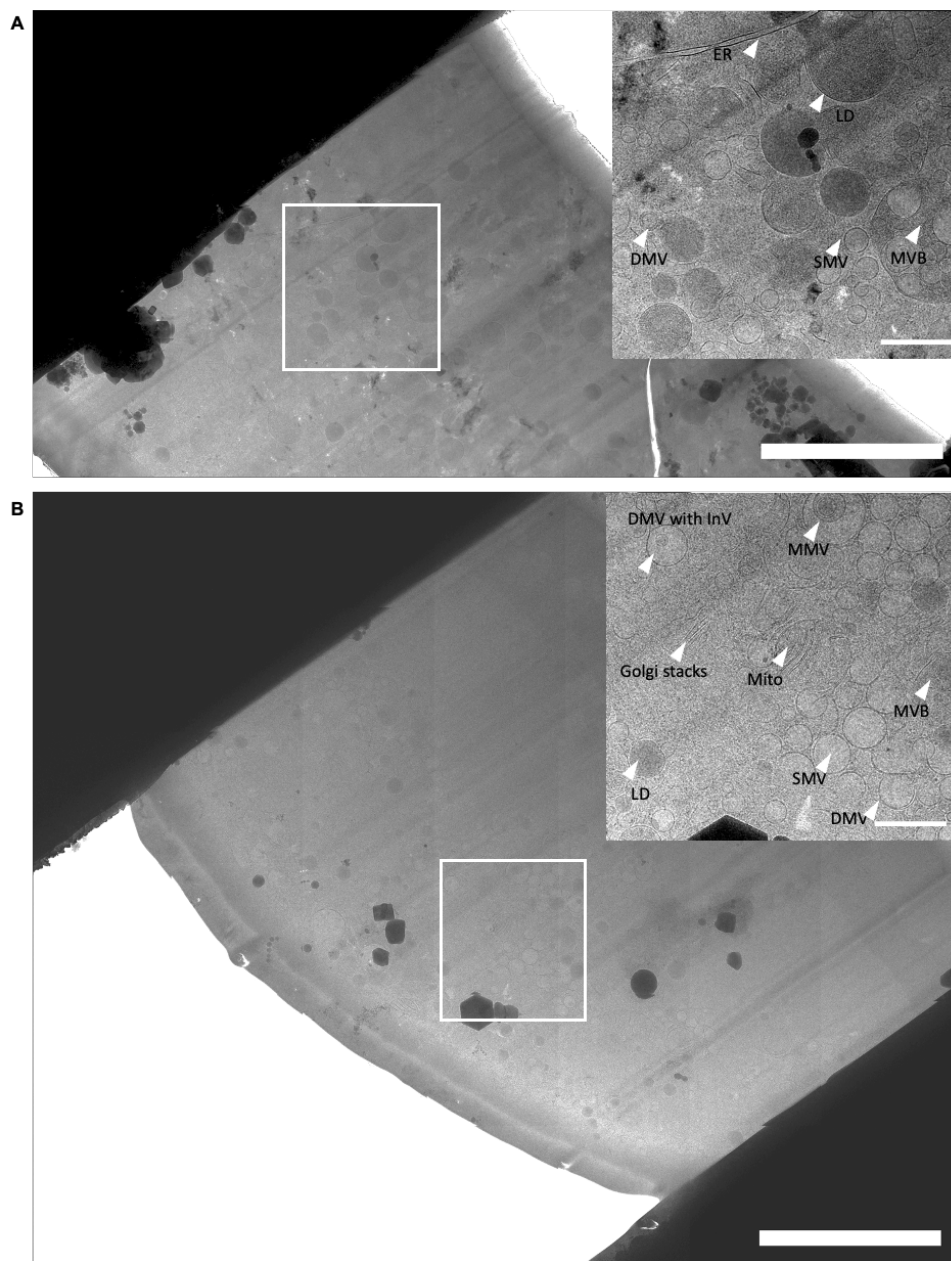


Figure 4.14: Representative images of cellular variations observed in search maps from cryo-FIB milled cells harbouring SGR JFH-1 NS5A-eGFP.

[A] Search map on a lamella illustrating replication organelles around lipid droplets, an area highlighting ROs is shown in the inset. [B] Search map on a lamella illustrating extensive membranous rearrangements, an area highlighting ROs is shown in the inset. White arrows indicate the various organelles identified on the lamella. Scale bars- Search map: 2 μ m, Inset: 500 nm. *In*: Inner, *InV*: Inner vesicle, *LD*: Lipid droplets, *MVB*: multivesicular bodies, *ER*: endoplasmic reticulum, *Mito*: Mitochondria.

4.2.5 Qualitative analysis of replication organelles

Due to the lack of fluorescence correlation of NS5A-eGFP with specific vesicles, the qualitative analysis performed below attempts to speculate the observed densities within the vesicles and connect them to previously published reports.

4.2.5.1 Differences in densities observed within DMV and SMV located close to each other may indicate their functional role in harbouring non-structural proteins

To characterise the various organelles observed in each tomogram, a detailed analysis was conducted on each type of organelle to determine whether a particular type is preferred as a replication site harbouring the assembly of the replication complex. Given the hypothesis that DMVs serve as the primary site of replication (Romero-Brey and Lohmann, 2016), the initial focus was on examining the architecture of DMVs and their surrounding environment. Closed DMVs were commonly found, often surrounded by other DMVs, double-membrane tubules (DMTs), SMVs, and MVBs. The majority of DMVs observed exhibited a closed structure (Figure 4.15), with rare instances of open DMVs, which were found in proximity to other open and closed DMVs (Figure 4.16A).

To identify the location of the replicase machinery, an analysis on the distribution of densities within both DMVs and SMVs were examined. DMVs observed in Figure 4.15 A that were surrounded by SMVs, contained densities compatible with being the replicase machinery. However, a single membrane inner vesicle (SMInV) within this DMV was observed which differed from a double membrane inner vesicle (DMInV) in Figure 4.15 B. It appears that the content of the SMInV is being released into the main DMV lumen, whereas the content of DMInV is compact and intact within this inner vesicle. The role of these inner vesicles was investigated in all tomograms and will be discussed later. A proportion of closed DMVs around MVB and SMV appeared to have no content, raising questions about their role in HCV replication (Figure 4.15 C).

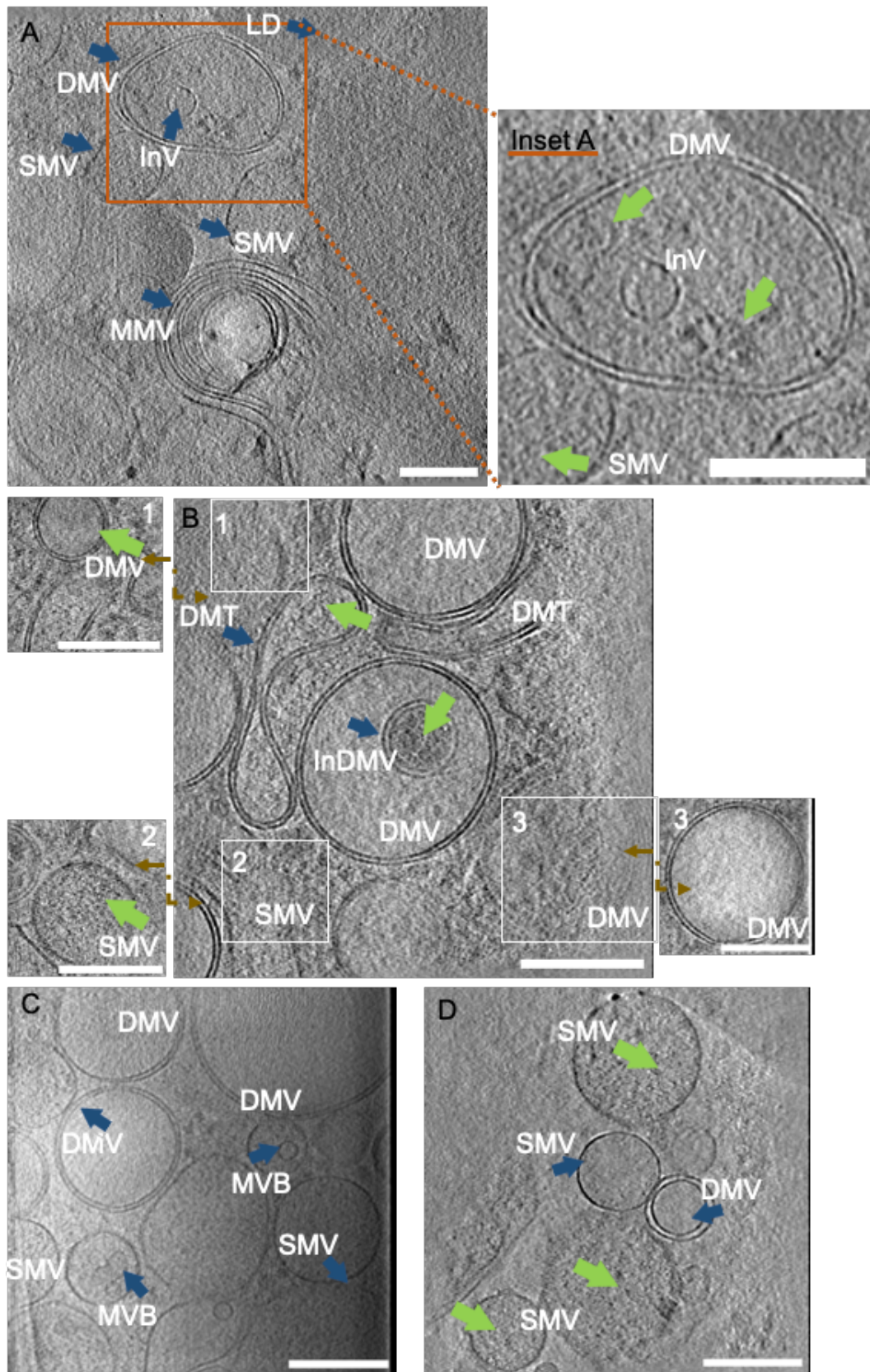


Figure 4.15: *In situ* cellular architecture of DMVs in cells harbouring SGR JFH-1 NS5A-eGFP from reconstructed tomograms.

[A] A representative tomographic slice displaying organelles surrounding closed DMVs, including SMVs, MMVs, LDs, and MVBs. The inset with an orange box highlights clustered densities within the DMV. [B] A representative tomographic slice,

illustrating various closed DMVs and double-membrane tubules (DMTs). Differences in densities within closed DMVs and DMTs are observed. Densities in the case of DMV with an inner vesicle (InV) are compactly packed in the inner DMV (InDMV). [C] A representative tomographic slice of closed empty DMVs observed around SMVs and MVBs. [D] A representative tomographic slice of DMVs near multiple SMVs (with dense lumen). Blue arrows indicate organelles, and densities within the organelles are indicated with green arrows. ROs with and without densities in different Z slices are marked within numbered white boxes. ROs present in different Z-slices are connected by a brown double-arrow connector. Scale bars: 200 nm. *In*: Inner, *InV*: Inner vesicle, *MT*: microtubule

In contrast, the densities observed in open DMVs appeared to be distributed in a thread-like pattern rather than forming clustered densities, suggesting the potential presence of double-stranded RNA (dsRNA) filaments (Figure 4.16 A), as observed in case of SARS-COV2 (Klein et al., 2020). Complex and distinctive higher-order double-membrane vesicles (DMVs) were categorized as multimembrane vesicles (MMVs) (Figure 4.16 B). The densities observed within the lumen of MMVs, which encompass multiple DMVs, were predominantly concentrated in the innermost compartment (Figure 4.16 B, C). The formation mechanisms of these higher-order structures and whether they contain similar densities observed in closed DMVs and double-membrane tubules (DMTs) remain unclear.

The DMVs present around mitochondria were also examined, as a fraction of the viral replication complexes are thought to be located at the mitochondria-associated ER membranes (MAMs), initiating the HCV RNA replication at MAMs, providing an environment rich in enzymes involved in cholesterol, triglyceride, fatty acid and phospholipid metabolism (Duponchel et al., 2023). Mainly closed DMVs and SMVs around mitochondria were observed (Figure 4.16 D). The association of mitochondria with ER and the overall integrity of the mitochondria was examined to identify whether the membranes of the DMVs were derived from these mitochondria. No close contacts between mitochondrial membrane and ROs were observed suggesting they were around the mitochondria possibly waiting for transfer of cellular and non- structural proteins NS3/4a (Duponchel et al., 2023).

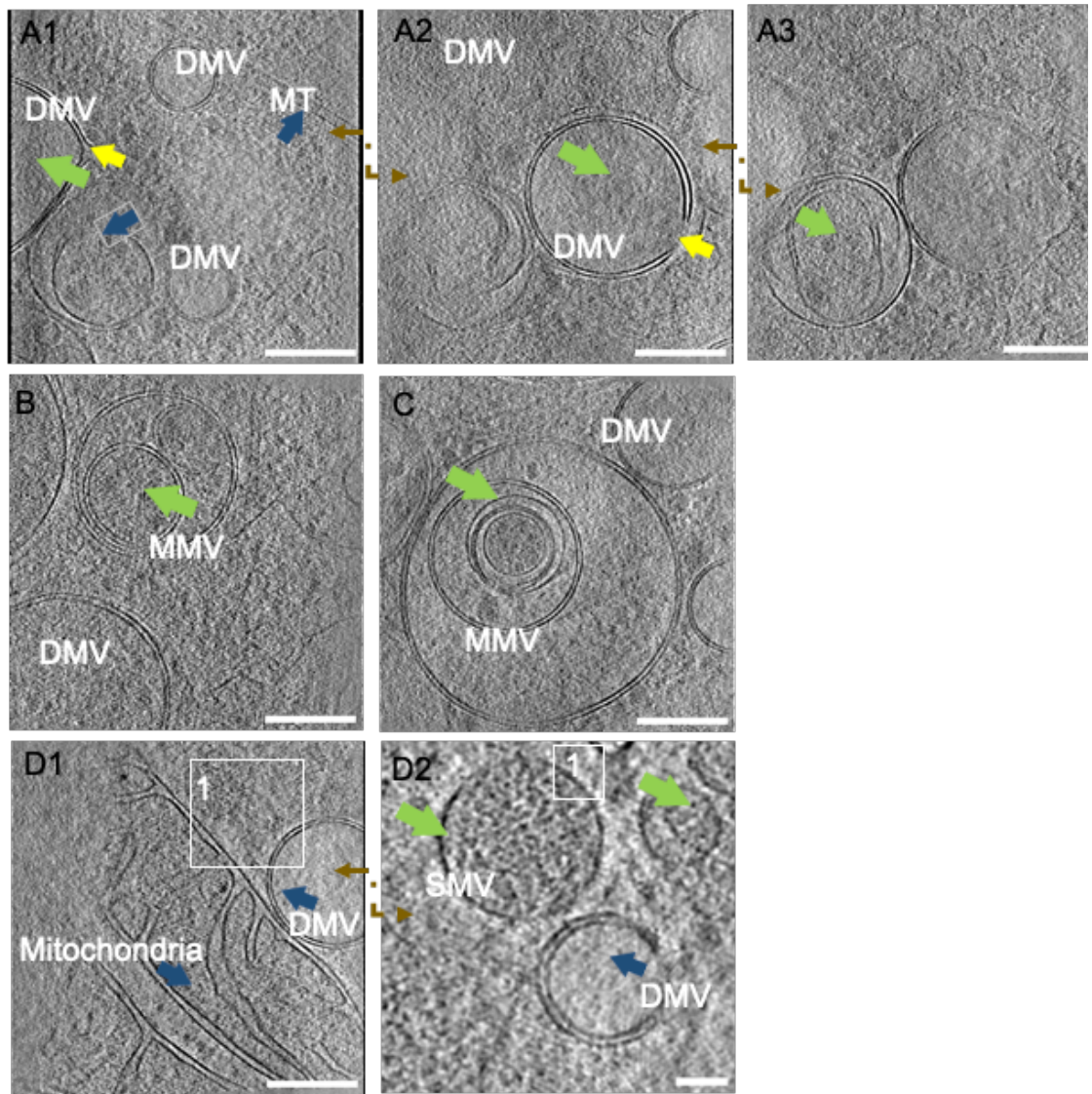


Figure 4.16: *In situ* cellular architecture of open DMVs, MMVs and ROs present near mitochondria in cells harbouring SGR JFH-1 NS5A-eGFP from reconstructed tomograms.

[A1-A3] A representative tomographic slice showcasing rare observation of open DMVs present around closed DMVs is shown. The open area in the DMV is highlighted with yellow arrows. [B, C] Exemplars of unique and complicated MMVs. The densities observed in unique MMVs were always found in the inner DMVs. [D1-D2] A representative tomographic slice of closed DMVs present around mitochondria. Blue arrows indicate organelles, and densities within the organelles are indicated with green arrows. RO present in different Z-slice in the tomogram is indicated by a brown double arrow connector. ROs with and without densities present in a different Z-slice in the tomogram are shown within the numbered white boxes. Scale bars: A-D: 200 nm; D (area1): 50 nm

Additionally, whilst examining DMVs, densities within SMVs present near the DMVs were assessed. Immunogold labelling of thawed cryo sections at 16 hour post infection identified NS5A to associate with SMVs of 50-70 nm (Romero-Brey et al., 2012). The diameters of the majority of SMVs observed in this study were in the range of 50-300 nm. The densities within SMVs observed proximal to DMVs were more pronounced and differed to the densities observed within DMVs as observed in Figure 4.15 B(2), Figure 4.16 D(1). Thus, SMVs may play a role in facilitating the transfer of NS5A proteins from one area of the cell to the other. Notably, the SMVs present around DMVs had content exclusively within the membrane, whereas the SMVs observed around LDs and ER differed and is discussed further.

Three categories of DMVs were observed to summarise the densities: DMVs with clustered densities indicating a possible formation of replication complex, DMVs with thread like structures indicating possible accumulation of dsRNA intermediate filaments, empty DMVs with no content visible, which possibly function as a decoy for immune evasion (Figure 4.17 A-D). The comparison of intensities within SMVs and DMVs located close to each other is shown in Figure 4.17 B, C.

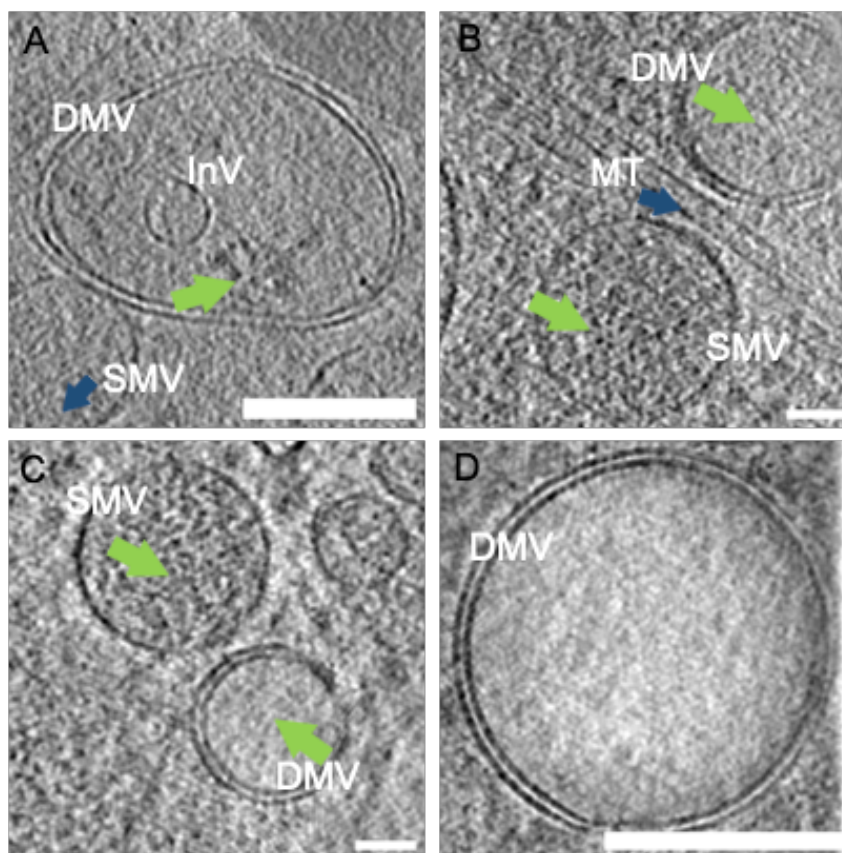


Figure 4.17: Summary of in situ cellular architecture of DMVs and SMVs in cells harbouring SGR JFH-1 NS5A-eGFP from reconstructed tomograms.

[A-C]: A representative tomographic slice of DMVs; DMV with clustered densities [A], thread like intensities in DMV [B, C] and empty DMV [D]. [B-C]: Comparison of densities between DMV and SMV with well distributed pronounced densities within SMV in comparison to thread like densities in DMV. Scale bars: [A, D]: 200 nm; [B, C]: 50 nm. Blue arrows indicate organelles, and densities within the organelles are indicated with green arrows. *InV*: Inner vesicle, *MT*: microtubule

4.2.5.2 SMVs were predominantly observed around LDs and ER in contrast to DMVs from previous literature

Lipid droplets play a crucial role in the assembly of the HCV viral particles. For example, it has been shown that NS5A and envelope protein E2 localise around LD and that these LDs are wrapped with ER in proximity to DMVs in infected cells (J.Y. Lee et al., 2019). Therefore, the architecture of LDs and their surrounding organelles were analysed in the dataset (Figure 4.18). The general architecture around LDs in this study observed SMVs compared to previous reports of DMVs decorating LDs wrapped by ER (J.Y. Lee et al., 2019). SMVs have electron dense particles decorated towards the luminal side, as well as inside which might be important in facilitating the transfer of both host and viral proteins to LD (the site of assembly) as shown in Figure 4.18.

By fractionation experiments and immunomicroscopy, it was determined that all NS proteins were associated with ER, thereby suggesting ER as the site for membrane associated HCV replication (Mottola et al., 2002). The ER around LDs appeared to be decorated with electron dense particles which could be speculated as an accumulation of non- structural proteins or just ribosomes indicating the ER to be rough ER (Figure 4.18 A).

LDs were predominantly surrounded by ER, SMVs and large single membrane compartments (SMC). Two such sizable compartments around the LD are illustrated in Figure 4.18 B. One SMC harbouring an SMV with clustered densities (and an InV within the SMV), whereas the other SMC harbours an empty vesicle. The observation of SMVs near LDs, in contrast to DMVs could be attributed to the differences in the preservation methods used in prior studies, which potentially compromised the integrity of SMVs while preserving the structure of DMVs. This analysis is consistent with EM examinations of liver biopsy specimens where membranous web was composed entirely of clusters of SMVs derived from the ER and located close to lipid droplets (Blanchard and Roingeard, 2018). The study proposed that DMVs could be hallmark of laboratory-adapted JFH-1 strain, facilitating high *in-vitro* replication while preventing a dsRNA-triggered antiviral response (Blanchard and Roingeard, 2018).

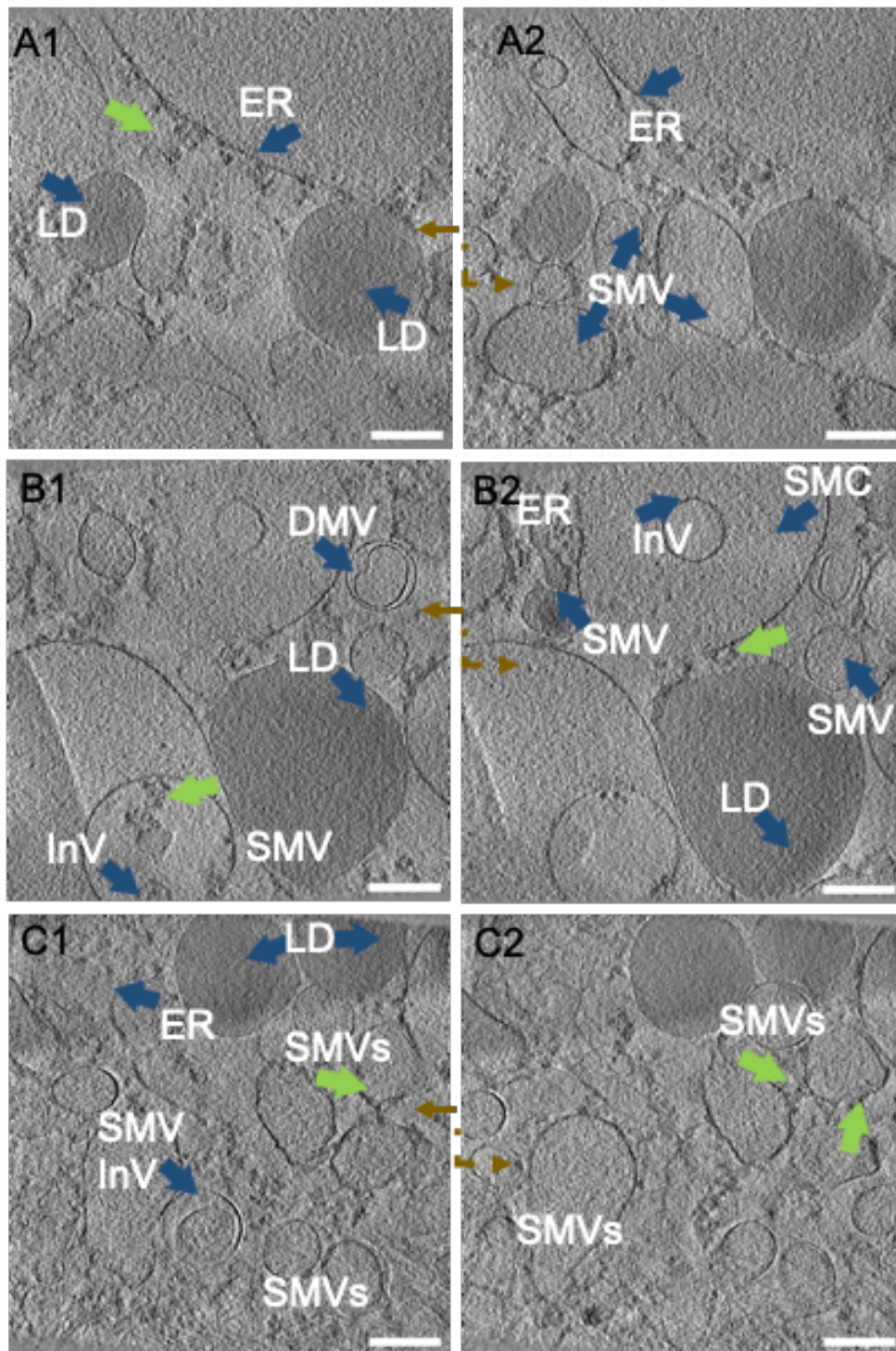


Figure 4.18: *In situ* cellular architecture of SMVs around LDs and ER in cells harbouring SGR JFH-1 NS5A-eGFP from reconstructed tomograms.

[A1-A2] A representative tomographic slice of SMVs present around the ER. Densities near ER are depicted by green arrows. [B1-B2] A representative tomographic slice illustrating densities present around LD and SMC. The SMV inside the SMC harbours cluster of densities indicating a possibility of assembly of replicase machinery. This SMV also harbour a InV within. [C1-C2] A representative tomographic slice illustrating several SMVs observed around LDs. Blue arrows indicate organelles, and densities

within the organelles are indicated with green arrows. RO present in different Z-slice in the tomogram is indicated by a brown double arrow connector. Scale bars: 200 nm

The ER structure is often dramatically rearranged in positive-strand RNA viruses due to the interaction of host and viral proteins and is the main hub for RO formation. For example, viruses such as beet black scorch virus (BBSV) and tobacco necrosis virus (TNV-W) induce ER membrane dilations and invaginations within the ER cisternae that are rounded structures up to 400 nm (Cao et al., 2015; Nguyen-Dinh and Herker, 2021). Whilst investigating the SMVs near the ER in the HCV dataset, an SMV within the ER that appeared to be dilated (Figure 4.19) was observed. In Dengue virus replication, vesicle packets (VPs) are membranous structures derived from dilated ER cisternae containing SMVs with a diameter of 80-150 nm. These SMVs originate from the invagination of the ER membrane into the ER lumen (Welsch et al., 2009; Nguyen-Dinh and Herker, 2021). Previously, in the case of HCV, DMVs appeared as exvaginations, connected via a short neck like structures to the ER (Romero-Brey et al., 2012). In the present study, SMVs were also noticed budding from the ER (Figure 4.19 B). Due to the sample preparation methods employed in the previous studies, SMVs were not studied extensively. Further investigations on how SMVs are formed (if within the dilated ER) and how they are released into the cytoplasm would shed light into SMV biogenesis and their role in HCV replication.

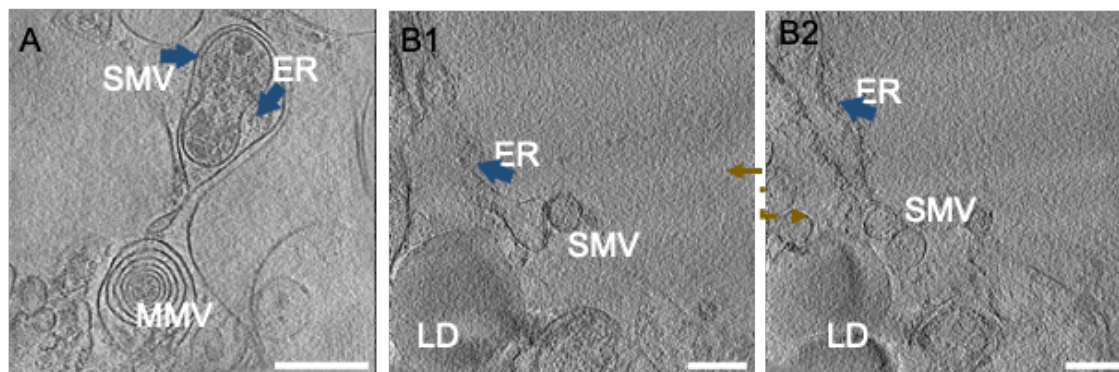


Figure 4.19: *In situ* cellular architecture of SMVs observed within the ER and released from ER in cells harbouring SGR JFH-1 NS5A-eGFP from reconstructed tomograms.

