Single-Molecule Super-Resolution Microscopy Reveals Formation of NS2B3 Protein Clusters on Mitochondrial Network Leading to its Fragmentation during the Onset of Dengue (Denv-2) Viral Infection

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NS2B/NS3 complex is a key protein complex essential for proteolytic activity and processing of viral polyprotein during Dengue (Denv-2) infection. The underlying mechanism involved in the early onset (first 24 hrs) of Dengue pathogenesis was studied using single molecule-based super-resolution studies to understand the Denv-2 infection. The study was conducted on transfected NIH3T3 cells using two distinct photoactivable fusion plasmid DNAs ($mEos3.2-NS2B/NS3$ and $paGFP-NS2B/NS3$). Studies demonstrated that the formation of NS2B/NS3 clusters ($mEos3.2-NS2B/NS3$ and $paGFP-NS2B3$) on the mitochondrial network induces mitochondrial fragmentation. The NS2B/NS3 complex acts as a protease that clips specific sites of mitofusin (MFN1/2) proteins, responsible for fusion which holds the network together, disrupting the mitochondrial network. Statistical analysis of super-resolution data (images) estimates an average NS2B/NS3 cluster size of 250 nm with a density of $5.82\times10^2 \#mol./\mu m^2$, and an average of 164 molecules per cluster. Based on the present study, we hypothesize that the formation of clusters and the associated cluster-related parameters are critical in promoting mitochondrial fragmentation. Overall, the single molecule-based super-resolution study helped reveal the basic mechanism of single-molecule (NS2B/NS3) clustering during the onset of Dengue viral infection. Understanding the underlying biophysical mechanism of NS2B/NS3 clustering at the single molecule level may help decipher potential drug targets and the mechanisms of action to disrupt the NS2B/NS3 clusters, which may ultimately usher the way to contain/treat Dengue viral infection.

I. INTRODUCTION

Dengue virus (Denv) is an enveloped virus that consists of capsid protein (C) and a positive single-stranded RNA molecule. The Denv genome encodes a polyprotein which ultimately produces ten proteins after cellular processing, three structured proteins (C, prM/M, E) and seven non-structured proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Out of these proteins, it is the non-structural proteins that are directly involved in viral replication and assembly [13] [14] [15]. NS2B, a non-structural protease subunit, promotes conformational changes in the NS3 structure [16]. Specifically, NS3 protein has the distinct ability to cleave parts of polyprotein precursor and is actively involved in viral replication due to its ATPase/helicase and RNA triphosphatase domain in the C-terminal region [20] [18] [19]. Together, NS2B/NS3 (in short, NS2B3) is a highly conserved protein and forms a weak non-covalent complex. The NS2B3 complex is responsible for proteolytic activity and processing of viral polyprotein at the junctions with other non-structured proteins (NS2B-NS3, NS2A-NS2B, NS3-NS4A, NS4B-NS5) [17]. The NS2B-NS3 protease is crucial in correctly processing viral polyproteins during Dengue viral infection.

The NS2B3, a unique protease, helps the Dengue virus enter, mature, replicate, and exit from the infected cell. Like most flaviviruses, Dengue virus (Denv) uses the host cellular mechanism to multiply and infect other healthy cells [1] [2]. However, little is known about what transpires at the single molecule level, their interactions, and their distribution during infection. Such information is vital to understand the underlying mechanism and selectively drug targets to disrupt the onset of these processes, hoping to inhibit the chain of viral infection.

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Studies over the years suggest that the NS2B3 protein complex interacts with several cellular proteins, which is essential for Den entry to new Denv virion exit [5] [6] [7]. Denv infection begins with virion E protein binding to the host cell surface ligands [8]. The Denv up on receptor-mediated endocytosis releases viral RNA genome into host cytosol and starts producing single viral polyprotein. The polyprotein using host cellular machinery produces ten vital viral structural and non-structural proteins. In conjunction with host cellular proteins, the viral proteins facilitate RNA replication, budding, and complete virion maturation [11]. Denv virion synthesis, maturation to release utilizes all the host organelles involved in protein expression to maturation. At the endoplasmic reticulum (ER) membrane, the NS3 protein interacts with fatty acid synthase resulting in enhanced fatty acid biosynthesis, which increases virion packaging [12]. The virion matures in the Golgi bodies, and the Golgi network helps it release into the extracellular region [21] [10].

