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A simple method for Alexa Fluor dye labelling of dengue virus

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A B S T R A C T

Dengue virus causes frequent and cyclical epidemics throughout the tropics, resulting in significant morbidity and mortality rates. There is neither a specific antiviral treatment nor a vaccine to prevent epidemic transmission. The lack of a detailed understanding of the pathogenesis of the disease complicates these efforts. The development of methods to probe the interaction between the virus and host cells would thus be useful. Direct fluorescence labelling of virus would facilitate the visualization of the early events in virus–cell interaction. This report describes a simple method of labelling of dengue virus with Alexa Fluor succinimidyl ester dye dissolved directly in the sodium bicarbonate buffer that yielded highly viable virus after labelling. Alexa Fluor dyes have superior photostability and are less pH-sensitive than the common dyes, such as fluorescein and rhodamine, making them ideal for studies on cellular uptake and endosomal transport of the virus. The conjugation of Alexa Fluor dye did not affect the recognition of labelled dengue virus by virus-specific antibody and its putative receptors in host cells. This method could have useful applications in virological studies.

1. Introduction

Dengue is a significant disease globally. With rapid urbanization, lack of vector control and increase in air travel, dengue has become the most common and rapidly spreading arthropod-borne viral disease (Gubler, 1998). An estimated 50–100 million dengue infections occur annually, and more are at risk of being infected with 2.5 billion people living in dengue endemic countries (WHO, 2007). Despite research efforts, however, there remains an incomplete understanding on the mechanism underlying pathogenesis, which limits antiviral and vaccine development. Among these gaps in knowledge are the specific cell-surface binding motifs and early viral entry processes. The availability of tools to visualize these early events can accelerate this area of research.

Dengue virus (DENV) is an enveloped, positive strand RNA virus that belongs to the family of *Flaviviridae*. Its structure consists of an external icosahedral scaffold of 90 envelope glycoprotein (E) dimers protecting the nucleocapsid shell, which contains the RNA genome (Kuhn et al., 2002). Dengue virus surface has several identical protein subunits and can therefore be labelled at multiple sites using an amine reactive dye; the resulting fluorescence intensity may be sufficient to track the virus under a fluorescence microscope.

A simple procedure of labelling dengue virus with a fluorescent Alexa Fluor succinimidyl ester that results in minimal loss of infectivity post-labelling is described. Alexa Fluor dye was chosen as it has better photostability and signal intensity compared to common fluorophores such as fluorescein isothiocyanate (FITC) or cyanine 3 bihexanoic acid (Cy3). In addition, Alexa Fluor dyes are stable at a lower pH compared to the common fluorophores, which could be useful for visualizing labelled viral particles as they move through the acidic compartments of endosomes post-infection. Furthermore, the permanence of covalent conjugation of the fluorophores to the surface proteins allows for storage of batch-labelled virus at −80 °C, providing uniformity across multiple experiments. It also opens up the possibility of performing live cell imaging to compliment the 'snapshot' imaging of conventional fluorescence microscopy.

2. Materials and methods

2.1. Cells and antibody

All cells and hybridomas were obtained from The American Type Culture Collection (ATCC, Manassas, VA), and all cell culture media and supplements were purchased from Gibco (Invitrogen, Singapore). Baby hamster kidney BHK-21 cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640), supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C with 5% CO_2. African green monkey kidney Vero cells were maintained in Medium-199 (M199)

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supplemented with 10% fetal bovine serum and 4 mM L-glutamine at 37 °C with 5% CO_2. Mouse anti-dengue virus serotype 2 E protein monoclonal antibody hybridoma, 3H5 (ATCC: HB46), was maintained in RPMI-1640 supplemented with 10% fetal bovine serum. Dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) transfected-Raji B cell line was a kind gift from Timothy H. Burgess (Naval Medical Research Centre, US) and maintained in RPMI medium supplemented with 10% fetal bovine serum. Alexa Fluor 488 (AF488) anti-mouse IgG antibody was purchased from Molecular Probes, Invitrogen and used at 1:100 dilution.

2.2. Virus culture and purification

DENV serotype 2 (DENV-2) (ST) strain was first isolated from a clinical sample in the Singapore General Hospital using an *Aedes albopictus* mosquito cell line C6/36 and subsequently propagated in Vero cells. The culture supernatant was harvested when 75% of the cells showed cytopathic effect and clarified by centrifugation at 1000 × *g* for 30 min at 4 °C. Virus in the supernatant was concentrated by centrifugation at 30,000 × *g* for 2 h at 4 °C. The pellet was resuspended in 5 mM Hepes, 150 mM NaCl, 0.1 mM EDTA (HNE buffer, pH 7.4) and purified further on a 30% sucrose cushion by ultracentrifugation in a Beckman SW41Ti rotor at 80,000 × *g* for 15 h at 4 °C. Purified virus was resuspended in HNE buffer and stored in 100 μl aliquots at −80 °C. A limiting dilution plaque assay was performed on BHK cell line to determine the viral titre in plaque forming units per millilitre (pfu/ml).

