Dengue virus cell entry
Ayala Nunez, Vanesa

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Monitoring virus entry into living cells using DiD-labeled dengue virus particles
Abstract

A variety of approaches can be applied to investigate the multiple steps and interactions that occur during virus entry into the host cell. Single-virus tracking is a powerful real-time imaging technique that offers the possibility to monitor virus-cell binding, internalization, intracellular trafficking behavior, and the moment of membrane fusion of single virus particles in living cells. Here we describe the development and applications of a single-virus tracking assay based on the use of DiD-labeled dengue virus (DENV) in BS-C-1 cells. In addition -and using the same experimental setup- we present a binding and fusion assay that can be used to obtain a rapid insight into the relative extent of virus binding to the cell surface and membrane fusion. Details of virus labeling and characterization, microscopy setup, protocols, data analysis, and hints for troubleshooting are described throughout the paper.
Introduction

Virus cell entry is a complex process that includes the interaction of viral and cellular structures in a series of complex events. The process starts by binding of a viral protein to a receptor molecule (protein, carbohydrate, or lipid) located at the cell surface. Subsequently, some viruses directly penetrate or fuse with the plasma membrane of the cell whereas other viruses are first internalized via endocytosis. Endocytosed viruses must penetrate or fuse with the endosomal membrane for their genome or capsid to be released into the cell cytoplasm. Usually, entry of endocytosed viruses is dependent on the acidification of the lumen of the endosome, triggering a low-pH-dependent conformational change in the virus that will lead to fusion, penetration and/or uncoating. In other cases, virus entry relies on proteolytic cleavage and activation of viral proteins, and/or association with cellular proteins. For enveloped viruses, membrane fusion is an essential step during entry into cells.

To study the route of virus entry into living cells, different approaches can be used. These include the use of chemical endocytic inhibitors, dominant-negative mutants, iRNA screening, and siRNA silencing of specific components of endocytic pathways. Virus cell entry can be visualized by use of electron microscopy and fluorescent labeling of viruses. Furthermore, in vitro fusion assays can be performed to characterize the basic features of membrane fusion of enveloped viruses.

Fluorescent imaging provides an important tool for studying the route of virus entry virus in living cells. More specifically, single-virus tracking is a real-time imaging technique that can be used to monitor individual virus particles or viral components in living cells. Single-virus tracking can be used to obtain a detailed insight in the binding, entry, and membrane fusion properties of single virus particles, since it allows the imaging of fluorescently labeled particles with high sensitivity and time resolution. In addition, by tracking the fate of a single virus particle, the dynamic interactions between viruses and cellular structures during the entry process can be studied in detail. However, single-virus tracking is technically demanding, since (i) the virus and cellular structures of interest must be fluorescently labeled with sufficient numbers of fluorophores without affecting viral infectivity and cell functions, (ii) the microscope must be sufficiently powerful for the detection of small single viruses or viral components in real time, and (iii) the acquired image series must be analyzed to extract useful information. For an extensive review on the challenges and technical aspects of single-virus imaging we would like to refer to Brandenburg and Zhuang.

Multiple fluorescent probes, like chemical labels (amino-reactive dyes, lipophilic dyes, metabolic labeling, etc.) and fluorescent proteins can be used to visualize individual virus particles. In our studies we have used the lipophilic fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) to label the viral membrane. DiD is a far-red fluorescent lipophilic carbocyanine (maximum excitation at 644 nm and emission
at 665 nm) with a high extinction coefficient, moderate fluorescence quantum yield, and short excited-state lifetime in lipid environments\textsuperscript{14}. An advantage of the use of DiD is that at longer excitation wavelengths the cell auto-fluorescence background levels are low, reducing the signal-to-noise ratio\textsuperscript{13}. When incorporated in the viral membrane at a relatively high surface density, the emitted fluorescence level is largely quenched, but single DiD-labeled virus particles can still be clearly detected\textsuperscript{15}. Membrane fusion of virus particles labeled with a relatively high surface density of DiD can be observed as a fluorescence dequenching due to the dilution of the DiD probe from the viral membrane into the target cell membrane.