Recent studies reported that NS2B3 protease targets host mitochondria, which induces matrix-localized GrpE protein homolog 1 (GrpEL1) cleavage. The GrpEL1 protein, a cochaperone of the Hsp-70 protein, cleavage led to the dysfunction of the Hsp-70 protein, hampering overall protein folding. Thus, GrpE1 cleavage leads to mitochondria and mitochondrial-associated membranes (MAMs) disruption and micro-fusion (MFN1 and 2), causing mitochondrial fragmentation [31]. NS2B3 proteases induce mitochondrial disruption responsible for inducing apoptosis in human medulloblastoma cells. It also blocks RIG-1 and MDA-5 translocation to the mitochondria from the cytosol leading to suppression of the host immune response [32]. However, the exact consequences of NS2B3 protease dysfunction at the molecular level have not been studied. Here we studied the interaction of NS2B3 protease with the mitochondrial matrix and evaluated its fragmentation mechanism at the single-molecule level. Most of the existing studies are performed

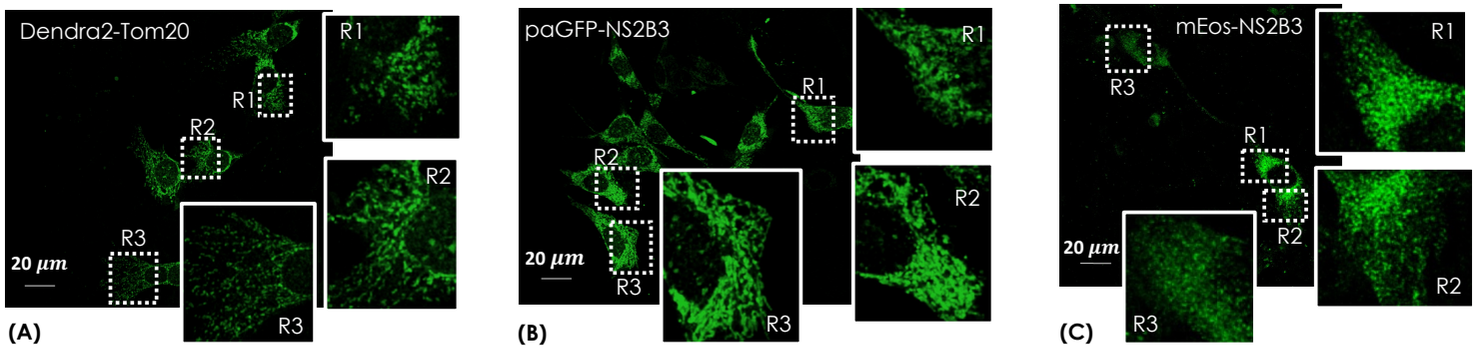

FIG. 1: **DNA Plasmid Transfection Study:** (A) Localization of Dendra2-Tom20 protein on mitochondrial network. (B,C) Transfected NIH3T3 cells show localization of complex viral proteins, $paGFP-NS2B/NS3$ and $mEos-NS2B/NS3$ on mitochondrial network. Few enlarged sections (R1, R2 and R3) are also shown. Scale bar = 20 μm .

by diffraction-limited microscopy techniques known to give ensemble information. Hence, the dynamics and related kinetics at a single molecule or cluster level were never revealed.

This report presents the Denv infection mechanism at a single molecule level for the first time using single-molecule localization microscopy (SMLM). Specifically, we study the dynamics of NS2B/NS3 complex mechanism of action post-entry in the cellular system. We investigate the collective dynamics of single molecules by suitably fusing the NS2B3 protein with a photoactivable protein. Studying the NS2B3 interaction mechanism at the molecular level has allowed us to decipher processes both at the single-molecule and cluster levels. Related biophysical parameters (cluster size, density, and copy number) related to the viral protein complex are critical in assessing the progress of the Denv-2 infection process.

II. RESULTS

Over the last few decades, disease biology of Dengue has advanced substantially with the advance of modern microscopes and biomolecular analysis. Mitochondrial damage due to dengue infection is revealed in confocal microscopy as reported by many researchers [21] [22] [23] [25]. Dengue protease, NS2B3 interferes mitochondrial fusion and mitochondrial dynamics by cleaving mitochondrial outer membrane protein mitofusin 1 and mitofusin 2 (MFN1/2) [21]. Specifically, mitofusins help in mitochondrial fusion by tethering adjacent mitochondria [24], and decreased mitofusin activity leads to mitochondrial fragmentation [26]. It is found that, MFN1 positively regulates host antiviral signaling and MFN2 is responsible for mitochondrial membrane potential during DENV infection. The cleavage of both MFNs by DENV protease would attenuate interferon production and promotes dengue-induced cell death [21]. But still, it is not well known about the mode of attachment of NS2B3 protease complex on mitochondrial membrane and its fate after attachment. There is no information available at the single molecule level and thus the underlying mechanism is unknown. It requires sophisticated super-resolution microscopy and single-molecule-based biological protocols. A better understanding of the infection process may lead to precision drug targeting and selective therapy for the disease.

