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Invited Review

Microscopy techniques in flavivirus research

Mun Keat Chong[a,1], Anthony Jin Shun Chua[a,b,1], Terence Tze Tong Tan[a], Suat Hoon Tan[c], Mah Lee Ng[a,b,c,*]

[a] Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore, 5 Science Drive 2, MD4 Level 3, Singapore 117545, Singapore
[b] NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Centre for Life Sciences (CeLS), 28 Medical Drive, #05-01, Singapore 117456, Singapore
[c] Electron Microscopy Unit, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore, 12 Medical Drive, MD5, Singapore 117597, Singapore

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ABSTRACT

The Flavivirus genus is composed of many medically important viruses that cause high morbidity and mortality, which include Dengue and West Nile viruses. Various molecular and biochemical techniques have been developed in the endeavour to study flaviviruses. However, microscopy techniques still have irreplaceable roles in the identification of novel virus pathogens and characterization of morphological changes in virus-infected cells. Fluorescence microscopy contributes greatly in understanding the fundamental viral protein localizations and virus–host protein interactions during infection. Electron microscopy remains the gold standard for visualizing ultra-structural features of virus particles and infected cells. New imaging techniques and combinatory applications are continuously being developed to push the limit of resolution and extract more quantitative data. Currently, correlative live cell imaging and high resolution three-dimensional imaging have already been achieved through the tandem use of optical and electron microscopy in analyzing biological specimens. Microscopy techniques are also used to measure protein binding affinities and determine the mobility pattern of proteins in cells. This chapter will consolidate on the applications of various well-established microscopy techniques in flavivirus research, and discuss how recently developed microscopy techniques can potentially help advance our understanding in these membrane viruses.

Contents

1. Introduction

The Flavivirus genus within the *Flaviviridae* virus family comprises numerous medically important pathogens such as Yellow Fever virus (YFV), Dengue virus (DENV), West Nile virus (WNV) and Japanese Encephalitis virus (JEV). Currently, more than half of the world's population (3.6 billion), in over 100 tropical and subtropical

* Corresponding author at: Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore, 5 Science Drive 2, MD4 Level 3, Singapore 117545, Singapore. Tel.: +65 65163283.
E-mail addresses: micngml@nus.edu.sg, mah_lee_ng@nuhs.edu.sg (M.L. Ng).
[1] Co-first authors.

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countries are at risk of Dengue infection. Approximately 390 million Dengue cases are reported annually, of which 96 million patients suffer from the more severe form of the disease – Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS) (Bhatt et al., 2013). West Nile virus infection causes neuroinvasive diseases such as encephalitis, meningitis, dyskinesis, and acute flaccid paralysis that may potentially lead to death. West Nile virus infection has remained the leading cause of viral encephalitis in the United States (Centers for Disease and Prevention, 2012).

Despite recent advances in flavivirus research, vaccine and antiviral therapy for some of these infectious diseases are yet to be available. This highlights the critical need in forwarding our understanding in flavivirus replication; how they infect and subjugate host cells for their own replication, and cause pathological changes. As the average diameter of flaviviruses is approximately 50 nm, microscopy techniques are proven to be one of the most powerful tools in acquiring knowledge on these nanoscopic pathogens. Through direct visualization, the induced pathological changes within infected cells can be dissected. Together with complementary biochemical and molecular studies, microscopy can expedite the development of promising vaccines and antiviral candidates to treat these potentially fatal diseases. In this review, we will provide brief introductions to the principles behind various microscopy techniques, updates on their latest applications in the context of flavivirus research, and discuss how recently developed microscopy techniques can potentially help advance our understanding in these enveloped viruses.

2. Contents

2.1. Bright field light microscopy – morphology of flavivirus-infected cells

The field of microscopy started in the 17th century with the invention of the first microscope by Antonie van Leeuwenhoek. Since then, light microscopes have remained the principle workhorse in Microbiology to date. In Virology, conventional light microscopes are commonly used to examine the stage of virus infection at the cellular level. This is no exception to the study of flaviviruses. Generally, cell death is the ultimate fate of flavivirus-infected cells although persistent infection has been reported in certain cell types (McLean et al., 2011). Using the conventional inverted light microscope, Chu and Ng (2003) demonstrated that in Vero cells, WNV-induced necrosis occurred when the multiplicity of infection (M.O.I.) exceeded 10 whereas apoptosis was initiated when a lower M.O.I. was used. Hallmarks of necrosis and apoptosis were observed at various stages of infection in WNV-infected cells. In C6/36 mosquito cells, DENV serotype 2 produces the signature syncytial phenomenon during infection (Fig. 1), a tell-tale sign of ongoing DENV serotype 2 replication.

2.2. Fluorescence microscopy – study of flaviviral protein localization and live cell imaging of flavivirus

An extension to bright field light microscopy is immunofluorescence microscopy (IFM), which also has a long history in flavivirus research. Even before the development of enzyme-linked immunosorbent assay (ELISA) (Dittmar et al., 1979; Nawa et al., 1985) or polymerase chain reaction (PCR) (Deubel et al., 1990) to detect the presence of DENV, immuno-fluorescence microscopy has already been utilized for diagnostic detection and identification of DENV in infected cell culture and mice (Atchison et al., 1966; Kuberski and Rosen, 1977). One main advantage of IFM is that fluorophore-tagged antibodies can be used to identify specific proteins-of-interest and their locations inside infected cells. This is

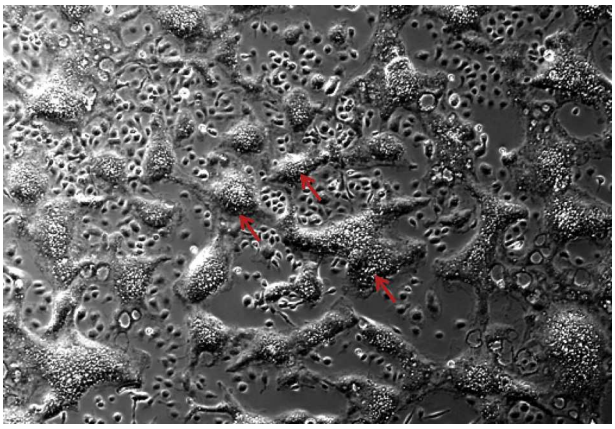

Fig. 1. Dengue virus serotype 2-induced syncytial in C6/36 mosquito cells. At 48-h post infection, multinucleated masses of cytoplasm are detected among C6/36 cells as indicated by red arrows. This signature phenomenon indicates that DENV serotype 2 infection of C6/36 cells is ongoing.

in contrast to bright field light microscopy whereby localizations of proteins-of-interest can only be inferred provided they reside in predetermined subcellular organelles observable under the light microscope. Prior information from complementary biochemical experiments are also mandatory in order for such inference to be made.

