Video Article

Visualizing Dengue Virus through Alexa Fluor Labeling

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Abstract

The early events in the interaction between virus and cell can have profound influence on the outcome of infection. Determining the factors that influence this interaction could lead to improved understanding of disease pathogenesis and thus influence vaccine or therapeutic design. Hence, the development of methods to probe this interaction would be useful. Recent advancements in fluorophores development[1-3] and imaging technology[4] can be exploited to improve our current knowledge on dengue pathogenesis and thus pave the way to reduce the millions of dengue infections occurring annually.

The enveloped dengue virus has an external scaffold consisting of 90 envelope glycoprotein (E) dimers protecting the nucleocapsid shell, which contains a single positive strand RNA genome[5]. The identical protein subunits on the virus surface can thus be labeled with an amine reactive dye and visualized through immunofluorescent microscopy. Here, we present a simple method of labeling of dengue virus with Alexa Fluor succinimidyl ester dye dissolved directly in a sodium bicarbonate buffer that yielded highly viable virus after labeling. There is no standardized procedure for the labeling of live virus and existing manufacturer's protocol for protein labeling usually requires the reconstitution of dye in dimethyl sulfoxide. The presence of dimethyl sulfoxide, even in minute quantities, can block productive infection of virus and also induce cell cytotoxicity[6]. The exclusion of the use of dimethyl sulfoxide in this protocol thus reduced this possibility. Alexa Fluor dyes have superior photostability and are less pH-sensitive than the common dyes, such as fluorescein and rhodamine[2], 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 labeled dengue virus by virus-specific antibody and its putative receptors in host cells[7]. This method could have useful applications in virological studies.

Protocol

1. Alexa Fluor labeling of dengue virus

1. Before the labeling reaction, purify dengue virus with sucrose cushion and prepare the necessary reagents and equipment as indicated in the protocol.
2. Prepare fresh 0.2M sodium bicarbonate buffer, pH 8.5 (labeling buffer), and 1.5M hydroxylamine buffer, pH 8.3 (stop reagent), just before labeling and filter sterilize with 0.2µm syringe filters.
3. Dilute approximately $3x10^8$ plaque forming units (pfu) of purified dengue virus in 1ml of labeling buffer in a 2ml tube. This can be scaled up proportionally for batch labeling of virus.
4. Reconstitute the lyophilized Alexa Fluor 594 (AF594) succinimidyl esters to 1mM in labeling buffer immediately prior to the labeling reaction. Other fluorochromes from the Alexa Fluor dye series may be used according to one's needs. Minimize exposure to light from this step onwards.
5. Add 100µl of the 1mM AF594 dye to the diluted virus while stirring gently with the pipette tip.
6. Incubate the labeling reaction mix at room temperature for 1hr in the dark. Mix by gentle inversions every 15mins.
7. Spin the tube briefly in a tabletop centrifuge and add 100µl of stop reagent to the reaction mix while stirring gently with the pipette tip.
8. Incubate at room temperature for an additional hour in the dark. Mix by gentle inversions every 15mins.

2. Purifying Alexa Fluor labeled dengue virus

1. In the meantime, equilibrate the purification column with buffer of choice. In this experiment, a PD-10 column is equilibrated with 25ml of HNE buffer (5mM Hepes, 150mM NaCl, 0.1mM EDTA), pH 7.4, before use.
2. Apply the labeled virus to the top of the column and start collecting the flow-through once the labeled virus enters the matrix. Fill the column with HNE buffer once all the labeled virus has entered the matrix. Discard the first 2.5ml of flow-through and collect the next 2ml of labeled virus fraction.
3. Aliquot and store purified AF594 labeled dengue virus in -80°C, away from light source.

1. Thaw one aliquot and determine the titer of the labeled virus by plaque assay before using the batch of labeled virus.
2. Seed $5x10^4$ per well of Vero cells, grown in M-199 growth medium, in a 4-well plate with a coverslip on the bottom of well a day before infection.
3. Remove the culture supernatant in well and infect the cells with multiplicity of infection of 1 of the labeled virus in 100µl volume (diluted in M-199 maintenance medium as required) for 10min at 37°C.
4. Remove the inoculums and wash the coverslips twice in 1xPBS.
5. Fix the cells in 3% paraformalydehyde for 30mins.
6. Wash the coverslips 3 times in 1xPBS.
7. Permeabilize the cells with permeabilization solution containing 0.1% saponin and 5% BSA in 1xPBS for 30mins.
8. Incubate the cells with undiluted centrifugation-clarified supernatant of 3H5 monoclonal antibody hybridoma culture for 1hr in a humid chamber, protected from light.