In the current paper, we will discuss the development and applications of a single-virus tracking assay based on the use of DiD-labeled dengue virus (DENV). Also, we will describe the use of a fluorescent bulk assay to obtain a quick insight into the relative extent of virus binding to the cell surface and membrane fusion. DENV will serve as a model virus since we have previously analyzed the dynamics and route of DENV cell entry by this method\textsuperscript{7, 16}. However, it is important to note that this methodology can be adapted to other enveloped viruses as well.

**Experimental procedures**

The use of DiD-labeled DENV particles to monitor and study viral entry into living cells comprises several steps. First, the experimental procedures for virus production, purification and DiD-labeling will be explained. Secondly, the characterization of the resulting labeled particles is presented. Third, a detailed protocol of the single-virus tracking setup, procedure, and data analysis is described. Finally, the bulk binding and fusion assay is explained. Hints for troubleshooting of the procedures are given in each section.

*Virus particle production*

C6/36 cells, derived from *Aedes albopictus* mosquitoes, are used for propagation of DENV serotype 2 strain 16681. C6/36 cells are cultured at 30°C and 5% CO\textsubscript{2} in minimal essential medium ([MEM] Gibco) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, 0.075% sodium bicarbonate, penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM glutamine and 100 μM nonessential amino acids. Cells are infected with a small volume of DENV at a MOI of 0.1 in 2% FBS culture media with no HEPES. A low FBS concentration in the media is used to limit protein interference in the binding of the virus to the cells. After a 2 h incubation period at room temperature, complete medium with HEPES buffer is added. HEPES prevents acidification of the medium during virus growth and thus prevents low-pH-induced inactivation of newly released virions. Incubation is continued at 30°C for 3 days after which the medium is harvested and cell debris is
removed by low speed centrifugation. For tracking experiments, we usually generate a large batch of virus particles, by infecting 16 flasks (225 cm²) at once.

**Virus purification and characterization**

Directly upon the removal of cell debris, the virus particles present in the media are pelleted by ultra-centrifugation (Beckman type 19 rotor, 15 h, 30,000 x g, 4°C). The media of 16 225 cm² flasks is distributed over two type 19 centrifuge bottles. Upon centrifugation, the virus pellets are resuspended in 1 mL of cold HNE buffer (5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) each. It is important to avoid air bubbles. The concentrated virus is then pooled and purified on a potassium-tartrate step gradient (10 to 35%, wt/vol) by ultracentrifugation (Beckman type SW41 rotor, 2 h, 125,000 x g, 4°C). This method has shown to preserve infectivity and morphology of different enveloped viruses. DENV will be at the interface of the 20% and 25% layers. The purified virus is aliquoted and stored at -80°C.

During purification, samples are taken at all steps to evaluate the specific infectivity of the virus by measuring the number of physical and infectious virions present in solution. Viral infectivity is determined with a standard plaque assay (plaque forming units, PFUs) and/or an infectious center assay (infectious units, IUs). The physical number of viral particles present is determined by quantitative PCR (qPCR), which detects the number of genome-containing particles (GCPs). For qPCR, the viral RNA is extracted by use of a QIAamp Viral RNA mini kit (Qiagen). Next, cDNA is synthesized from viral RNA with reverse transcription-PCR (RT-PCR) using the forward primer 5'-ACAGGCT ATGGCAGTGTACGAT-3', the reverse primer 5'-TGCAGCAACACCCTC TCTTG-3', deoxynucleoside triphosphates, RNasin (Promega), and Omniscript (Qiagen). For the qPCR, cDNA is mixed with the TaqMan probe (5'-FAM-AGTGCTCTCAAGAACGGGCCTCG-TAMRA- 3', where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine) (Eurogentec), the same primers as for the RT-PCR, deoxynucleoside triphosphates, and HotStarTaq DNA polymerase (Qiagen). DNA is amplified for in 40 cycles (15 s at 95°C and 60 s at 60°C) on a Smart-Cycler (Cepheid). Determination of the number of genomic RNA copies is performed with a standard curve (correlation coefficient of >0.995) of a quantified DNA plasmid containing the DENV prM and E sequence (pcDNA3-prM/E), which was constructed using standard DNA techniques. The purified virus preparation is suitable for DiD labeling when the PFU to particle ratio is similar to that of unpurified virus.