Currently, IFM has been widely used to study the presence and localization of viral proteins in infected cells, and in viral–host protein interaction (Mackenzie et al., 1998; Westaway et al., 1999). Through IFM assay, viral protease complex (NS2B and NS3) and NS4A protein of Kunjin virus were shown to be localized in the convoluted membranes (CM) and paracrystalline arrays (PA) whereas the NS1, NS2A, NS3 and NS4A proteins and double-stranded RNA co-localized in flavivirus-induced vesicle packets as the viral replication complex (Mackenzie et al., 1998; Westaway et al., 1997b, 1999). By using various organelle markers, host *trans*-Golgi membrane was found to be the source for the flavivirus-induced CM and PA (Mackenzie et al., 1999). Although flavivirus is a positive-stranded RNA virus, some of the viral proteins were found in the nucleus. The capsid and NS4B proteins, for instance, were found to localize in the infected cell nucleus (Westaway et al., 1997a). Fig. 2 shows the localization of transfected flavivirus capsid and pre-membrane proteins in the cell. Localizations of the viral proteins in different subcellular compartments may hint at their roles and functions in the host cells.

Although conventional light microscopes cannot be used to visualize flaviviruses directly, fluorescent dyes can be used to label flaviviruses to enable their observation under the fluorescence microscope. Zhang et al. (2011) labelled DENV directly with Alexa Fluor succinimidyl ester dye and studied virus entry. Imaging of labelled virus particles can be achieved using real-time time-lapse confocal microscopy. van der Schaar et al. (2007) carried out single-particle tracking (SPT) or real-time fluorescence microscopy of DENV by labelling virus with lipophilic fluorescent probe DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt). The study demonstrated that DENV exhibited three stages of transport behaviour: (1) DENV entered cells and moved at the cell periphery slowly at a speed of less than 0.2 μm/s. (2) DENV then moved towards the nucleus in a unidirectional manner with a speed of more than 0.5 μm/s. (3) Before fusing with the acidic endosomal membrane, DENV either moved at the perinuclear region intermittently with lower speed or travelled back and forth along the same track. Using fluorescently labelled DENV and time-lapse confocal fluorescence microscopy, Teo and colleagues (2012) demonstrated in real-time

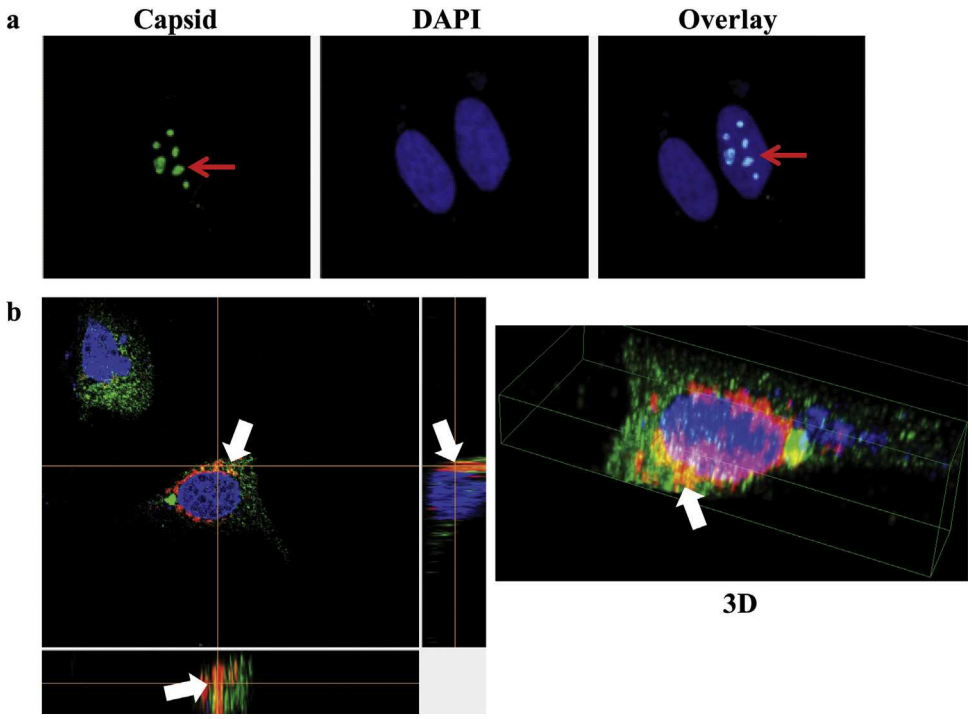

Fig. 2. Localization of flavivirus capsid and pre-membrane proteins using a conventional fluorescence microscope and confocal microscope, respectively. (a) Human embryonic kidney (HEK)-293 cells are transfected with vectors expressing FLAG-tagged capsid protein and the localization of the protein is detected with anti-FLAG antibody followed by Alexa Fluor-488 antibody. DAPI is used to stain the nuclei. Capsid proteins localize in the cell nucleus as indicated by the red arrows. (b) The localization of pre-membrane protein in Baby Hamster Kidney (BHK)-21 cells is detected with anti-pre-membrane protein antibody countered stained with Alexa Fluor-488 secondary antibody. Organelle-Light Golgi N-acetylgalactosaminyltransferase-2 (NGAT2)-RFP is used to label the Golgi apparatus. Co-localization of West Nile virus pre-membrane protein with the Golgi apparatus is indicated by white bold arrows in three dimensional views.

the direct neutralization of DENV by a fluorescent-labelled anti-DENV-1 human antibody, HM14c10.

Although direct fluorescence labelling of flaviviruses primarily enabled the live recording of interactions between wild-type flavivirus virions and host cells, there are still limitations to this technique that users should be aware of. IFM is essentially a light microscopy technique and has a light diffraction limit of approximately 200 nm. Thus, fluorescence-labelled virions will still appear as fluorescent punctae of approximately 200 nm in diameter. This implies that differentiation cannot be made between labelled single virions and labelled virions that are clustered together.

2.3. Advanced fluorescence microscopy techniques – further flaviviral protein characterization

Based on the principle of IFM, more advanced fluorescence techniques in microscopy were developed over time and applied in flavivirus research. Fluorescence resonance energy transfer (FRET), for instance, is useful in detecting molecular interactions between two proteins. This method employs the principle of energy transfer between two fluorophores to determine if the two molecules were within a certain distance of each other. When the two molecules are in close proximity due to association, emission from the donor fluorophore is transferred to the acceptor chromophore through long-range dipole-dipole intermolecular coupling. When this occurs, emission by the acceptor fluorophore is observed, instead of that by the donor fluorophore. The FRET efficiency can be measured by examining increased fluorescence emission by the acceptor fluorophore, measuring the change in fluorescence lifetime or the photobleaching rate of the donor in the presence and absence of the acceptor. The most common pair of fluorophores used in FRET is cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP).

In flavivirus research, FRET has being successfully used to study protein oligomerization, viral–viral or viral–host protein–protein interactions, and protease-substrate activities. For instance, the fusogenicity of the fusion peptide of tick-borne encephalitis virus (TBEV) envelope protein was investigated using FRET and the important residue for trimer stability and membrane fusion was identified using this strategy (Pan et al., 2010). In the study of hydrolytic behaviour of YFV NS2B/NS3 protease, FRET substrate was exploited as a tool to determine the catalytic efficiency of the viral protease (Kondo et al., 2011). Through FRET analysis, Yu et al. (2013) also demonstrated the associations among all seven non-structural proteins of West Nile virus. From these studies, it is evident that FRET can be used in an assay to screen potential antiviral drugs that disrupt oligomerization between viral protein(s), pro-viral replication viral–host protein interactions or key viral protease-substrate activities. In addition to capturing FRET changes using microscopy, fluorescence plate readers can also be used to measure kinetic parameters such as K_m, K_{cat}, and K_{cat}/K_m (Liu et al., 1999).