In the present study, we investigate the role of NS2B/NS3 complex post entry to the host cell. To accomplish this, we need appropriate fluorescent probes in order to localize the site or organelle of activity. Two photoactivable probes are designed : paGFP-NS2B/NS3 and mEos-NS2B/NS3. Recombinant plasmid pPAGFP-NS2B3 was generated by subcloning of NS2B3 protease from $pcDNA-DENV2-NS2B3-V5$, a Gift from Alan Rothman [27] at Pst1- BamH1 site of PAGFP C1 plasmid, a gift from Jennifer Lippincott-Schwartz [28]. NS2B3 protease with PstI- BamHI overhangs were generated by polymerase chain reaction (PCR) using NS2B3-specific oligonucleotide primers, NSPstFP with Pst 1 site as forward primer and NSBamRP with Bam H1 site as reverse primer. Recombinant plasmid pmEos-NS2B3 was generated by subcloning of mEos fragment from mEos3.2-TOMM20-N-10, a gift from Michael Davidson [29] at Nhe I- Pst I site of pPAGFP-NS2B3 by removing PAGFP fragment. mEos with NheI-PstI overhangs were generated by PCR using oligonucleotide primers, mEosNheFP with Nhe 1 site as forward primer mEosPstRP with Pst 1 site as reverse primer. PCR was performed using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, India). All restriction enzymes used were purchased from Thermo Fisher Scientific, USA. Sub-cloning and transformation were performed using the standard protocol [30].

The presence of NS2B3 fragment in pPAGFP-NS2B3 was confirmed by PCR and restriction digestion analysis. PCR of pPAGFP-NS2B3 using NS2B3-specific primers gave an amplicon around 2300bp and restriction analysis using the restriction enzyme pair Pst 1 and Bam H1 released a fragment of 2300 bp along with 1Kb ladder. Similarly, PCR of pmEos-NS2B3 using mEos-specific primers gave an amplicon around 700 bp along with 1 Kb ladder confirming the presence of mEos fragment in recombinant vector. The recombinant nature of both pPAGFP-NS2B3 and pmEos-NS2B3 was further confirmed by sequencing. Sequence analysis confirmed the intactness of the reading frame while fusing NS2B3 with the fluorophore (PAGFP/mEos).

The recombinant plasmids, both pPAGFP-NS2B3, and pmEos-NS2B3 were used to transfect 3T3 cells (mouse embryonic fibroblast cell line) to study the interaction of dengue NS2B3 with the cellular organelles. Transfection was performed using 1 μg of plasmid DNA with Lipofectamine

