Micron 59 (2014) 33–43

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

Invited Review

Microscopy techniques in flavivirus research

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ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form
11 December 2013
Accepted 11 December 2013

Keywords:
Flavivirus
Fluorescence microscopy
Transmission electron microscopy
Scanning electron microscopy
Correlative light electron microscopy
Super resolution microscopy

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.

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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

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http://dx.doi.org/10.1016/j.micron.2013.12.006

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 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.

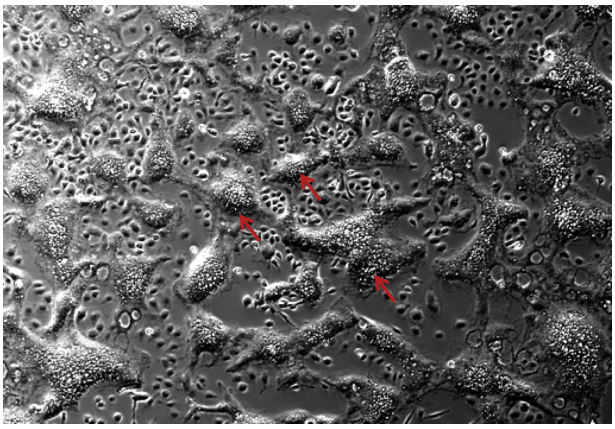

Fig. 1. Dengue virus serotype 2-induced syncytial in C6/36 mosquito cells. At 48-h post infection, multinucleated masses of cytoplasm are detected among C6/36 cells as indicated by red arrows. This signature phenomenon indicates that DENV serotype 2 infection of C6/36 cells is ongoing.

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