Deciphering virus entry with fluorescently labeled viral particles

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Running title: Fluorescent Labeling of Viruses

i. Abstract

To infect host cells, viruses have to gain access into the intracellular compartment. The infection process starts with the attachment of viruses to the cell surface. Then a complex series of events, highly dynamic, tightly intricate, and often hard to investigate, follows. This includes virus displacement at the plasma membrane, binding to receptors, signaling, internalization, and release of the viral genome and material into the cytosol. In the past decades, the emergence of sensitive, accurate fluorescence-based technologies has opened new perspectives of investigations in the field. Visualization of single viral particles in fixed and living cells as well as quantification of each virus entry step have been made possible. Here we describe the procedure to fluorescently label viral particles. We also illustrate how to use this powerful tool to decipher the entry of viruses with the most recent fluorescence-based techniques such as high-speed confocal and total internal reflection microscopy, flow cytometry, and fluorimetry.

ii. Key Words

Endocytosis, Flow cytometry, Fluorescent dyes, Fluorescently labeled viral particles, Fluorimetry, Intracellular trafficking, Membrane fusion, Microscopy, Single viral particle tracking, Virus entry.

1. Introduction

Animal viruses present an apparent diversity, not only in size, structure, tropism, and mode of replication, but also at the level of host cell entry. To transfer their genome and proteins into the cytosol, enveloped viruses make use of membrane fusion, while non-enveloped viruses induce membrane lysis or pore formation [1,2]. The penetration can occur directly through the plasma membrane (Fig. 1) [3]. However a large majority of viruses enters the cytosol through endosomal membranes following binding to cellular surface receptors and sorting into the endocytic machinery (Fig. 1) [4,1]. A few viruses have been shown to penetrate cells from the endoplasmic reticulum [2]. The requirements and details differ in each case.

In the last decades, major advances in fluorescence microscopy and other fluorescence-based techniques have significantly contributed to improve our understanding of early virus-host cell interactions. Nevertheless the big picture of virus entry remains largely incomplete. Our knowledge of these complex processes, which involve hundreds of cellular factors with many functions, are limited to a sprinkle of viruses. Here we will provide general protocols to study virus entry in fixed and living cells. We will first describe a method to label viruses with fluorescent dyes. We next present a set of basic experimental procedures involving the use of fluorescently labeled viruses in combination with advanced fluorescence microscopy, flow cytometry, and fluorimetry technologies to investigate virus binding, uptake, intracellular trafficking, and fusion. To illustrate our points, Uukuniemi virus (UUKV) will serve as a model system, but the procedures described here can be readily applied to influenza virus.

1.1 The arthropod-borne virus Uukuniemi

UUKV is an arthropod-borne virus (arbovirus) from the genus *Phlebovirus* in the *Phenuiviridae* family (order *Bunyavirales*) [5]. UUKV was originally isolated from the *Ixodes ricinus* tick reservoir in the 1960s [6], and later, from seabirds [7]. UUKV constitutes the viral model system to study the highly pathogenic tick-borne human phleboviruses that have recently emerged in different parts of the world [8,9]. Examples are Heartland virus in North America [10] and severe fever with thrombocytopenia syndrome virus (SFTSV) in Asia, with the latter causing a fatality rate of up to 60 % in several outbreaks [11].

Similar to other phleboviruses, UUKV particles are enveloped and roughly spherical (*ca.* 100 nm in diameter) with a tri-segmented single-stranded ribonucleic acid (RNA) genome (Fig. 2A) that replicates exclusively in the cytosol [12]. The three RNA segments encode four structural proteins in a negative-sense orientation: the nucleoprotein N, the RNA-dependent RNA polymerase L, and two transmembrane glycoproteins (G_N and G_C) [5,13]. The protein N

is associated with the RNA genome and, together with the viral polymerase, constitutes the ribonucleoproteins.

On viral particles, G_N and G_C are responsible for the virus attachment to target cells [12]. Many open questions remain concerning the receptors, cellular factors, and pathways used by phleboviruses to enter their host cells. However, the human C-type lectins DC-SIGN and L-SIGN have been shown to act as receptors for UUKV and many human pathogenic phleboviruses [14,15]. Both lectins are interesting candidate molecules to explain the tropism of phleboviruses for dermal dendritic cells (DCs) and liver [5]. DC-SIGN is expressed on the surface of dermal DCs whilst L-SIGN is present on the endothelium lining hepatic sinusoids.