**DiD labeling**

**Procedure of virus labeling**

To ensure efficient DiD labeling, we first remove the potassium-tartrate of...
the purified virus solution and replace it with HNE buffer by use of a 100 kDa filter device (Millipore). Approximately $10^{10}$ GCPs of purified DENV (strain 16881) particles, equivalent to ~$10^8$ PFUs, are added to the filter and concentrated to an end volume of 50 μl. While vortexing, 1 μL (= 2 nmol) of a 2 mM DiD solution prepared in DMSO (Molecular Probes) is added to the virus and incubation is continued for 10 min at room temperature. Vortexing is critical and the settings should be optimized depending on the machine used (not too hard not too soft). Given its hydrophobic nature, DiD spontaneously integrates into the viral membrane. The DiD solution should be at room temperature when added to the virus. To remove unbound dye, the DiD-labeled virus is filtered by use of a Sephadex G-50 Fine (Pharmacia) column prepared in a short glass pasteur pipette. HNE is used to equilibrate the column. The virus goes through the column as a blue band (unincorporated DiD stays on top of the column). Different fractions are collected, stored at 4ºC and used within 2 days.

**Characterization of the DiD-labeled virus**

It is important to first determine the specific infectivity of the labeled virus preparation, as outlined above. This should be similar to that of unlabeled purified virus preparations.

The incorporation of DiD molecules in the viral membrane can be assessed by fluorescence spectroscopy. The fluorescence intensity of the collected fractions is measured at 650-750 nm in the absence and presence of the detergent octaethyleneglycol monododecyl ether ([C12E8] Sigma Aldrich). Addition of C12E8 detergent should significantly increase the DiD fluorescence intensity (Figure 1A). The fraction that has the highest number of particles and the highest DiD fluorescence intensity measured after adding C12E8 is used in the subsequent experiments.

The efficiency of DiD labeling is evaluated by comparing the number of physical particles present in solution by qPCR with the number of detected DiD-labeled particles by fluorescence microscopy. To analyze the number of viral spots, a fixed small volume of DiD-labeled DENV is added to a glass coverslip and excited with a 633-nm helium-neon laser. Fluorescent emission is collected by an oil immersion objective with a numerical aperture of 1.45 and imaged through a 665-nm long-pass filter (Chroma) onto a CCD camera. Background and noise are removed by convolving with a Gaussian spatial filter. The virus spots are detected by recursively integrating over bright regions connected to each local maximum. The number of viral spots are counted in five random image areas, and the concentration of DiD-labeled DENV is calculated according to the following formula: number of spots/mL = $N \times \left( \frac{S_{\text{CoverSlip}}}{S_{\text{ImageArea}}} \right) \times \text{dilution factor} \times \frac{1}{V}$, where $N$ is the average number of virus spots, $S_{\text{CoverSlip}}$ is the surface area of the glass coverslip, $S_{\text{ImageArea}}$ is the surface of the image area, and $V$ is the volume. The majority of DiD-labeled particles should display a low DiD fluorescence intensity, indicating that, under these conditions, DiD does not spontaneously integrate into the viral envelope.
conditions, the DiD fluorescence is largely quenched (Figure 1B-C). In our tracking experiments, only those particles that fall into the peak with the lowest fluorescence intensity (range of 0 to 500 arbitrary units [AU] in the histogram in Figure 1B) are selected for data analysis since these most probably represent single virus particles. For an overview of typical physical properties of single DiD-labeled particles upon microscopic examination, see Van der Schaar et al.\textsuperscript{16}.

![Figure 1. Characterization of DiD-labeled virus. (A) Fluorescence emission spectra of DiD-labeled DENV with or without C12E8 detergent. An increase in fluorescence intensity indicates incorporation of DiD into the viral membrane. (B) Intensity distribution of DiD-labeled DENV2 particles. Particles with low fluorescence intensity (0 to 500 AU) were selected for image analysis. (From ref.\textsuperscript{16}, with permission) (C) DiD-DENV particles bound to BS-C-1 cells. Images were taken before starting to record a movie with an oil-immersion 100x objective with a numerical aperture of 1.46, and an EM CCD camera. The white arrows indicate suitable labeled particles with a high incorporation of DiD in the viral membrane.]