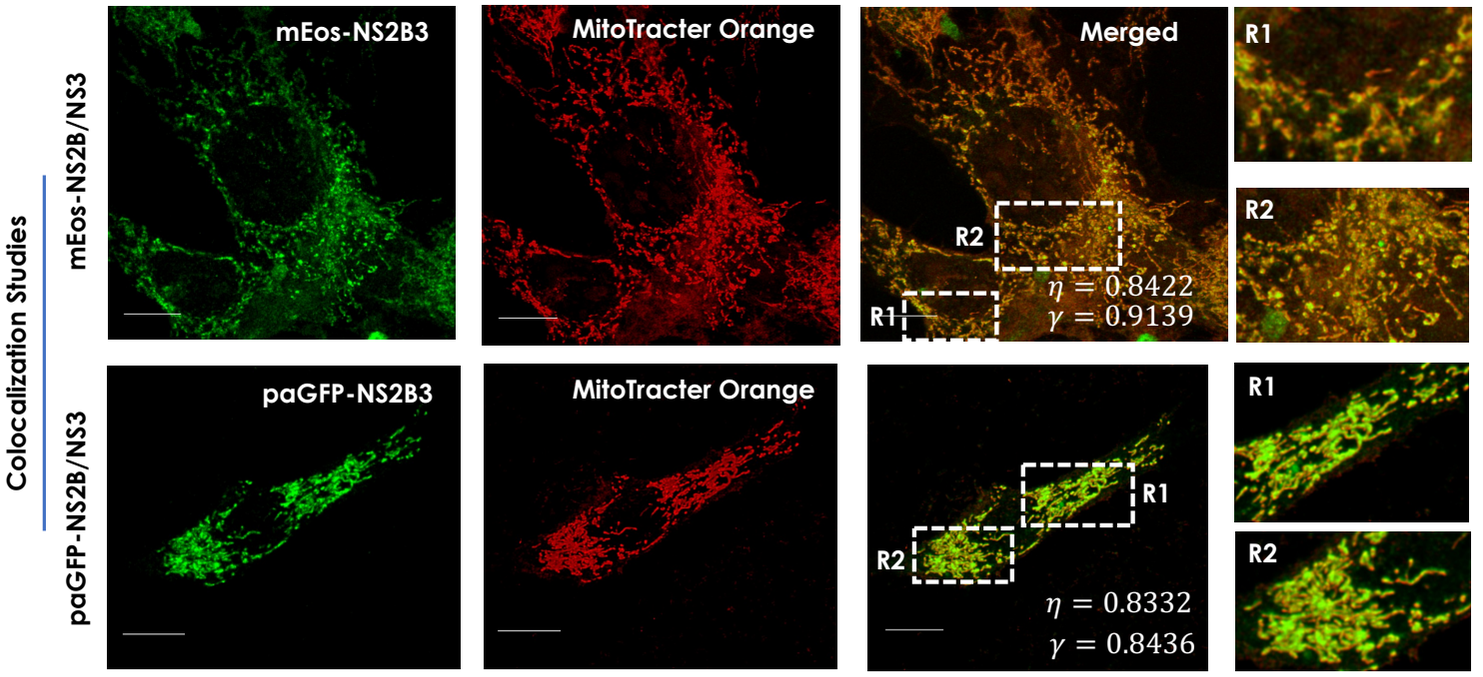

FIG. 2: **Colocalization Study:** (A) Fluorescence images of mEos-NS2B3 transfected NIH3T3 cells labelled with MitoTracker Orange dye, along with the merged image (Pearson's coeff (η) = 0.8422; Overlap coefficient ($\gamma = 0.9139$)). (B) Fluorescence images of paGFP-NS2B3 transfected NIH3T3 cells labelled with MitoTracker Orange dye, along with the merged image ($\eta = 0.8332$; $\gamma = 0.8436$)). Few enlarged sections (R1, R2 and R3) are also shown. Scale bar = 10 μm.

3000 (Invitrogen, USA) according to manufactures instructions. The cells were cultured on coverslip in a 35mm dish with a density of $10^5 cells/ml$. After 12h incubation cells were transfected with respective plasmids. Cells transfected with Dendra2-Tom20 (A gift from Samuel T. Hess, University of Maine, Orono, USA) was used as control. Cells were incubated for 24 hrs and fixed with 3.7% (W/V) paraformaldehyde for 15 min.

Fig.1 shows NIH3T3 cells transfected with paGFP-NS2B/NS3 recombinant plasmids DNA followed by standard protocol of washing, culture and incubation for 24 hrs. As a control, cells were also transfected by a standard Dendra2-Tom20 plasmid in a separate experiment. The transfection was confirmed by exposing it to blue light and observing the fluorescence in green. It is clear that paGFP-NS2B/NS3 protein complex is expressed and located on mitochondrial membrane. This can be ascertained by the control experiment where a known and targeted protein Dendra2-Tom20 is expressed on mitochondrial membrane. Few enlarged images of individual cells are also shown in Fig. 1. To ascertain the presence of paGFP-NS2B/NS3 protein complex on the mitocondrial network, we have carried out colocalization studies. The experiment involves transfecting NIH3T3 cells with mEos-NS2B3 recombinant plasmid. Upon expression of the mEos-NS2B3 recombinant protein, the cells were washed and labelling with MitoTracker orange (Invitrogen, USA). The specimen is then fixed following standard protocol and imaged using confocal microscopy as shown in Fig. 2. It is evident that, mEos-NS2B3 recombinant protein and MitoTracker orange show high degree of colocalization. This can be visually confirmed from the merged images (see, Fig.2A) and the enlarged section (R1 and R2). The corresponding statistical analysis based on Pearson's coefficient ($\eta = 0.8422$) and overlap coefficient ($\gamma = 0.9139$) further confirm strong colocalization of mEos-NS2B3 and MitoTracker orange. A similar experiment is repeated with paGFP-Ns2B3 plasmid as shown in Fig.2B. Again, colocalization of paGFP-Ns2B3 protein and MitoTracker orange demonstrate strong colocalization both visually (see, Fig.2B, merged and enlarged images) and statistical analysis ($\eta = 0.8332$ and $\gamma = 0.8436$). Overall, these experiments confirm the location of viral complex $NS2B3$ on mitochondrial network confirming mitochondria as its target organelle during the onset of Denv-2 viral infection (post 24 hours).

The next step is to probe and analyze the confocal images for accessing the effect of paGFP-NS2B/NS3 protein complex on mitochondrial network. Fig. 3 shows mitochondrial network for paGFP-NS2B3 transfected cell and the control experiment (Dendra2-Tom20 transfected cells). The acquired images are subjected to edge-detection using standard inbuilt Matlab scripts as shown in Fig. 3. Visually, the first observation suggests the presence of small segments suggesting fragmentation of mitochondrial network in paGFP-NS2B/NS3 transfected cells. This can be further confirmed by comparing it with control experiment that shows long connected mitochondrial network. To quantize, we carried out statistical analysis related to segment size histogram, number of fragments and image entropy. Segment size analysis clearly indicate the presence of shorter segments for paGFP-NS2B/NS3 (average size $\sim$ 7.66 μm) and mEos-NS2B/NS3 (average size $\sim$ 9.29 μm) transfected cells as compared to control (average size $\sim$ 14.64). This confirms breakdown of mitochondrial network by the viral complex NS2B/NS3. In addition, the number of segments have increased by more than 2-fold for NS2B/NS3 transfected cells (see, Fig. 3D). Moreover, we have used Shannon entropy measure to access randomness (distribution of fragments)