[A] A representative tomographic slice of a SMV inside a dilated ER cisterna. The SMV is composed of variable densities, possibly combination of host and viral proteins. [B1-B2] A representative tomographic slice highlighting the release of SMV from ER stalk. Blue arrows indicate organelles. RO present in different Z-slice in the tomogram is indicated by a brown double arrow connector. Scale bars: 200 nm

4.2.5.3 MVBs harbour DMVs and SMVs which may play a role in egress of non-structural proteins and dsRNA as a non-canonical secretory route to evade host immune system

Employing an HCV replicon in which NS5A is fused with mCherry (CLEM study), it was determined that 35% of NS5A-mCherry was localised within DMVs and SMVs, while 20% was situated in endosomal compartments and MVBs (Grünvogel et al., 2018). In the current study, a combined dataset consisting of 112 tomograms, revealed that 50% of tomograms contained MVBs or late endosomes. These were identified based on their morphological appearance as described in the literature (Yu et al., 2021). The examination of the contents and architecture of MVBs in this dataset aimed to identify specific features within them. SMVs and DMVs are frequently observed around the MVBs (Figure 4.20 A, B). SMVs, DMVs and MMVs were also identifiable within MVBs (Figure 4.20 C, D). The number of MVBs harbouring organelles was not quantifiable as the low contrast inside the MVBs did not allow to clearly differentiate specific individual vesicles. Generally, the components within the MVBs differed ranging from small vesicles, organelles with densities, degraded elements. This difference in contents inside the MVB could indicate the possible functional role. MVBs where degraded elements were observed could be speculated as lysosomes whereas MVBs where organelles such as SMV and DMV were observed could be speculated as MVBs *en-route* to outside of the cells.

Two predominant shapes of MVBs were noticed. For categorisation, one was termed as spherical MVB (Figure 4.20 A,B) and the other as elongated (Figure 4.20 C,D). Elongated MVBs were large and mostly beyond the field of view. Elongated MVBs could correspond to MVBs in the process of endocytosing vesicles (e.g. Figure 4.20 D). A third of MVBs were mostly observed alongside contiguous network of ER, almost wrapping the MVB and are near LDs (e.g. Figure 4.21 A,B), indicating their potential role in carrying HCV ROs harbouring either non-structural proteins or the HCV genome.

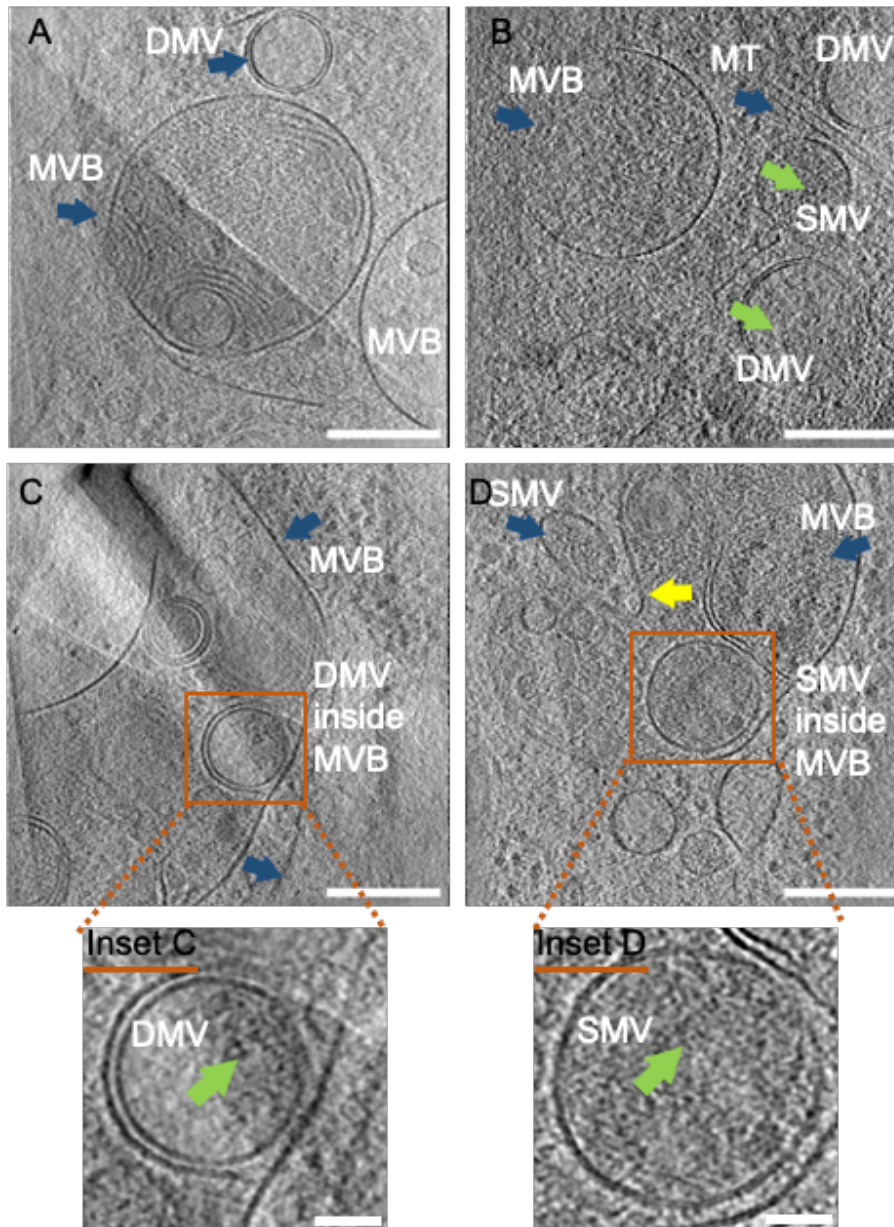


Figure 4.20: *In situ* cellular architecture of MVBs in cells harbouring SGR JFH-1 NS5A-eGFP from reconstructed tomograms.

[A-B] A representative tomographic slice depicting SMVs and DMVs harbouring densities located near spherical appearing MVBs. [C-D] A representative tomographic slice depicting MVBs (appeared to be elongated) containing multiple ROs within. DMV and SMV (within the MVB) with densities are highlighted in Inset C, Inset D from corresponding image C and D. Blue arrows indicate organelles, and densities within the organelles are indicated with green arrows. Yellow arrow indicated the bend in the MVB indicating a process of engulfing a SMV. Scale bars: A-D: 200 nm, Inset C, D: 50 nm

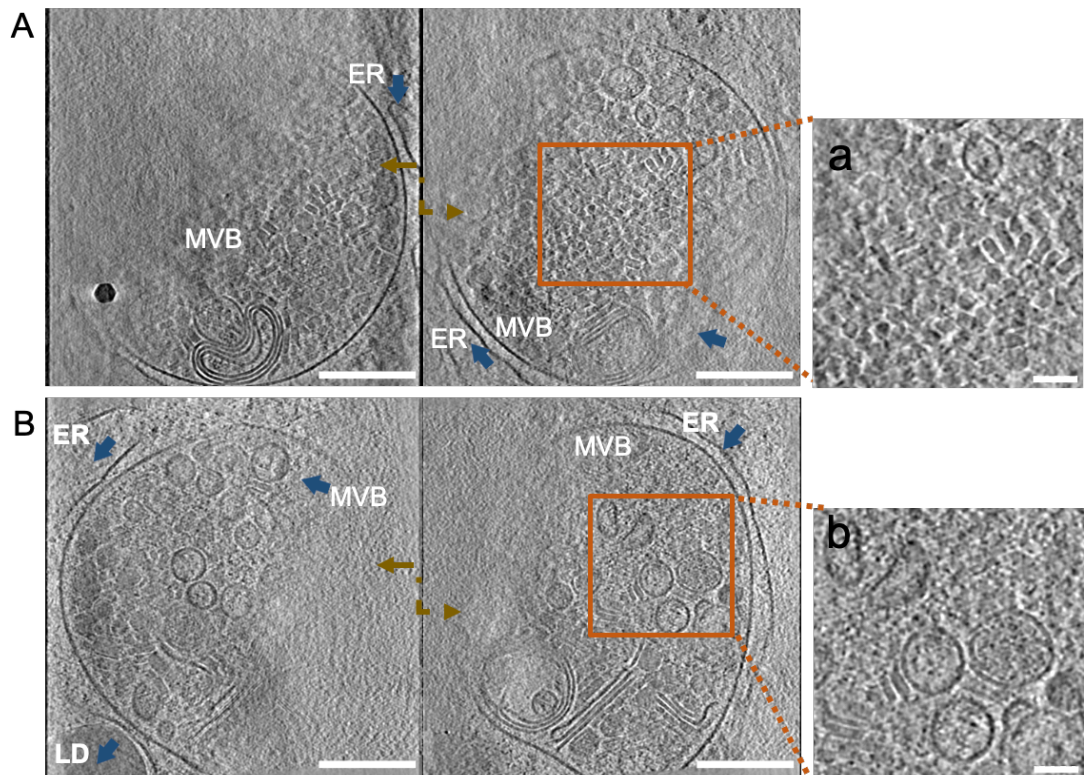


Figure 4.21: *In situ* cellular architecture of MVBs surrounded by ER in cells harbouring SGR JFH-1 NS5A-eGFP from reconstructed tomograms.

[A] A representative tomographic slice showing a spherical MVB. [B] A representative tomographic slice showing an elongated MVB. Both type of MVBs are observed to be wrapped by ER, however, the content of these two MVBs differ in general. Inset a and b to shows the content within the respective MVBs are shown. Blue arrows indicate organelles. ROs present in different Z-slices are connected by a brown double-arrow connector. Scale bars: A, B: 200 nm, a, b: 50 nm

4.2.5.4 Fusion event of small vesicles into SMVs observed in membrane rearrangements

Out of all the reconstructed tomograms, approximately one-tenth of the total had extensive membrane rearrangements, making it challenging to categorise distinct cellular compartments such as DMVs and SMVs. This truly highlights the complexity of host cell membrane rearrangements. A notable feature observed in these membranous compartments, as well as in previously mentioned tomograms, was the presence of InVs within the different types of vesicles. The InVs appeared to have a dense lumen and were captured at various stages of entry to a vesicle (Figure 4.22 A-C). For example: the InV observed in Figure 4.22 A and Figure 4.22B was classified as an SMV undergoing a self-invaginating process to form a DMV (Ferraris et al., 2013). It appears that one vesicle had completed its fusion, moving towards the centre of the SMV, while the other vesicle had recently fused. Clusters of SMVs and DMVs with InVs within some of them are

illustrated in Figure 4.22 C. These were previously observed and termed as Vesicles in Cluster (ViCs) (Ferraris et al., 2013). Given that exosomes can transfer HCV RNA to uninfected Huh7 cells in HCV SGR systems lacking structural proteins (Ramakrishnaiah et al., 2013), a plausible hypothesis is that the InVs observed in the dataset may represent exosomes. This could potentially be a mechanism through which the HCV genome is transferred to different ROs within the cells.

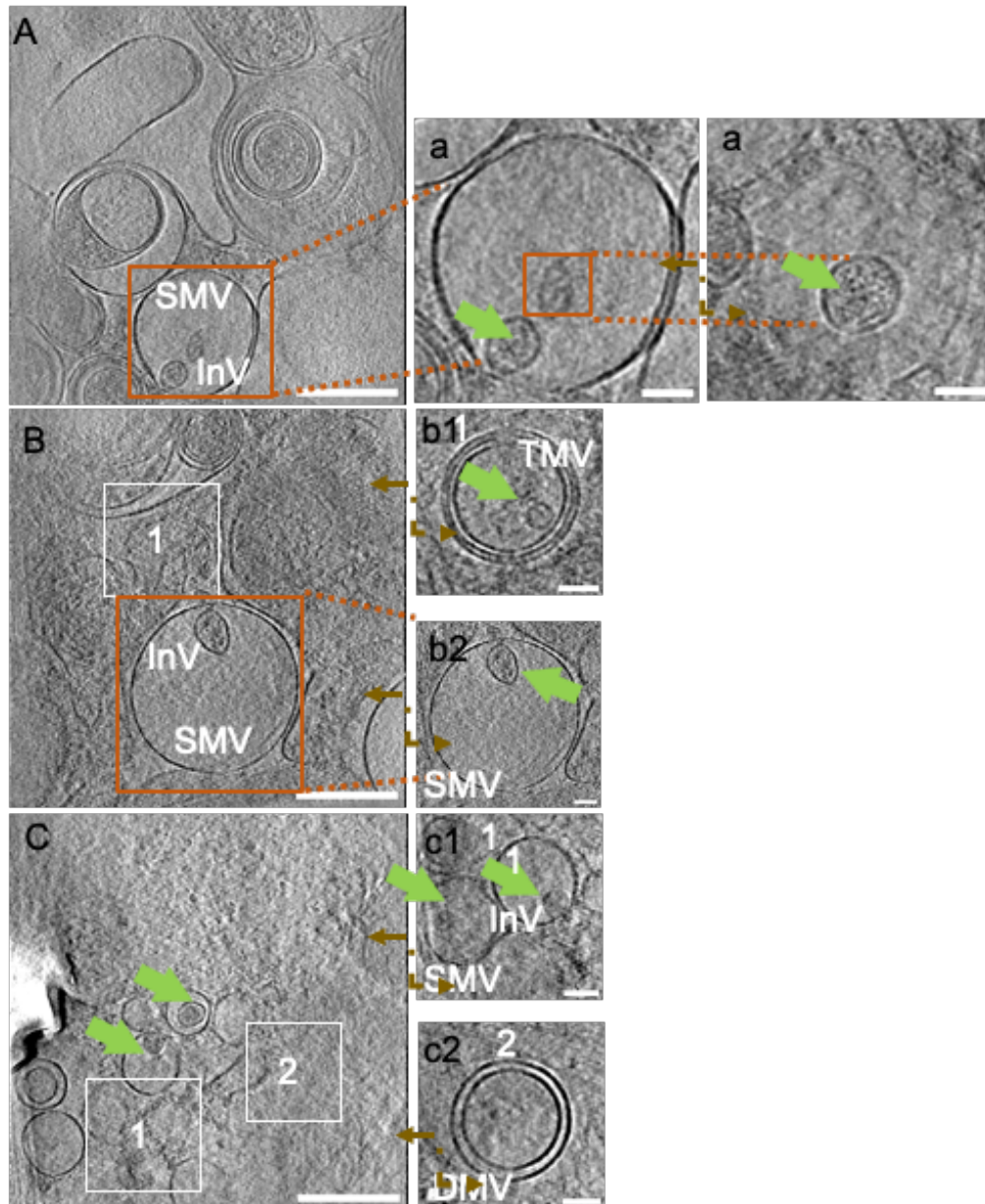


Figure 4.22: *In situ* visualisation of extensive membrane rearrangements in cells harbouring SGR JFH-1 NS5A-eGFP from reconstructed tomograms.

[A] A representative tomographic slice of MW, consisting of a SMV with a vesicle in the process of internalisation and internalised [a]. [B] A representative tomographic slice of MW, consisting of a SMV (with InV) (b1) and a triple membrane vesicle (TMV) captured with an InV, which appeared to be releasing its content into the lumen of the

TMV [b1]. [C] Vesicles in clusters (SMVs and DMVs) observed with InV specifically in SMV either in the process of internalisation or already internalised I. ROs with and without densities in different Z slices are marked within numbered white boxes (b1, c1, c2). ROs present in different Z-slices in the tomogram are connected by a brown double-arrow connector. Blue arrows indicate organelles, and densities within the organelles are indicated with green arrows. Scale bars: [A-C]: 200 nm, [b1, b2, c1, c2]: 50nm

4.2.6 Quantitative analysis revealed that the majority of SMVs and DMVs were between 50 – 300 nm in diameter

To assess the size of each RO, identifiable vesicles and cellular features based on previous literature were summarised per tomogram. The data was acquired without any correlative guidance specific with the NS5A-eGFP foci, therefore all membrane rearrangements (SMV, DMV, MMV, MVB, LD) that were measurable out of 112 tomograms were analysed. The predominant organelles that were in the field of view in the tomogram and measurable were SMVs, followed by DMVs, LDs, MVBs and mitochondria.

The different types of organelles imaged in all the tomograms was then quantified. Major and minor axis measurements were made for 178 organelles: 95 SMVs, 36 DMVs, 11 MVBs, 20 inner vesicles (InV, inside DMV or SMV) and 16 LDs were measured (Figure 4.23 A). To determine whether there was any significant difference in the shape of these vesicles, an aspect ratio of major vs minor axis was measured. In general, an aspect ratio close to one (value=1) suggests the shape of the object as circular (Figure 4.23 B). To identify the percentage of vesicles that were circular in the dataset, vesicles with an aspect ratio limited between the range of 0.9-1.1 were counted. About 25% of SMVs, 19% DMV, 18% MVB, 85% InV, 50% of LD indicated as circular in shape than others.

A normal distribution (normality versus lognormality) test was conducted to examine the distribution of the data and to quantify how far the distribution is in terms of asymmetry and shape. If the observations are normal, they are symmetric suggesting the mean is equal to the median. A Quantile-Quantile plot (QQ plot) suggested the dataset to be left-skewed, which means the smallest observations are smaller than you would expect as well as the largest observations are smaller than you would expect from a normal distribution curve (Figure 4.23 C). Based on the non-Gaussian distribution of data, with a single variable (shape-circular) in more than three groups with distinct means and medians, a Kruskal-Wallis test (a non-parametric test equivalent of one-way Anova) was used to determine any significant difference observed between the groups. An approximate p value of 0.0015 was noticed where the medians varied significantly ($p < 0.05$). A significant difference on circularity was observed between SMVs and InVs

($p=0.0052$), DMVs and InVs ($p=0.0052$) and MVBs and InVs ($p=0.0452$), which correlates with the percentage of vesicles observed as circular in shape.

The diameter of the 178 organelles that were measured (average of minor and major axis measurement) suggested about 89 % of them were within the range of 50-300 nm. The average diameter of each of these vesicles and a standard deviation for vesicle type were calculated; SMVs were 144.7 ± 68 nm ($n=95$), InVs were 75.35 ± 33.4 nm ($n=20$), LDs were 290.6 ± 36.3 nm ($n=166$), DMVs were 193 ± 78.7 nm ($n=36$), spherical MVBs 487 ± 67.4 nm ($n=9$), and elongated MVBs 628.5 ± 22.25 nm ($n=2$). Therefore, while the sizes of SMVs, DMVs and InVs are comparable and in the region of 50 to 300 nm, MVBs tend to be larger and are normally above 400 nm in diameter. Additionally, LDs appeared to possess a wide range of diameters (Figure 4.23 D).

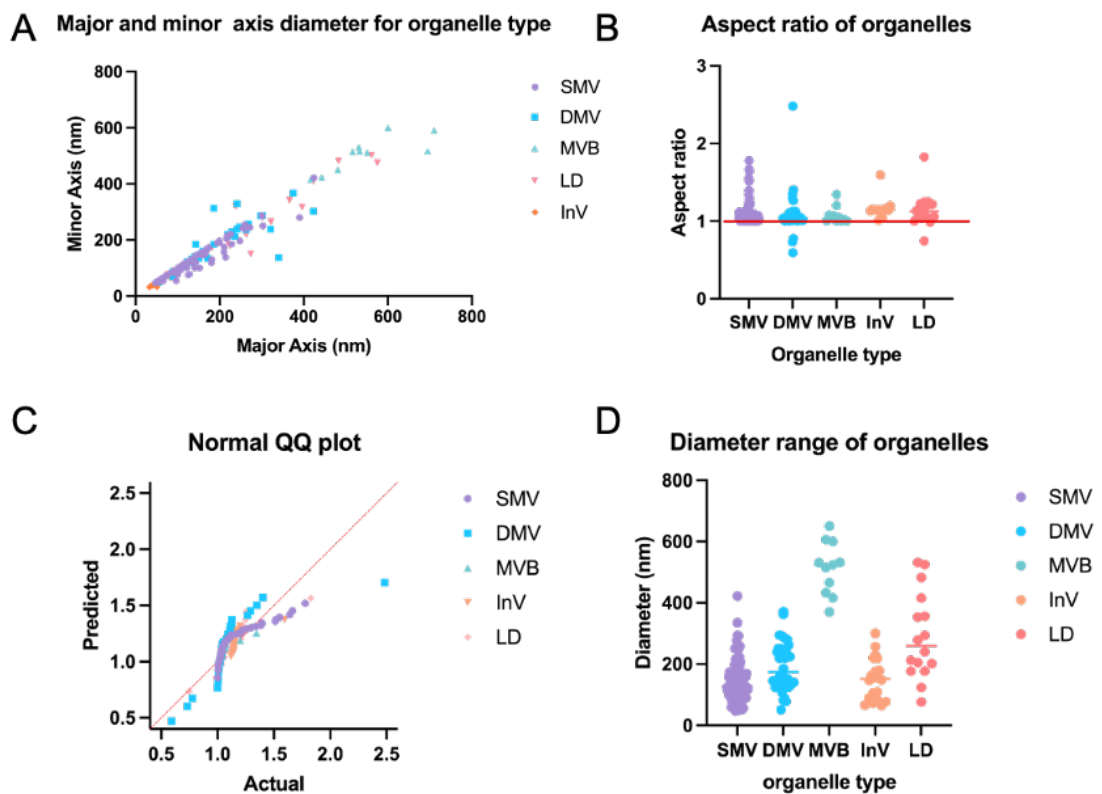


Figure 4.23: Quantitative analysis of ROs in cells harbouring SGR JFH-1 NS5A-eGFP

[A] A scatter plot of major and minor axis measurements for all measured vesicles. [B] A plot of aspect ratio (major axis divided by the minor axis) for the organelle type. An aspect ratio of 1 would suggest that the shape of the organelle is circular and is denoted by a red line across the plot. [C] Normality and lognormality test to identify the distribution of data. Since, it did not follow a gaussian distribution, Kruskal-Wallis test

was conducted to observe any significant differences in the circularity between the groups. [D] Graph depicting the range of diameter of all the organelles. Majority of SMVs, DMVs and InVs fall in the range of 50- 300 nm whilst LDs displayed a wide range of diameter. MVBs were much larger and mostly above 400 nm in diameter.

4.3 Cryo-correlative light and electron imaging

4.3.1 Integration into the cryo-FIB-ET workflow

To identify the location of the ROs, eGFP fluorescence fused with NS5A protein was utilised (Moradpour et al., 2004). Initially, cryo-FM images of the entire grid followed by specific grid squares of interest, were captured using a Thunder Leica microscope under cryogenic conditions. Although the cryo-FM images were intended for use as a guiding tool for milling through the MAPS software (licensed by Thermo Fisher), this approach was impractical due to changes in the stage geometry between cryo-FM and FIB-SEM. Both Scios and Aquilos, utilised for FIB-SEM in this study, lacked cryo-FM capabilities as present in the newer models. LDs, known for their crucial role in HCV assembly (Bley et al., 2020) and located in close proximity to ROs (J.Y. Lee et al., 2019), were abundant in these cells harbouring SGR JFH-1 NS5A-eGFP and served as markers to identify ROs around them. Therefore, LDs were used as markers along with search maps to identify organelles of interest. Post-correlation on lamella after data acquisition (Klein et al., 2021) was also attempted but was unsuccessful likely as the lamellae are fragile in nature and are contaminated with ice in the cryo-holder of the cryo-FM microscope (Figure 4.24). In these lamellae, green cells can be observed, however, the limited magnification and the high levels of ice contamination prevented correlating green fluorescence back to the tomogram.

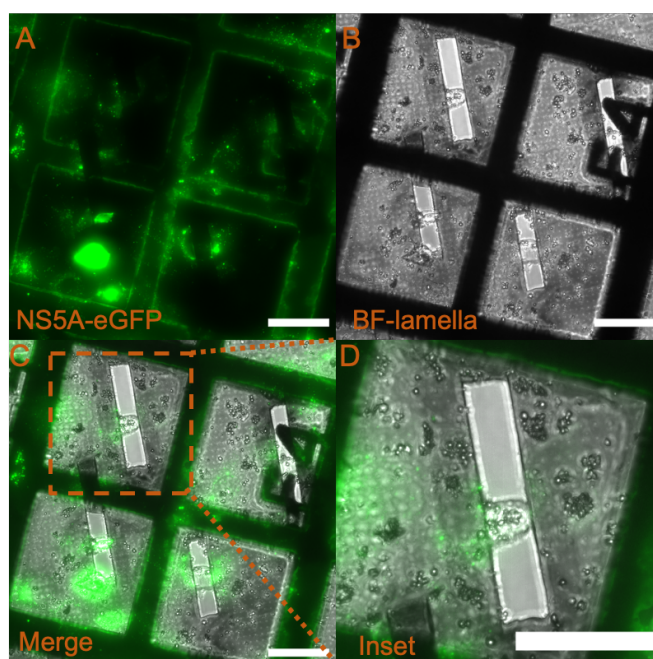


Figure 4.24: Representative cryo-FM images of FIB-milled lamellae on cells harbouring SGR JFH-1 NS5A-eGFP, acquired on Thunder Leica.

[A] Cryo-FM image [B] Cryo- bright field image [C] Merge of cryo-FM and cryo-bright field images. [D] Inset of one lamella from(C). Scale: 50 nm

4.3.2 CEMOVIS- employing NS5A-eGFP guidance revealed a MMV harboring the heterogenous arrangements of the non-structural proteins

Thus far, a definitive organisation of the replicase machinery within specific RO has not been identified with the cryo-FIB milled datasets. Due to constraints in using NS5A-eGFP fluorescence for guided tilt series collection via FIB-SEM, an alternative workflow was pursued, employing CEMOVIS (Chlanda and Sachse, 2014). The workflow was established to gain evidence of the type of organelles possibly harbouring replication complex in cells harbouring SGR JFH-1 NS5A-eGFP. Sample preparation optimisation, high-pressure freezing, ultrathin sectioning, and data collection were carried out by Dr. Tom Sullivan at the Astbury Biostructure Laboratory Electron Microscopy Facility.

Using this approach, cryo-electron tomograms were generated with guidance from the fluorescent signal of eGFP. The software MAPS was effectively utilised in conjunction with the TOMO software on the TEM (Krios) for collecting tilt-series. Ultrathin sections of three different thicknesses (100 nm, 70 nm, and 40 nm) were tested, with 40 nm sections revealing clear cellular features, while the other thicknesses resulted in low contrast. Tilt-series were collected with a pixel size of 2.4 Å and a total dose of 138 electrons per Å². From one 40 nm thin section, a total of 23 tomograms were collected, with 12 positions

guided by MAPS on green foci locations. The architectural features observed in 7 out of 12 tomograms revealed predominantly MMVs with densities present within. The other organelles observed included ER, SMV, LD, MVB and mitochondria, with similar densities within and around them as observed in the FIB-milled dataset. DMVs were a rare observation. In the cryo-FIB milled dataset, MMVs were infrequently observed compared to the CEMOVIS dataset.

The CEMOVIS workflow is illustrated in Figure 4.25, along with one tomogram where a specific MMV appears to harbour a heterogeneous arrangement of proteins, potentially indicating the assembly of the replication complex. While the heterogeneous arrangements may pose challenges for further sub-tomogram averaging at this stage, it is noteworthy to identify NS5A in the organised cryo-densities. This represents the most organised assembly of cryo-densities observed across all datasets, emphasising the significance of using fluorescence-guided tilt-series collection on a thin cryo-specimen. Representative images from 5 other tomograms from this dataset is shown in Appendix Figure 4.5.

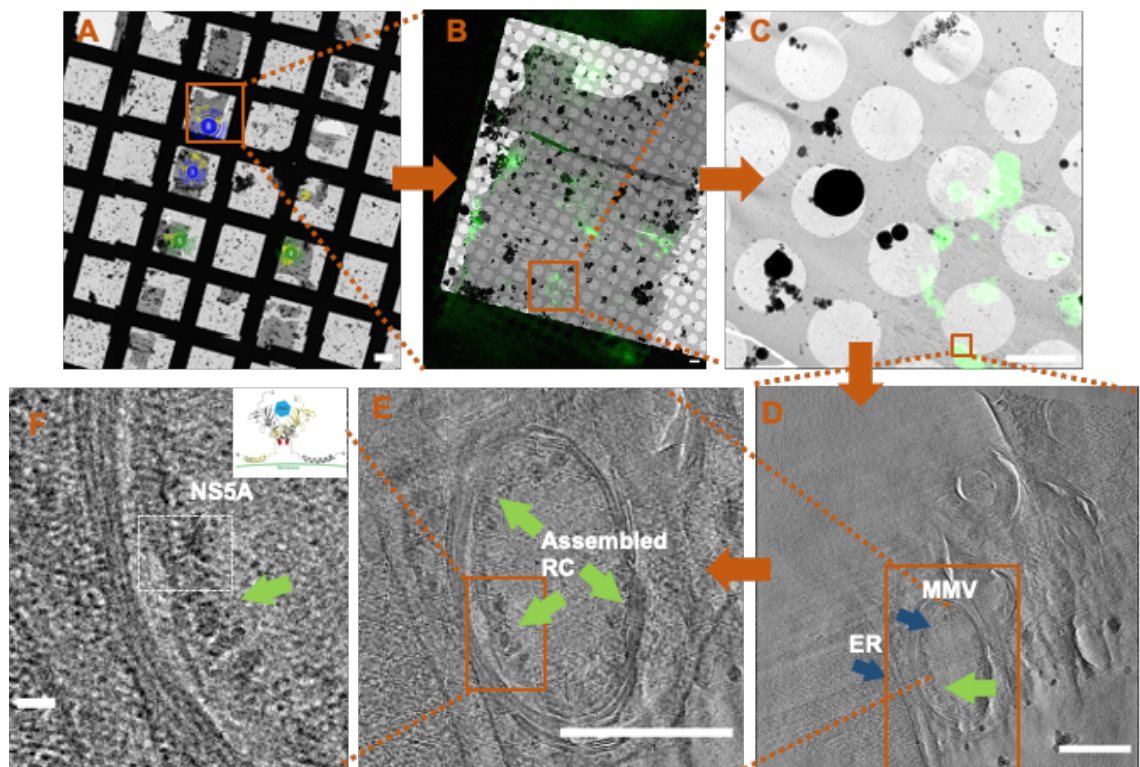


Figure 4.25: Overview of workflow for deducing cellular architecture from 40 nm ultrathin sections prepared cryo-ultramicrotomy.