Although acceptor fluorophore emission is an indirect function of the distance between the donor and acceptor fluorophores, FRET does not necessarily always occur when protein partners tagged with the respective donor and acceptor fluorophores interact. The donor and acceptor fluorophores have to be positioned at a certain angle when the protein partners bind in order for FRET to happen (Miyawaki, 2003). Thus, while one can deduce with confidence that two proteins-of-interest are binding when FRET occurs, one cannot exclude the possibility that the protein partners are interacting when FRET is absent. Complementary biochemical experiments such as co-immunoprecipitation should also be performed to confirm the lack of binding between the two proteins-of-interest.

Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are two other advanced IFM

techniques to measure the locomotion of fluorescence particles inside cells. Green fluorescence protein (GFP) is most commonly used to tag a protein-of-interest and a confocal microscope is used to photobleach a defined region-of-interest within a cell where the GFP-tagged protein localizes. By examining the rate at which the green fluorescence is replenished in the photobleached region, the diffusion coefficient of the protein-of-interest or the kinetic coefficient of protein's binding/unbinding reactions can be determined. In cases where the fluorescence level does not recover to the initial level, it implies that some portion of the green fluorescence is immobile and cannot be replenished by diffusion. Likewise, in protein–protein interaction studies whereby one of the interacting partners is static, the recovery of green fluorescence depends on the association and disassociation coefficient of the interaction. Nevertheless, it is worth noting that strong laser photobleaching and long exposure time may result in the quenching of some fluorophores in the region-of-interest, thereby leading to incomplete recovery after photobleaching and affecting the outcome of the quantitative data (Tan et al., 2010).

Rather than measuring the recovery of fluorescence after a single photobleaching event, FLIP monitors the loss of fluorescence in the surrounding of a defined multiple-photobleached area. Like FRAP, FLIP can also be used to study the continuity of membranous organelles using specific fluorescent dyes. As compared to FRAP which is confined to the defined region of photobleaching, FLIP is able to examine protein trafficking between different regions within a cell. For instance, FLIP can be used to measure the exchange rate of proteins shuttling between the nucleus and cytoplasm. In flavivirus research, FRAP technique was employed to track the fate of newly synthesized TBEV genomic RNA. It was found that the newly synthesized RNA was not able to freely diffuse in the cytoplasm and was confined within a defined area of juxtaposed endoplasmic reticulum cisternae during replication (Miorin et al., 2013).

Total internal reflection fluorescence (TIRF) microscopy is another technique used to observe a biological phenomenon within a very shallow depth (usually less than 200 nm) from the surface of a specimen. It uses the evanescent wave produced by total internal reflection at the interface between two media (e.g. specimen and coverslip), to excite fluorophores. As a result, only fluorophores near the surface of a specimen are excited to emit fluorescence. Minimal fluorophores outside the evanescent field are excited, thereby yielding very good signal-to-noise ratio and minimal signal loss during illumination. This technique will be useful in studying molecular events on the cell surface, such as virus-receptor binding, internalization, and egression.

2.4. Super-resolution light microscopy – improved spatial resolution beyond 200 nm

The resolution of light microscopes is limited by the diffraction limit of light. Thus, the highest resolution attainable by any light microscope (fluorescence microscopes included) is only approximately 200 nm. Development of super-resolution microscopy allowed microscopists to break the resolution limit of conventional optical microscopes. Various super-resolution microscopy techniques such as structured illumination microscopy (SIM), spatially modulated illumination (SMI), stochastic optical reconstruction microscopy (STORM), photo-activated localization microscopy (PALM), spectral precision distance microscopy (SPDM) and stimulated emission depletion (STED) have been developed in recent years. Some of these techniques can even be combined. For instance, 3D light microscopical nanosizing (LIMON) microscopy is a combination of SMI and SPDM to achieve a high resolution of approximately 25 nm in all three (x, y and z) planes.

In SIM, patterned light is used to illuminate the sample to generate interference patterns (moiré fringes), which are then used collectively to reconstruct super-resolution images with enhanced spatial resolution. With this technique, images with resolutions up to 100 nm can be obtained and thus more information can be derived from the images. Yi et al. (2012) employed 3D-SIM super-resolution microscope to demonstrate distinct Yellow Fever virus replication complexes comprising dsRNAs, NS3 and NS4B proteins as a clustered protein network. DNAJC14, an HSP40 family protein, was found associated with the replication complexes via direct interaction. The direct interaction between DNAJC14 and the replication complex was further confirmed via co-immunoprecipitation. Super-resolution microscopy can thus provide additional confidence to researchers that two different overlapping fluorescence signals under confocal microscopy indeed belong to two interacting proteins, and not just that the two proteins happen to localize within 200 nm of each other.

Sample preparation for SIM is similar to that for conventional fluorescence microscopy, without the need for complicated labelling processes and/or use of specific dyes. This allows fluorescence microscope users to easily adopt this super-resolution technique. As moiré fringes are generated by wide-field fluorescence illumination, this further allows the technique to capture fast events. Our group has reported its use in imaging the localization of flavivirus-derived cell-penetrating peptides in the membrane of endosomes (Fig. 3). Through SIM, additional information on the specific localization of a cell-penetrating peptide-of-interest in vesicular membrane was obtained, which would otherwise not be possible with conventional fluorescence microscopy methods (Chua et al., 2012). SIM thus holds the promise of imaging fast events during flavivirus internalization, infection and trafficking, and provide unprecedented information about these processes. However, one major drawback users should keep in mind when planning SIM experiments is that SIM quickly photobleaches fluorophores, an inevitable result of the use of direct wide-field fluorescence illumination. We recommend users to use fluorophores that are more resistant to photobleaching, such as the Alexa Fluor series of dyes or quantum dots instead of earlier generations of fluorescent dyes such as fluorescein.

In order to further push the limit of resolution attainable by light, more super-resolution microscopy techniques were developed to obtain higher quality images with resolutions in the nanometer-range. Photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) are two common stochastic functional super resolution microscopy techniques (Huang et al., 2010) achievable with commercially available microscopes. Both techniques utilize photo-switchable fluorophores that can switch between fluorescent and dark states. During imaging, only a small fraction of the fluorophores will be in the fluorescent state such that the precise positions of the fluorophores can be determined from the centroid position of the fluorescent spots. The fluorophores in the snapshot are then deactivated and another subset of the fluorophores is activated. This process is repeated and a final super-resolution image is reconstructed from the image data.