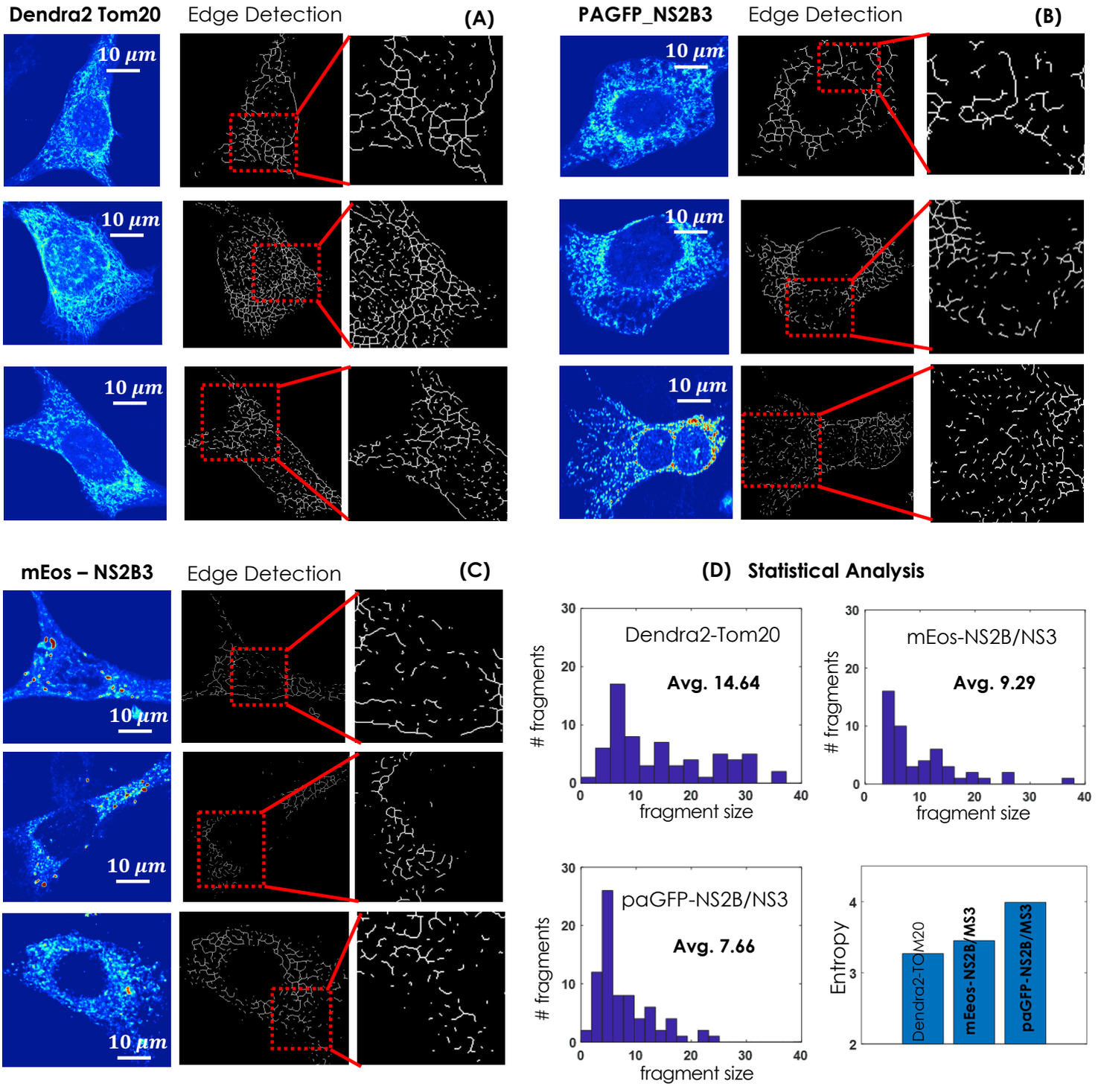

FIG. 3: **Mitochondrial Network Fragmentation Study:** (A) Dendra2-Tom20 transfected NIH3T3 cells along with edge-detection analysis suggests an intact connected mitochondrial network (enlarged sections are also shown). (B,C) Viral plasmid DNA ($paGFP-NS2B/NS3$ and $mEos-NS2B/NS3$) transfected cells and edge-detection analysis strongly suggests fragmented mitochondrial network. This is better visualized in the enlarged view of selected region. (D) Statistical analysis based on the fragment length indicate an an average decrease in the mitochondrial segment length for ... ($mEos-NS2B/NS3 \sim 9.29\mu m$ and $paGFP-NS2B/NS3) \sim 7.66\mu m$ as compared to the control ($Dendra2-Tom20 \sim 14.64\mu m$) with a substantial increase in the fraction of smaller segments. The entropy plot shows increase in randomness for viral transfected cells ($mEos-NS2B/NS3$ and $paGFP-NS2B/NS3$) as compared to control due to fragmentation of mitochondrial network scattered randonly throughout the cell cytoplasm.