Overview of workflow for deducing cellular architecture from 40 nm ultrathin sections prepared cryo-ultramicrotomy. [A-C] Demonstrates the cryo-CLEM workflow using MAPS software to guide tilt-series collection at specific green foci of NS5A-eGFP. [D] Illustrates a representative tomographic slice of the MW in cells harbouring SGR NS5A-eGFP, acquired by guided by NS5A-eGFP fluorescence. [E] Inset of the MMV in (D), illustrating the heterogenous regular arrangements of proteins indicating the assembly of replication. [F] Inset of proteins arranged in the MMV (D) and identifiable NS5A shown in dashed box. Blue arrows indicate organelles, and densities within the organelles are indicated with green arrows. *RC: Replication complex*, Scale bars: A-C: 2 μ m, D, E: 200 nm, F: 20 nm

4.4 Comparison of image quality acquired from lamella vs ultrathin sections

Cryo-ET on cryo-ultrathin sections (CEMOVIS) is challenging primarily because of difficulties in securely attaching the entire section flat on the grid and the notable formation of crevasses in sections thicker than 60 nm (Sachse et al., 2019). However, overcoming these challenges is not impossible (Bouchet-Marquis and Hoenger, 2011). In contrast, the preparation of cryo-lamella by FIB-SEM over the past few years has addressed these issues for cryo-ET of vitreous sections (Sachse et al., 2019). Therefore, similar architecture of membranous web constituting MMVs, DMVs, SMVs acquired from CEMOVIS datasets that were guided by NS5A-eGFP fluorescence were compared to FIB-milled datasets. A side-by-side comparison on the contrast of images from lamella vs ultrathin cryo sections from similar cellular architecture observed by both methods suggest both methodologies developed can elucidate the cellular architecture well. The MMVs observed in cryo-FIB dataset (Figure 4.26 A) were compared with MMVs observed in CEMOVIS dataset (Figure 4.26 B). Similarly, SMVs with densities present on the cytoplasmic side were compared in cryo-FIB dataset (Figure 4.26 C) and with SMVs observed in CEMOVIS dataset (Figure 4.26 D).

The compression of the membranes of the organelles, knife cutting artefacts and ice contamination are visible in the ultrathin sections (Figure 4.26 B and D) from the CEMOVIS derived tomograms, however the overall integrity of cellular information is maintained. Some of the FIB-milled tomograms also had artifacts of Gallium ion deposition on the surface of the lamella and curtaining that resulted in poor reconstruction of the tomogram (not shown) (Lucas and Grigorieff, 2023). The visualisation and categorisation of all the ROs was easier in the FIB-milled dataset

compared to the CEMOVIS dataset, due to the presence of membrane compression in the latter. Overall, both the methods established yield intact structural information of the organelles induced by HCV SGR in cells harbouring SGR JFH-1 NS5A-eGFP.

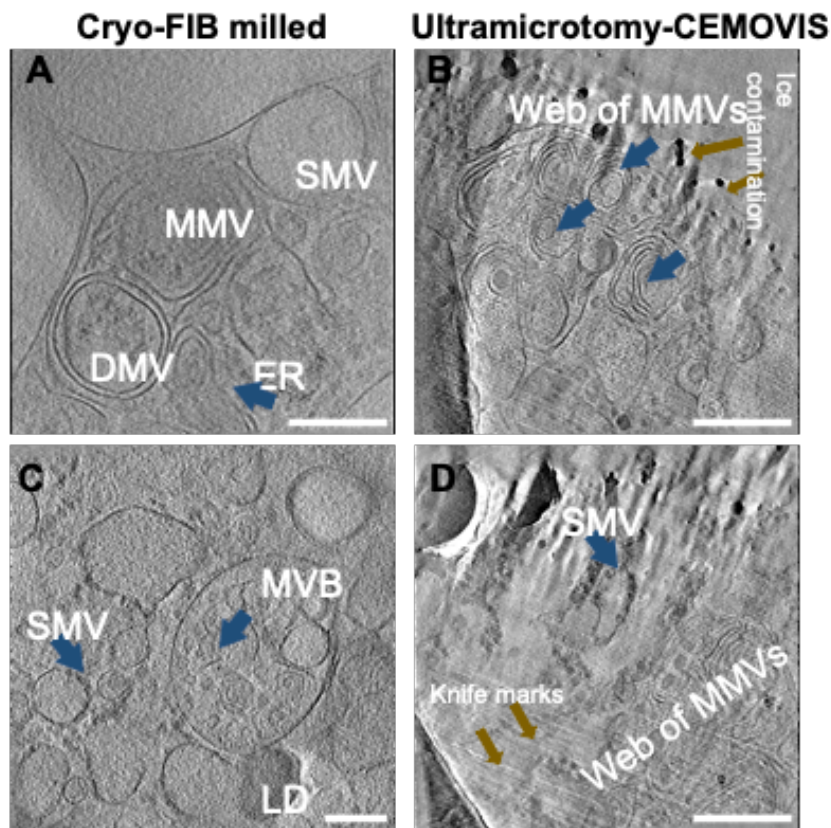


Figure 4.26: Comparison of *in situ* cellular architecture observed in cells harbouring SGR JFH-1 NS5A-eGFP from FIB-milled datasets and CEMOVIS.

Comparison of *in situ* cellular architecture observed in cells harbouring SGR JFH-1 NS5A-eGFP from FIB-milled datasets and CEMOVIS. [A] A representative tomographic slice of MW, including DMV, SMV, MMV, ER, derived from cryo-FIB-milled datasets [B] A representative tomographic slice of MW, mostly containing MMV, derived from CEMOVIS dataset [C] A representative tomographic slice illustrating MVB, LD, SMV harbouring densities facing the cytosol, derived from FIB-milled datasets. [D] A representative tomographic slice illustrating MW, and SMV harbouring densities facing the cytosol, derived from CEMOVIS datasets. Blue arrows indicate organelles, brown arrows indicate the artefacts. Scale bars: 200 nm

4.5 Discussion

4.5.1 *In-situ* workflow development and challenges

In this chapter, the primary objective was to develop a cryo-CLET workflow and explore the localisation of the HCV replication complex within the replication organelles. A comprehensive workflow, encompassing sample preparation to data analysis, was devised, implementing the latest cryo-ET techniques. Two distinct sample preparation methods were employed for an *in-situ* examination of the ultrastructure of HCV replication organelles in cells harbouring an SGR (HCV genotype 2a).

To prepare samples using the FIB-milling approach, the necessary steps for obtaining well-frozen cells with vitreous ice on an Au EM grid were established. Au grids equipped with holey carbon layers of a thickness of 49 nm (Quantifoil) and 17 nm (C-flat) were identified as suitable for cell culturing (Cho et al., 2013). The methodology was optimised using support films with a 200-mesh design to enable high-tilt tomography (Thompson et al., 2016). A hole pattern featuring 1.2 μm holes and 1.3 μm hole spacing was employed, although these parameters are more crucial when acquiring data near the cell periphery for cryo-ET and are less relevant for imaging cell lamellae. Generally, a higher incidence of broken grid squares was observed with C-flat grids compared to Quantifoil, possibly attributed to the weight of multiple cells on individual grid squares. This difference may be influenced by the thickness of the carbon support in Quantifoil grids, providing enhanced stability.

A systematic approach to preparing a series of grids (Quantifoil and C-flat), using two seeding density: 5×10^3 and 10×10^3 cells, yielded a final success rate of 50% of grids containing an optimal number of vitrified cells. On average, losses were incurred at each stage, including cell adherence, plunge freezing, and clipping, resulting in 75% of grids reaching the screening stage. Typically, cells exhibited a tendency to adhere to the grid bars rather than attaching at the centre of the grid square, leading to a reduction in the number of usable grids. The combination of grid functionalisation with fibronectin and subsequent seeding of a homogenous population of cells harbouring SGR JFH-1 NS5A-eGFP improved distribution and adherence. However, further improvement could be achieved through the micropatterning of grids (Sibert et al., 2021). With the availability of an in-house FIB-SEM, micropatterning will become a standard practice for culturing cells on grids, enabling the seeding of a single cell at the center of each grid square. This approach is expected to consistently improve the success rate of optimal grids.

The access of FIB-SEM instruments was a major bottleneck in the workflow, as they were located at eBIC (Diamond Light Source) and time on these instruments was limited to 48 hours per session. The logistics of shipping the grids in dry shipper introduced some

ice contamination before milling. Datasets generated in this study were derived from 200 nm and 160 nm thick lamellae. However, thinner lamellae would have resulted in better contrast providing better information on the arrangement of proteins or dsRNA within the ROs. Lamellae are fragile in nature and the choice of lamella thickness to keep it above 160 nm was considered due to the logistics of transportation. One of the key artefacts observed in the dataset from milling was curtaining. Curtaining manifests as ridges and grooves on the surface of the lamella parallel to the milling axis (Lam and Villa, 2021). Tilt-series acquired near curtained area resulted in poor tomograms, hindering all the information equivalent of a grid bar obstruction.

The biggest limitation was the inability to utilise the eGFP foci to guide tilt series collection in the FIB-SEM workflow. Due to the difference in the geometry of the stages between FIB-SEM, cryo-FM, and cryo-TEM, the MAPS software to correlate EM-FM images was deemed unsuitable. The integrated multi-modality where FIB-SEM is equipped with in column FM would be the next steps in this investigation (Li et al., 2023).

To determine any variations in cellular architecture when tilt-series are acquired by guided eGFP fluorescence, CEMOVIS workflow was employed. The facility's sustained access to ultramicrotomy facilitated efficient optimisations. The utilisation of MAPS software (Thermo Fischer) allowed straightforward correlation of cryo-FM and cryo-EM images, guiding tilt-series collections based on NS5A-eGFP. Common artefacts such as compression and knife marks were observed (Chlanda and Sachse, 2014); however, the overall information was retained and informative. The guided tilt-series provided crucial insights, revealing an abundance of MMVs vesicles around the ER with densities within. However, only one MMV exhibited organised arrangements within the inner leaflet, suggesting a potential assembly of the replicase machinery. This highlights the importance of fluorescence guided cryo-ET. Despite guided collection, identifying the assembly of the replicase machinery remains challenging. Technically, locating the replicase machinery with just one fluorescence marker is possible. However, a substantial number of tilt-series would be needed to deduce the organelle with regular arrangements of the machinery. This is due to the versatile characteristics of NS5A, which is distributed throughout the cytoplasm. Live FM demonstrated the dynamic behaviour of NS5A as static and mobile foci, indicating the role of NS5A in interactions with several cellular factors important for both replication and its role as part of the replication machinery (Eyre et al., 2014; Fiches et al., 2016).

The processing pipeline to solve the structure of the replication complex *in-situ* from this dataset needs to be established, however may be extremely difficult. Tomograms

generated in cryo-ET suffers from 'missing wedge' and signal to noise ratio (SNR) issues, which make particle picking for subtomogram averaging (STA) *in-situ* very challenging (Kim et al., 2023). For STA, multiple copies of identifiable particle of interest must be present within the tomograms to allow manually picking coordinates of each particle accurately (Pyle et al., 2022). The dataset gathered in this study suffers from imprecise alignment as required for particle picking due to the absence of fiducial markers. Even though tomograms generated by Aretomo software (Zheng et al., 2022) provide sufficient accuracy to align membranes to visualise the organelles, the alignment of the proteins on the membrane may have loss of information. However, the location of lipid bilayers can assist in finding the particles. The high SNR of membranes helps with the identification of regions with membrane associated particles. Additionally, these membrane-associated particles typically adopt consistent orientation relative to the membrane helping with the speed and accuracy of the alignment process (Pyle et al., 2022). *De Novo* structural pattern mining (Xu et al., 2019) and convolutional neural network (CNN)-guided picking (de Teresa-Trueba et al., 2023; Rice et al., 2023) can be explored for STA on these datasets.

4.5.2 Correlating densities in replication organelles: insights drawn from previous literature

The induction of host endomembrane rearrangements necessary for virus replication is facilitated by a set of viral proteins. In the case of HCV, a SGR consisting of non-structural proteins NS3-5B has been demonstrated to effectively induce host membrane rearrangements, exhibiting no significant difference from those induced by HCV infection (Romero-Brey et al., 2012).

Between 2002 and 2019, several research groups investigated the membranous web in the context of HCV infection or SGR, utilising a combination of electron and light microscopy approaches (Romero-Brey and Lohmann, 2016). However, their method of cellular preservation employed chemical fixatives with resin-embedded sections (Table 1) which essentially limited direct visualisation of any contents like dsRNA or the arrangement of proteins forming the replication complex within these organelles. Despite DMVs being implicated as the most likely organelle to host the replication machinery (Wolff et al., 2020a), the role of SMVs and MMVs were still under question.

For the first time, the possible location of the replication complex and dsRNA intermediates was observed directly within the ROs, providing insights into the functional role of these organelles induced in cells harbouring SGR. A comprehensive qualitative and quantitative analysis was conducted.

Firstly, DMVs were proposed as main replication compartment used by HCV (Paul et al., 2014b), prompting a detailed examination of all DMVs in the cryo-FIB milled dataset. Three distinct types of DMVs were identified. (1) DMVs exhibiting clustering of densities indicating a possible formation of the replicase assembly. (2) DMVs with thread like structures were observed, could only be speculated to be intermediate dsRNA filaments. (3) DMVs devoid of any visible structures. One hypothesis regarding the biogenesis of DMVs proposed that HCV proteins induce tubulation of ER membranes, which then undergoes secondary invagination, leading to the formation of DMVs. These DMV may be open and connected to the ER via stalk or released as free closed DMV (Romero-Brey et al., 2015). Closed DMVs were predominantly present in the FIB-milled dataset and their proximity to the ER was limited.

DMVs were noted in proximity to LDs (J.Y. Lee et al., 2019), however, in this study SMVs were observed predominantly around LDs. Additionally, SMVs were also the only vesicles that appeared to be budding from ER. The observation of a higher number of SMVs around LDs and the ER is consistent with EM analyses conducted on HCV-infected liver biopsy samples (Blanchard and Roingeard, 2018). This finding is also consistent with the hypothesis that HCV proteins induce ER tubulation, leading to the formation of free SMVs in the case of SGR JFH-1 (Romero-Brey et al., 2012). Collectively, these findings support the notion that DMVs might be generated in a cell culture environment allowing JFH-1 replication, as the infection of HCV in chronic liver biopsies predominantly revealed SMVs near LDs and the ER (Blanchard and Roingeard, 2018).

A distinction in vesicle contents was noted between SMVs located around DMVs and MVBs and those around ER and LDs. SMVs in proximity to DMV and MVB (including within MVB) generally exhibited internal densities. In contrast, SMVs surrounding ER and LDs displayed densities both internally and, on the surface, exposed to the cytoplasm. A previous immunofluorescence study identified that membranes for replication organelles in HCV-infected cells originate from the ER but also colocalise with markers for early and late endosomes, coat protein complex (COP) vesicles, mitochondria, and LDs (Romero-Brey et al., 2012) and lysosomes (Matsui et al., 2021). The measured diameter of SMVs fell within the range of 50-300 nm. This size range aligns with that of endosomes (100-500 nm) and lysosomes (200-300 nm), although lysosomes may increase in size depending on the vesicle they fuse with. If these SMVs were derived from early endosomes, the internal densities could be attributed to NS3, NS5A, with surface exposed densities marked by GFP-Rab21 ((Romero-Brey et al., 2012). Alternatively, if derived from lysosomes, the internal densities could be NS5A and LAMP-2A (Matsui et al., 2021).

InVs, ranging from 20-160 nm, were observed in both SMVs and DMVs. These small vesicles were previously termed self-invaginations of SMVs (Romero-Brey et al., 2012) or vesicles in cluster (ViCs) (Ferraris et al., 2013). However, these vesicles might also be exosomes containing either dsRNA or non-structural proteins that fuse with larger SMVs (potentially an endosome in the process of becoming MVB) or DMVs, serving as potential replication sites (Ramakrishnaiah et al., 2013; Bukong et al., 2014; Yin et al., 2022).

NS5A-foci-driven acquisition from samples prepared by CEMOVIS predominantly revealed the presence of MMVs, LDs, and ER, indicating their involvement as a replication organelle. In contrast, the FIB-SEM-derived dataset captured fewer MMVs. Notably, all MMVs observed in the CEMOVIS dataset exhibited densities within them. Particularly noteworthy was a specific MMV in the CEMOVIS dataset, displaying regular arrangements on its innermost membrane, suggestive of the assembly of the replicase machinery. In contrast, other MMVs appeared to have scattered densities within. This suggests the possibility that a fraction of DMVs, MMVs, and SMVs may be involved in the assembly of the replicase machinery. Another proportion of DMVs, MMVs, SMVs might be used to conceal the dsRNA intermediates from the host immune system. Lastly, a fraction of these RO may serve as a decoy mechanism for evading the host immune system. As HCV is classified as a category 3 pathogen in UK, this study utilised Huh7 cells stably harbouring SGR JFH-1. This led to a significant drawback in using the methods to compare the membrane rearrangements in the context of an infection in a spatio-temporal manner.

4.5.3 The role of MVBs in releasing replication organelles alongside exosomes and HCV viral lipoproteins.

A significant finding in this investigation is the aggregation of multi-vesicular bodies (MVBs) or lysosomes, along with the cooccurrence of vesicles like SMVs, DMVs, and MMVs within and around them. MVBs were primarily located in proximity to SMVs, DMVs, and LDs. The measured sizes of MVBs in the tomograms ranged from 400-800 nm. However, a considerable number of MVBs were too large to be fully captured within the tomogram area, and their dimensions, measured in the 'search maps,' revealed sizes ranging from 1000-1200 nm across. These MVBs were previously described as a large single membrane containing several circular units. The units were observed as concentric membranes surrounding a dense core (Ferraris et al., 2010). The presence of densities inside SMV and DMVs in the dataset correlates with NS5A-mcherry observed inside MVB (Grünvogel et al., 2018) as well as the dense lumen within

concentric units (Ferraris et al., 2010). In rare instances, the tomograms captured the invagination of a SMV into a MVB, and another tomogram depicted a vesicle in the process of being released from the MVB. The accumulated MVBs with intact ROs observed around the ER may potentially serve to transport ROs containing either the replicase machinery or double-stranded RNA (dsRNA) to another cell.

Moreover, the smaller SMVs within the range of 30-150 nm could be exosomes, as their size aligns with typical exosome dimensions. These smaller SMVs were observed within MVBs and match the size range of InVs observed within certain SMVs and DMVs. Studies have shown that exosomes derived from cells infected with HCV contain complete viral genome, particles, and viral proteins (Yin et al., 2022). Exosomes were noticed to transfer RNA to uninfected cells, even in presence of neutralizing antibodies. It has been demonstrated that exosomes are capable of transferring HCV RNA to uninfected Huh7 cells in HCV SGR that lack structural proteins (Ramakrishnaiah et al., 2013). Again, this indicates that MVBs can play a role in transferring HCV RNA to another cell.

Based on the differences in the content of MVBs observed in the dataset, some appeared to have intact putative RO within them, while others appeared to be contained degraded membranes which could be designated as lysosomes. The types of organelles and the content observed within MVB in this study supports the growing evidence HCV that viral particles might be released by a non-canonical endosomal pathway rather than the canonical secretory route. A multitude of Rab proteins direct the particles towards late endosomes (Rab7a-dependent), where they are either sorted for release by fusing to MVBs with the plasma membrane (Rab 9a and 7a dependent) alongside the release of exosomes (Rab 3d- and Rab 27a -dependent) or for auto lysosomal degradation (Elgner et al., 2018). Lysosomes vary in size and increase their size after fusion events with other components like endosomes and autophagosomes (de Araujo et al., 2020), which explains the differences in size of the MVBs. It would be interesting to study cell culture media to detect the presence of MVB markers, HCV non-structural proteins and dsRNA. A positive detection of all of these in the cell culture supernatant will further highlight the endosomal route of egress as a mode of action by HCV to evade the immune system.

4.5.4 Hypothetical models of replication complex models

Four hypothetical models of replication complex assembly were proposed earlier (Romero-Brey et al., 2012). Model A indicated that HCV proteins induce extensive invagination, causing local ER lumen shrinking, where replicase proteins remain active until the vesicle connects to the cytosol; however, this phenomenon was not observed in

our dataset. Model B suggested HCV proteins induce ER membrane tubulation, leading to secondary invagination and DMV formation. Evidence supporting this model was observed in this study, with ER tubulation resulting in closed SMVs. Model C proposed a similar pathway to B, but the proteins reside on the cytosolic surface. In this study, densities were exclusively within DMVs but within and on the surface of SMVs. Model D suggested SMVs or DMVs as the primary replication site, with the other as an epiphenomenon; however, observed clustered densities in a fraction of SMVs, DMVs, and MMVs suggested the formation of a replication complex, making Model D less relevant. Consistent with previous hypothesis, it is likely that HCV induces various vesicles to evade the innate immunity system (Romero-Brey and Lohmann, 2016; Neufeldt et al., 2016). This explains why both immunogold labelling and cryo-ET detected proteins in some but not all vesicles. Immunogold labelling detected NS5A in some LDs and 50-70nm SMVs (Romero-Brey et al., 2012), consistent with the results of densities within some SMVs and around LDs in this study. A revised hypothetical model based on this study is illustrated in Figure 4.27.

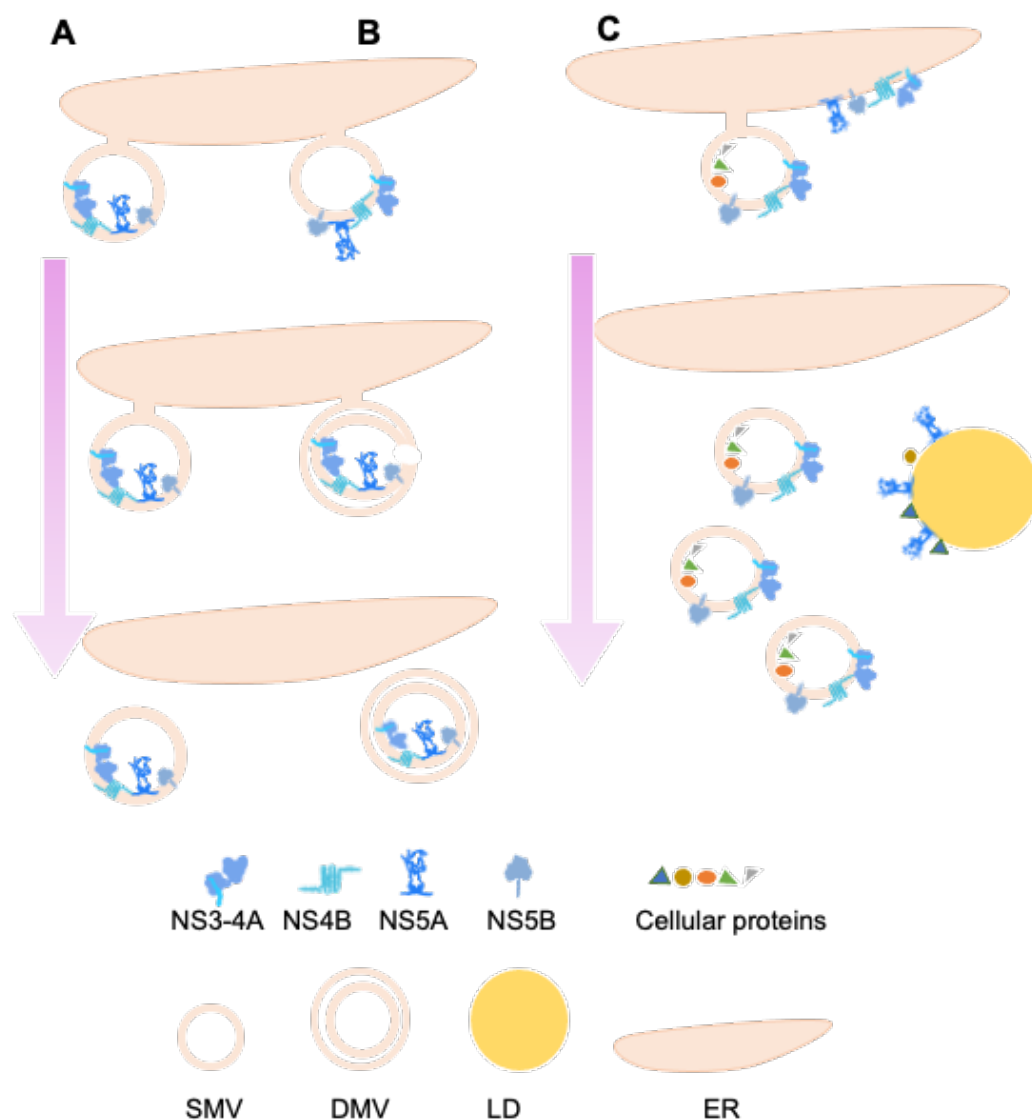


Figure 4.27: Revised hypothetical model of formation of ROs harbouring replication complex.

[A] Model A: SMV budding from ER harbouring the replicase machinery inside. [B] Model B: ER tubulation leading to secondary invagination and formation of DMV with replicase machinery inside. [C] SMVs budding from ER harbouring the replicase machinery on the surface with host proteins residing inside.

The overall cryo-ET methodologies established in this chapter elucidates the architecture of all the compartments (previously observed in the literature) induced in Huh7 cells expressing SGR in their native environment. The future work should focus on integrating the correlative techniques to identify the specific compartments harbouring the replication complex and the densities within them. The workflow established in this study lays the groundwork and paves a way to deduce the architecture of ROs in other genotypes of HCV SGR.

Chapter 5| Development of fluorescence microscopy tools to study the assembly of the HCV replicase

5.1 Introduction

5.1.1 Labelling methods compatible for correlative light and electron microscopy

To understand where viral proteins are situated within their host cells over time, it is crucial to mark these proteins with fusion tags that can be utilised in both fluorescence microscopy (FM) and electron microscopy (EM) (Sosinsky et al., 2007). There are various options for tagging proteins for visualisation with FM. The most widely employed method involves the utilisation of intrinsically fluorescent proteins (FPs) that share a similar sequence and structure with the green fluorescent protein (GFP) (Chalfie et al., 1994). GFP is approximately 27 kDa in size and is known to minimally disrupt the fusion protein when attached to the N or C terminus, folds well within biological temperature ranges, matures quickly, and can remain fluorescent in many sub-cellular compartments (Crivat and Taraska, 2012).

Self-labelling enzyme tags such as the Halo (Los et al., 2008), SNAP (Dreyer et al., 2023), and CLIP tags (Wilhelm et al., 2021) are fused to the protein of interest. These enzyme tags are detected by introducing a cell permeable substrate molecule, often a benzyl guanine derivative, linked to a fluorophore of choice. Upon covalent attachment of the fluorescent ligand to the enzyme, the labelled proteins can be visualised with the appropriate wavelength of light (Wilhelm et al., 2021). However, these are as large as FPs in size.

The tetracysteine biarsenical system FIAsh (Griffin et al., 1998) and ReAsH (Adams et al., 2002) are smaller peptide tags genetically encoded for covalent fluorophore labelling, where the short peptide sequence, Cys-Cys-Xaa-Xaa-Cys-Cys (where X is any amino acid other than Cys), is fused with the target protein and then detected by a covalent reaction with a membrane permeable biarsenical dye. However, the toxicity of these peptides and labelling complexity provides limitations in using these systems (Pomorski and Krężel, 2020).

The majority of EM tags suitable for correlative light and electron microscopy (CLEM), such as APEX (Martell et al., 2012; Lam et al., 2015), MiniSOG (Shu et al., 2011), tetracysteine tags (Gaietta et al., 2002), and FLIPPER (Kuipers et al., 2015) rely on the process of oxidizing diaminobenzidine (DAB) to produce a polymer which is then stained with osmium tetroxide to generate contrast in EM images. However, these methods are only suitable when the cells are fixed for FM and then processed for EM (Beatty and López, 2023). Furthermore, CLEM compatible reporters such as quantum dots labelled with antibodies (Killingsworth and Bobryshev, 2016) and nanogold particles (Miles et al.,

2017) have also been used for CLEM imaging. The goal of this chapter was to identify an appropriate labelling system which could be incorporated into the cryo-CLEM methodology established in the previous chapter. However, labelling a viral protein with a fluorescent tag for cryo-CLEM methodology posed two challenges. Firstly, the insertion of a tag with the viral protein must be tolerated and allow replication of the viral genome in the target cells. Secondly, the fluorescence from the tag should be retained in live cell imaging and subsequently retained in cryogenically preserved conditions (Kaufmann et al., 2014).

The essential proteins involved in HCV replication are non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B (Tabata et al., 2020). At present, the only fluorescent marker used to examine the spatiotemporal location of the HCV replicase, is eGFP fused within the disordered third domain (D3) of NS5A (Moradpour et al., 2004). Live-cell imaging revealed two distinct NS5A-eGFP punctate structures: larger, static foci and smaller, motile puncta, illustrating the multifunctional role of NS5A. The static foci were proposed to be components of the replication complex and thus occupying fixed positions, whereas the motile foci were tracked for movement between putative replication and assembly sites (Wölk et al., 2008; Fiches et al., 2016). As illustrated in the previous chapter, it was validated that the fluorescence of eGFP (NS5A-eGFP), maintained its brightness, even in cryogenic environments. Hence, one of the objectives was to discover fusion tags different from eGFP, and suitable for other NS HCV proteins which could be used in conjunction with cryo-CLEM. This aimed to narrow down the identification of the replication complex assembly within the hypothesised ROs.