Using the PALM/STORM technique, previously vague morphologies of organelles can now be visualized in great details, thus revealing dynamic processes inside cells. These techniques are useful for single-virus tracking from entry and intra-cellular trafficking for assembly and egress (Brandenburg and Zhuang, 2007). Although the application of PALM/STORM in flavivirus research has not been reported yet, Muranyi et al. (2013) demonstrated the feasibility of capturing the assembly and budding events of HIV-1 using PALM/STORM technique together with TIRF microscopy. Each individual assembly site was imaged as a single punctum with a diameter of approximately 130 nm near the plasma membrane. Using the same super-resolution microscopy techniques,

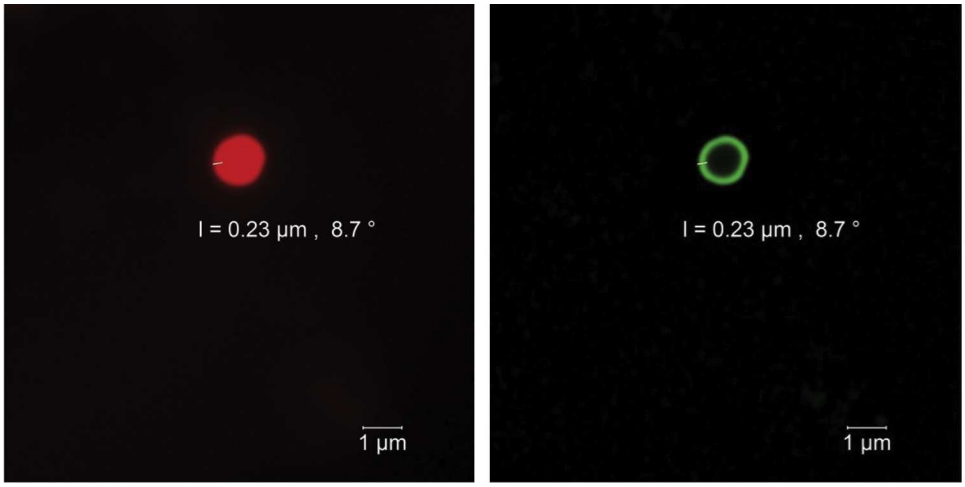

Fig. 3. Elucidation of additional information such as localization of molecules by SIM, which would otherwise not be possible via conventional fluorescence microscopy techniques. The Left Panel shows a micrograph on an endosome containing a fluorescence-tagged flavivirus-derived cell-penetrating peptide (CPP) obtained using a conventional wide-field fluorescence microscope. The Right Panel shows the same endosome after reconstruction via the SIM algorithm. Note that while conventional wide-field fluorescence microscopy can only show the presence of the CPP in the endosome, SIM is able to show specifically its localization in the endosomal membrane.

more information on similar events in the flavivirus replication life-cycle can definitely be elucidated.

2.5. Atomic force microscopy – study of flavivirus-induced events on infected cell surface and measurement of binding force between flaviviral proteins and their interacting partners

Atomic force microscopy (AFM) is an entirely different microscopy technique that is not based on the principle of light microscopy. It is a form of high-resolution scanning probe microscopy which can be used for imaging and probing mechanical properties of cells or receptor–ligand interactions. The major advantages of AFM are that the biological specimen can be studied in its native environment and at a resolution comparable to electron microscopy (EM). No additional chemical treatment or harsh sample preparation procedure is required. The basic principle of AFM is to scan the specimen surface with a cantilever. The cantilever has a nanoscale sharp tip that acts as a probe. When the tip approaches the specimen surface, forces between the tip and the specimen will deflect the cantilever. The deflection will be measured using a laser spot reflected from top of the surface of the cantilever into an array of photodiodes.

Using AFM, Lee and Ng (2004) demonstrated WNV (Sarafend) budding at the infected cell surface in three-dimensions at high resolution (Fig. 4). Thickening of clusters of cytoskeletons and formation of filopodia was also imaged at the periphery of WNV-infected cells (Fig. 5). This yields important information on virus-induced changes in host cells during infection. Other than imaging infected cells, the morphology of DENV particle could also be imaged *in air* using AFM (Ferreira et al., 2008). In this study, the extrusion of viral RNA between DENV particles were visualized and recorded, confirming the phenomenon of cyclization of flavivirus genome during replication.

Other than imaging, AFM can also be used to measure the binding force between two molecules. In flavivirus research, AFM was used to determine the binding force between WNV envelope domain III protein and its putative host cell receptor, $\alpha_V\beta_3$ integrin (Lee et al., 2006) (Fig. 6). Using AFM, it was also found that the DENV capsid protein interacted strongly with lipid droplets and the interaction was dependent on intracellular potassium ion concentration but not sodium ion concentration (Carvalho et al., 2012).

2.6. Electron microscopy – direct visualization of flaviviruses, and flavivirus-induced ultra-structures

Having discussed about the use of conventional light microscopy and super-resolution microscopy techniques in flavivirus research, we will next move on to the application of the most classical microscopy technique used in Virology – electron microscopy – in flavivirus research. Electron microscopy (EM) is a very useful method to visualize ultra-structures in biological specimens and is by far the most powerful imaging tool to visualize virus structures. With the wavelength of electrons being 100,000 times shorter than that of light photons, EM can resolve structures down to 50 pm using magnifications of up to approximately 10^7 times. This allows for elucidation of nanoscopic structures that will otherwise be impossible by light microscopy techniques. Similar to optical lenses in light microscopes, the electron microscope uses electrostatic and electromagnetic lenses to control the electron beam and focusing. When the electrons interact with the specimen, information is obtained.

The first commercial electron microscope produced in 1939 was the transmission electron microscope (TEM). With the help of electrostatic and electromagnetic lenses, it transmits a high voltage electron beam in vacuum, through an ultra-thin specimen. The unscattered electrons are then captured on a fluorescent screen to give an image of the specimen structure. The darkness of the image varies according to the density of the specimen. The denser the specimen, the lesser the number of electrons passing through, the darker the image.

The second type of electron microscope is the scanning electron microscope (SEM). Similar to TEM, SEM directs an electron beam towards the specimen. When the electron beam strikes the specimen surface, some of the electrons will be backscattered. At the same time, X-rays and secondary electrons will be emitted from underneath the specimen surface and from atoms within the specimen, respectively. These backscattered electrons, secondary electrons and X-rays are then detected by special detectors and the information is used to construct the surface topography of the specimen. The most commonly used mode of detection for SEM is via secondary electrons whereby the number of emitted secondary electrons varies according to the angles of collision between the incident electron beam and the specimen surface.