2.3. Plaque assay

Tenfold serial dilutions of virus were added to BHK-21 cells in 24 well plates and incubated for 1 h at 37 °C, with gentle rocking every 15 min. The medium was then aspirated and replaced with 0.8% methyl-cellulose in maintenance medium (RPMI-1640, 2% fetal bovine serum, penicillin and streptomycin). After 4 days at 37 °C, the cells were fixed with 25% formaldehyde and stained with 0.5% crystal violet. The plates were washed, dried and the plaque forming units per ml (pfu/ml) calculated.

2.4. Virus labelling

For the initial experiment aimed at determining the optimal concentration of dye needed to label DENV, Alexa Fluor 594 succinimidyl ester (AF594SE, Molecular Probes, Invitrogen) was reconstituted in 0.2 M sodium bicarbonate buffer, pH 8.3 (SBB) immediately before labelling, and added to approximately 3×10^7 pfu DENV diluted in SBB at final concentrations of 10, 50,100, 200, 500 and 1000 μM of dye, while stirring gently. The dye-virus mixture was incubated at room temperature for 1 h with gentle inversions every 15 min. The labelling reaction was stopped by adding freshly prepared 1.5 M hydroxylamine, pH 8.5 (Sigma–Aldrich) and incubated at room temperature for 1 h with gentle inversions every 15 min. Labelled DENV was re-titrated after labelling and tested for fluorescence using immunofluorescence assay.

For subsequent AF594SE or AF488SE labelling involving larger batches of virus, the same approach was employed and the labelled DENV was purified using Sephadex G-25 columns (Amersham, GE Healthcare, Singapore) to remove the excess dye. The labelled virus was stored in 100 μl aliquots at −80 °C, re-titrated and tested for fluorescence before use.

2.5. Immunofluorescence assay

Equal volume of AF594-labelled DENV was incubated with Vero cells plated on coverslips for 10 min at 37 °C, washed, fixed with 3% paraformaldehyde (Sigma–Aldrich, Singapore) and permeabilized with 0.1% saponin (Sigma–Aldrich, Singapore). The cells were then incubated for 1 h with undiluted centrifugation-clarified supernatant of 3H5 monoclonal antibody hybridoma culture, at room temperature. The cells were washed three times in wash buffer (PBS containing 1 mM calcium chloride, 1 mM magnesium chloride and 0.1% saponin), followed by incubation with AF488 anti-mouse IgG antibody for 45 min at room temperature. Cells were then washed three times with the wash buffer, rinsed once with deionized water and mounted on glass slides with Mowiol 4-88 (Calbiochem, San Diego, CA) with 2.5% Dabco (Sigma–Aldrich, Singapore). Cells were visualized at 63× magnification using a Leica Microsystem TCS SP5 confocal microscope and merged images exported for processing. Processing of the images involved adjustment of the contrast of the images on a whole for clarity and exported in individual colours for unmerged images using Adobe Photoshop CS3 version 10.

2.6. Flow cytometry determination of labelled DENV

Raji B cells transfected with DC-SIGN were counted, aliquoted, centrifuged and resuspended in AF488-labelled DENV at multiplicity of infection (MOI) of 1 and incubated for 10 min at 37 °C, then fixed with 3% paraformaldehyde. The cells were then washed twice with FACS buffer consisting of PBS with 0.1% fetal bovine serum and permeabilized with 0.1% saponin in PBS. Cells were stained subsequently for the presence of DENV using anti-E protein monoclonal antibody, 3H5, and PE anti-mouse anti-IgG antibody before reading on BD FACS Calibur machine and analysed with CellQuest software, version 3.3 (Becton Dickinson).

2.7. Detection by SYBR green-based real-time PCR

Real-time detection of viral RNA was conducted as described previously (Lai et al., 2007) using pan-dengue forward and reverse primers with some modifications. Briefly, RNA extraction was carried out using QIAamp viral RNA mini kit for purified non-labelled and AF594-labelled DENV (AF594-DENV), or RNeasy mini kit (both from QIAGEN, Hilden, Germany) for total RNA extraction from cells according to the manufacturer's instructions. Complementary DNA was synthesized using SuperScript III First Strand Synthesis System (Invitrogen, Singapore) with random hexamers and according to the manufacturer's instructions. RNA copy number was then determined by SYBR green-based real-time PCR on LightCycler 480 Real-Time PCR System (Roche Diagnostics, Penzberg, Germany).

2.8. Growth kinetics

Vero cells were seeded at 2×10^5 per well in 24 well plates and incubated at 37 °C for 4 h before infecting at a MOI of 0.1 with either purified DENV or AF594-DENV for 1 h at 37 °C, with gentle rocking every 15 min. The cells were then washed twice with 1 ml per well PBS and replaced with maintenance medium (M199 supplemented with 2% FBS). Supernatant was harvested from wells and stored at −80 °C for plaque assay, and cells were washed twice in PBS and lysed with RLT buffer provided in RNeasy mini kit at 0, 6, 8, 12, 24, 48 and 72 h post-adsorption. The cell lysates were stored frozen until all samples were collected and subsequently extracted according to the kit's protocol. Viral RNA was detected using real-time PCR.

2.9. Statistical analysis

Statistical analyses were performed using unpaired Student's *t* test (Graphpad Prism 5.0). *P* value < 0.05 was considered significant.