To achieve this, a class of tags for multiscale microscopy termed Versatile Interacting Peptide CoilE-CoilR (VIPER) was explored. These tags were shown to be compatible with both fixed and live FM, EM and CLEM (Doh et al., 2018, 2019, 2020; Morgan et al., 2019). The tag consists of a coiled-coil heterodimer, formed between the 5.2 kDa genetic tag CoilE, and a 7.5 kDa probe-labelled peptide, CoilR (e.g. conjugated with a fluorescent dye or a quantum dot). The modified MiniVIPER system was created by removing the fifth heptad of the previously described VIPER tag. The MiniVIPER has a MiniE tag with a molecular mass of 4.3 kDa and a MiniR probe which is 7.4 kDa. (Doh et al., 2020). The principle and the application of VIPER and MiniVIPER, along with the size of the fusion tag and the probe is shown in Figure 5.1.

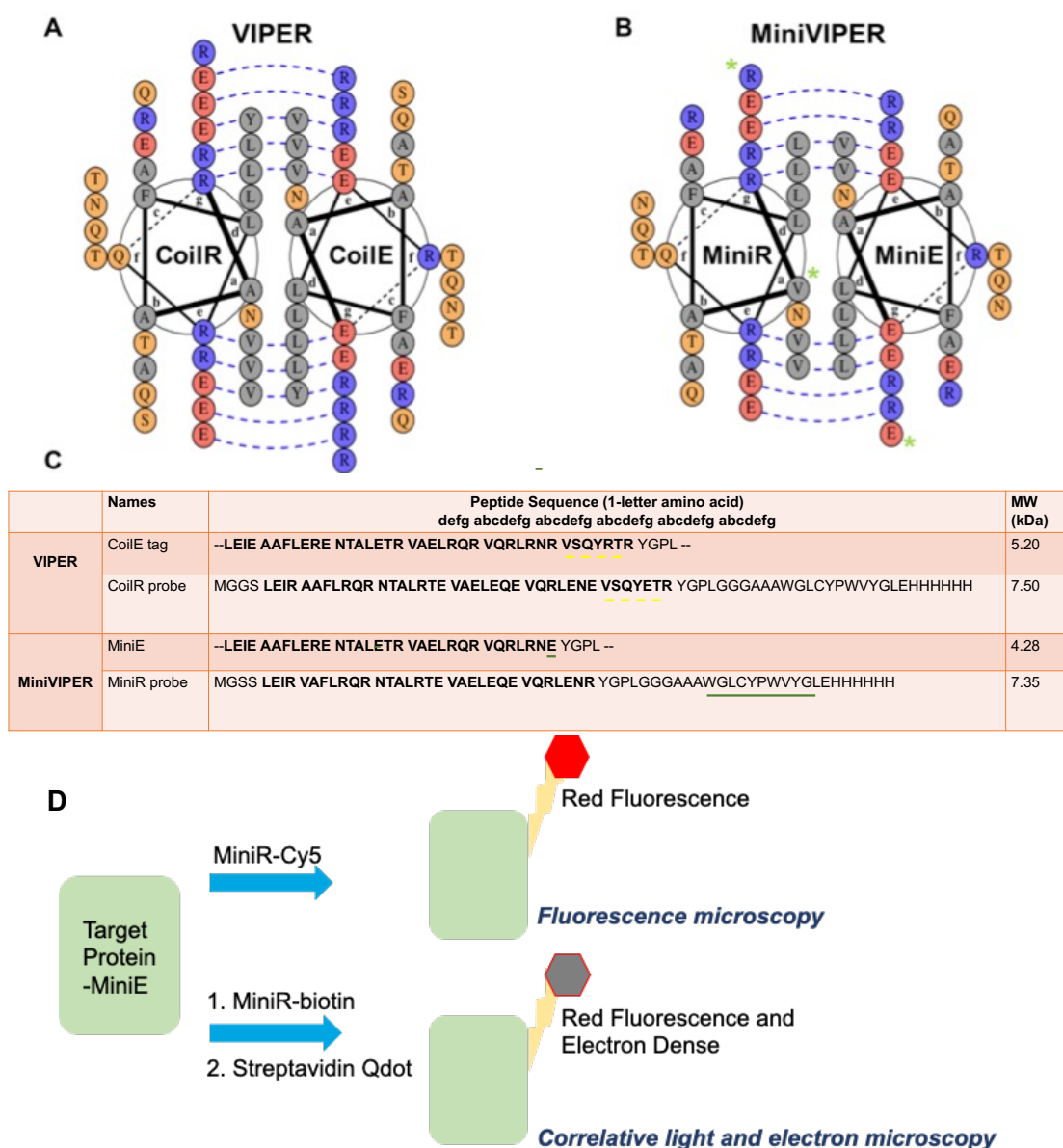


Figure 5.1: Overview of the VIPER and MiniVIPER system.

[A-B] Helical wheel diagrams of VIPER [A] and MiniVIPER [B], taken from (Doh et al., 2020). Charged residues are represented as red and blue, polar residues are orange, hydrophobic residues are grey, and salt bridges are highlighted with dashed lines. Altered residues in MiniVIPER are denoted with a light green asterisk (*). [C] Sequences of VIP tags and probes with highlighted amino acid residues in bold involved in the coil formation. The fifth heptad present in the VIPER tag and probe, which are deleted in the MiniVIPER tag and probe are denoted with yellow dashed lines. Any other changes made are highlighted with a green line. [D] A schematic illustration of the MiniVIPER system showing target detection by FM and CLEM. [C and D]: Adapted from (Doh et al., 2020).

There are a lack of genetic fusion tags for NS3 for direct FM investigations of the replicase complex *in-vivo* using the SGR polyprotein. Previously, the N-terminus of NS3

was His₆-tagged *for in-vitro* protein purification to investigate the functional interdependence of the NS3 protease and helicase domains, as well as the modulation of NS3 helicase by NS5B (Zhang et al., 2005). In literature, transposon mutagenesis has been utilised to investigate tolerable insertion sites into the HCV genome (Arumugaswami et al., 2008; Remenyi et al., 2014). In this study, Insertion sites that replicated with small 15-nucleotide insertions in NS3 in JFH-1 (Arumugaswami et al., 2008) were investigated for fusion strategy with MiniVIPER tags.

In this chapter, the objective was to create a dual-tagged system for the precise localisation of the RO containing the replicase machinery using FM. Once successful, the intention was to explore its potential integration into the cryo-CLEM workflow. Therefore, VIPER and the modified MiniVIPER systems were examined for their suitability for tagging another non-structural protein alongside NS5A-eGFP in the SGR JFH-1.

5.2 Results

5.2.1 Addition of the CoiE and MiniE genetic tags at the eGFP insertion site in NS5A DIII was compatible with genome replication.

To test the suitability of VIPER and MiniVIPER tags, the tolerance of the full-length genetic tag CoiE (7.35 kDa) and its smaller modified version MiniE (4.35 kDa) was examined by evaluating their replication fitness by a luciferase assay. The codon sequences for the CoiE and MiniE tags were optimised to avoid any restriction sites. Sequences were flanked by 5' and 3' BglII sites and synthesised in the pUC19- vector by Genewiz. These tags were integrated at the C-terminus of NS5A within the domain III region, downstream of the eGFP tag in the SGR NS5A-eGFP construct (Moradpour et al., 2004). This integration was achieved through a single-site BglII restriction digestion, resulting in the generation of SGR NS5A-eGFP-CoiE and SGR NS5A-eGFP-MiniE constructs. A schematic of the SGR NS5A eGFP-MiniE sequence is illustrated in Figure 5.2A

To verify the compatibility of VIPER and MiniVIPER tag insertions with genome replication, the replication of SGR NS5A-eGFP-CoiE and SGR NS5A-eGFP-MiniE was assessed through a Luciferase assay in Huh7.5 cells up to 72 hours post electroporation (h.p.e) and compared with other SGRs (Figure 5.2B). Despite a 10-fold lower replication rate at 72 hours compared to SGR NS5A-eGFP, stable cell lines were successfully established for both SGR NS5A-eGFP-MiniE and CoiE using geneticin sulphate (G418)

selection. SGR NS5A-eGFP-MiniE was chosen for future applications based on its smaller size compared to the CoilE tag (Figure 5.1C)

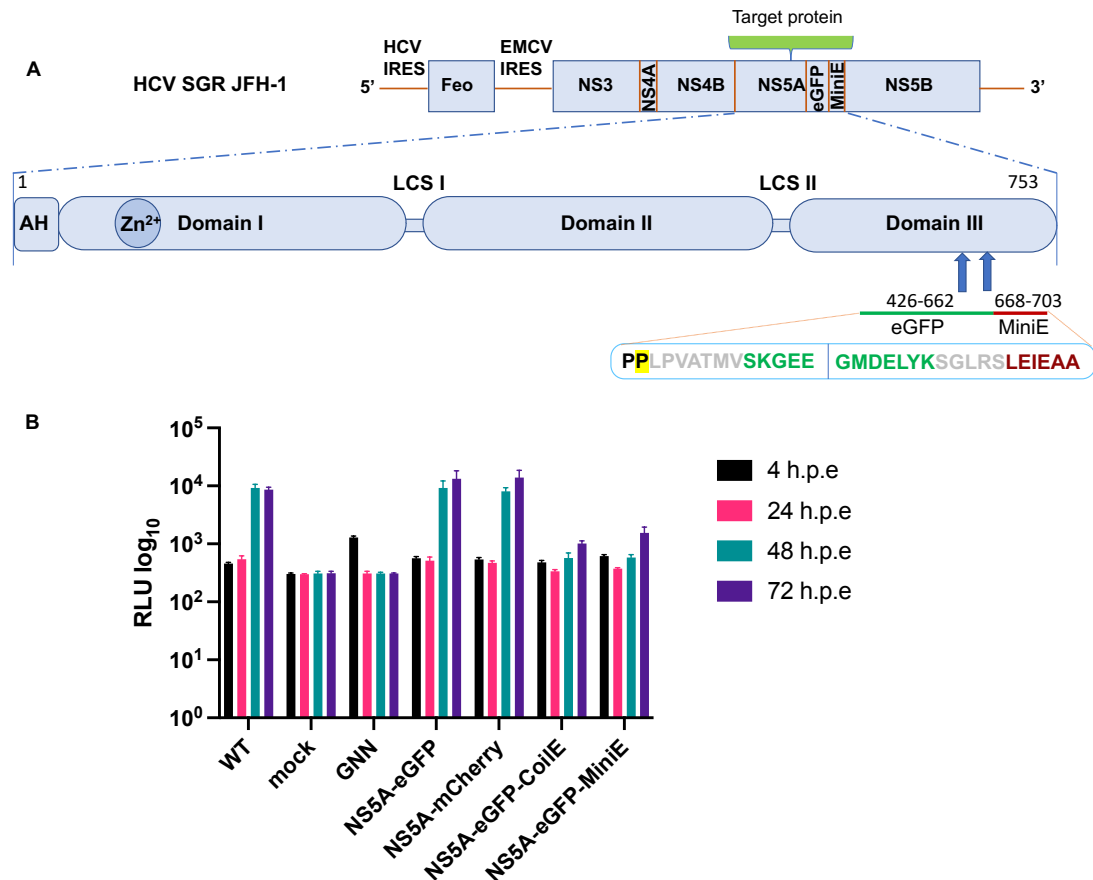


Figure 5.2: Assessment of replication: VIPER and MiniVIPER tags.

[A] Schematic of HCV SGR JFH-1 with eGFP and MiniE fused to NS5A. The amino acid sequence (1- 753) is numbered including the eGFP and MiniE sequence. Regions in domain III where eGFP and MiniE are incorporated at P418 is highlighted in yellow, eGFP sequence is represented in green, MiniE in red and any linkers in gray. [B] Bar graph showing the replication of CoilE and MiniE tagged SGR constructs (WT, NS5A-eGFP, NS5A-mCherry, and polymerase deficient (GNN, negative control)) measured using a Luciferase assay up to 72 h.p.e from transient electroporation of 2 μ g of SGR RNA into Huh7.5 cells. Relative luciferase units (RLU) shown. Data represents the mean $\pm$ SD, n=1 (with three technical repeats).

5.2.2 Distinct NS5A cytoplasmic foci: comparative analysis between SGR NS5A-eGFP and SGR NS5A-eGFP-MiniE

The subcellular localisation of NS3 in hepatocytes during chronic HCV infection was predominantly observed in the cytoplasm, with a minority showing localisation in both the cytoplasm and nucleus (Errington et al., 1999). The subcellular localisation of NS5A was observed throughout the cytoplasm as two distinct puncta - static/big and small/mobile (Fiches et al., 2016). To investigate the cytoplasmic localisation of NS5A and NS3 in SGR NS5A-eGFP-MiniE compared to SGR NS5A-eGFP, the area of NS5A and NS3 foci were examined through antibody labelling and immunofluorescence detection.

NS3 distribution was assessed using anti-NS3 antibody staining in both stable cell lines and in cells post electroporation at 72 hours. The distribution of NS3 differed between SGR NS5A-eGFP (Figure 5.3 A) and SGR NS5A-eGFP-MiniE (Figure 5.3 B and C). In SGR NS5A-eGFP expressing cells, NS3 exhibited a punctate patterning throughout the cytoplasm. However, in cells harbouring SGR NS5A-eGFP-MiniE, both stable and transiently electroporated cells displayed a diffuse NS3 distribution. Interestingly, not all cells expressing NS5A-eGFP-MiniE exhibited a NS3 signal (Figure 5.3 B). This observation suggests that the flexible nature of the helical MiniE tag, fused with NS5A, may hinder antibody access to the NS3 epitope, resulting in an indistinct NS3 distribution phenotype.

Next, any differences in NS3 and NS5A foci in SGR harbouring NS5A-eGFP and NS5A-eGFP-MiniE were evaluated (Figure 5.4). Colocalisation of NS3 and NS5A foci with Pearson's coefficient(R) was evaluated using the Colocalisation Finder (plugin in Fiji), and is as illustrated in Figure 5.4. To test any significant differences in NS3 and NS5A foci, an unpaired t-test (parametric, Welch's test) was conducted. Results suggested no significant differences in the mean area of NS3 foci ($p = 0.1827$) between the two SGRs. In contrast, a significant difference was observed in the mean area of NS5A foci ($p = 0.0003$). The frequency distribution of NS3 and NS5A foci, descriptive statistics, the lognormal QQ plot, the lognormal distribution of the area of NS3 and NS5A, are shown in appendix (Figure 6, Table 8). Since, the number of NS5A area values in the SGR NS5A-eGFP-MiniE dataset were ~2.4 times more than SGR NS5A-eGFP, an unpaired t-test (non-parametric, Mann Whitney test) was performed. A significant difference in the median of the area of NS5A was observed ($p = 0.0006$).

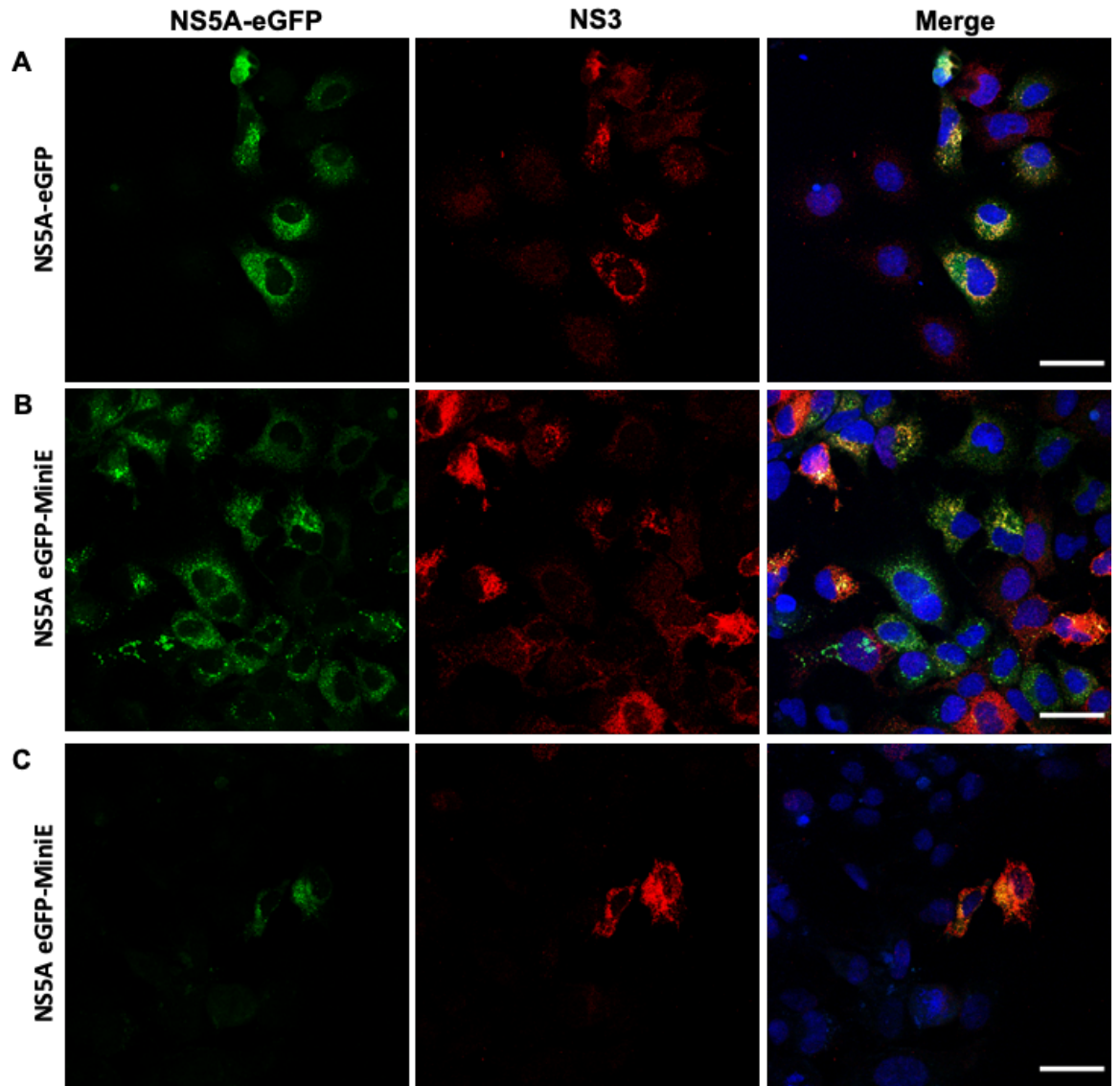


Figure 5.3: Variations in NS3 protein localisation in HCV SGRs.

Immunofluorescence images of NS3 antibody staining (red) in [A] Huh7 cells expressing SGR NS5A-eGFP [B] Huh7.5 cells harbouring SGR NS5A-eGFP-MiniE, and [C] Huh7.5 cells transiently replicating with SGR NS5A-eGFP-MiniE at 72 h.p.e. Nuclei of cells stained with DAPI (blue). Scale bar = 40 μ m. Imaged on LSM880 confocal microscope with 40X objective (oil immersion) with N.A of 1.4.

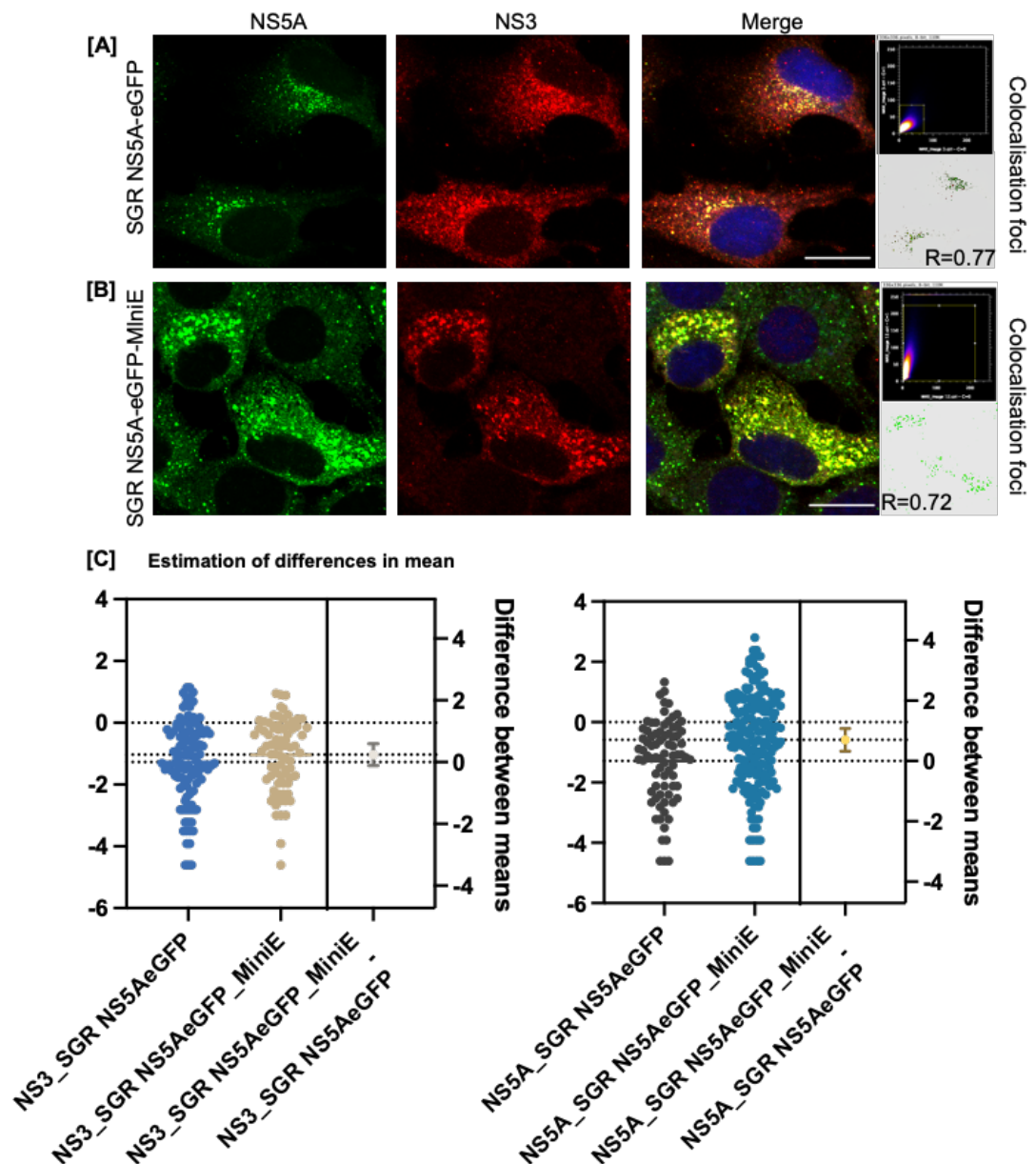


Figure 5.4: Comparison of NS3 and NS5A foci between SGR NS5A-eGFP and SGR NS5A-eGFP-MiniE.

[A] Huh7 cells expressing SGR NS5A-eGFP [B] Huh7.5 cells expressing SGR NS5A-eGFP-MiniE. Colocalisation of NS3 and NS5A was determined by Pearson's correlation using Colocalisation Finder in ImageJ. The Pearson's R value is indicated on the colocalised foci selected on the pixel scatterplot. Nuclei of cells stained with DAPI (blue). [C] Unpaired t-test (Welch's test, GraphPad), calculated differences in the mean of NS5A foci from the lognormal distribution. Area of NS5A and NS3 were determined from n=2 (3 ROI 250 x 250 pixels). Imaged on LSM880 confocal microscope with 40X objective (oil immersion) with N.A of 1.4.

5.2.3 Generation of purified, fluorescently labelled miniR probe

The subcellular localisation of the MIniVIPER tag is detected by the MiniE tag forming a heterodimer with the MiniR probe, thus allowing the target protein to be visualised (Figure 5.1 B). To examine the subcellular localisation of NS5A-eGFP-MiniE and validate colocalisation of the MiniE-MiniR signal with the eGFP signal, it was necessary to prepare the MiniR probe.

The MiniR protein (7.35 kDa) was expressed and purified as per published protocol (Doh et al., 2020). MiniR protein was expressed using bacterial system and purified using Ni-NTA chromatography via the C-terminal His6-tag (methods). Eluted fractions from the Ni-NTA affinity purification were analysed on a 12% Bis-tris SDS-PAGE gel. On a denatured SDS-PAGE gel, MiniR was observed as a monomer ~ 6 kDa and a dimer at ~14 kDa, as previously reported (Doh et al., 2020) and is shown in Figure 5.5. The elutions were concentrated using a 3 kDa molecular weight cut off (MWCO) concentrator, and then buffer exchanged into TBS-Urea. The final yield of MiniR protein was 31.5 mg at 0.5 mg/ml concentration.

The purified protein sample was also tested for degradation due to carbamylation (Sun et al., 2014) by native mass spectrophotometry to assess the quality of the protein. The sample was found to contain a heterogenous population, with the major peak corresponding to 7349 Da (appendix Figure 7). A common modification of 305 Da mass increase was also observed indicating seven locations for carbamylation, presuming there are no other post-translation modifications. Nonetheless, the protein was labelled with the Sulfo-Cyanine5 maleimide (Cy5) dye and used for downstream experimentation.

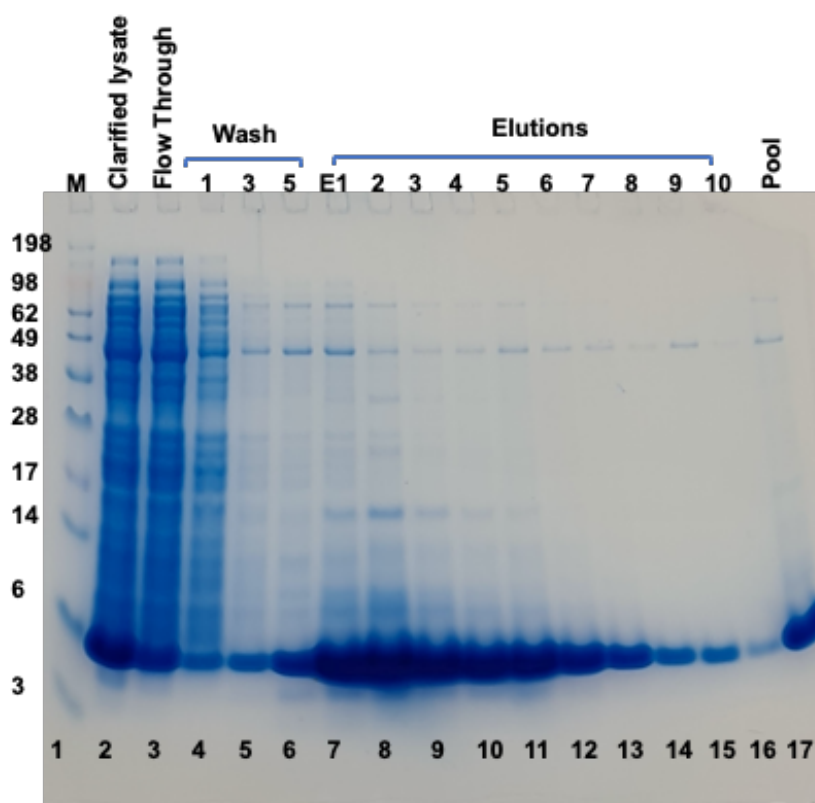


Figure 5.5: MiniR protein purification.

MiniR protein purification. Ni-NTA affinity purification steps of MiniR protein analysed on a reduced 12% Bis-tris SDS-PAGE. Protein was eluted in 5 ml elutions (E1-E6), and 15 ml elutions (E7-E10), and pooled (90 ml).

To generate the MiniR probe, purified MiniR protein was labelled with Cy5 using thiol-maleimide chemistry. Two methods for thiol-maleimide labelling of MiniR-Cy5 were evaluated to identify an efficient and fast way of labelling with reliable reproducibility. The first was previously documented in (Doh et al., 2020) , and the second was using an in-house protocol employed by the University of Leeds BioScreening Technology Group (BSTG) (Kindly provided by Christian Tiede, BSTG_SOP_006). The key differences of how the molecules are reduced using the two approaches are depicted in Figure 5.6. Both methods were assessed for efficiency and suitability in downstream applications, with differences noted in protein amounts and dye concentrations for conjugation.

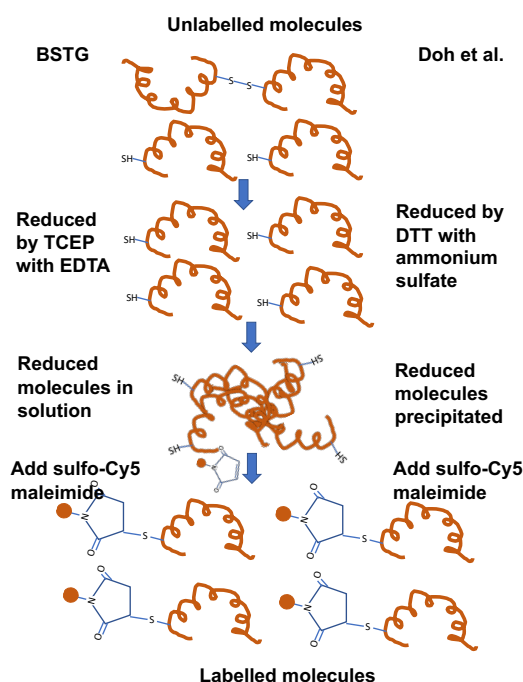


Figure 5.6: A schematic principle of thiol-maleimide labelling and the differences in the methodologies.

Image adapted from (Kim et al., 2008).