Troubleshooting

The preparation of DiD labeled virus particles is relatively complex. Different viruses may need to be subjected to different purification and labeling procedures. If the purification or labeling procedure fails this may be caused by the presence of virus aggregates, loss of viral infectivity, and incorrect labeling of the particles. Virus aggregates may form when the K-tartrate gradient is not well prepared (make sure all the gradient layers are clearly seen in the tube), when too much virus is loaded on one gradient (for 16 225 cm\textsuperscript{2} flasks we typically load 4 distinct gradients (tubes)), and if a cold DiD solution is used (it should be at room temperature). If the specific infectivity drops as a consequence of the purification procedure one can consider to pellet the
virus through a sucrose cushion and/or test other solutions for the density gradient such as Optiprep. Incorrect DiD-labeling can result in single particles with a high fluorescence intensity (low concentration of DiD, thus no fluorescence quenching) or almost undetectable fluorescent particles (excessive amount of DiD, thus highly quenched fluorescence). If that is the case, optimize the conditions for vortexing or increase or lower the concentration of DiD to define the adequate labeling procedure. While optimizing, it is important to analyze the specific infectivity of the virus as this may be affected during the labeling procedure.

**Single-virus tracking**

**Technical details of the microscopic setup**

A typical single-virus tracking setup includes an inverted microscope with a temperature-controlled stage, several lasers, optics, and a sensitive detector. A temperature regulator is needed throughout the whole experiment to keep the cells metabolically active so that virus uptake and trafficking can take place. As light sources, laser lines such as Argon ion, Krypton ion, Helium-Neon, and Nd:YAG can be used according to the fluorophore requirements. The imaging geometries that are commonly employed for single-virus tracking are epifluorescence, confocal and total internal reflection fluorescence (TIRF) microscopy (for details, we refer to the scheme in Box 2 of Brandenburg and Zhuang, 2007). As a complement to these microscope setups, fluorescence emission from the sample should be collected by a high numerical aperture objective and detected by a charge-coupled device (CCD) camera.

In our current experimental setup, tracking of DiD-labeled DENV particles includes detection by epifluorescence microscopy in a Leica Biosystems 6000B instrument. The advantages of this scheme include a low signal loss, rapid wide-field detection, and a large excitation depth, allowing single particles to be tracked in a large sample volume. The disadvantage is its poor rejection of the fluorescence background signal from the cell. However, for tracking experiments with DiD-labeled particles, epifluorescence can be readily used since auto-fluorescence background levels are low at longer excitation wavelengths. DiD-labeled viruses are excited with a 635-nm helium–neon laser. The fluorescent emission is collected by an oil-immersion 100x objective with a numerical aperture of 1.46 (Leica Microsystems) and imaged using an EM CCD camera (Hamamatsu 9100-02). A thermostatted stage and objective heater are used to keep the temperature at 37°C throughout the whole experiment.

**Procedure of tracking experiments**

A 100x glucose oxidase (GLOX) solution is prepared beforehand. It is composed of 25-50 μL of glucose oxidase (Type VII from *Aspergillus niger*, Sigma
Aldrich No. G2133), 200 μL of 1x PBS, and 50 μL of catalase (from beef liver, Roche No. 10106810001). The GLOX solution is centrifuged for 5 min at 13,000 rpm in a 1.5 mL tube, until a clear yellow supernatant and a dark brown pellet can be observed. The supernatant is then used for the tracking experiments. GLOX is used to prevent laser-induced phototoxicity of the cells.

Due to the epifluorescence setup, it is important to select relatively flat cells for the tracking experiments. In thick cells, the virus particles will easily go out of focus. Here, we describe the use of BS-C-1 cells (African Green Monkey kidney cells). BS-C-1 cells are maintained at 37°C and 5% CO₂ in 1x high glucose, L-glutamine-enriched DMEM (PAA) with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). For the tracking experiments, BS-C-1 cells are seeded in a 8-wells Lab-Tek II Chambered Coverglass (Nunc No. 155409) such that the well is 50 to 70% confluent at the day of tracking. When cells are 100% confluent, they become thicker, thus less suitable for tracking experiments. The cells are then washed twice with cold serum-free, phenol red-free MEM (Gibco), after which cold phenol red-free MEM containing 1% glucose is added. Phenol red-free media is used to reduce background fluorescence. GLOX is added to the media at a final concentration of 1x and the chamber slide is swirled. Subsequently, DiD-labeled DENV (~10⁸ GCPs) is added to the cells (total volume 150 μL/well) and incubated for at least 15 min at 4°C. Unbound virus is removed by washing the cells with cold phenol red-free MEM, The Lab-Tek is then placed in the microscope and the temperature is rapidly elevated to and kept at 37°C throughout the tracking experiment using the thermostatted stage and objective heater. The moment of the temperature shift to 37°C is referred to as time point 0 min. Alternatively, the virus can be added to the cells in situ at 37°C.