Numerous studies have reported the use of electron microscopy in the identification of novel flaviviruses and in characterizing

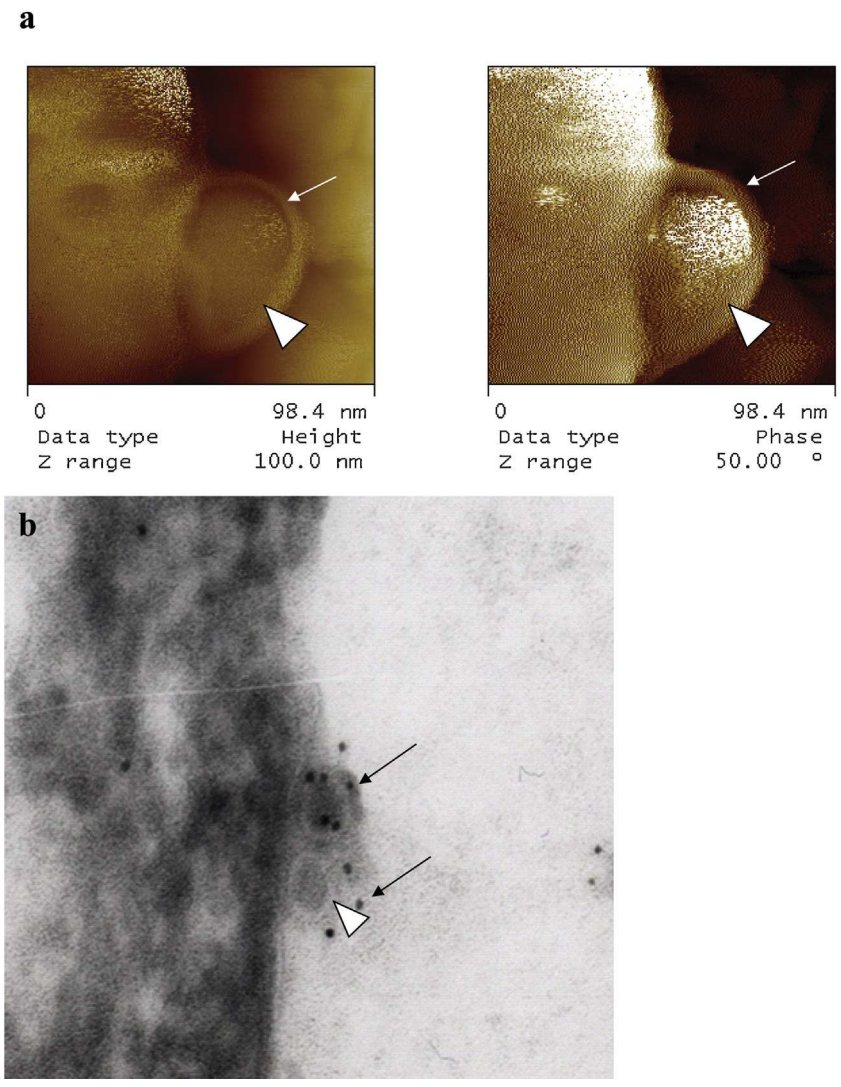

Fig. 4. Atomic force microscopy (AFM) image of West Nile virus (Sarafend) suggests viral budding from Vero cells. (a) Atomic force microscopy (AFM) height and phase data of a WNV-infected Vero cell at 24-h post infection (p.i.). Arrowheads show the emerging progeny virion as it egresses from the plasma membrane of the host cell, while the arrows show the envelope surrounding the virion. Scan size: 98 nm × 98 nm. The images are reproduced with permission from BioMed Central (Lee and Ng, 2004). (b) Immuno-transmission electron microscopy (TEM) image of immunogold-labelled (arrow) WNV (arrowhead) budding from an infected Vero cell at 24 h p.i. Tokuyasu cryosectioning method is employed during processing. This image is included for comparison with the AFM image.

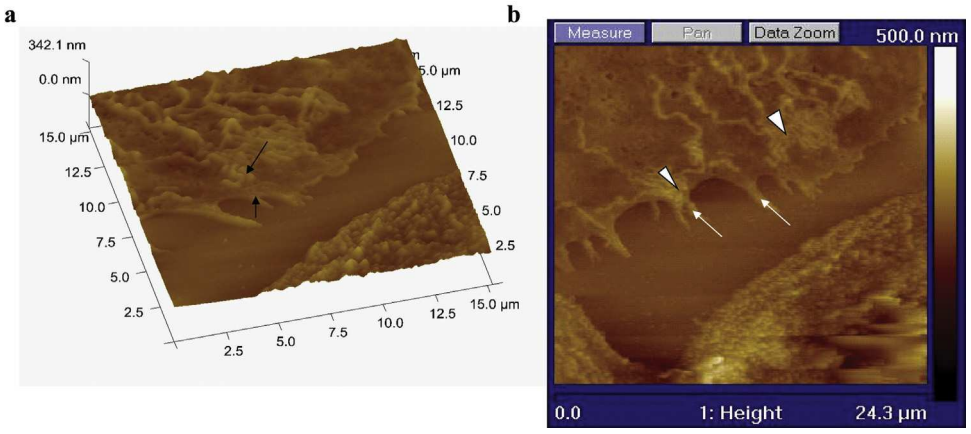

Fig. 5. Atomic force microscopy (AFM) image of West Nile virus (Sarafend)-induced cytoskeletal structures and filopodia. (a) AFM 3D image of two adjacent WNV-infected Vero cells at 16 h p.i. The arrows show the close association of the induced cytoskeletal structures and the filopodia. Scan size: 15.3 µm × 15.3 µm. (b) AFM Height data of a WNV-infected Vero cells at 16 h p.i. The arrowheads show sub-surface cytoskeleton network induction at the plasma membrane. Arrows show the filopodia formed at the edge of the plasma membrane. Scan size: 24.3 µm × 24.3 µm.

The images are reproduced with permission from BioMed Central (Lee and Ng, 2004).

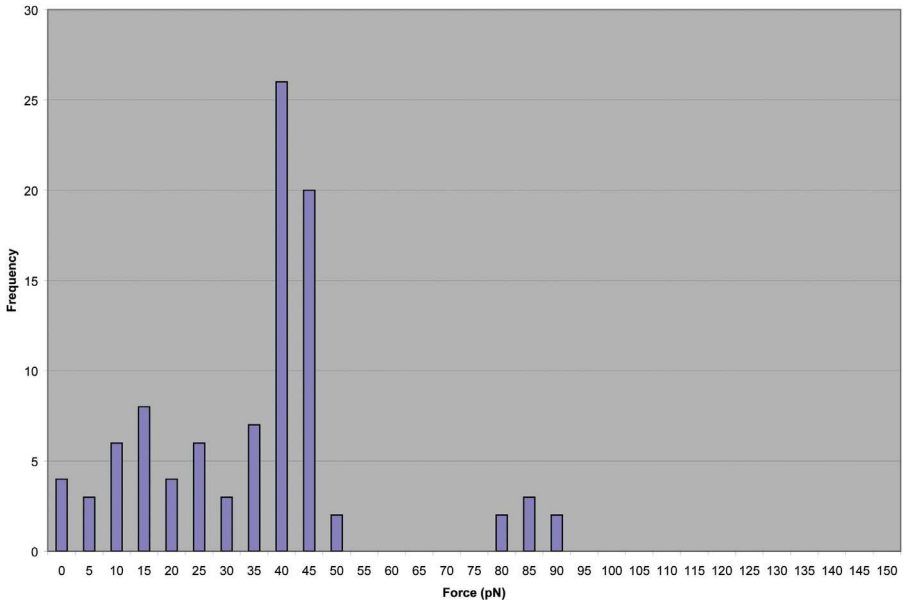

Fig. 6. Force distribution frequency between WNV envelope domain III protein and integrin $\alpha_V\beta_3$. The WNV envelope domain III protein and integrin $\alpha_V\beta_3$ are coated on the probe and the wafer substrate, respectively. The histogram shows that majority of the measured binding force values between WNV E protein domain III and integrin $\alpha_V\beta_3$ are of 45 ± 5 pN.