The BSTG method utilised 150 μ l of MiniR (0.5 mg/ml, 10 nmoles) and 6 μ l of 2 mM Cy5 (final concentration: 76.9 μ M). Due to MiniR's reduced stability at room temperature (RT), adaptations to the BSTG method were explored. MiniR molecules were reduced with TCEP for 1 hour at 4 $^{\circ}$ C, with reactions quenched at 2 and 22 hours. SDS-PAGE gel analysis revealed excess unlabelled fluorophore at 2 hours, compared to completely labelled protein at 22 hours, as observed on white lightbox (Figure 5.7 A). Subsequently, all proteins were visualised after Quick Coomassie staining (Figure 5.7 B).

The Doh et al. method used 1 ml of MiniR protein (0.7 mg/ml, 100 nmoles), which was reduced with 20 mM DTT at 4 $^{\circ}$ C for 30 minutes. The reduced peptide was precipitated with ammonium sulphate for 2 hours at 4 $^{\circ}$ C. After washing, the pellet was resuspended in 200 μ l labelling buffer with a 10-fold molar excess of Cy5 and TCEP and incubated at 4 $^{\circ}$ C for 12 hours. The labelled protein underwent pelleting, washing, and resuspension in TBS-urea buffer. SDS-PAGE gel analysis showed successful labelling, as observed on white lightbox (Figure 5.7 C). Subsequently, all proteins were visualised with Quick Coomassie stain (Figure 5.7 D).

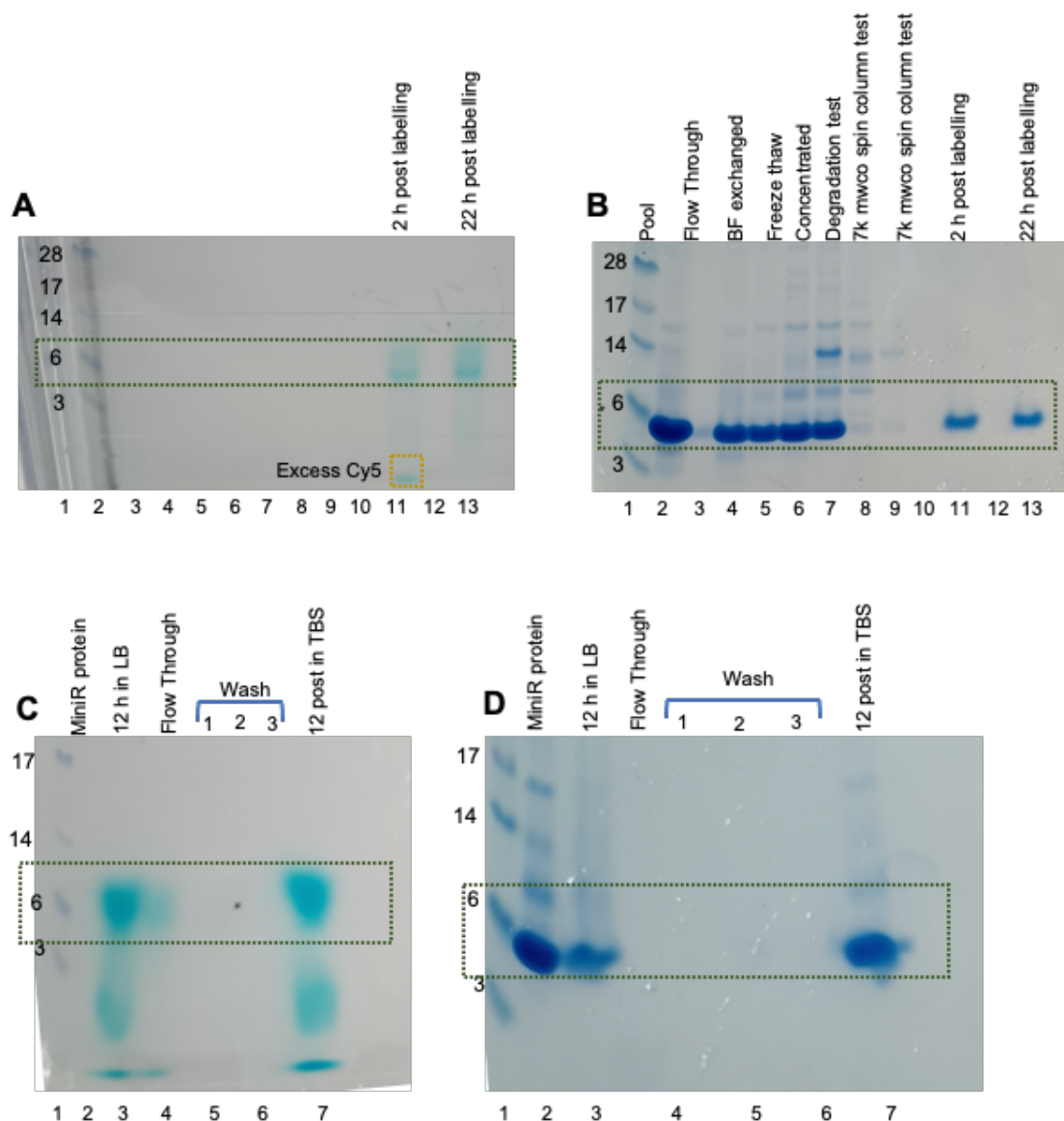


Figure 5.7: MiniR conjugation with Sulfo-Cy5 maleimide.

MiniR conjugation with Sulfo-Cy5 maleimide. [A] Visualisation of MiniR-Cy5 labelling on 12% Bis-tris SDS-PAGE after 2 and 22 hours using the BSTG method, displayed on a white light box. Conjugated proteins appear blue compared to unconjugated proteins. [B] Analysis of MiniR-Cy5 labelling on a 12% Bis-tris SDS-PAGE after 2 and 22 hours post-staining with Quick Coomassie (BSTG method). Lane 1: molecular weight ladder. Lane 2: Pooled protein (8 μ g), Lane 3: Flowthrough from concentrator after buffer exchange, Lane 4: Protein after buffer exchange into TBS-Urea, stored at -20° C (2 μ g), Lane 5: Freeze-thawed MiniR protein at 0.5mg/ml used for the labelling reaction (2 μ g, highlighted in the gel), Lane 6: 8 ml of freeze thawed protein solution concentrated to 1 ml using Mini concentrators (3 kDa MWCO, 2.5 μ g), Lane 7: Investigation of protein degradation overnight at 4° C (2.5 μ g), Lane 8-9: Testing a 7 kDa MWCO column to assess protein retention, with no protein detected in the elution,

Lane 11: MiniR-Cy5 labelled protein after 2 hours (5 µg), Lane 13: MiniR-Cy5 labelled protein after 22 hours (5 µg). [C] Visualisation of MiniR-Cy5 labelling on 12% Bis-tris SDS-PAGE after 12 hours in labelling buffer (LB) and 12 hours in TBS buffer using the Doh et al. method on a white light box. Conjugated proteins appear blue compared to unconjugated proteins. [D] Analysis of MiniR-Cy5 labelling on a 12% Bis-tris SDS-PAGE post-staining with Quick Coomassie using the Doh et al. method. Lane 1: molecular weight ladder. Lane 2: MiniR protein (2.8 µg), Lane 3: 100 nmoles of MiniR protein labelled by Cy5 for 12 hours in labelling buffer (2.8 µg)

The intended applications of the MiniR-Cy5 probe in this study were for confocal and live cell microscopy. To eliminate background fluorescence from unlabelled fluorophores in the sample mixture, the most effective method to purify the crude labelling mixture was investigated. Desalting spin columns with a 5 kDa MWCO proved better than dialysis cassettes with a 3.5 kDa MWCO, and Ni-NTA columns. Dialysis cassettes resulted in sample loss due to the small volume of labelled protein, while the Ni-NTA column retained the labelled protein without elution. The MWCO of the desalting columns was close to the molecular weight of MiniR, therefore the devised methodology was sensitive to the volume and concentration added to the spin column.

5.2.4 Subcellular localisation of SGR NS5A-eGFP MiniE using MiniR-Cy5

In order to investigate the subcellular localisation of SGR NS5A-eGFP-MiniE using the MiniR-Cy5 probe, three dilution ratios of MiniR-Cy5 (1:5, 1:10, and 1:50) were tested for staining cells expressing SGR NS5A-eGFP-MiniE. These dilution ratios were evaluated concurrently under different staining conditions at both RT and 4 °C. Notably, enhanced, and specific staining results were observed in cells stained at RT compared to overnight at 4 °C, particularly for the 1:10 and 1:50 concentrations (Figure 5.8). The colocalisation (overlap coefficient) of NS5A-eGFP-MiniE foci was found to be $R = 0.67$ (1:50) and $R = 0.72$ (1:10) shown in appendix Figure 8, 9.

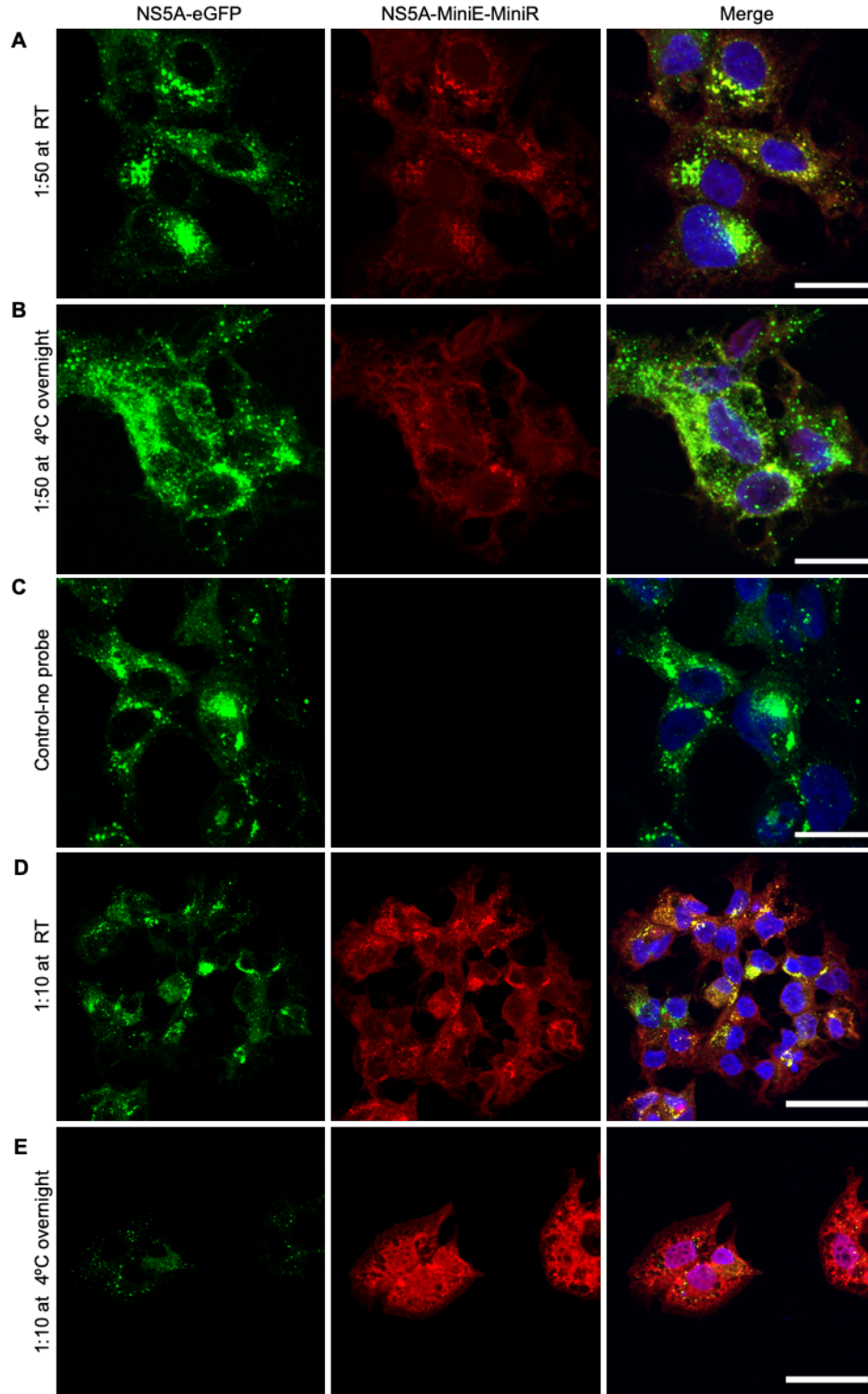


Figure 5.8: Optimisation of MiniR-Cy5 probe for MiniE detection in Huh7.5 cells expressing SGR NS5A-eGFP-MiniE.

[A-B] Immunofluorescent images evaluating MiniR-Cy5 staining (red) at a 1:50 dilution at RT (1 hour) [A], and overnight at 4 °C [B]. Scale bar = 20 µm. [C] Negative control with no probe added to cells to account for autofluorescence (1 hour), scale bar = 20 µm. [D-E]: Immunofluorescent images evaluating MiniR-Cy5 staining (red) at a 1:10 dilution at RT (1 hour) [D], and overnight at 4 °C [E]. Scale

bar = 40 μ m. NS5A-eGFP staining shown (green) and nuclei of cells stained with DAPI (blue). Imaged on LSM880 confocal microscope with 40X objective (oil immersion) with N.A of 1.4.

5.2.5 Design of a Dual SGR Construct: examining potential tolerable sites in NS3 for MiniE tag integration

To narrow the search of ROs harbouring the replicase machinery, generating a dual construct where two non-structural proteins are tagged with different fusion tags was explored using the MiniVIPER tag system. In order to identify putative tolerated insertion sites in NS3, a combination of literature mining, structural information of NS3 and structural prediction tools were employed.

The accessibility of the MiniE fusion tag in the cytoplasm is essential for its interaction with the corresponding MiniR probe, as outlined previously (Doh et al., 2020). Thus, the introduction of the MiniE tag into the NS3 protein is limited to either the N or C terminal ends of the proteins, or longer loops devoid of known binding partners. The sequence and structure of JFH-1 NS3 is depicted in Figure 5.9.

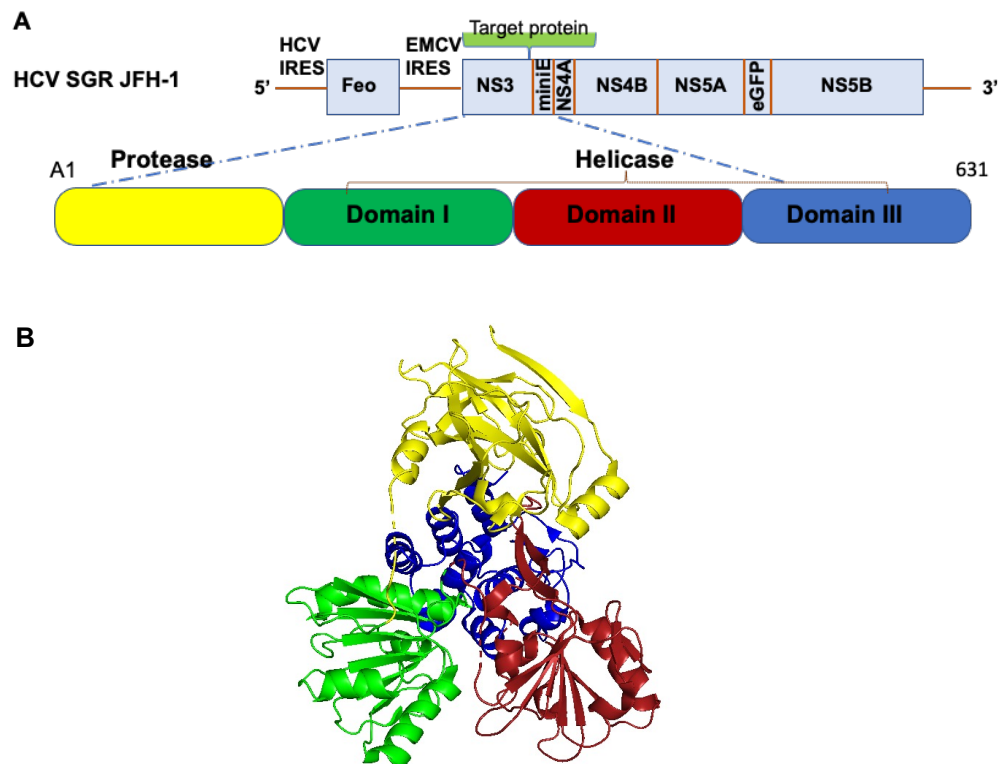


Figure 5.9: JFH-1 NS3 Sequence and structure.

[A] Domain schematic of the SGR NS5A-eGFP and MiniE fusion to NS3. The NS3 amino acid sequence (1-631) is annotated with alanine as amino acid 1 (A1). [B] Visualisation of NS3 crystal structure from PDB file 5DWX, with color-coded domains corresponding to the schematic in (A).

In the SGR NS5A-eGFP polyprotein construct, the initiation of the NS3 protein begins with a methionine (referred as M0 in this study). Previously, an N-terminal histidine tag was added to NS3 (Vishnuvardhan et al., 1997). The N-terminal insertion site M0, was chosen as the initial site for incorporating the MiniE tag. Subsequently, protease sites near the N-terminus of NS3, known to tolerate a 15-nucleotide sequence based on transposon mutagenesis (Figure 5.10 A) (Arumugaswami et al., 2008), were considered. However, the sequence ‘APITAY’ was identified as being conserved in 138 NS3 sequences stored in the PDB (Frick, 2007), and was therefore deliberately avoided as an insertion point. Following this, attention was directed to a loop region near the N-terminus. Since no binding partners were reported for this region in the literature, the second residue (D25) within the loop was explored as a potential novel insertion site.

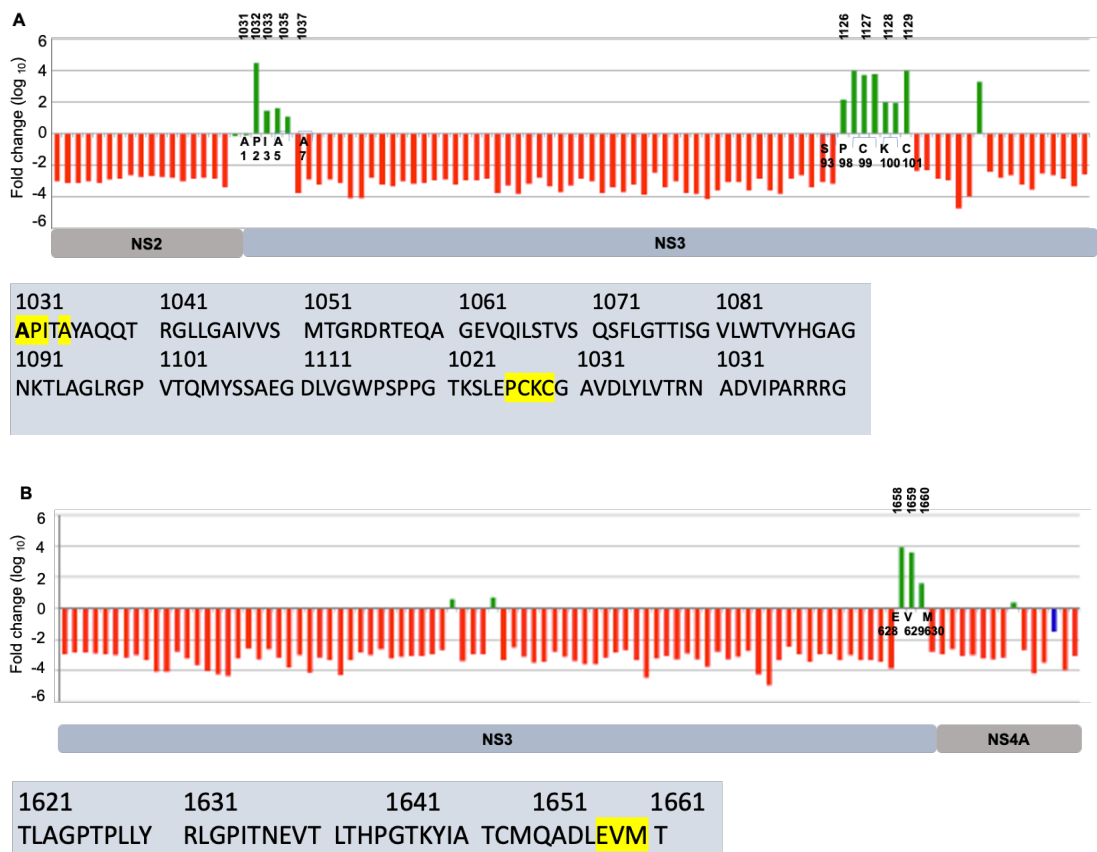


Figure 5.10: Insertion sites in NS3 identified by transposon mutagenesis.
[A] Regions of transposon insertion in the NS2-NS3 coding region, with highlighted NS3 sites located towards the N-terminal and loop regions denoted by amino acid numbers from the whole JFH-1 genome (top values), and numbers corresponding to

the NS3 protein amino acid sequence (bottom values with single-letter amino acids). [B] Transposon insertion regions in the NS3-NS4A coding region, with highlighted NS3 sites towards the C-terminus identified by amino acid numbers from the whole JFH-1 genome (top values), and numbers corresponding to the NS3 protein amino acid sequence (bottom values with single-letter amino acids). Lethal, attenuated, and tolerated sites are indicated by red, blue, and green colors, respectively. Adapted from (Arumugaswami et al., 2008).

By employing the overlap PCR method, two dual SGR constructs, NS3-M0-MiniE and NS3-D25-MiniE, were generated with SGR NS5A-eGFP as the plasmid backbone. Huh7 and Huh7.5 cells electroporated with RNA derived from these constructs exhibited no signs of replication as they were comparable to the polymerase-deficient control (GND), where no luciferase signal was detected as shown in Figure 5.11. The positive controls SGR NS5A-eGFP and NS5A-eGFP-miniE replicated as observed previously (Figure 5.2).

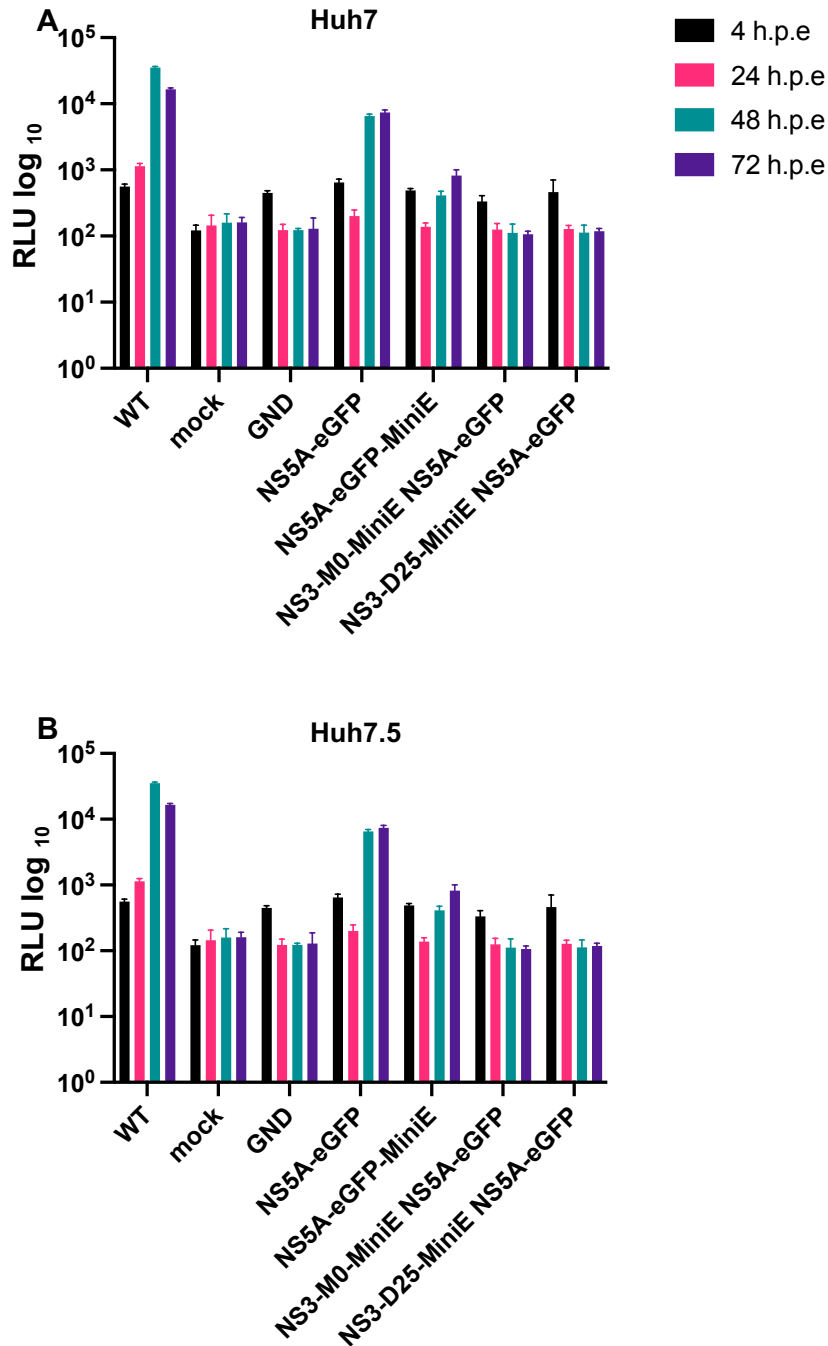


Figure 5.11: Assessment of the replication of NS3-MiniE tagged SGR constructs.

RLU was recorded up to 72 h.p.e following the transient electroporation of 2 μ g of SGR RNA into both Huh7 [A], and Huh7.5 cells [B]. The MiniE tagged constructs were compared to SGR: WT, NS5A-eGFP, NS5A-eGFP-MiniE, and the polymerase-deficient variant (GND, serving as a negative control). The presented data reflects the mean $\pm$ SD, n=1 (with three technical repeats).

Following the release of AlphaFold's structural predictions (Jumper et al., 2021), the JFH-1 NS3 sequence structure was predicted using ColabFold, and superimposed onto the established crystal structure 5DWX (Tunyasuvunakool et al., 2021; Mirdita et al., 2022). The predicted region of NS3 protease structure by ColabFold is shown in Figure 5.12 A (pLDDT and PAE shown in appendix Figure 10). Corresponding regions from the 5DWX structure were overlaid with the predicted NS3 structure to identify structural disparities (Figure 5.12 B). An evident difference emerges in the amino acid sequence 'ITAYQQ,' which forms a beta sheet in the crystal structure but adopts a loop configuration in the predicted structure. This dissimilarity may be attributed to additional sequences upstream of NS3 enhancing crystal structure stability. Since no replication was detected (Figure 5.11), it can be suggested that the residues possibly form a beta sheet conformation in the physiological environment, and that introducing a fusion tag at M0 and D25 may have disrupted the structural integrity of NS3 essential for the replication of the SGR.

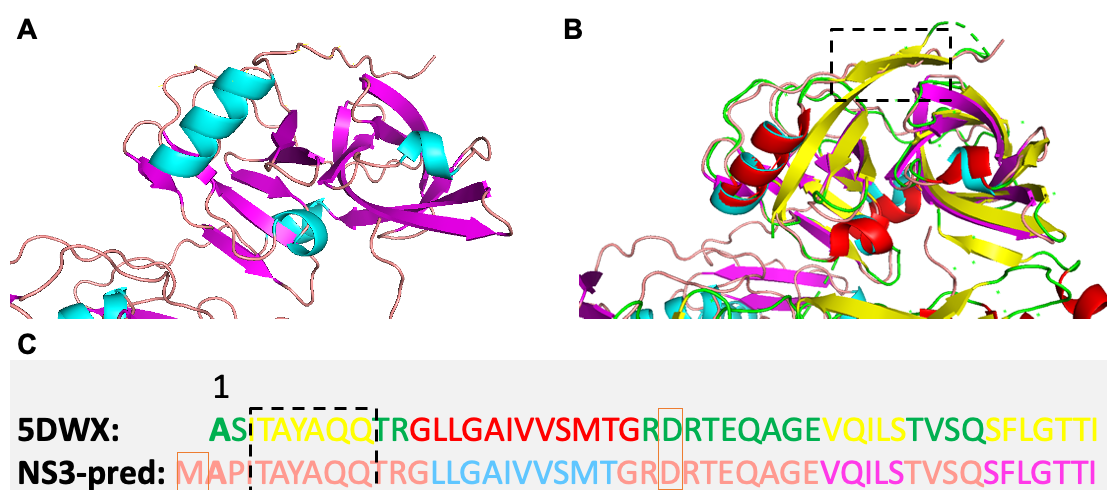


Figure 5.12: Predicted and crystal structures of JFH-1 NS3 (region of protease domain shown).

[A] Predicted structure of the NS3 by ColabFold (part of protease domain shown). [B] Overlay of predicted ColabFold and crystal structure of NS3 protease domain (5DWX). Black dashed box highlights the differences in the two structures and the corresponding sequence is shown in [C]. [C] Sequence of NS3 protease domain. Differences in the structures highlighted in the two sequences. Red boxes show the insertion site for SGR NS3-MiniE (M0) and (D25). Residues forming helices are shown in red and blue, loops are indicated in green and salmon and beta sheets are denoted as yellow and pink in both the 5DWX and the NS3 predicted (NS3-pred) structures.

Henceforth, a more comprehensive approach in designing additional constructs was employed. This approach incorporated insights from transposon mutagenesis research to identify potential insertion sites (Figure 5.10). This information was integrated with the ColabFold tool to evaluate the suitability of these insertion sites in terms of structural folding for both the NS3 protein and the MiniE tag. The findings from the transposon mutagenesis indicated specific locations within the protease domain, including A1, P2, I3, A5, located near the N terminus, and, within one of the loop regions, P98, C99, K100, C101 (Figure 5.10 A). Furthermore, potential sites were identified towards the C-terminus, namely E628, V629, and M630 (Figure 5.10 B). Various sites, each with distinct linker sequences and lengths, were subjected to evaluation using ColabFold. It's worth noting that the MiniE-MiniR complex is a synthetic construct, so the accuracy of structural predictions by ColabFold may have limitations.