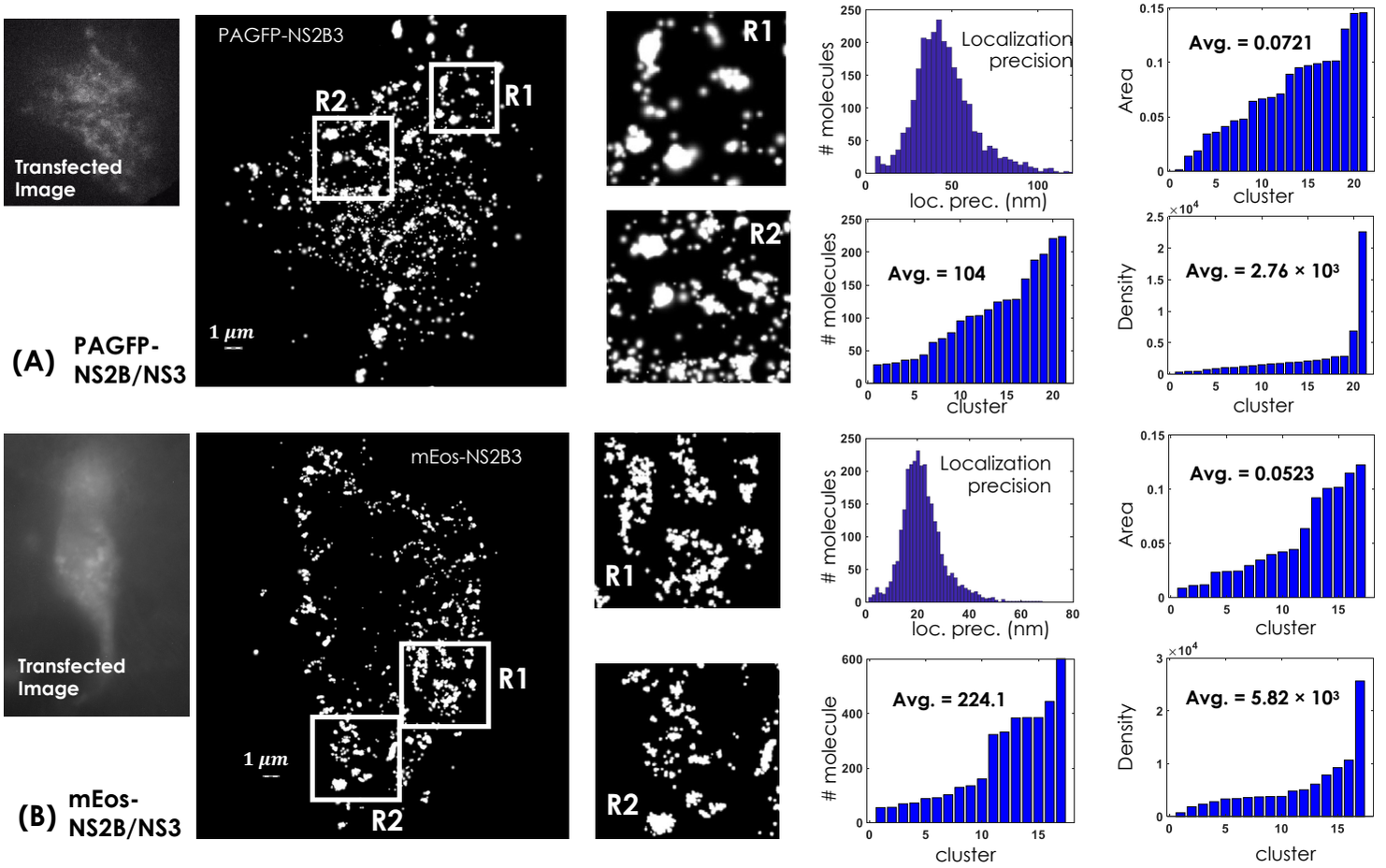

FIG. 4: **NS2B/NS3 Clustering on Mitochondrial Network:** Super-resolution study on the viral plasmid DNA ($paGFP-NS2B/NS3$ and $mEos-NS2B/NS3$) transfected cells suggests clustering of viral proteins on mitochondrial network. This is further confirmed by enlarged section of selected regions (R1 and R2). The corresponding localization plot indicate an average localization precision of < 20 nm. The estimated biophysical parameters suggests an average cluster size of 0.065 μm^2 with a cluster density between 2.76 to 5.82 $\times 10^3$ $mol./\mu m^2$.

in each case. For control, non-treated cells are expected to preserve the connectedness in mitochondrial network which is quite evident for control suggesting low entropy. On the other hand, paGFP-NS2B3 and mEos-NS2B3 transfected cells show relatively high entropy. Clearly, the confocal experiments show short segments of randomly-distributed mitochondrial network bring about by the viral complex NS2B/NS3.