The images are reproduced with permission from BioMed Central (Lee and Ng, 2004).

pathological changes in the morphology of infected cells during viral replication. TEM micrographs of cells infected with these unknown flaviviruses and/or their negatively-stained culture medium containing extracellular flaviviruses are frequently used to provide visual confirmation through virus morphology typical of flaviviruses (Haddow et al., 2013; Roiz et al., 2012; Yun et al., 2012). Zargar et al. (2011) further capitalized on the application of TEM in visualizing DENV-infected cellular morphology by following the pathological changes step-by-step in a time-dependent manner. Through EM analysis, Barth et al. (1994) found that Brazilian DEN-2 isolates were morphologically different in infected mosquito cells as compared to the New Guinea C reference strain.

Sample preparation protocols for EM studies vary depending on the type of samples to be viewed and the type of analysis to be performed. The most common sample preparation method is by chemical fixation using formaldehyde, glutaraldehyde, and/or osmium tetroxide. Araldite is the most commonly used embedding resin during sample preparation although LR White is preferred when preservation of antigens for immuno-gold labelling in EM is required. Other than conventional chemical fixation, cryo-techniques can also be used to prepare samples for TEM studies. Cryo-substitution is able to better preserve the structure of flavivirus nucleocapsids than chemical processing, and the flavivirus envelope can be clearly differentiated from the nucleocapsid (Ng et al., 1994b). In addition, virus-induced structures are also preserved well (Mackenzie et al., 1996). The advantage of using cryo-technique is that it is milder than chemical treatment, thus preserving the immuno-reactivity of the specimen for immuno-labelling.

Using immuno-electron microscopy (IEM), DENV envelope (E) proteins were shown to accumulate at the plasma membrane in infected Vero and C6/36 mosquito cells while NS1 and NS3 proteins were found to localize at the cytoplasm and perinuclear regions, respectively (Ng and Corner, 1989). Unlike DENV, the NS3 protein of WNV (Kunjin) was found to associate with virus-induced microtubule paracrystals during infection instead (Ng and Hong, 1989; Westaway et al., 1997b). Using IEM, NS4A protein of flavivirus was shown to be responsible for inducing membrane rearrangement whereas NS5 protein was found co-localized with double-stranded RNA in vesicle packets (Mackenzie et al., 2007; Miller et al., 2007; Roosendaal et al., 2006). DENV RNA and virus particles were detected in the flavivirus-induced smooth membrane structures within the ER via EM in situ hybridization analysis (Grief et al., 1997). With the combinatory use of TEM, SEM and IEM, it was also discovered that maturation of WNV (Kunjin) particles occurred within virus-induced vesicles near the ER regions along the secretory pathway (Mackenzie and Westaway, 2001; Ng, 1987). In contrast to other flaviviruses (Hase et al., 1987a,b), WNV (Sarafend) was found to egress at the plasma membrane rather than mature intracellularly (Ng et al., 1994a) (Fig. 7). Taken together, IEM indeed plays an important role in deciphering the replication cycle of flavivirus.

In addition to facilitating our understanding of the replication process of flaviviruses in infected cells, EM also enables atomic structures of flaviviral structural proteins to be determined in the form of protein shells on the wild-type virus itself. Using cryo-EM and fitting with the known atomic structure of tick-borne encephalitis virus (TBEV) envelope (E) glycoprotein, the three-dimensional E protein structure of DENV was first revealed by Kuhn et al. (2002). Subsequently, WNV was found to follow a similar protein structure via the same technique (Mukhopadhyay et al., 2003). Cryo-EM also allowed further refinements to the structure of DENV membrane envelope glycoprotein domains (Zhang et al., 2003). As structures of proteins were determined in their native states through cryo-EM, it showcased how the E and membrane (M) proteins associate with each other within the lipid bilayer. Through this technique, it was also suggested that the nucleocapsid core is asymmetrical, with the capsid proteins randomly orientated within the icosahedral envelope.

Cryo-EM has indeed played an instrumental role in flavivirus research. Other than determining the structure of flaviviruses at the virion level, cryo-EM is also used to identify protein structures, and study protein–protein and protein–lipid interactions. With a combination of cryo-EM and amphipathic properties analysis, the structure of secreted DENV NS1 hexamer protein complex was first revealed to be a barrel-shaped high-density lipoprotein (Gutsche et al., 2011). Investigation on the lipid membrane of mature and immature flaviviruses using cryo-EM demonstrated