A cell is focused and selected with the differential interference contrast (DIC) optics. Avoid single isolated cells which are not attached to others, since they have a greater chance of dying during the experiment. Try to find a flat cell of intermediate size. Before and after fluorescence imaging, a DIC image is taken to register the localization of the nucleus and plasma membrane of the cell, damage, movement, or a general change in cell morphology. A movie of the fluorescence emission is then recorded with an EM CCD camera at least 1 frame per s for a total of 25 – 30 min after the temperature shift. During this period, several DiD-labeled single particles will be seen in the field. Fusion of the virus within the endosome will be evident as an increase in fluorescence intensity of a single particle (see Figure 2A). For a real-time movie of single-particle entry and membrane fusion of DiD-DENV in BS-C-1 cells see the supplemental material of Van der Schaar et al.¹⁶.

Ammonium chloride can be included as a negative control if the virus to study is expected to fuse within acidic endosomes. The virus will then be able to bind and be internalized into the cells, but fusion is prohibited due to neutralization of the endosomal pH. In case of DENV, cells are treated with 20 mM ammonium chloride before and after binding of virus to the cells¹⁶.
Chapter 2

After single DiD-virus tracking has been completed, analysis of the trajectories can provide a detailed insight into the dynamics of virus cell entry. The movies produced during a tracking experiment consist of a time series of approximately 1500 images that may show several fluorescent particles in the same imaging field. The identification of the particles to be analyzed is done by eye, by first pinpointing possible fusion events at the end of the movie, and then going backwards to determine which of those fusion events were originally small single DiD-labeled viruses. Only those virus particles that move roughly within the same focal plane and show more than a fivefold increase in fluorescence intensity after membrane fusion are used for single-particle tracking analysis (Figure 2A).

The analysis consists of the conversion of the raw data (stack of fluorescence images) into a set of trajectories by use of computational algorithms. In general, what these algorithms do is to (i) remove noise, (ii) identify virus particles by locating isolated fluorescent peaks, and (iii) establish trajectories by connecting particles from one frame to the next using nearest-neighbor association and the motion history of individual particles. The “Particle Detector and Tracker” ImageJ plugin, for instance, can be used for the automated detection and analysis of two-dimensional particle trajectories. Other examples of these software tools (both commercial and open-source) are summarized by Hand et al.

The resulting trajectories can be analyzed to study transport mechanisms.
and to detect interactions with cellular structures. A single-particle trajectory can be represented as a plot of a specific particle parameter (e.g., fluorescence intensity, coordinates) as a function of time or space. Time trajectories of fluorescence intensity, for example, show the specific fusion time-point of DiD-labeled virus within endosomes, which is defined as the moment at which the DiD fluorescence intensity starts to increase (Figure 2B). For DiD-DENV infecting BS-C-1 cells, the majority of particles fuse within 17 min after the temperature shift in both the periphery and perinuclear regions of the cell\(^\text{16}\). Other features that can be analyzed from the obtained trajectories include total and net distance traveled, directional changes, instant and average velocities, accelerations, and mean square displacement (MSD). The latter enables to compute diffusion parameters of individual particles, resulting in an MSD-time curve that can be used as an indicative of the mode of motion (Brownian, active transport, impeded or confined)\(^\text{19}\).

**Troubleshooting**

If little or no bound DiD-labeled particles are observed, not enough virus is being applied to the cells, or the binding capacity of the virus to the cells is too low or most particles are washed away. In this case, perform parallel attachment assays based on qPCR to determine the number of GCPs bound per cell. When optimizing the tracking procedure, it is important to work with a cell line that is adherent, thin, and highly permissive to infection, since flat cells allow the tracking of one virus particle in one focal plane. If a cell is highly permissive to infection, multiple fusion events per cell can be observed. Yet, not all bound particles will fuse; we previously observed that 1 out of 6 bound DiD-DENV2 particles in BS-C-1 cells undergo membrane fusion\(^\text{16}\). Other cell lines that have been used for single-virus tracking include TZM-bl and DFJ-8 cells for human immunodeficiency virus type 1 (HIV-1)\(^\text{20, 21}\), CHO cells for influenza virus\(^\text{15}\), Huh-7.5 cells for hepatitis C virus (HCV)\(^\text{22}\), 3T6 Swiss albino fibroblasts and mouse lung fibroblasts for polyoma virus-like particles\(^\text{23}\), among others. We have also used CV1, P388D1 and PMA-treated U937 cells for tracking of DiD-labeled DENV2 (unpublished data).