Next we set on to understand the behaviour of these viral particles (NS2B/NS3 complex) at single molecule level. We employed super-resolution localization microscopy to undertand the distribution of these viral particles on mitochondria that lead to its fragmentation. Fig. 4 shows super-resolved images of paGFP-NS2B/NS3 and mEos-NS2B/NS3 transfected cells along with the respective transfected images. The studies were performed on fixed cells after 24 hours of incubation post transfection using standard protocol. One can readily see the appearance of single molecule clusters indicating formation of NS2B3 clusters. The fact that viral particles accumulates on mitochondrial network and form clusters suggest clustering as a precursor that ultimately leads to the fragmentation of mitochondrial network. Corresponding localization precision analysis show a mean resolution of 20 nm. It is due to the high-resolvability of super-resolution microscopy we are able to see and analyse distinct disconnected viral clusters. Further analysis show a cluster size (Area) of $0.0622\mu m^2$, with a cluster density of 4.29×10^3 $\#mol/\mu m^2$ and an average of 164 $mol/cluster$. Overall, these biophysical parameters related to viral clustering on mitochondrial network has a direct bearing on Dengue infection.

Based on the limited results and ongoing experiments, we propose the underlying biological mechanism leading to Dengue infection. Fig. 5 shows the detailed mechanism based on the existing literature and present findings. In our experiment, lipo-complex containing the plasmid enters the cell through endocytosis, followed by uncoating of lipo-complex [25]. Then the plasmid DNA enters the nucleus and the synthesis of mRNA takes place via the process of transcription. mRNA will be carried to the Ribosomes located on ER followed by translation and post-translation modifications (expression of NS2B/NS3 protease). The mature protease are then directed to mitochondrial membrane protein (Mitofusions 1/2) that regulates mitochondrial fusion. The viral complex (NS2B3) form clusters on mitochondrial network, and upon reaching a critical number and density leads to its fragmentation. Specifically, NS2B3 acts on specific sites of mitofusions (region between coiled-coil domain 1 (HR 1) and the transmembrane domain (TM)), leading to its cleavage. This separates the GTPase domain of mitofusins from coiled-coil domain 2 (HR 2), which leads to