9. Wash the coverslips 3 times in wash buffer (1xPBS containing 1mM calcium chloride, 1mM magnesium chloride and 0.1% saponin).
10. Incubate the cells with AF488 anti-mouse IgG antibody, 1:100 diluted in permeabilization solution, for 45mins in humid chamber, protected from light.
11. Wash the coverslips 3 times in wash buffer and rinse once in deionized water.
12. Dab the edge of coverslip against a paper towel to drain excess water and mount on to glass slide with 8µl Mowiol 4-88 containing 2.5% Dabco.
13. Allow the mounting solution to set overnight at 4°C before viewing using a Zeiss confocal microscope. Sequential acquisitions should be performed exciting one fluorophore at a time and switching between the detectors concomitantly.
14. Images are then analyzed for co-localization of the E protein antibody staining with the labeled virus using the Zeiss LSM Zen software to estimate the degree of labeling.

3. Representative Results

An example of the yield of dengue virus labeled with AF594 dye is shown in Figure 2. Normally, less than 10-fold drop from the initial titer should be observed following successful labeling. However, it should be noted that all buffers have to be prepared fresh for the labeling to be successful and the Alexa Fluor succinimidyl esters should be used immediately upon reconstitution as they hydrolyze into nonreactive free acids in aqueous solutions[8].

Next, the labeled virus has to be checked for sufficient fluorescence before use in experiments. A simple immunofluorescence assay was done on Vero cells and the degree of labeling can be estimated from the co-localization of the labeled virus with anti-E protein antibody staining. Several cells were examined and a typical confocal image is shown in Figure 3. Co-localization analysis of the images using the LSM Zen software demonstrated overlap coefficients ranging from 0.65 to 0.8, suggesting that approximately 65 to 80% of the virions were labeled with the dye.