If little or no fusion events are detected, the incubation time of the virus with the cells may not be enough for the DiD particles to fuse. In that case, a longer movie can be recorded but always taking into consideration laser-induced phototoxicity of the cells, as evidenced by damaged or dying cells.

The temperature of the medium during tracking should be kept constant at 37°C. We previously experienced with DiD-DENV that a drop of 5°C abolishes all membrane fusion events. If all conditions are met but no membrane fusion events are seen, the temperature of the medium inside the Lab-Tek should be checked for the duration of the experiment.
Binding and fusion assay

Procedure of the assay

The experimental setup described for the single-virus tracking experiment (virus preparation, characterization and DiD-labeling, microscopic setting, and BS-C-1 cells seeding and culture) can be readily adapted to a bulk assay to get a quick quantitative insight in the relative extent of virus binding to the cell surface and membrane fusion. An advantage of the bulk assay is that it does not require the use of an advanced real-time fluorescence microscope.

The relative extent of virus-cell binding can be estimated by measuring the number of membrane-bound DiD-labeled DENV particles to BS-C-1 cells. The BS-C-1 cells are seeded and treated as described above. Subsequently, the cells are incubated for 10 min at 4°C. DiD-labeled DENV is then added to the BS-C-1 cells and incubation is continued for 1 h at 4°C. Unbound virus is removed by gently washing the cells three times with cold phenol red-free MEM. Subsequently, cold phenol red-free MEM containing 1% glucose and GLOX is added to the cells and the microscopic analysis can start, as described below.

Besides determining the extent of virus-cell binding, this experimental setup can also be used to investigate the membrane fusion capacity of the virus. The only difference is that in this case the cells are kept at 37°C for the duration of the experiment. For DENV, the extent of membrane fusion is determined at 20 min post addition of the virus to BS-C-1 cells as single-particle tracking analysis revealed that more than 90% of the particles fuse within 17 min post-infection.

The microscopy analysis is done by taking 15-20 snapshots of randomly selected fields using both normal transmitted light (e.g. DIC) and DiD channels. DIC images are taken from each field to make sure that the visualized DiD-particles are bound to a cell and not lying in an empty space with no attached cells. In the binding assay, single fluorescent virus particles should be clearly detected as small fluorescent spots (Figure 3A). In the fusion assay, fusion should be evident as spots with increased fluorescence intensity (Figure 3B).

A set of negative controls should be included to confirm that viral fusion is, indeed, what is being measured. By single-particle tracking and infectivity assays we showed that addition of ammonium chloride to cells completely inhibits membrane fusion of DiD-labeled DENV with endosomes and therefore we use this treatment as our negative control.

Data analysis

The acquired images in the binding assay are analyzed by counting the total number of bound DiD-particles. The binding capacity should be presented relative to that of a control as it is unlikely that all particles present in solution are labeled.
Monitoring virus entry using DiD-virus properly (Figure 1).

The extent of membrane fusion is analyzed by measuring the total fluorescent signal per imaging field. Since the fusion assay quantifies total post-fusion fluorescence, it provides an indirect measurement of how much fusion took place during a particular infection. Simply counting the number of fluorescent spots by eye will lead to misinterpretation of the data, as upon membrane fusion, blebbing of DiD-labeled endosomes can occur. For this reason, the fusion assay does not quantify the absolute number of fusion events occurring in a single imaging view, but it only provides a relative extent of fusion. For estimation of the actual number of membrane fusion events in one focal plane, it is necessary to perform single-virus tracking as described above. The membrane fusion capacity of the virus should be related to that of a negative control (in case of pH-triggered viruses, for example, ammonium chloride treated cells). The quantitative results of the negative controls should be taken as background; hence, they should be subtracted from the total fluorescence result of the positive control.