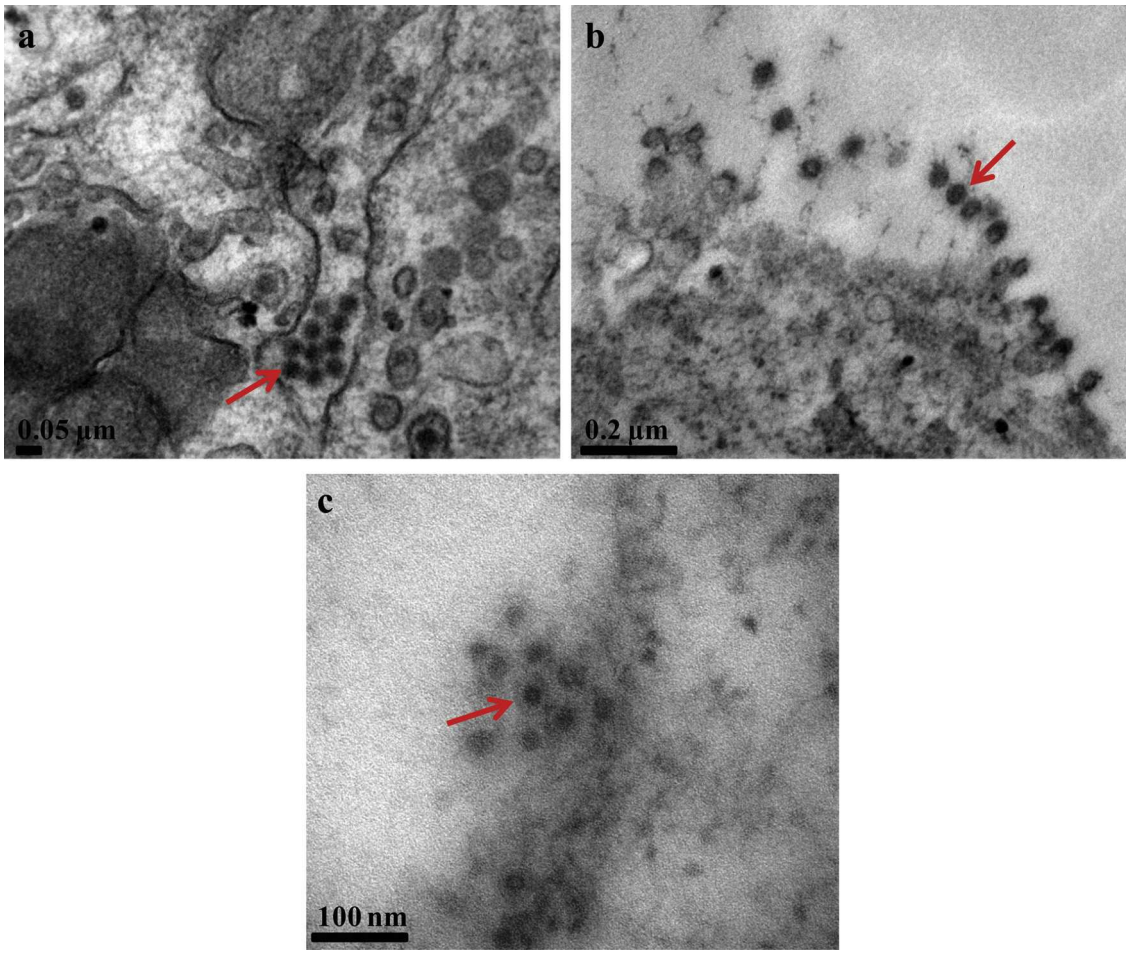

Fig. 7. Transmission electron microscopy images of DENV and WNV particles in infected cells. (a) Progeny DENV detected inside the swollen lumen of endoplasmic reticulum as indicated by the red arrow. (b) In contrast, WNV (Sarafend) egresses at the plasma membrane. The red arrow indicates progeny WNV budding from the infected cell. (c) Using a higher magnification, the envelope protein of WNV (Sarafend) can be seen surrounding the dense core nucleocapsid as indicated by red arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

that the organization of the envelope and membrane proteins mediated the formation of membrane curvature in flaviviruses (Zhang et al., 2013). In DENV-specific antibody development, cryo-EM was used to map out the interacting site between antibody and viral envelope protein (Teoh et al., 2012). Therefore, cryo-EM has been instrumental in providing crucial information on the structures of flaviviruses and facilitating developments in anti-flaviviral therapies.

The development of EM is still on-going for improved performance and further applications. Electron tomography (ET), for instance, is an extension of the conventional TEM technique to obtain three-dimensional structures of a biological specimen. It uses an electron beam to pass through the specimen at incremental degrees of rotation about the centre of the specimen. An array of two dimensional images are collected and used to construct a three dimensional image. To date, ET is able to achieve a resolution of down to 2.4-angstrom (Scott et al., 2012). Sample preparation for ET is similar to that for normal TEM and cryo-techniques provide better preservation of structures within specimens. This technique has been used widely to study the life cycles of different viruses such as HIV, herpesvirus, vaccinia virus and influenza virus (Fu and Johnson, 2011). In flavivirus research, ET was used to show the three-dimensional organization of multivesicular structures, autophagic vesicles, convoluted membranes, double-stranded viral RNA and virus particles in DENV- and WNV-infected cells (Gangodkar et al., 2010; Gillespie et al., 2010; Welsch et al., 2009).

Another recent EM technique developed to obtain high resolution three-dimensional images is serial block face scanning electron microscopy (SBEM, formerly known as SBF-SEM). In this technique, an ultramicrotome is placed in the vaccum chamber of an SEM and signal is detected using a backscattered electron detector. The instrument automatically alternates sample sectioning using the in-built ultramicrotome with SEM imaging of the fresh block face to obtain the three-dimensional ultra-structure of the specimen.

Focused ion beam (FIB) is another EM technique that can be used to obtain three-dimensional images. Instead of the focused beam of electrons in conventional SEM, it utilizes a focused beam of ions (usually gallium) to image samples. Signals from the secondary electrons and sputtered ions are collected for image construction. However, FIB is inherently destructive because it mills holes into the sample and sputters away atoms from the surface. By combining both FIB and SEM in one instrument, imaging can be done at higher magnification and better resolution in all three dimensions. This "DualBeam" technique images samples using the electron beam, which is non-destructive, while milling using the ion beam to remove materials at specific sites. Unlike SBEM, sample preparation for FIB-SEM is much easier because precise cross-sectioning can be performed. Another advantage of FIB-SEM is that the ion beam does not alter fluorescence signal from fluorophores, thus allowing image correlation between fluorescence microscopy and electron microscopy.

Fig. 8 shows the three-dimensional image of DENV-infected cells captured using FIB-SEM. A total of 420 planes of 9 nm thickness

each were obtained with alternate milling and imaging (Fig. 8a). After three-dimensional reconstruction, all the intracellular structures were revealed in a stereoscopic image [Fig. 8(b and c)]. A cluster of DENV particles was observed in the lumen of the endoplasmic reticulum [Fig. 8(d and e)]. This imaging procedure can potentially be performed on DENV-infected cells to visualize the signature network of virus-induced multi-vesicular structures and virus particles.

As mentioned earlier, IFM is commonly used to identify the localization of proteins-of-interest in cells. However, to visualize the localization of proteins at the ultra-structural level, the only feasible method previously was IEM. One major drawback is that the images obtained in IFM and IEM are not from the same cell as samples need to be prepared differently. Correlative light-electron microscopy (CLEM) is another EM technique developed recently to bridge the gap between IFM and EM, in which fluorescently-labelled proteins in cells can be imaged first by IFM, followed by EM to visualize ultra-structures at the subcellular level. This enables users to first locate the region-of-interest at the cellular level via conventional fluorescence microscopy before focusing EM efforts on the selected region-of-interest. Users can now minimize the time spent on viewing random EM sections in the hope to see one section with the desired biological phenomenon. Live imaging can be carried out using CLEM (van Rijnsoever et al., 2008) and three-dimensional structures determined subsequently via ET (Sartori et al., 2007).

Although applications of SBEM, FIB-SEM or CLEM in flavivirus research has not been reported so far, these techniques will provide unprecedented information on virus internalization, trafficking, assembly and budding. For instance, using CLEM technique, Romero-Brey et al. (2012) was able to pinpoint the localization of HCV NS5A protein at the double membrane vesicles near the ER. These double membrane vesicles were found to be protrusions from the ER via ET analysis and 3D reconstructions. Investigations on vaccinia virus entry using FIB-SEM 3D tomography revealed that vaccinia virus induced systemic blebbing at the plasma membrane to facilitate its entry via macropinocytosis (Mercer and Helenius, 2009). The vaccinia virus particle could be visualized clearly inside a bleb-associated macropinosome. Correlative microscopy was also employed to study HIV-infected cells. Jun et al. (2011) characterized HIV-infected HeLa cells first using confocal live-cell microscopy, followed by cryo-EM tomography analysis on the same region containing the virus particles.

One recent breakthrough in EM is the development of a technique to directly image biological specimen in situ in solution (Gilmore et al., 2013). Chemical fixation and cryo-techniques are not required and molecular events can be captured live in nanoscale resolution. This technology utilizes an affinity capture device or microchip made of silicon nitride coated with functionalized lipid monolayers, and a microfluidic-based specimen holder (Degen et al., 2012). Gilmore et al. (2013) demonstrated that assembly of rotavirus double-layered particles can be imaged in solution via TEM using affinity microchips coated with antibody against VP6 protein. This technology can therefore also be applied in flavivirus research to study the assembly of virus-like particles in solution.