At first, ColabFold was employed to predict the MiniE-MiniR structures. MiniE is composed of 36 amino acids and forms a structure characterised by a single helical strand. This helical strand subsequently interacts with MiniR, which is labelled with a fluorescent molecule. The predicted structure, as determined by ColabFold, is illustrated in the Figure 5.13.

To establish context, predictions from ColabFold were applied to NS5A-eGFP-MiniE, a construct that replicates and correctly folds the MiniE protein, serving as a positive structural reference. Conversely, NS3-M0-MiniE and NS3-D5-MiniE, which did not replicate and resulted in incorrect folding of the MiniE protein, were used as negative references. Insertion sites for MiniE guided by structural insights from ColabFold are illustrated in Figure 5.13, with confidence measures of the pLDDT and PAE plots shown in appendix Figure 10. With this integrated approach, five more constructs were generated using insertion sites P2, A5, G90, C99, and E628, each with respective linker sequences shown in appendix Table 9. Among these, P2, A5, C99, and E628 were previously identified as tolerating a 15-nucleotide (5-amino acid) insertion tag through transposon mutagenesis (Arumugaswami et al., 2008). In the loop region 'SPPGTKSLPCKCG,' the tested sites were P98, C99, K100, and C101. Given that the MiniE tag consists of 36 amino acids, which is significantly longer than the 5 amino acids tested for insertion, insertion site G90 towards the beginning of the loop was also tested. Replication was assessed through a Luciferase assay, which showed replication in SGR NS5A-eGFP but no replication in any of these constructs when electroporated into Huh7.5 cells (Figure 5.14).

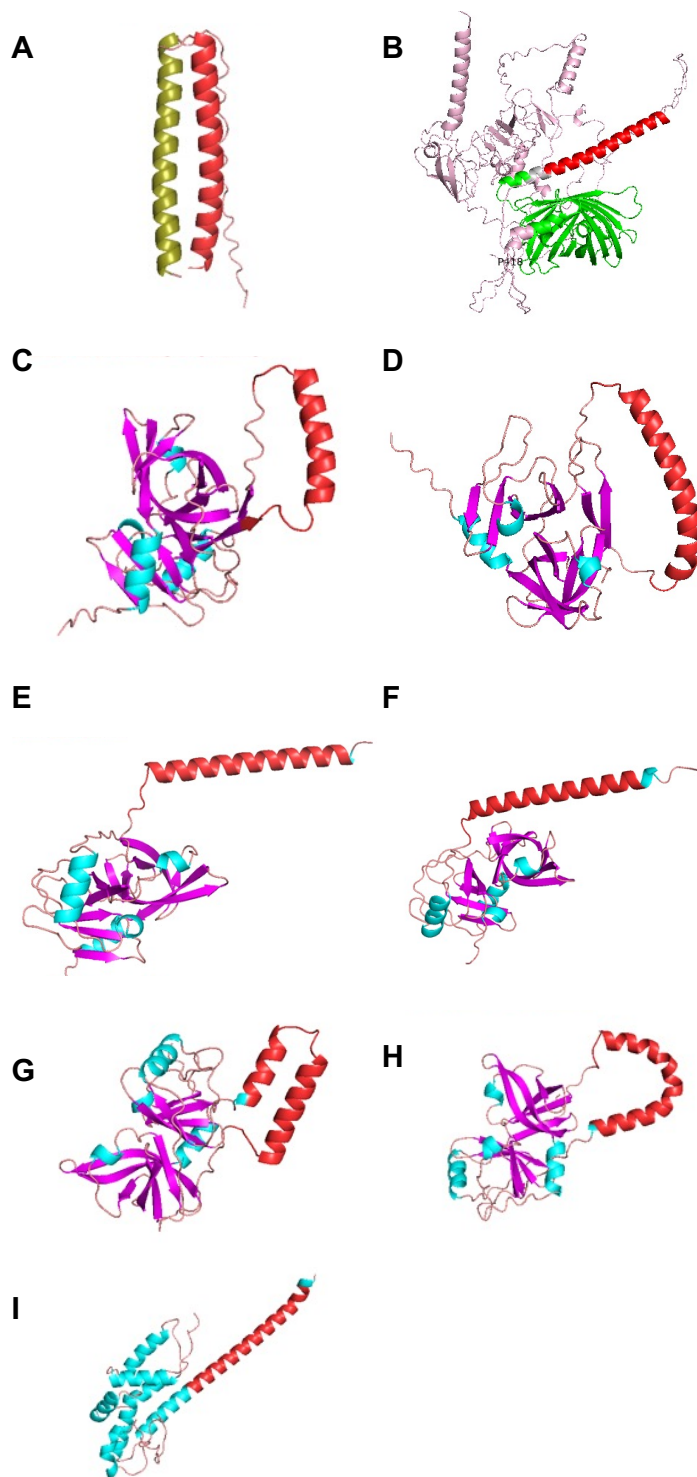


Figure 5.13: Structural predictions NS3 tagged with MiniE by ColabFold.

[A] Heterodimer structure of MiniE (red) and MiniR (olive). MiniE and MiniR residues forming a loop is depicted in salmon. [B] Structure of SGR NS5A-eGFP-MiniE with NS5A (light pink), MiniE (red), and eGFP (green) [C-H] Structures of NS3 protease domain tagged with MiniE at various insertion sites, after residue M0 (C), D25 (D), P2 (E), A5 (F), G90 (G), C99 (H). Beta sheets, helices, loops, MiniE are displayed in magenta, cyan, salmon, and red respectively. [I] Structure of the NS3 helicase

domain3 with NS3-MiniE inserted at the E268 site. Linker residues detailed in appendix Table 9.

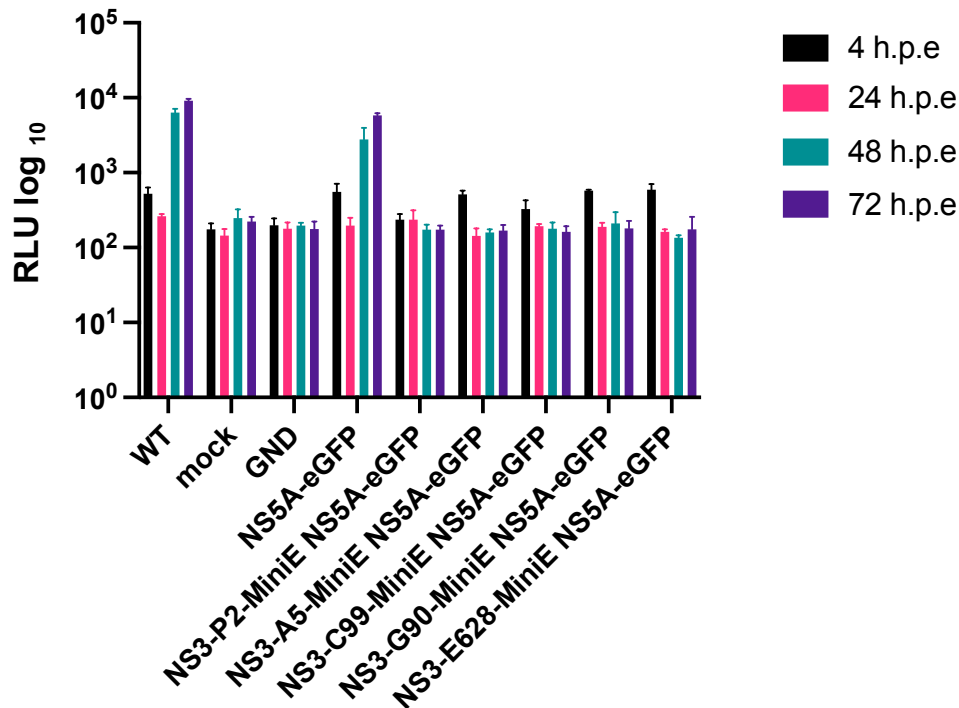


Figure 5.14: Assessment of the replication of NS3-MiniE tagged SGR constructs.

RLU was recorded up to 72 h.p.e following the transient electroporation of 2 µg of SGR RNA into Huh7.5 cells. The MiniE tagged constructs were compared to SGR: WT, NS5A-eGFP, and the polymerase-deficient variant (GND, serving as a negative control). The presented data reflects the mean ± SD, n=1 (with three technical repeats).

5.2.6 Detection of structural integrity of NS5A and NS3 expressed in HEK 293 cells

As the MiniE tag was not tolerated in any of the 7 insertion sites tested, the impact of MiniE insertion at NS3 sites on protein folding and structural integrity was evaluated using a pCMV10 expression plasmid (under the CMV promoter). The pCMV10 plasmid was utilised to express the NS3-5B polyprotein without eGFP fusion to NS5A. Recombinant constructs NS3-MiniE-M0 and NS3-MiniE-D25 were subcloned from SGR to the pCMV10 plasmid carrying the NS3-NS5B polyprotein. The resulting pCMV10 constructs, pCMV10 NS3-5B with NS3-MiniE-M0 and NS3-MiniE-D25, was used to

transfect HEK 293 cells. Transfected cells were subjected to western blot analysis to assess NS5A and NS3 expression. The overall cloning strategy is shown in methods (section 2.5.5, Figure 2.2). The structural integrity of NS5A was confirmed through western blot analysis, showing expression of both hyper and basal phosphorylated NS5A, indicating correct folding (Figure 5.15 A). Unfortunately, NS3 expression was not detected using the antibody against NS3 in the western blot analysis (Figure 5.15 B), although was successfully visualised in an immunofluorescence assay shown in (Figure 5.3).

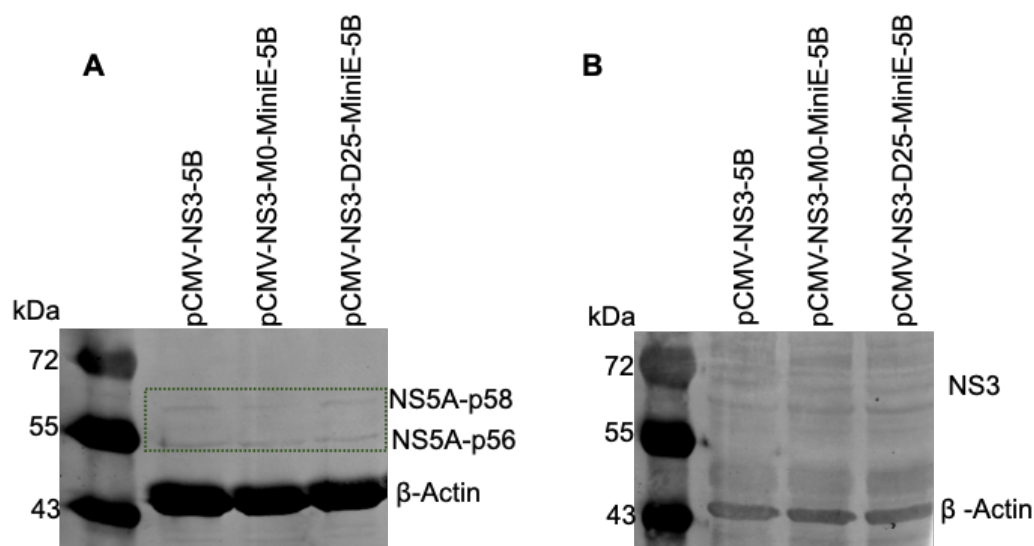


Figure 5.15: Expression of recombinant HCV proteins analysed by western blot.

[A] Detection of NS5A expression at 48 hours post transfection. Hyper (58 kDa) and basal (56 kDa) phosphorylated NS5A was detected in the pCMV construct, polyprotein NS3-NS5B cloned into pCMV10 plasmid (positive control), and the recombinant pCMV10 NS3 M0-MiniE and NS3 D25-MiniE constructs. [B] Detection of NS3 expression after 48 hours post-transfection of HEK 293 cells by lipofectamine2000. Expected band for positive control was at 68 kDa, and for recombinant construct at 72 kDa. Distinct bands showing expression of the NS3 protein were not detected.

5.3 Discussion

To facilitate the identification of the RO hosting the HCV replicase machinery for potential integration into cryo-CLEM, the MiniVIPER fusion tag was investigated. This included evaluating the MiniE fusion tag in NS5A and NS3 within the SGR JFH-1 NS5A-eGFP, and investigating the purification and conjugation procedures of the MiniR probe for detecting the fused tag.

The specificity of the MiniR has been tested on selected proteins such as transferrin receptor protein1 (TfR1), Histone2B (H2B) and mitochondrial import receptor subunit (TOMM20) (Doh et al., 2020). MiniE was fused with TfR1, a cell surface receptor, H2B, which localises to the nucleus, and TOMM20 which localises to the outer mitochondrial membrane (Doh et al., 2020). These are endogenous cellular components where tagging the N-terminus with MiniE did not disrupt their structural integrity and function. However, labelling viruses for scientific studies generally presents with practical challenges due to their highly organised compact structures, issues involving tag size, labelling methods, and precise placement of labels. Maintaining virus or SGR functionality and replication levels is crucial, requiring careful selection of labelling methods and components (Liu et al., 2023). This project aimed to identify the cellular compartments induced by HCV SGR within the host environment where replication assembly takes place. Therefore, controls are essential to confirm the replication process.

5.3.1 Characterisation of MiniE tagged NS5A protein and detection by MiniR-Cy5

To test the MiniVIPER tag system in HCV SGR, a known NS5A tolerated insertion site was used as a control system. NS5A is a multifunctional protein, of which domain III contributes to both HCV viral replication as well as assembly of the virion (Hughes et al., 2009). Domain III has also been indicated as dispensable for HCV replication, however is important for production and assembly of HCV particles (Hanouille, Verdegem, et al., 2009). The flexibility of domain III is able to tolerate epitope tag insertions (Moradpour et al., 2004; Appel et al., 2005; McCormick et al., 2006). It's worth noting that out of 8 HCV genotypes, thus far eGFP is only tolerated and is found to replicate in cell cultures in Con1 (gt1) or intergenotypic chimeras (Moradpour et al., 2004), and JFH-1 (gt2) NS5A (Ngo and Ridgway, 2009), suggesting the genome specific role of NS5A.

The MiniE tag was inserted downstream of eGFP with the hypothesis that a single helical coil will be tolerated at this known site and thus allow for the replication of the SGR. Even though the tag was tolerated, it reduced the replication capacity of the recombinant SGR by 10-fold. Although, the cellular localisation of NS5A-eGFP-MiniE remained punctate in

the cytoplasm, there was a significant difference in the mean and median size of NS5A foci between NS5A-eGFP-MiniE and NS5A-eGFP.

To gain insight into any subcellular differences caused by the MiniE tag, comparison of NS3 distribution in the cells harbouring SGR NS5A-eGFP and SGR NS5A-eGFP-MiniE was examined by using an NS3 antibody. It was evident that in cells harbouring SGR NS5A-eGFP-MiniE, only a proportion of cells were detected with the NS3 signal. Moreover, a diffuse NS3 signal was detected in a fraction of cells compared to cells harbouring only NS5A-eGFP. However, in cells that exhibited NS3 puncta, there was no significant differences in the NS3 size between SGR NS5A-eGFP and SGR NS5A-eGFP-MiniE. Additional experimental data is required to investigate the cause of the observed differences to identify whether these differences were due to the sensitivity of the NS3 antibody, or an indirect impact of the MiniE fusion tag to NS5A.

The generation of the MiniR probe for detecting the fusion MiniE tag also presented challenges, exhibiting variability and limited reproducibility. While the expression and purification of MiniR proved to be reliable and straightforward, the conjugation of Cy5 to MiniR using the devised methodology did not yield consistent fluorescence signals (data not shown). MiniR-Cy5 was reported to be labelled at $29\% \pm 2\%$ (J. K. Doh et al., 2020), indicating that not all MiniR protein molecules were efficiently labelled. This variability extended to the reported labelling efficiency of fluorophores (AF488, Cy5, Cy3) on coilR, MiniR, and MinE, suggesting that an exploration of which fluorophore yields optimal labelling is recommended. In an effort to reproduce MiniR-Cy5 labelling, an identified key factor was the assessment of protein concentration both before the labelling process (after freeze-thaw), and after the labelling process. The measurement of protein concentration was found to be sensitive to the method used, comparing BCA versus nanodrop (not shown). To address this challenge, employing more reliable and sensitive protein concentration methods before and after labelling, such as the Qubit protein assay (ThermoFisher Scientific) is recommended. This approach holds the potential to resolve the inconsistency in MiniR-Cy5 labelling and enhance the overall robustness of the experimental procedure.

The intended application of MiniVIPER system was for live cell imaging and incorporation in to the cryo-CLEM workflow, to study the dynamics of two proteins in the assembly of the replication complex. Therefore, designing a dual construct with MiniE fused with another non-structural protein alongside NS5A-eGFP was explored (section 3.2).

5.3.2 Characterisation of MiniE tagged NS3 protein

Presently, there are no reported fusion proteins for NS3 capable of *in-vivo* detection, aside from the His-tag at the N-terminus of the protease domain for expression in mammalian cells (Vishnuvardhan et al., 1997). In an effort to establish a dual tagged system guiding the investigation of putative ROs housing the replicase machinery, an integrated approach was employed. The NS3 protein was tagged with the MiniE fusion tag, and subsequent replication kinetics were explored.

Unfortunately, all seven designed SGR constructs (six in the protease region and one in domain3 of the helicase) were found to be non-viable. This suggests that the chosen insertion sites were crucial for replication, and the introduction of a helical tag impeded these interactions. Alternatively, the tag disrupted the structural integrity of the protein, rendering it incapable of replication.

To assess the hypothesis regarding the integrity of the protein structure, two recombinant pCMV10 NS3-5B (NS3-M0-MiniE and NS3-D25-MiniE) were examined under the CMV promoter, expressing the polyprotein NS3-MiniE-NS5B in a replication-independent manner. Notably, this expression construct lacked eGFP to facilitate exclusive NS5A expression and act as positive control. As such, any differences observed will primarily be due to the fusion of MiniE to NS3. Western blot analysis revealed the presence of both basal and hyper phosphorylated NS5A proteins, although in low concentrations, indicating that the structural integrity of NS5A was maintained in the cells expressing the pCMV10 NS3-5B (NS3-M0-MiniE and NS3-D25-MiniE) presence of MiniE tagged to NS3. It is essential to note that this was a singular experiment (n=1), and a series of experiments using control constructs may provide additional insights. A key question remains unanswered regarding the impact on both basal and hyperphosphorylated NS5A protein when NS5A-eGFP-MiniE is expressed under the CMV promoter, in comparison to when NS5A is fused with eGFP and NS3 with MiniE. Additional experiments, including western blot analysis of NS3-MiniE with an improved anti-NS3 antibody, are required to complement the findings mentioned earlier.

The aim was to create a dual tagged system was based on the hypothesis that as eGFP has a molecular mass of 27 kDa in size (Snapp, 2005) whereas MiniE is about 4.23 kDa size (Doh et al., 2020), the smaller tag may be tolerated in NS3 and allow replication of the SGR. However, this resulted in replication incompetent SGRs. A potentially more promising strategy forward could involve tagging essential cellular proteins such as Rabs (Salloum et al., 2013) and VAPs(Paul et al., 2013), which play crucial roles in HCV replication, with various VIPER tags. This alternative avenue may offer greater

adaptability and facilitate the successful integration of MiniVIPER into cryo-CLEM experimental methodologies.

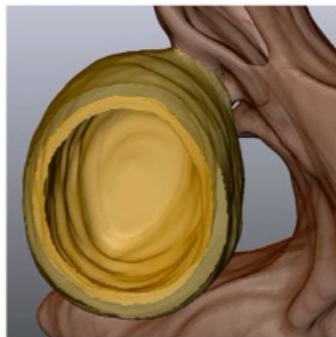
Other fusion strategies that could be explored, include the split-GFP system. This was used to characterise the intranuclear dynamics of PB2 protein of influenza virus by fluorescence correlation spectroscopy and to visualise the trafficking of influenza viral ribonucleoproteins (vRNPs) by dynamic light microscopy (Avilov et al., 2012). The 16 C-terminal amino acid of the GFP was fused with the viral PB2 whereas the large trans-complementing GFP was supplied by transiently or stable expression in cultured cells that were permissive to infection (Avilov et al., 2012). In recent years, Quantum Dots (QD) have become a promising fluorescent label for investigating virus trafficking due to their high brightness and electron dense properties suitable for integrating into a cryo-CLEM workflow (Wang et al., 2021). Dual labelling of the vRNP complexes and envelope of influenza virus with respective green and red QDs were achieved, maintaining 93% infectivity (Hong et al., 2018). This approach could be employed to identify the HCV replicase by attaching quantum dots to any available anti-non structure antibody, NS3 or NS5B.

In this chapter, the MiniVIPER tag was successfully cloned in the JFH-1 NS5A domain III and detected in cells harbouring the SGR NS5A-eGFP-MiniE by MiniR probe. This suggests the tag is accepted in unstructured regions of proteins and thus can be explored further.

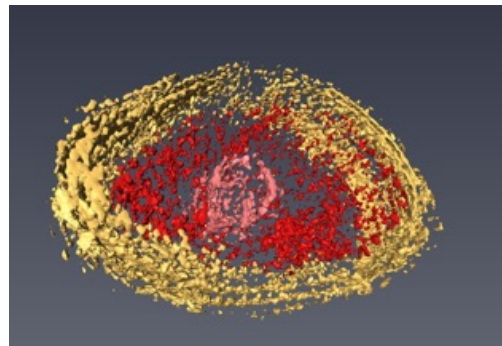
Chapter 6 Conclusions and future perspectives

Highlights

- (1) Two cryogenic sample preparation techniques and associated workflow for cryo electron tomography were established using HCV SGR JFH-1 (genotype 2a)
- (2) Novel insights into the native architecture of replication organelles (RO) were gained: heterogeneity within the various RO (SMV, DMV, MMV, and MVB) suggested that HCV employs an effective immune evasion strategy by employing a fraction of these ROs for replication, consistent with the literature.
- (3) Number of ROs observed were quantified, indicating presence of more SMVs than DMVs, which highlight the importance of preservation of cellular membranes by cryogenic methods.
- (4) The advancements in cryo-ET approaches in understanding the native architecture of DMV in just a decade can be visualised below. The study paves a way to ultimately deduce an *in situ* structure of HCV replication complex.



2012



2023

(Romero-Brey et al., 2012)

The membranous compartments induced by HCV virus and SGR have been characterised by many different EM approaches over the last decade. However, which membrane compartments are specifically active and harbour the replication complex remained elusive. This study set out to establish *in-situ* cryo-ET workflow to investigate where and how the replicase machinery is assembled and whether the hypothesised DMVs are the true replication sites.

Two cryogenic sample preparation techniques were explored and established: cryo-FIB milling (Lam and Villa, 2021) and CEMOVIS (Chlanda and Sachse, 2014). The cryogenic cell specimens were prepared using SGR JFH-1 NS3-NS5B, where NS5A is fused with eGFP. Datasets acquired by FIB-milling followed by cryo-ET of the resulting lamellae (Wagner et al., 2020) without any fluorescence guidance led to exploration of all membranous compartments induced by the SGR. Upon categorising all the DMVs observed in the dataset, it was evident that there are three different types of the closed DMVs present in the cells harbouring SGR JFH-1 NS5A-eGFP: DMVs with electron dense particles which appeared to form clusters and could indicate assembly of the replicase, DMVs and DMTs which appeared to contain thread like densities, indicating dsRNA intermediate filament most likely concealed from the immune system, and lastly empty DMVs with no apparent densities present within and on the surface.

Upon classifying SMVs, two types were noticed, SMVs with densities distributed within the vesicle and SMVs with densities within and on the surface. Since, this was an exploratory dataset, more SMVs were classified than DMVs. Based on the EM analysis of cells from chronically infected HCV patients, SMVs were noticed around LDs and ER (Blanchard and Roingeard, 2018). This study found similar results where SMVs were frequently observed around LDs and ER. These SMVs appeared to harbour densities within the vesicle and outside the surface, which could indicate that SMVs act as a coupling vesicle to transfer proteins from one site to the LDs. One of the intriguing and most frequently observed vesicles were the MVBs, suggesting their role in the process of release of the genome. EM analysis of cells with NS5A-mCherry noticed NS5A mainly present in DMVs and MVBs (Grünvogel et al., 2018). Consistent with this study, MVBs carrying SMVs and DMVs were observed, suggesting a role of MVB releasing encapsulated replicase elements into the extracellular space via exocytosis. Investigations of the media for non-structural proteins and cellular proteins associated with the replication process like VAP-A and cyclophilin might shed light into this process. Alternatively, the accumulation of MVBs may have been observed because there was no assembly process and therefore waiting to be degraded by the immune system. MVBs

containing various elements that appeared to be degraded indicate towards this hypothesis.

Dataset acquired on the ultrathin cryo-sections prepared by ultramicrotome (Chlanda and Sachse, 2014) with NS5A-eGFP fluorescence guidance revealed mostly SMVs, ER, LDs, DMVs, MMVs. DMVs and MMVs were clearly recognisable with the scattered or clustered densities observed in the FIB-milled datasets. However, out of 7 tomograms acquired based on eGFP fluorescence, only one MMV (from 1 tomogram) appeared to harbour densities in a regular arrangement, suggesting the assembly of the replicase machinery on the inner leaflet of the MMV. Acquisition of numerous particles arranged in the similar fashion will lead to the structure of the replication complex. This highlights the challenge in determining the *in-situ* structure of the replication complex. Due to the heterogenous and crowded nature of the cellular environments as well as the distortions caused by the missing wedge, particle picking poses a major challenge (Pyle et al., 2022). Unlike chikungunya virus, where the replication complex and the RNA was deduced from spherules regularly organised on the plasma membrane (Laurent et al., 2022; Tan et al., 2023), the assembly of the replication complex in HCV still remains an elusive process, which adds another layer of complexity in the particle picking processing pipeline.

Several strategies have been proposed in recent years to deduce the heterogenous protein structures in cryo-electron tomograms. Deep neural network program available in the software packages EMAN2.2 can be employed to annotate the features in the tomograms. Subtomograms of specific features can then be extracted from each of the tomogram and subtomogram averaging (STA) can be applied to test the usefulness of the annotations (Chen et al., 2017). Template matching can be used when an external reference is available (Böhm et al., 2000). However, a template free detection and classification of membrane bound complexes would be more suitable and should be explored on the dataset acquired in this study (Martinez-Sanchez et al., 2020). A comprehensive knowledge of the open source software package PySeg in Scipion (Martinez-Sanchez et al., 2020) is required to enable classification of the heterogenous population of densities observed within the various vesicles. Another strategy that can be explored for picking membrane associated particles within the STA workflow involves combination of various computationally available tools like Dynamo, Relion, chimera and Eman2 to deduce the architecture of the HCV replication complex (Pyle et al., 2022).

To overcome the challenge of pinpointing the organelle harbouring the HCV replication complex, a dual tagged system was examined. MiniVIPER with a tag of 4.5 kDa (33 amino acid) was tested at 7 different NS3 insertion sites. However, none of these sites

resulted in a viable construct that replicated in Huh7 and Huh7.5 permissive cells. Domain 3 of NS5A protein is the only NS protein that has ever tolerated tags and allowed replication. Since, several cellular factors are necessary for replication, possibly evaluating structures of known cellular factors that interact with other NS proteins could be another way to narrow the search for the replication organelle. For example Rab5 is involved in HCV replication and C-terminal of NS5B might be involved in recruitment of Rab5 (Aligo et al., 2009). Thus, suggesting early endosome contributes to the formation of the replication site (Manna et al., 2010). Rab5 with eGFP has been successfully employed to understand the dynamics of rab5 activation in endocytosis and phagocytosis (Roberts et al., 2000). Therefore, Rab5-eGFP could be employed to identify where NS5B is harboured in cells harbouring SGR JFH-1 NS5A-eGFP.

The cryo-CLET methodologies established in this study leads a pathway to investigate the morphological variations induced by other genotypes in HCV. Majority of studies have focussed using the JFH-1 strain. With the advanced cryo-FIB-SEM methods, cells harbouring SGR JFH-1 NS5A-eGFP from other genotypes could also be investigated. This will highlight whether DMVs are a lab-culture adapted features of JFH-1 used for the replication. The next strategy to integrate into the cryo-CLET methodology would be to tag host proteins associated with replication specifically VAP proteins and Rabs. These are vesicle associated proteins and would help identify SMVs, DMVs and MMVs specifically used as replication organelle. As the computational methods to mine data from the wealth of cryo-tomograms are rising (Kim et al., 2023), this study provides a foundation for deciphering the architecture of the HCV replication complex.

The main outcome of this project was developments of cryogenic *in-situ* workflows and identifying areas for future developments.

Appendix

Table 1: Descriptive statistics of cells transfected with SGR JFH-1 WT (NS3-5B).

Cells were stained with primary anti-NS5A antibody and visualised using Alexa Fluor (AF): AF-488 and AF-594. Area of NS5A foci were calculated by 'Analyse Particles' function in Fiji and analysed using in-built statistical tools in GraphPad (Prism).

	NS5A-AF488	NS5A-AF594
Number of values	121	100
Minimum	0.02000	0.02000
25% Percentile	0.1900	0.1700
Median	0.4300	0.6800
75% Percentile	1.450	1.575
Maximum	14.75	13.27
Range	14.73	13.25
Mean	1.261	1.258
Std. Deviation	2.209	1.774
Std. Error of Mean	0.2008	0.1774
Lower 95% CI of mean	0.8630	0.9055
Upper 95% CI of mean	1.658	1.609

Table 2: Descriptive statistics on replicon cells harbouring SGR JFH-1 NS5A-eGFP and SGR JFH-1 NS5A-mCherry.

Cells were stained with primary anti-VAP-A antibody and visualised using AF-488 and AF-594. Area of NS5A foci were calculated by 'Analyse Particles' function in Fiji and analysed using in-built statistical tools in GraphPad.