ImageJ offers the necessary tools for the image processing. To analyze the images, change them to 8-bit, subtract background, adjust the threshold using different controls as a reference, and use the Analyze Particles tool. In the binding assay, to avoid exclusion of small particles and inclusion of aggregates in the total particle count, the size range (“Analyze Particles…”) should be low, being the lower limit of at least 2 pixels. For the fusion assay, extend the size range (to include bigger objects) as well as the circularity range, since the image might include big groups of fusion events with uneven shapes (see Figure 3B). A macro can speed up the processing when a large number of images are analyzed. No particular plugin is needed for the analysis.

Applications

Single-virus tracking of DiD-labeled virus provides an excellent tool to
follow the fate of individual virus particles in cells. By means of this technique, virus-receptor binding, intracellular transport behavior, and membrane fusion characteristics can be investigated in living cells. Single-virus tracking of DiD-labeled virus has been used, for example, to dissect the route of entry of DENV, influenza virus, and hepatitis C virus.

The dynamic interactions between viruses and the cellular machinery can also be unraveled by tracking of DiD-labeled virus particles in cells expressing specific marker proteins that are tagged with a fluorescent protein. The use of tagged proteins can be challenging since it is difficult to obtain a sufficient level of protein expression without interfering with cellular processes or viral infectivity. Examples of tagged proteins that have been used to study the endocytic pathway of different viruses and do not adversely affect endocytic trafficking include clathrin-eYFP, Rab5-eCFP, Rab7-eYFP, caveolin-1-GFP, actin-GFP, Rab4-YFP, Rab5-YFP, Rab11-YFP, and Lamp1-YFP.

Another approach for studying viral entry into the cells is the use of biochemical inhibitors of different stages of endocytic trafficking. In this case, the described binding and fusion assays can serve as a tool to determine the effect of endocytic inhibitors on DiD-labeled virus infection. Examples of some of these inhibitors are listed in Table 1. This approach has also been applied by Zaitseva et al. to determine the effect of anionic lipids on DENV-mediated intracellular fusion and viral infection.

**Table 1. Biochemical inhibitors of endocytosis.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Inhibitor</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clathrin-mediated endocytosis</td>
<td>Chlorpromazine</td>
<td>Induces reversible translocation of clathrin and its adapter proteins from the plasma membrane to intracellular vesicles.</td>
</tr>
<tr>
<td>Caveolae-mediated endocytosis</td>
<td>Filipin</td>
<td>Sterol-binding agent that disrupts caveolar structure and function.</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>Sterol-binding agent that disrupts caveolar structure and function.</td>
</tr>
<tr>
<td>Phagocytosis and macropinocytosis</td>
<td>Cytochalasin D</td>
<td>Mycotoxin that inhibits actin polymerization and induces actin depolymerization.</td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>Inhibitor of sodium-proton exchange.</td>
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<tr>
<td></td>
<td>Wortmannin</td>
<td>PI3K inhibitor, impedes fusion of endosomes.</td>
</tr>
<tr>
<td>Acidic pH of endosomes</td>
<td>Bafilomycin A1</td>
<td>Specific inhibitor of vacuolar-type H(+) ATPase, inhibits acidification and protein degradation in lysosomes.</td>
</tr>
<tr>
<td></td>
<td>Ammonium chloride</td>
<td>Neutralizes the acidic compartments.</td>
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</table>
If the route of virus cell entry is known, the effect of an external stimulus can be studied by both single-virus tracking and the bulk binding and fusion assays. For example, the neutralizing or enhancing properties of antibodies on viral infections can be analyzed in detail by studying the cell entry mechanism of antibody-opsonized DiD-labeled virus particles. Furthermore, the mode of action of antiviral drugs that interfere with virus cell entry can be unraveled.

If labeling of the viral particle membrane is combined with labeling of the genome or capsid, the release of the genetic material or nucleocapsid from the endosome can be visualized. Labeling of viral genomes can be done by infecting cells in the presence of fluorescently labeled nucleotides or nucleic acid-binding dyes.

Concluding remarks

Single-virus tracking represents an excellent system to address - in real time - the transport dynamics of single virus particles and their membrane fusion characteristics in cells. Fluorescent labeling with lipophilic probes, like DiD, can in principle be done with any given enveloped virus, providing an important tool to explore the initial stages of viral infection processes. This technique can also be adapted to study dynamic interactions with cellular factors, providing a better insight in the multiple steps of viral entry.

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