With our limited understanding in flaviviruses, flavivirus research remained a fascinating research area for virologists, immunologists and molecular biologists alike. History has proven that microscopy techniques are indispensible tools in unravelling the mysteries in the flavivirus life-cycle, and have helped scientists to understand the complex interactions between flaviviruses and their hosts. As discussed in this review, significant findings in flavivirus research can be obtained with conventional optical microscopy, super-resolution microscopy, electron microscopy and correlative light electron microscopy, each with their own merits and shortcomings suited for different experiments (Fig. 9). Together with other contemporary molecular and biochemistry techniques, microscopy techniques will definitely continue to contribute to a

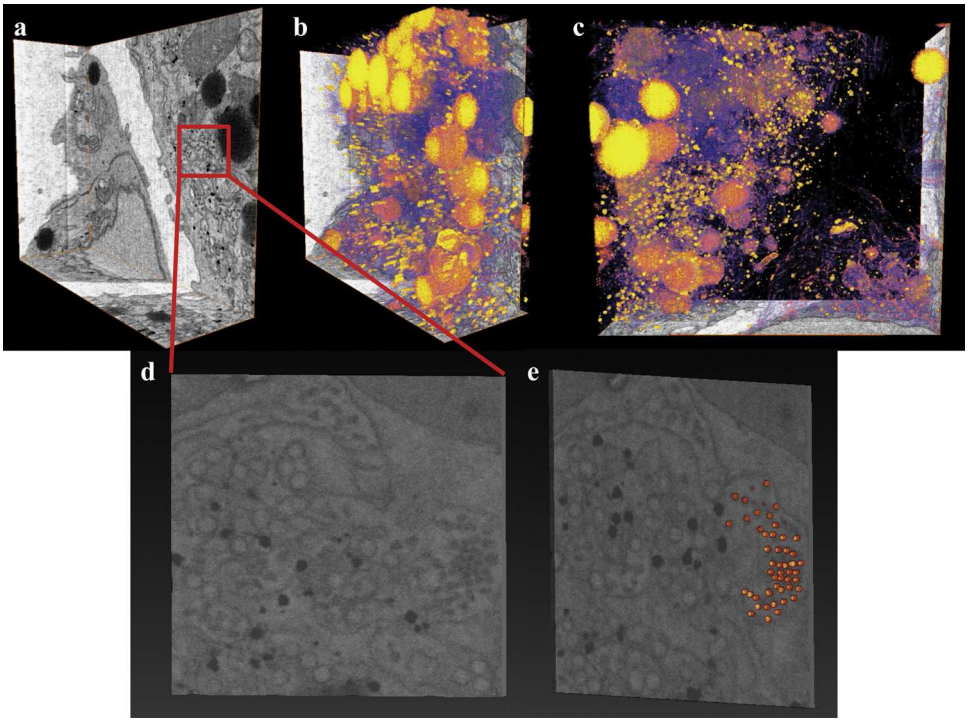

Fig. 8. Focused-ion beam (FIB)-scanning electron microscopy (SEM) images of Dengue virus-infected cells. (a) One section of two infected cells is chosen for milling and imaging using Carl-Zeiss Auriga CrossBeam workstation. 420 planes of 9 nm thickness each are obtained. Three-dimensional construction of all the planes is performed to generate a stereo-image of cells with pseudo colour illustrating the cellular components. The (b) front and (c) back of the 3D image are shown. (d) Enlarged section to show the virus particles which are presented three-dimensionally in (e).

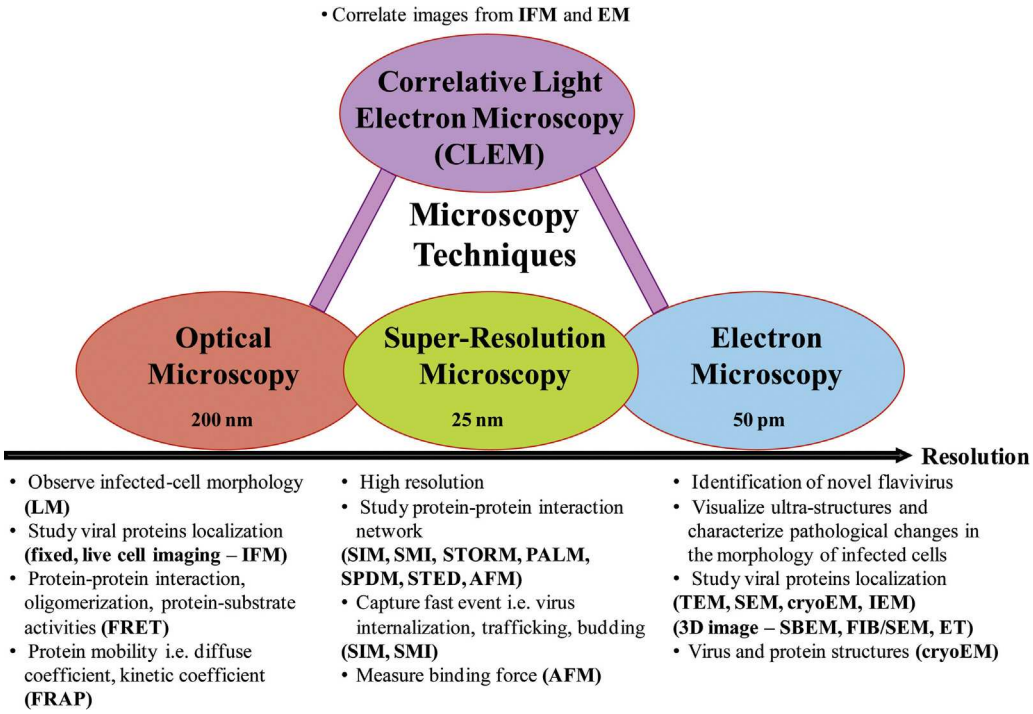

Fig. 9. A summary of microscopy techniques utilized in flavivirus research. The resolution of images increases from optical microscopy, through super-resolution microscopy, to electron microscopy. Correlative light-electron microscopy (CLEM) combines the advantages of both optical microscopy and electron microscopy. The applications of each microscopy technique are illustrated. (Abbreviations: LM – light microscopy; IFM – immuno-fluorescence microscopy; FRET – fluorescence resonance energy transfer; FRAP – fluorescence recovery after photobleaching; SIM – structured illumination microscopy; SMI – spatially modulation illumination; STORM – stochastic optical reconstruction microscopy; PALM – photoactivated localization microscopy; SPDM – spectral position determination microscopy; STED – stimulated emission depletion; AFM – atomic force microscopy; TEM – transmission electron microscopy; SEM – scanning electron microscopy; cryoEM – cryo-electron microscopy; IEM – immuno-electron microscopy; SBEM – serial block-face scanning electron microscopy; FIB/SEM – focused ion beam/scanning electron microscopy; ET – electron tomography.)

more complete understanding of flavivirus replication cycles, and the development of flavivirus vaccines and efficacious antiviral therapeutics in the future.

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