	NS5A-eGFP	NS5A-mCherry
Number of values	59	102
Minimum	0.02000	0.02000
25% Percentile	0.2300	0.2250
Median	0.5600	0.5700
75% Percentile	1.110	0.9750
Maximum	9.710	5.490
Range	9.690	5.470
Mean	1.147	0.8891
Std. Deviation	1.828	1.031
Std. Error of Mean	0.2380	0.1021
Lower 95% CI of mean	0.6705	0.6865
Upper 95% CI of mean	1.623	1.092

Table 3: Descriptive statistics of stable cells harbouring SGR JFH-1 NS5A-eGFP and SGR JFH-1 NS5A-mCherry.

Cells were stained with primary anti-PDI antibody and visualised using AF-488 and AF-594. Area of NS5A foci were calculated by 'Analyse Particles' function in Fiji and analysed using in-built statistical tools in GraphPad.

	NS5A-eGFP	NS5A-mCherry
Number of values	48	132
Minimum	0.02000	0.01000
25% Percentile	0.06000	0.09250
Median	0.1500	0.2050
75% Percentile	0.3225	0.4050
Maximum	2.430	3.940
Range	2.410	3.930
Mean	0.2604	0.3288
Std. Deviation	0.3863	0.4388
Std. Error of Mean	0.05576	0.03820
Lower 95% CI of mean	0.1482	0.2532
Upper 95% CI of mean	0.3726	0.4043

Figure 1: Assessment of the subcellular distribution of NS5A in SGR NS5A-eGFP and NS5A-mCherry which were fixed and stained with anti-PDI antibody and DAPI.

[A-B] Subcellular distribution of NS5A in Huh7 cells harbouring SGR NS5A-eGFP and NS5A-mCherry. Scale bar = 20 μm [C] Calculated differences in the mean of the area determined of the NS5A foci by unpaired t-test (Welch's test, GraphPad), revealing no significant differences. The left hand side of the plot denotes all the NS5A focus points detected by NS5A-eGFP and NS5A-mCherry, distributed logarithmically. The right hand side of the plot denotes the differences in the area of NS5A mean between NS5A-eGFP and NS5A-mCherry. Imaged on LSM880 confocal microscope with 63X objective (oil immersion) with N.A of 1.4

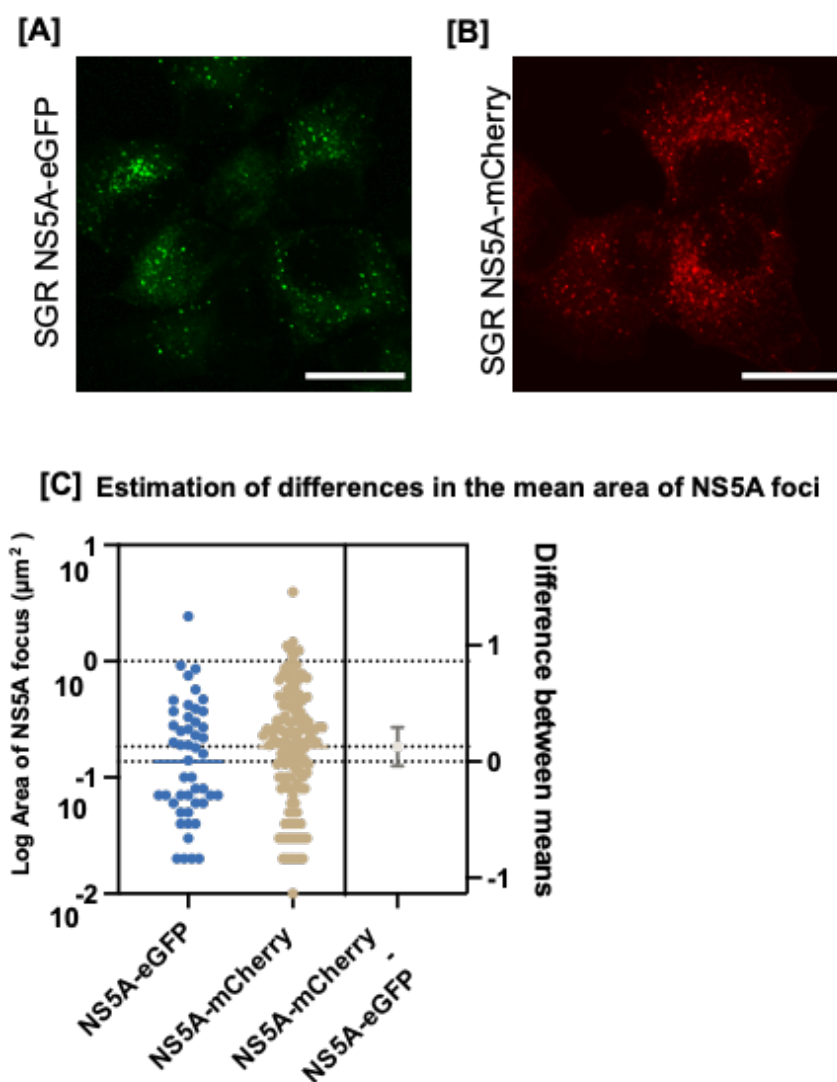


Figure 2: Assessment of the subcellular distribution of NS5A in SGR NS5A-eGFP and NS5A-mCherry which were fixed and stained with anti-TGN-26 and DAPI.

[A-B] Subcellular distribution of NS5A in Huh7 cells harbouring SGR NS5A-eGFP and NS5A-mCherry. Scale bar = 10 μm [C] Calculated differences in the mean of the area determined of the NS5A foci by unpaired t-test (Welch's test, GraphPad), revealing no significant differences. The left hand side of the plot denotes all the NS5A focus points detected by NS5A-eGFP and NS5A-mCherry, distributed logarithmically. The right hand side of the plot denotes the differences in the area of NS5A mean between NS5A-eGFP and NS5A-mCherry. Imaged on LSM880 confocal microscope with 63X objective (oil immersion) with N.A of 1.4 .

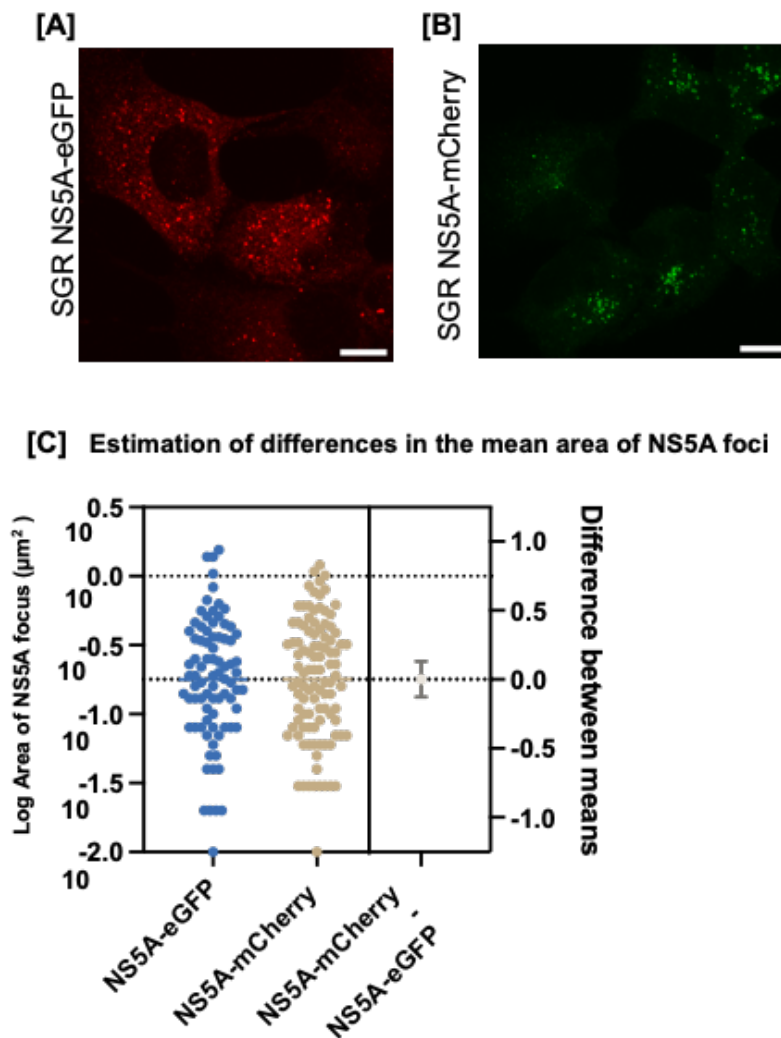


Table 4: Descriptive statistics of stable cells harbouring SGR JFH-1 NS5A-eGFP and SGR JFH-1 NS5A-mCherry.

Cells were stained with primary anti-TGN-46 antibody and visualised using AF-488 and AF-594. Area of NS5A foci were calculated by 'Analyse Particles' function in Fiji and analysed using in-built statistical tools in GraphPad.

	NS5A-eGFP	NS5A-mCherry
Number of values	82	102
Minimum	0.01000	0.01000
25% Percentile	0.08750	0.08000
Median	0.1900	0.1950
75% Percentile	0.3650	0.3925
Maximum	1.550	1.200
Range	1.540	1.190
Mean	0.2837	0.2766
Std. Deviation	0.2994	0.2532
Std. Error of Mean	0.03306	0.02507
Lower 95% CI of mean	0.2179	0.2268
Upper 95% CI of mean	0.3494	0.3263

Figure 3: Subcellular localisation of NS5A-eGFP in proximity to LDs in replicon cells harbouring SGR JFH-1 NS5A-eGFP.

Cells were fixed in 4% formaldehyde and stained with 1:1000 dilution of BODIPY (558/568) for 1 hour at room temperature. NS5A-eGFP (green), LDs (red) and nuclei of cells stained with DAPI (blue). Acquired on 40x/1.4 oil objective and merged using Fiji. Scale: 20 μ m.

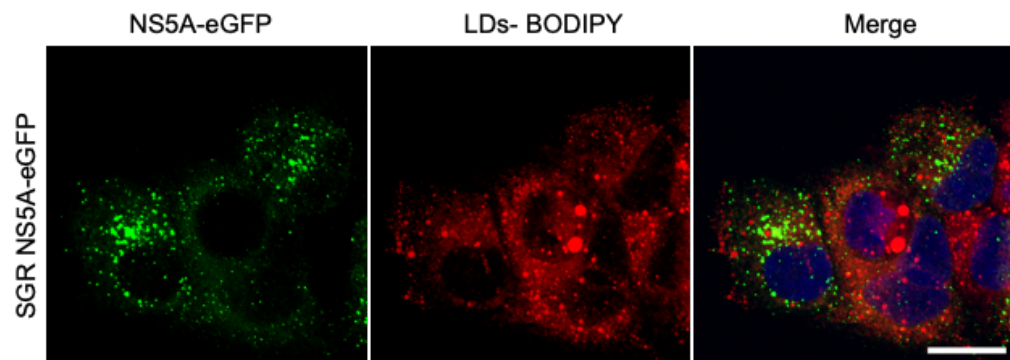


Table 5: Cryo-ET parameters for Dataset 1 (from cryo-FIB milled lamellae) on Krios1.

Hardware	Value
Microscope	Krios 1
Detector (mode)	Falcon 4 (counting)
Accelerating voltage (kV)	300
Software	
TOMO version	5.11
Data acquisition parameters	
Pixel size (angstrom)	3.00
Nominal magnification	42000
Spot size	9
Illuminated area (um)	2
Dose calculations	
Square pixel (Å ²)	9
Dose per physical pixel/second	7.2
Dose per Å ² /second	0.8
Exposure time	1.94
Total dose/Å ²	1.552
Manual Fractions	4
Dose per fraction (e-/Å ²)	0.388
Number of images in tilt series	61
Total dose (Å ²)	94.672
TOMO parameters	
Session name	
Defocus range	3-5um
Autofocus period	
Adjust ZLP	
Wait after tilt	
Exposures per hole (quantifoil) or Spacing of exposures (lacey)	
Apertures (size in microns)	
C1 aperture	2000
C2 aperture	50
C3 aperture	2000
Objective aperture	100

Table 6: Cryo-ET parameters for Dataset 2(from cryo-FIB milled lamellae on Krios1.

Hardware	Value	Notes
Microscope	Krios 1	
Detector (mode)	Falcon 4i (counting)	
Accelerating voltage (kV)	300	
Software		
TOMO version	5.13	
Data acquisition parameters		
Pixel size (angstrom)	1.90	Get this value from Tomo > Image information
Nominal magnification	64,000	Get this from Tomo > preparation > Presets > Exposure
Spot size	9	
Illuminated area (um)	1.5	
Dose calculations		
Square pixel (Å2)	3.61	
Dose per physical pixel/second	4.83	Get this from Tomo > Preparation > Presets > Exposure> Measure (with parameters above over vacuum).
Dose per Å2/second	1.337950139	
Exposure time	2.2	Usually 3 - 5 seconds- tune this to get appropriate total dose
Total dose per image (e-/Å2)	2.943490305	
Manual Fractions	8	Tomo > preparation >Presets > Exposure > Fractions Manual > number of fractions
Dose per fraction (e-/Å2)	0.367936288	
Number of images in tilt series	41	
Total dose for the tilt series (Å2)	120.6831025	Total dose within tilt series
TOMO parameters		
Session name	Bi3607_5_20230512	In TOMO > Tomography > Session setup
Defocus range	.5, -3.75, -4, -4.25, -4.5, -4.7	In TOMO > Data Acquisition > Batch Positions
Adjust ZLP	Yes	In TOMO > Data Acquisition > Other
Adjust ZLP Period	1	In TOMO > Data Acquisition > Other > flap
Max Negative Angle	-46.8	In TOMO > Data Acquisition > Acquisition
Max Positive Angle	71.2	
Tilt Span	59	
Tilt Step	3	
Start angle	12.14	
Apertures (size in microns)		
C1 aperture	2000	
C2 aperture	50	
C3 aperture	2000	
Objective aperture	100	

Table 7: Cryo-ET parameters for dataset on ultrathin sections (40 nm samples) by CEMOVIS

Hardware	Value	Notes
Microscope	Krios 1	
Detector (mode)	Falcon 4i (counting)	
Accelerating voltage (kV)	300	
Software		
TOMO version	5.13	
Data acquisition parameters		
Pixel size (angstrom)	2.40	Get this value from Tomo > Image information
Nominal magnification	53,000	Get this from Tomo > preparation > Presets > Exposure
Spot size	9	
Illuminated area (um)	1.85	
Dose calculations		
Square pixel (Å ²)	5.76	
Dose per physical pixel/second	4.86	Get this from Tomo > Preparation > Presets > Exposure: Measure (with parameters above over vacuum).
Dose per Å ² /second	0.84375	
Exposure time	4.01	Usually 3 - 5 seconds- tune this to get appropriate total dose
Total dose per image (e-/Å ²)		
Manual Fractions	6	Tomo > preparation > Presets > Exposure > Fractions Manual > number of fractions
Dose per fraction (e-/Å ²)	0.37	
Number of images in tilt series	41	
Total dose for the tilt series (Å ²)	0	Total dose within tilt series
TOMO parameters		
Session name	Tom_Cells_3_20230429	In TOMO > Tomography > Session setup
Defocus range	.5, -3.75, -4, -4.25, -4.5, -4.7	In TOMO > Data Acquisition > Batch Positions
Adjust ZLP	Yes	In TOMO > Data Acquisition > Other
Adjust ZLP Period	1	In TOMO > Data Acquisition > Other > flap
Max Negative Angle	-60	In TOMO > Data Acquisition > Acquisition
Max Positive Angle	60	
Tilt Span	60	
Tilt Step	3	
Start angle	0	
Apertures (size in microns)		
C1 aperture	2000	
C2 aperture	50	
C3 aperture	2000	
Obiective aperture	100	

Figure 4: Representative images of MW from reconstructed tomograms, derived from tilt-series collection at specific green foci of NS5A-eGFP in cells harbouring SGR JFH-1 NS5A-eGFP (CEMOVIS dataset).

Blue arrows indicate organelles and densities within the organelles are indicated with green arrows. Insets from the tomogram are shown in orange box. RO present in different Z-slice in the tomogram is indicated by a brown double arrow connector.

Scale bars: A1, B1, C1: 2 μ m. A2, B2, C2, C4: 200 nm. A3, B3, C3, C5: 100 nm. *MMV*: multimembrane vesicle, *MW*: membranous web, *ER*: Endoplasmic reticulum

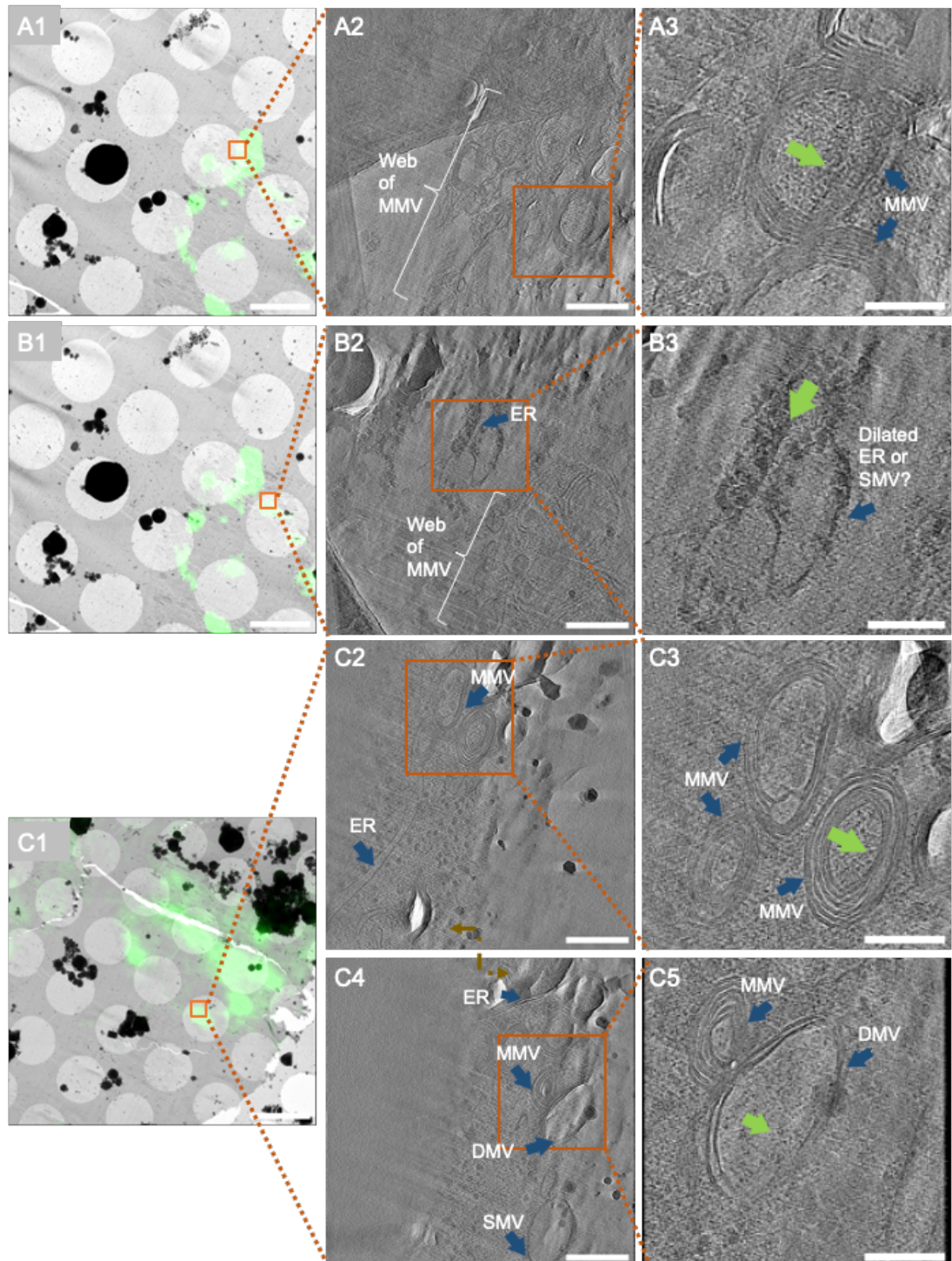


Figure 5: Representative images of MW from reconstructed tomograms, derived from tilt-series collection at specific green foci of NS5A-eGFP in cells harbouring SGR JFH-1 NS5A-eGFP (CEMOVIS dataset).

Blue arrows indicate organelles and densities within the organelles are indicated with green arrows. Insets from the tomogram are shown in orange box. RO present in different Z-slice in the tomogram is indicated by a brown double arrow connector.

Scale bars: A1, B1: 2 μ m. A2, A3, B2, B3: 200 nm. A4, B4: 100 nm. *MMV*: multimembrane vesicle, *MW*: membranous web, *ER*: Endoplasmic reticulum, *LD*: Lipid droplets

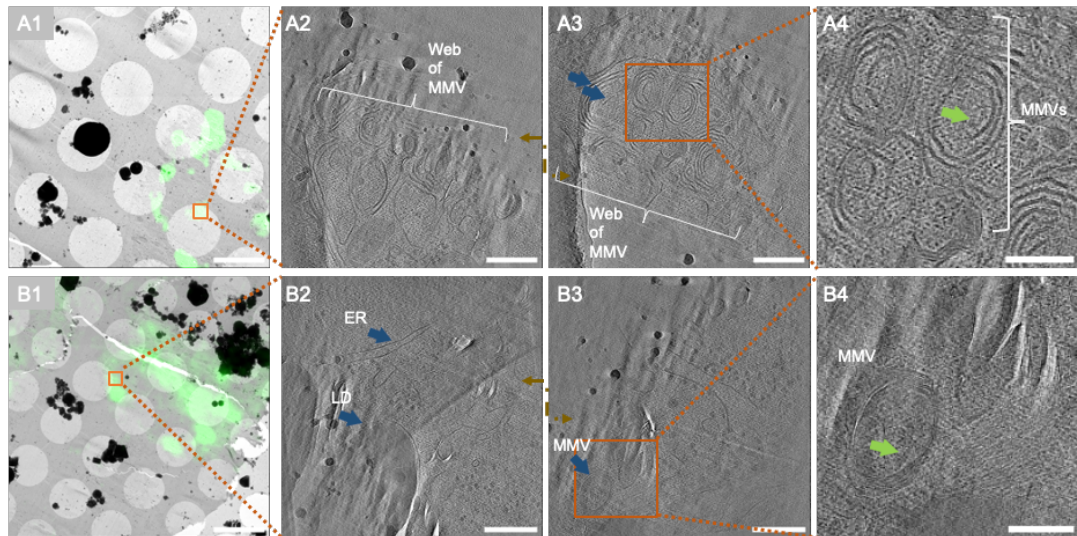


Figure 6: Assessment of the subcellular distribution of NS5A and NS3 in SGR NS5A-eGFP and NS5A-eGFP-MiniE.

[A] Assessment of the frequency distribution of NS5A foci areas (n=2, ROI with 250 x 250 pixels). The bin center denotes area ranges, e.g., 1 indicates the number of foci falling in the range of 1-2 μm^2 . [B] Assessment of the frequency distribution of NS3 foci areas (n=2, ROI with 250 x 250 pixels). [C] Lognormal distribution of NS5A-eGFP and NS5A-mCherry. Areas with same denoted by red line. [D] Logarithmically transformed area values plotted on a logarithmic axis to achieve a symmetrical distribution of values (Gaussian).

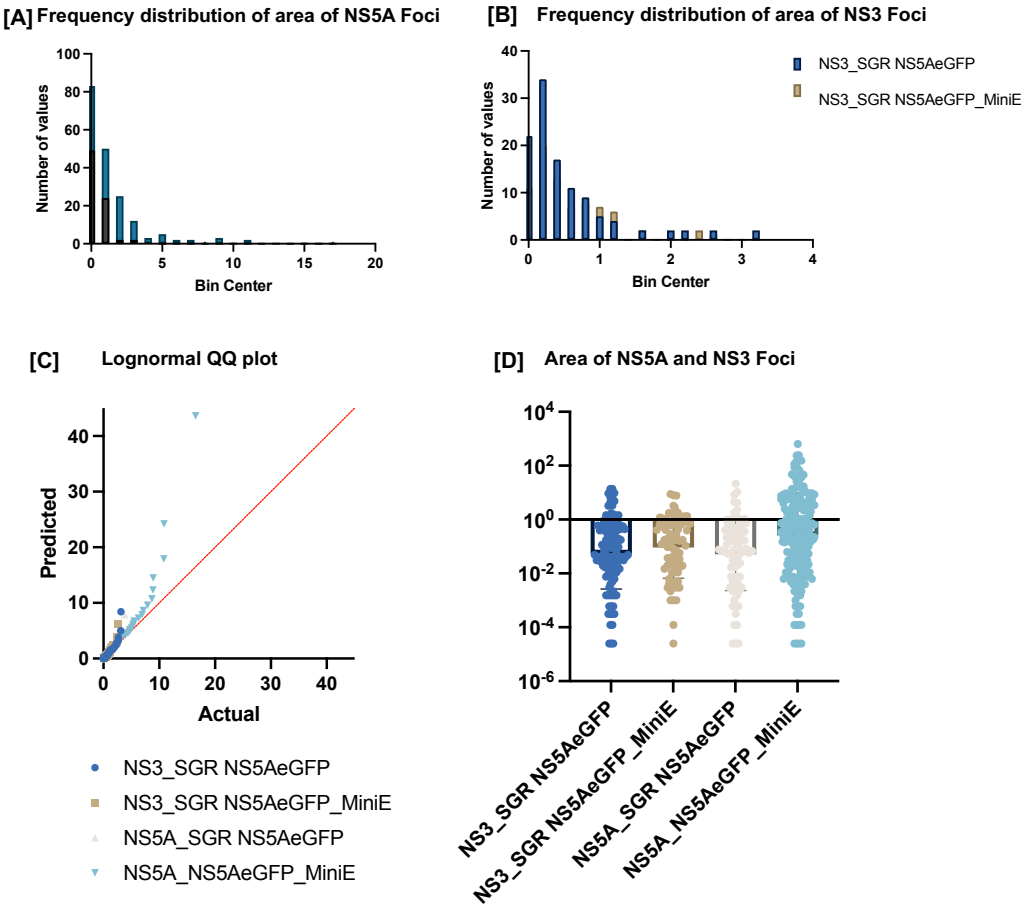


Table 8: Descriptive statistics of cells harbouring SGR JFH-1 NS5A-eGFP and SGR JFH-1 NS5A-eGFP-MiniE.

Area of NS5A and NS3 foci were calculated by 'Analyse Particles' function in Fiji and analysed using in-built statistical tools in GraphPad.

	NS3_SGR NS5AeGFP	NS3_SGR NS5AeGFP_MiniE	NS5A_SGR NS5AeGFP	NS5A_NS5AeGFP_MiniE
Number of values	112	77	78	189
Minimum	0.01000	0.01000	0.01000	0.01000
25% Percentile	0.1325	0.1650	0.1150	0.1900
Median	0.3050	0.4600	0.3350	0.5700
75% Percentile	0.7075	0.8750	0.7325	1.845
Maximum	3.120	2.570	3.780	16.50
Range	3.110	2.560	3.770	16.49
Mean	0.5521	0.5922	0.5491	1.482
Std. Deviation	0.6594	0.5604	0.6568	2.287
Std. Error of Mean	0.06231	0.06387	0.07436	0.1663
Lower 95% CI of mean	0.4287	0.4650	0.4010	1.154
Upper 95% CI of mean	0.6756	0.7194	0.6972	1.810

Figure 7: Native mass spectrometry analysis of purified MiniR protein.

MiniR is 7.25 kDa in size. Analysis suggested heterogenous molecules of MiniR, probably due to either carbamylation or any other post-translational modification. Performed by Rachel at the Biomolecular Mass spectrometry (University of Leeds).

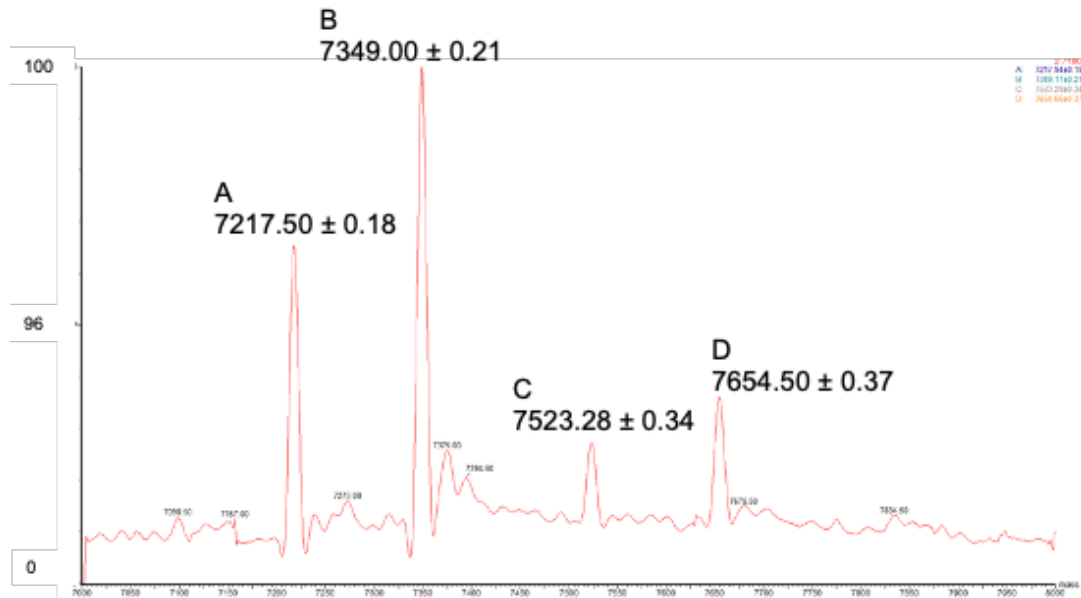


Figure 8: Assessment of colocalisation of NS5A-eGFP-MiniE foci detected by MiniR probe. Evaluation of 1:50 dilution ratio of MiniR-Cy5 at RT (1 hour). NS5A-eGFP (green), NS5A-MiniE-MiniR (red) and nuclei of cells stained with DAPI (blue) Pearson's coefficient (R) value was measured by the Colocalisation plugin (Fiji). Acquired on 40x/1.4 oil objective and merged using Fiji. Scale: Merge: 20 μ m.