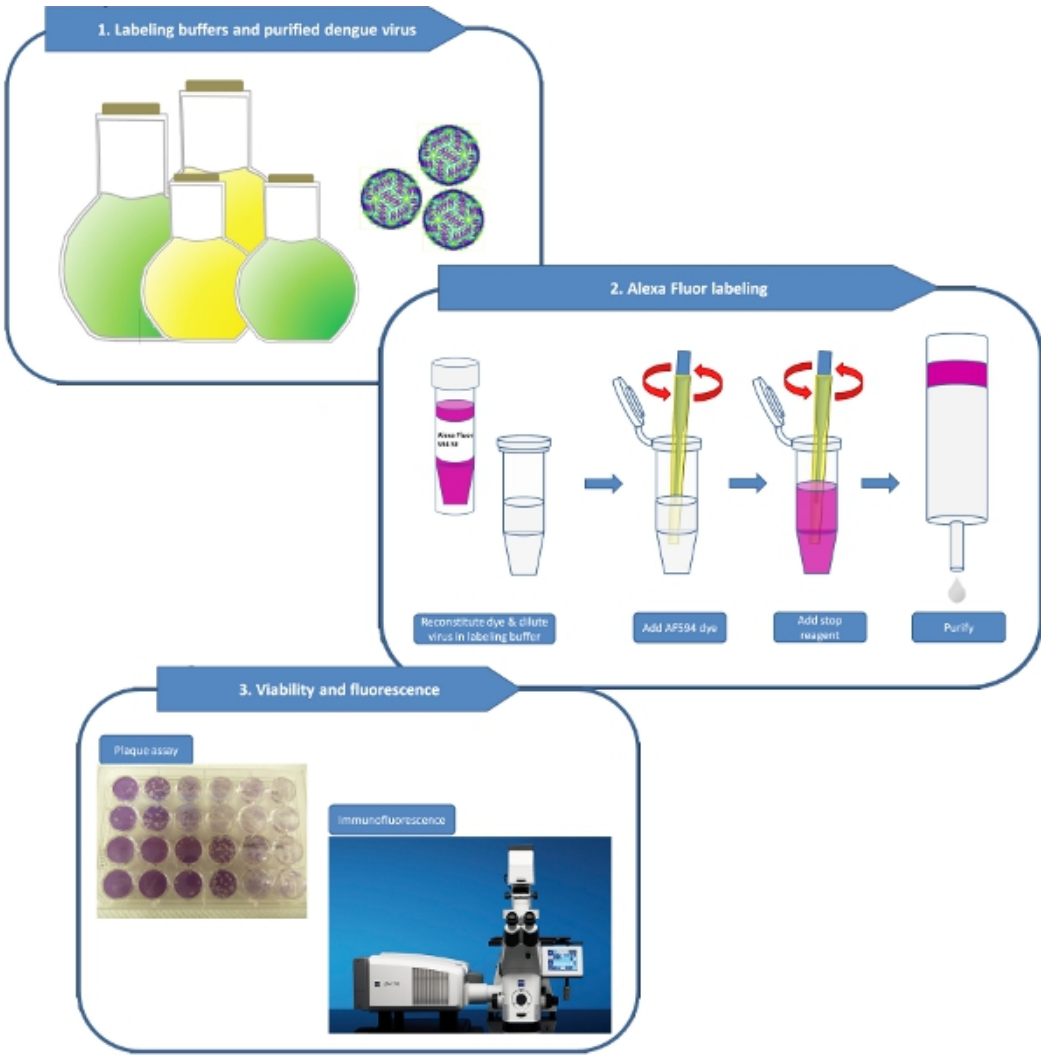

Figure 1.Overall scheme depicting the Alexa Fluor dye labeling of dengue virus procedure. First, the relevant buffers and purified dengue virus are prepared. The Alexa Fluor dye is reconstituted and added to the dengue virus diluted in labeling buffer. The reaction is then stopped 1 hour later with the addition of stop reagent. Subsequently, the labeled virus is purified through a size exclusion column to remove free dye. Finally, the labeled virus is re-titrated by plaque assay and tested for fluorescence.

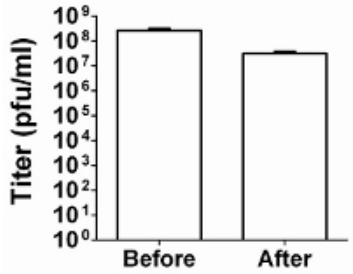

Figure 2. Mean number of viable virions (pfu/ml) as determined on a plaque assay before and after AF594 labeling. An aliquot of the AF594 labeled dengue virus is thawed and re-titrated by plaque assay and it typically shows less than 10-fold drop from the starting titer. Error bars indicate standard deviation of duplicates.

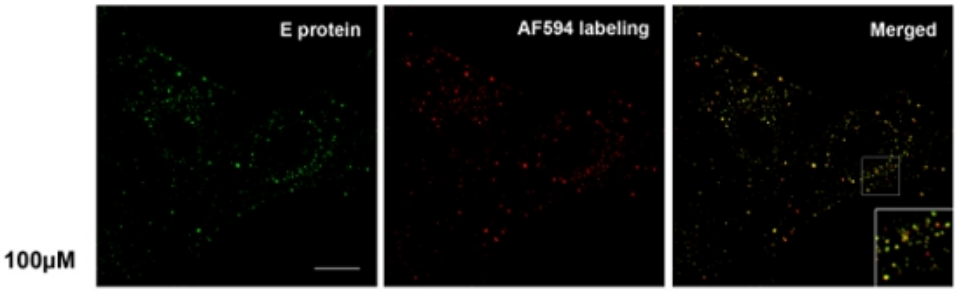

Figure 3. Co-localization of AF594 labels with dengue virus E proteins in Vero cells. cells grown on coverslips the day prior were infected with AF594 labeled dengue at MOI of 1 for 10 minutes at 37°C. The cells were subsequently fixed and labeled with anti-E antibody, and examined for colocalization of E protein (green) and AF594 labeling (red). Fluorescent signals were visualized under 63X magnification using Zeiss LSM 710 confocal microscope. Scale bar is 10μm. Yellow indicate areas of colocalization, as shown in the inset.

Discussion

Although AF594 dye was used in this report, a wide range of fluorophores in the Alexa Fluor succinimidyl esters series is available with similar labeling chemistry. This could extend the labeling application beyond imaging. Flow cytometry can be used as an alternative to confocal microscopy for estimating the degree of labeling for fluorophores that can be excited and detected by the FACS machine.

Alexa Fluor dyes are small molecules that react with free amino groups, primarily arginine and lysine[9], normally outward facing residues of proteins. In our laboratory, conjugation of dengue virus with 100μM of Alexa Fluor 594 dye provided sufficient brightness for imaging with minimal loss in the viral titer. Different brightness may be achieved by varying the concentration of dye used. However, increasing the dye concentration can reduce virus viability[7,10]. One possible limitation is the interference in receptor binding due to the blockade of access by the fluorophores. Therefore, depending on application, an optimal level of labeling should be determined to ensure a balance between the degree of labeling and functional abrogation10. Do note that the concentration can also be affected by the post-packaging reactivity of the Alexa Fluor succinimidyl esters[8].

The direct labeling of dengue virus with Alexa Fluor dye presented here does not require any additional labeling steps to visualize the virus, thus removing the possibility of non-specific staining from indirect antiviral antibodies. It also allows for real-time tracking of post-internalization events in live cell imaging. This method is relatively simple, and because the conjugation is stable, it can be used to produce and store batch-labeled virus for multiple experiments as opposed to lipophillic fluorescent dyes, such as long-chain carbocyanine1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine (DiD) or styryl dyes, which cannot be stored in the cold for more than 3 days.

Disclosures

We have nothing to disclose.

Acknowledgements

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