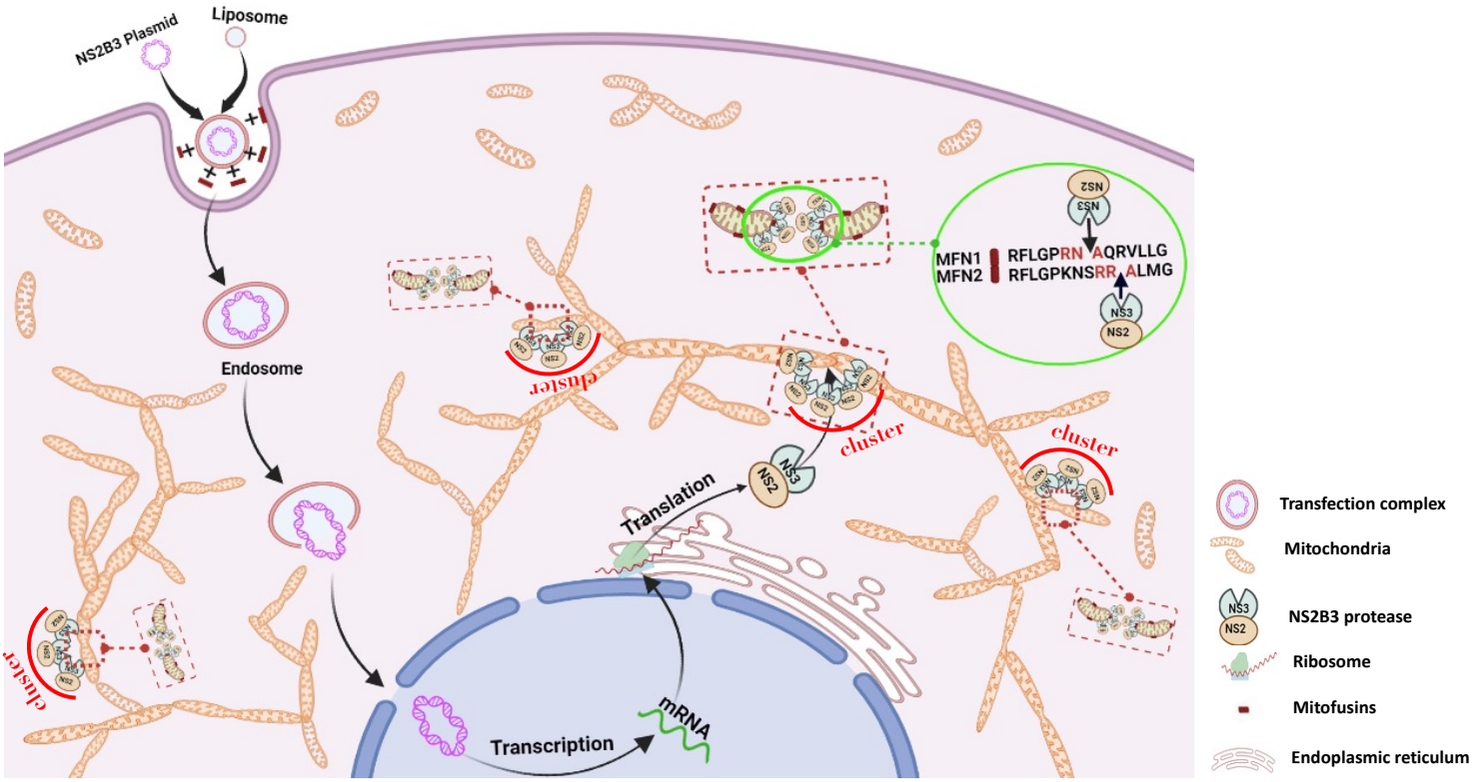

FIG. 5: **The Biological Mechanism:** The experimental evidence suggests entry by endocytosis, followed by uncoating, transcription, translation of the viral complex (NS2B/NS3). These complex protease are then guided to the the mitochondrial network where it forms cluster (marked by red semi-circle) and subsequently cleaves MF1/2 fusion proteins at GTPase domain (shown by green circle) leading to its disintegration (shown by red dotted box). This cause an avalanche effect leading to the fragmentation of mitochondrial effect, ultimately dismantling its function. All other organelles and proteins are also shown for completeness.

its disintegration. This ultimately cause the fragmentation of mitochondrial network leading to cell apoptosis.

Discussions

NS2B3, a protease, acts on the mitochondrial membrane fusion proteins (MFN1/MFN2), leading to its breakdown. Previous studies have shown NS2B3 using innate mitochondrial transport signal (MTS) to get into mitochondrial network [31]. In the present study, we report the accumulation and clustering of the NS2B3 complex on the mitochondrial membrane that leads to the fragmentation of the mitochondrial network. The in-vitro study, using purified proteins, reduced levels of matrix-localized GrpEL1, a cochaperone of mitochondrial Hsp70 (mtHsp70), noticed in NS2B3 expressing Denv-infected cells is due to GrpEL1 cleavage. However, in-situ details studies about the biophysical properties of NS2B3 leading to the mitochondrial matrix have not been studied due to diffraction-limited resolution of existing microscopes. In this study, we used super-resolution microscopy that can decipher details at single-molecule level to reveal the formation of NS2B3 protein clusters on the mitochondrial network, leading to its fragmentation.

Our study based on single molecule imaging indicates that the clustering of NS2B3 single molecules occurs at the mitochondrial matrix leading to matrix fragmentation. Further, transient-transfection and protein import studies demonstrate that having a critical number (≥ 164) is essential for cleaving the mitochondrial network. Additionally, a relatively large number of short fragments of the matrix (7.66 μm for PAGFP-NS2B3 and 9.29μm for $mEos-NS2B3$ transfected cells) was observed in NS2B3 expressing cells, compared to long-sized fragments (14.64μm) in control ($Dendra2-Tom20$). (Figure 3). Super-resolution microscopic study estimated critical values for the biophysical parameters (cluster size (Area) of 0.0622 μm^2, cluster density of $4.29 \times 10^3 \#mol/\mu m^2$ and an average of 164 $mol./cluster$) that are essential for the mitochondrial fragmentation initiation in NS2B3 transfected cells. The study is critical since this mimics the role of the NS2B3 complex during actual Denv-2 viral infection.

It may be noted that clustering of the viral complex is a prerequisite for the fragmentation of mitochondrial network. Previous studies have indicated that NS2B3 complex-induced cleavage of GrpEL1, a matrix protein is a major cause of mitochondrial matrix fragmentation [31]. GrpEL1 cochaperones are essential for the activity of mtHsp70, which is involved in various functions such as protein import, folding, redox homeostasis, and Fe-S cluster biogenesis [33]. During the early phase of Denv infection, partially GrpEL2 replaces the functions of GrpEL1. However, NS2B3 protease cleavage depletion of GrpEL1 led to dysfunctional mitochondria [34] ultimately, take-over mitochondrial machinery critical roles in the cells, such as innate immunity [35], apoptosis [35], cellular metabolism [36], and aging [37] to favor viral production.

Although more direct evidence may be necessary to establish the mechanism, the basic understanding of clustering can be of the therapeutic potential that may be exploited to design new drug targets or may help explore existing FDA-approved drugs. Specifically, any drug candidate that can intervene in the process of viral clustering or reverse it may be an effective strategy to stop/contain severe Dengue infection with the hope that the immune system will take rest care.

Supplementary Information

The work is supported by 4 supplementary detailing information related to recombinant plasmids, the plasmid map, amino acid sequence, and Materials & Methods.

Contributions

PPM conceived the idea. JMV and PPM designed the experiments. JMV, PJ, PPM carried out the experiments. JVM, PJ and AS analyzed the data. PJ and AS wrote the Matlab codes for analysis. PPM and JMV wrote the manuscript by taking inputs from others.

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