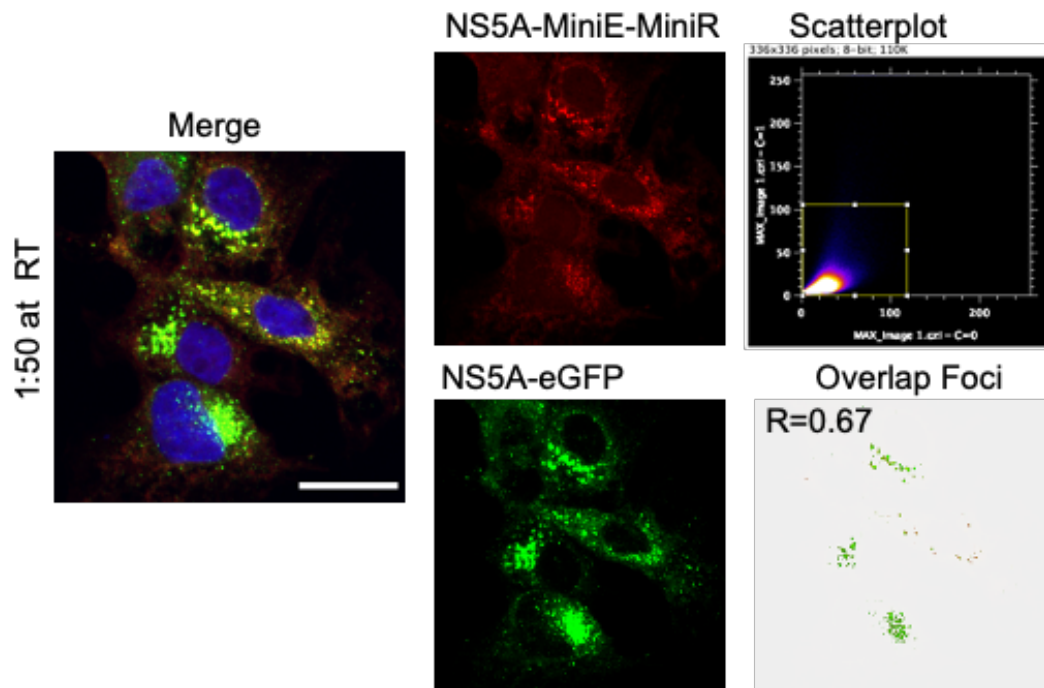


Figure 9: Assessment of the colocalisation of NS5A-eGFP-MiniE foci detected by the MiniR probe. Evaluation of a 1:10 dilution ratio of MiniR-Cy5 at RT (1 hour). NS5A-eGFP (green), NS5A-MiniE-MiniR (red) and nuclei of cells stained with DAPI (blue). Pearson's coefficient (R) value was measured by the Colocalisation plugin (Fiji). Acquired on 40x/1.4 oil objective and merged using Fiji. Scale: Merge: 40 μ m. Insets for MiniE-MiniR and NS5A-eGFP: 10 μ m

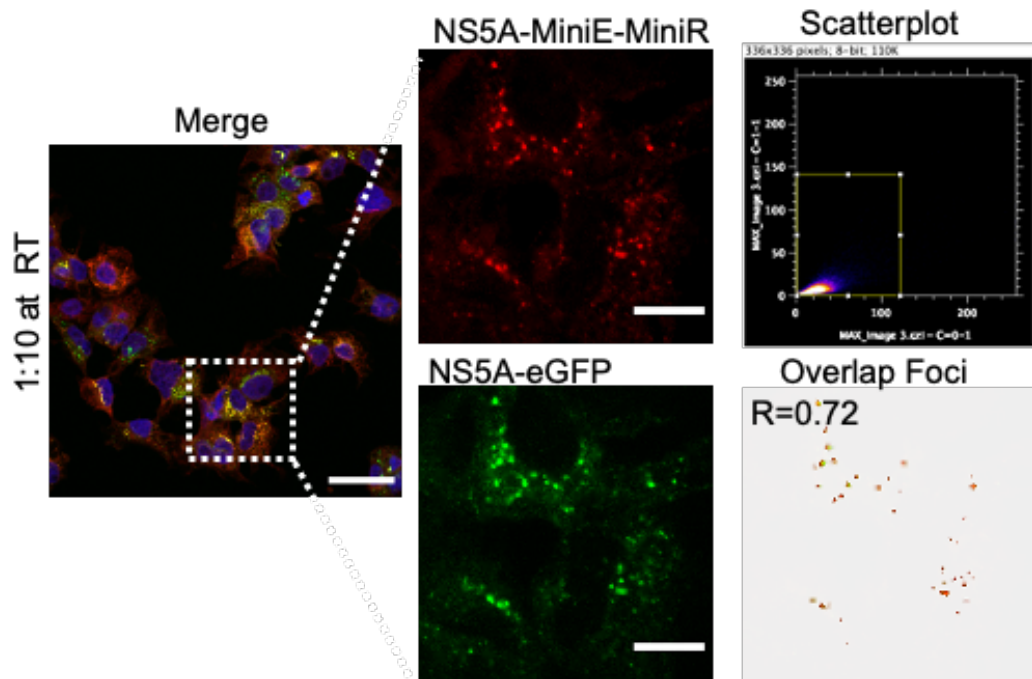
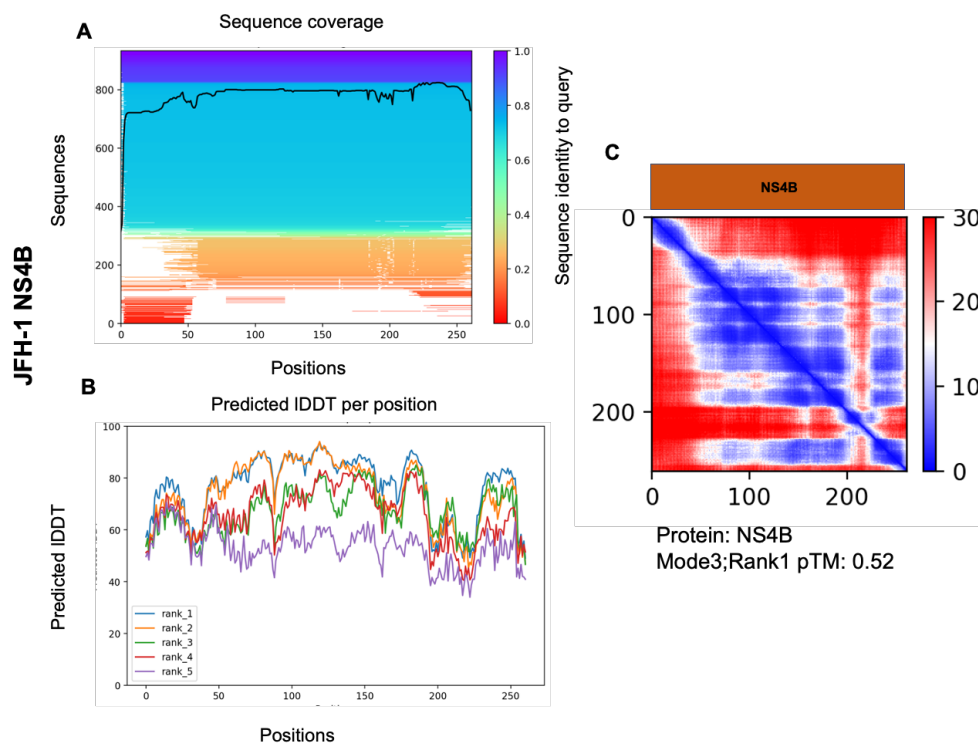
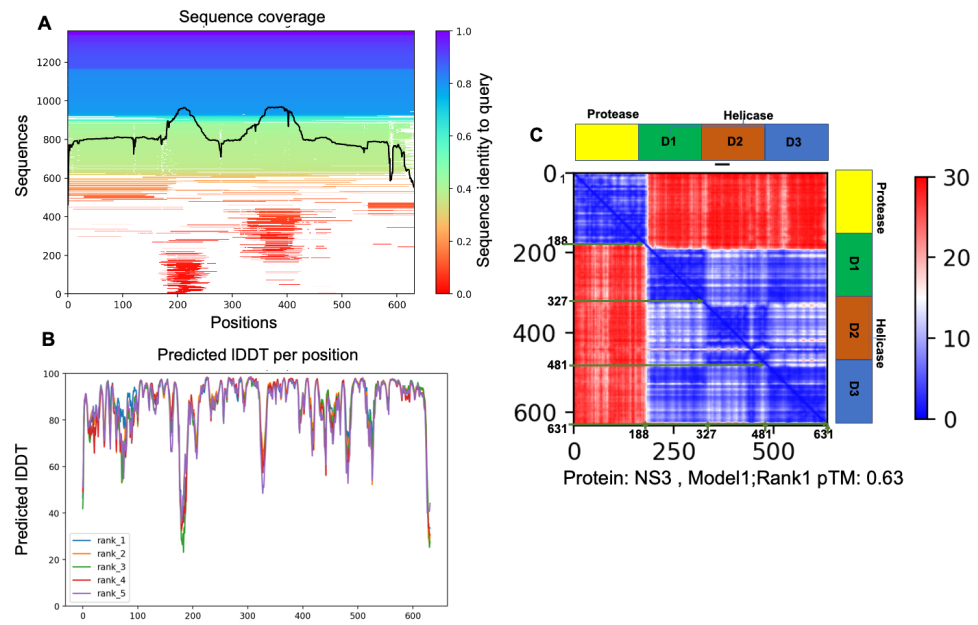


Figure 10: Structural prediction by ColabFold of JFH-1 NS proteins.

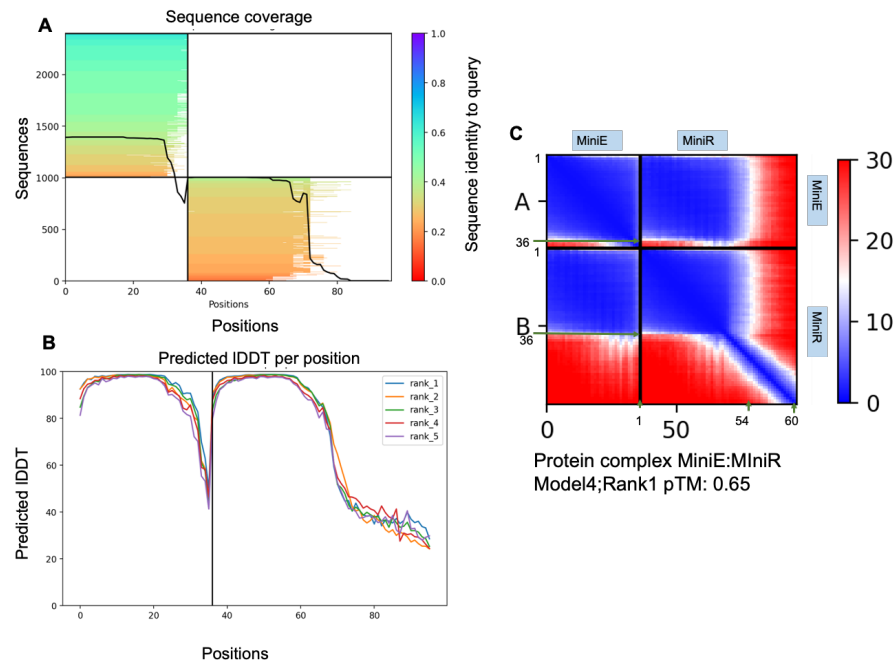
[A] Sequence coverage of the NS3 protein query. Black curve indicates the number of sequences it found at each residue position. The Green and red colour indicates high and low sequence identity (see colour key). [B] Predicted local difference test (pLDDT) of predicted 5 models. pLDDT shows confidence score per residue. pLDDT >90 signified high confidence, between 70-90 is good, and <70 depicts low confidence [C] Predicted aligned error (PAE) indicates the error in position between pairs of residues (x,y) and provides a global accuracy. The colour at position (x,y) indicates the expected position error at residue x, when the predicted and true structures are aligned on residue y. Low error is denoted with blue and a high error is denoted with red. Predicted template modelling (pTM) score measures the structural similarity between two folded protein structures. The pTM ranges from 0 to 1 and provides a framework for ColabFold to rank its predicted output. A pTM score >0.5 indicates usually strong correlation to supposed native structure.



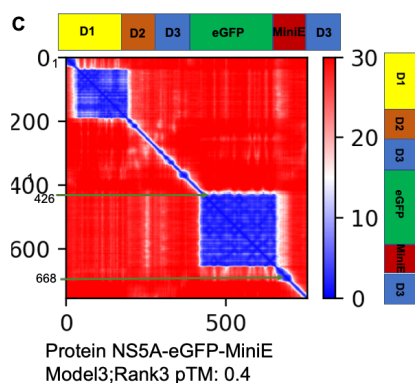
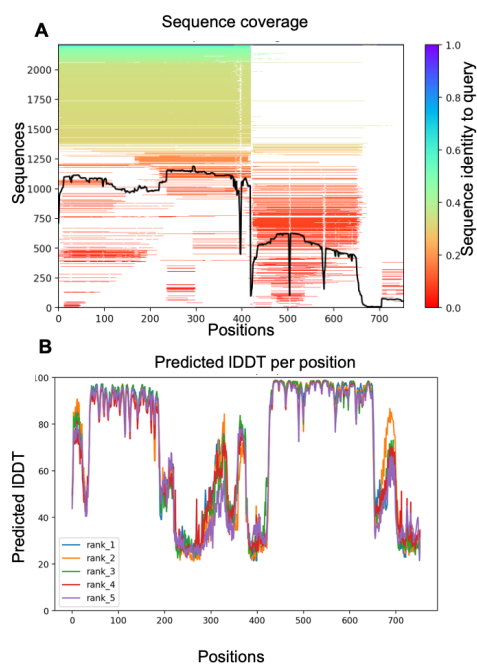
JFH-1 NS3 full protein



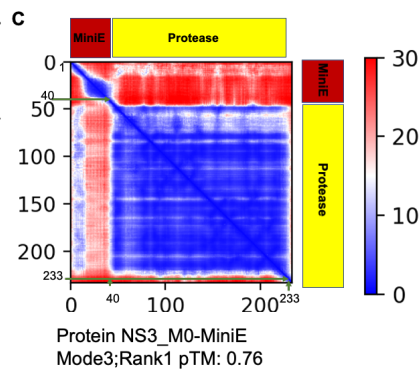
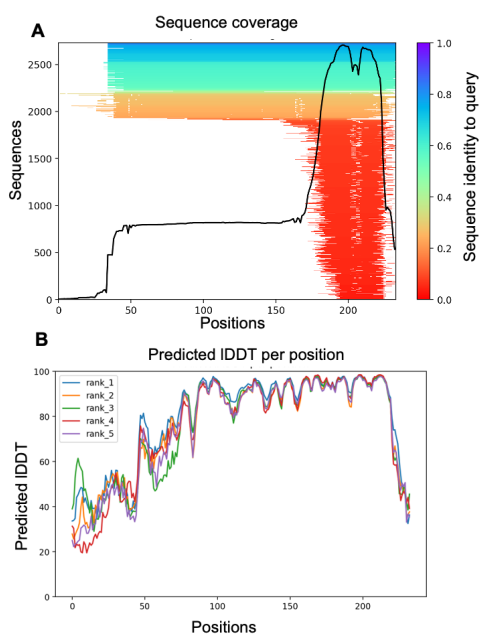
MiniE : MiniR complex



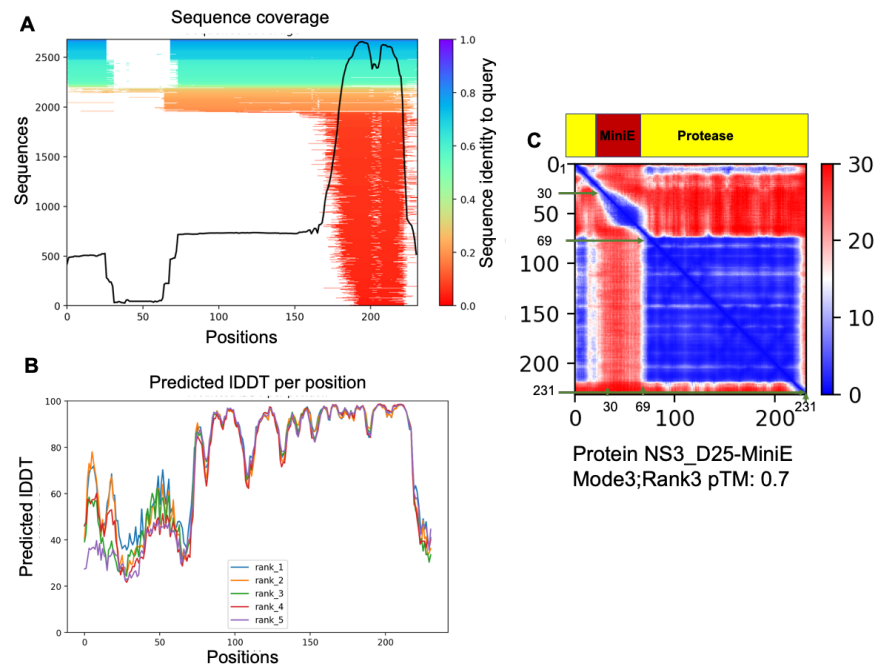
JFH-1 NS5A-eGFP-MiniE



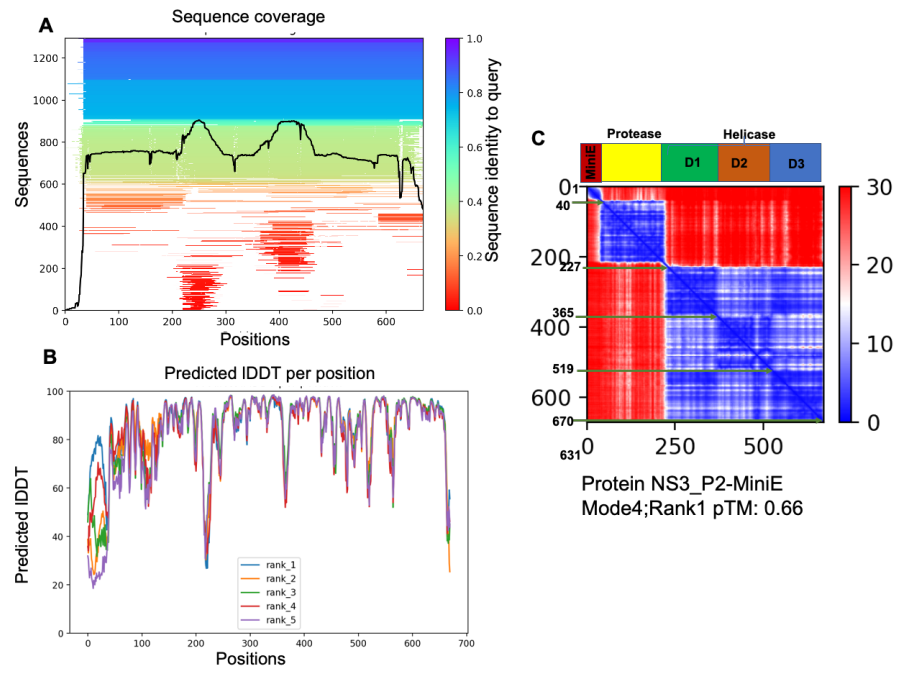
JFH-1 NS3_M0-MiniE



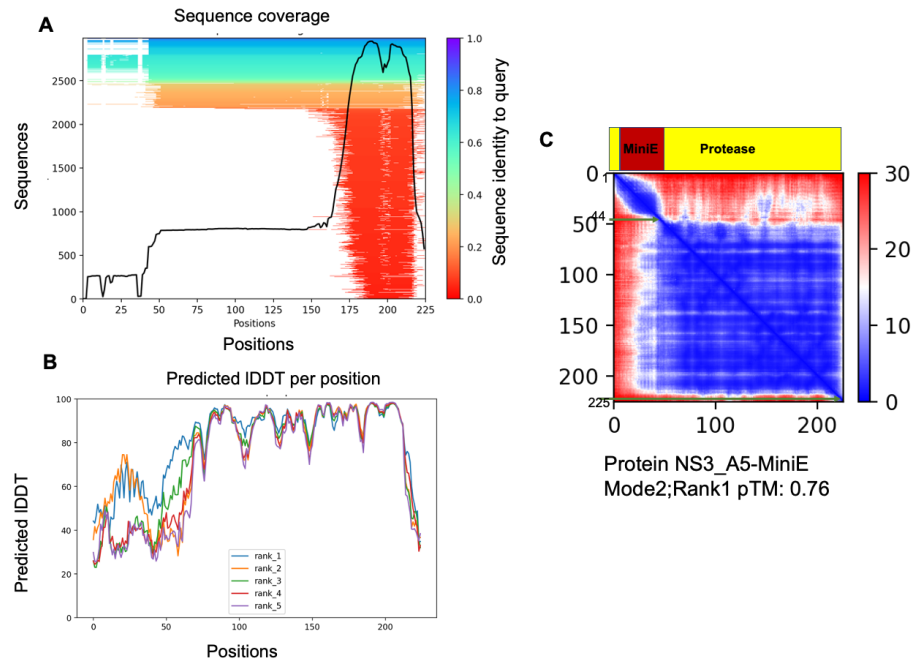
JFH-1 NS3_D25-MiniE



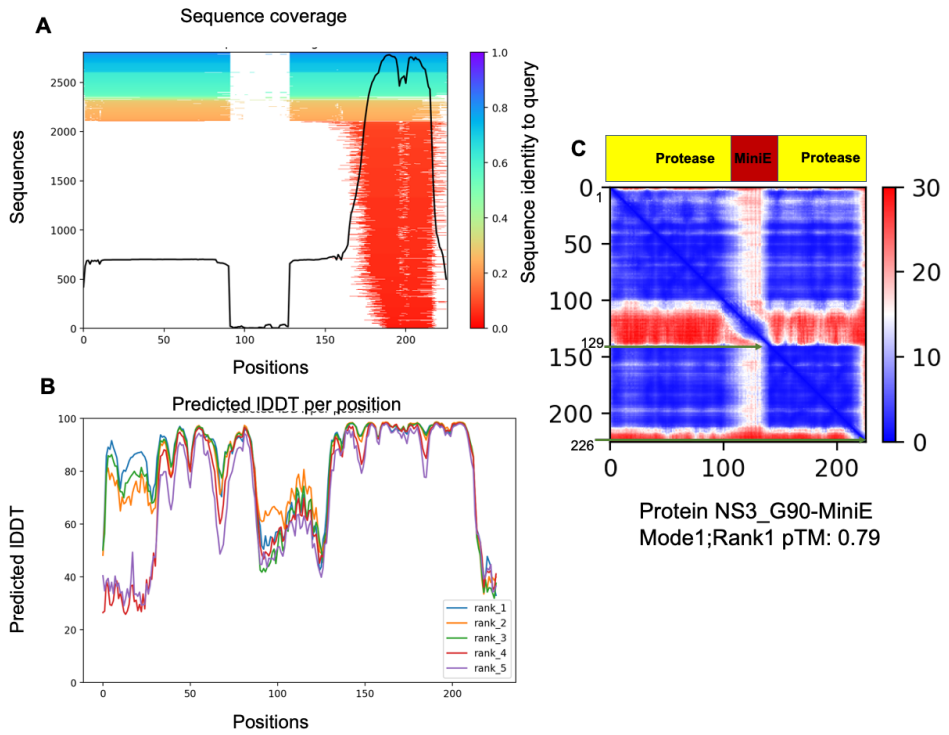
JFH-1 NS3_P2-MiniE



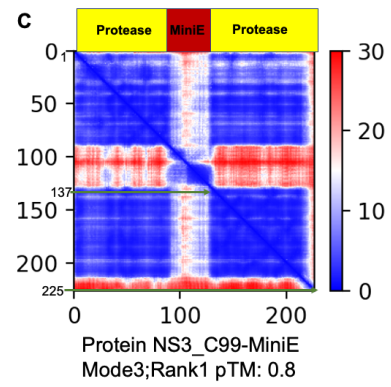
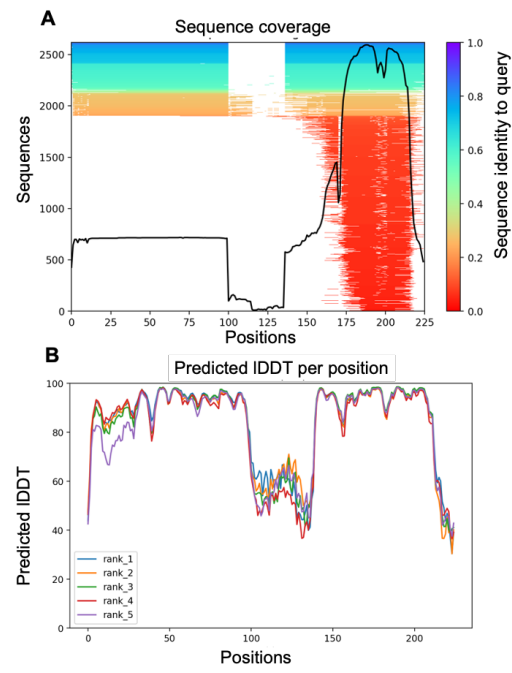
JFH-1 NS3_A5-MiniE



JFH-1 NS3_G90-MiniE



JFH-1 NS3_C99-MiniE



JFH-1 NS3_E628-MiniE

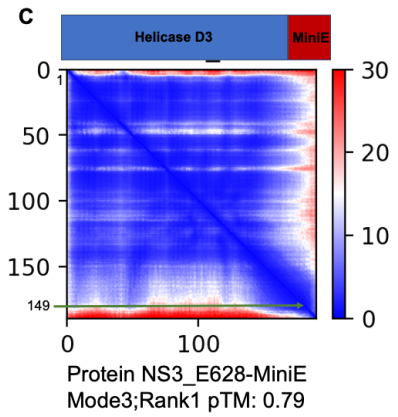
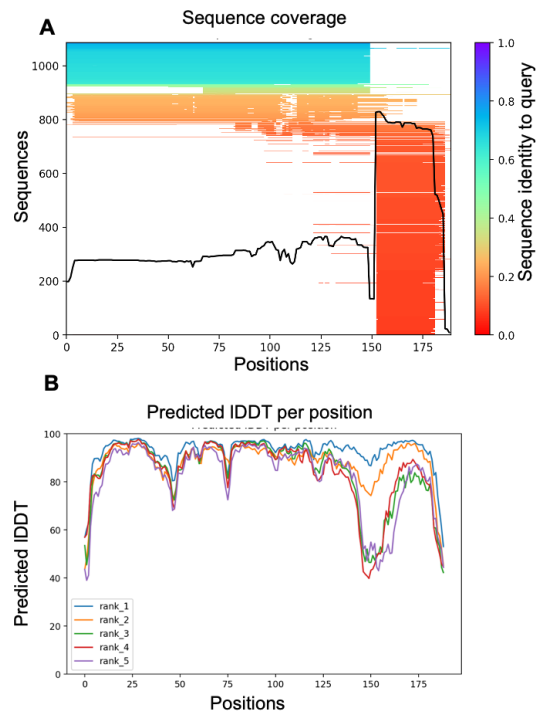


Table 9: Insertion sites with the linkers used for the MiniE tag in NS proteins (corresponds to structural predictions shown in Chapter 5 Figure 5.13). [A] Heterodimer structure of MiniE (red) and MiniR (olive). MiniE and MiniR residues forming a loop is depicted in salmon. [B] MiniE (red) inserted downstream of eGFP (green) residues connected with linker(grey). [C] MiniE (red) inserted in NS3 protein at position M0 (bold-black) residues connected with linker (light green), followed by NS3 sequence. [D] MiniE (red) inserted in NS3 protein at position D25 (bold-black) residues connected with linker (light green), followed by NS3 sequence. [E] MiniE (red) inserted in NS3 protein at position P2 (bold-black) residues connected with linker (light green), followed by NS3 sequence. [F] MiniE (red) inserted in NS3 protein at position A5 (bold-black) residues connected with linker (light green), followed by NS3 sequence. [G] MiniE (red) inserted in NS3 protein at position G90 (bold-black) residues connected with linker (light green), followed by NS3 sequence. [H] MiniE (red) inserted in NS3 protein at position C99 (bold-black) residues connected with linker (light green), followed by NS3 sequence. [I] [D] MiniE (red) inserted in NS3 protein at position E628 (bold-black) residues connected with linker (light green), followed by NS3 sequence.

A	MiniE tag (36 aa):	LEIEAAFLERENTALETRVAELRQRVQRLRNEYGPL
A	MiniR probe (60 aa):	LEIRVAFLRQRNTALRTEVAELEQEVQRLENRYGPL GGGAAAWGLCYPWVYGLEHHHHHH
B	NS5A: insertion site P418-eGFP-MiniE	GMDELYKSGLSR(MiniE)
C	NS3: insertion site M0	MSGL(MiniE)GLSGTAPI
D	NS3: insertion site D5	MAPITAYAQQTRGLLGAIVVSM TGRD SGL(MiniE)GL SRT
E	NS3 insertion site P2	MAP(MiniE)APITRT
F	NS3: insertion site A5	MAPITAG(MiniE)AQQT
G	NS3: insertion site G90	SPPGG(MiniE)TKSLE
H	NS3: insertion site C99	SPPGTKSLEPCKC(MiniE)GAVD
I	NS3: insertion site E628	ADLEG(MiniE)VMT

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