Following virus binding to cells, UUKV is sorted into the endocytic machinery and enters the cytosol by acid-activated membrane fusion from late endosomal compartments [4,15,16]. Therefore, UUKV is a late-penetrating virus, a large group of viruses that share dependence on late endosomal maturation for productive infection, including influenza virus [4]. Upon endosomal acidification, UUKV glycoproteins undergo multiple conformational changes resulting in the fusion of the virus envelope with the endosomal membrane, and then, the subsequent release of the viral genome and associated proteins into the cytosol [17,16]. While acidification below pH ~5.5 is sufficient to trigger UUKV fusion [18,16], the efficiency of the whole process depends on additional factors such as the lipid composition of target membranes. For instance, the late endosomal lipid bis-(monoacylglycero)-phosphate facilitates UUKV fusion [18]. Once the virus gains access to the cytosol, replication and infection begin.

UUKV represents an interesting model to study arbovirus entry. **Large amount of virus can be produced in the absence of serum, which largely facilitates the purification steps and subsequent fluorescence-labeling procedures.** Bright fluorescent viral particles can be obtained without impairing the infectivity of the virus using a minimal molecular ratio of dyes to viral envelope glycoproteins. Finally, UUKV is a validated biosafety laboratory level 2 surrogate for arboviruses of higher biosafety classification. The use of UUKV allows approaches such as live-cell imaging that are nearly impossible for pathogenic human arboviruses, most of which must be handled in higher biosafety laboratories.

1.2 Small fluorescent dye molecules to label viral particles

There is a diverse range of methods to make viral particles fluorescent. Many viruses have successfully been genetically engineered to carry fluorescent protein reporters [19], stainable small tags such as the tetracysteine peptide with biarsenical fluorescent compounds [20], or clickable amino-acids, a novel technology with minimal invasiveness [21]. Fluorescent dye

molecules are also commonly employed to non-specifically stain the transmembrane proteins and envelope membranes in viral particles (Fig. 2B) [19]. All of these technologies present their own set of advantages and limitations, which are all discussed in recent reviews [19-21].

In this chapter, we will limit our experimental labeling procedures to the use of the fluorescent dyes Alexa Fluor (AF) succinimidyl esters and octadecyl rhodamine B chloride (R18). **Labeling of viral particles with those dyes is arguably the least invasive method** and is compatible with analysis of fixed and live-cell samples, in addition to exhibit bright fluorescence and great photostability. While the dye AF reacts with free amine groups to form covalent links on viral transmembrane proteins such as UUKV G_N and G_C, R18 inserts in the lipid bilayer membrane of virions (Fig. 2B). AF-conjugated viral particles are often used to monitor virus binding, uptake, and intracellular trafficking. AF dyes exist in different versions that cover a broad range of emission colors from ultraviolet to far red. The lipophilic dye R18 is rather employed to assess and quantify viral membrane fusion. The high density of R18 dye molecules in the virus envelope results in the auto-quenching of the fluorescence signal. After fusion, the release of R18 in the endosomal membrane is followed by a fluorescence increase that can be quantified with a spectrofluorometer (Fig. 2C).

1.3 Fluorescence microscopy to track virus entry events

Fluorescence microscopy offers a powerful tool to investigate virus entry. Laser scanning confocal microscopy allows for scanning cells from the bottom to the top, and therefore, for imaging the inside of cells. Derived technologies have enabled ultra-fast confocal imaging such as spinning disc microscopy, which are particularly appropriate to study the dynamics of virus entry into cells. Total internal reflection (TIRF) microscopy is another example of a recently developed fluorescence microscopy technique. TIRF microscopy permits the visualization of the plasma membrane and virus motion at the cell surface with unprecedented accuracy. Super-resolution microscopy as well as correlative light and electron microscopy (CLEM) provide access to ultrastructural details of virus penetration into cells. Multiphoton microscopy will arguably open the possibility to follow virus entry in live animals in the near future. For a more complete picture of these microscopy techniques, we recommend recent reviews [22-27].

1.4 Quantification and definition of the main virus entry steps

Development of image analysis and deep-learning algorithms have enabled the tracking of a larger number of virus entry events from microscopy pictures, and in turn, the quantification of the different virus entry stages [28,29]. In many cases, flow cytometry and fluorimetry

represent an interesting alternative to measure virus binding, internalization, and fusion. Both methods offer a good compromise between the number of samples to analyze and the rapidity of analysis. Furthermore, the monitoring of fluorescently labeled viral particles by fluorescence microscopy, flow cytometry, and fluorimetry can be combined with the use of perturbants of the endocytic machinery such as drugs, dominant negative and constitutively active mutants, and small interfering RNAs [30]. In addition, prior exposure to viruses and subsequent analysis, cells can also be transfected with plasmids encoding endocytic markers fused to fluorescent proteins [19]. Together these approaches have been proven useful to provide new insights into the entry program of a number of viruses [31,32,15,16,28,33-35].

2. Materials

2.1 Virus production

1. 175-cm² cell culture flasks with filter screw caps.
2. Glasgow's Minimum Essential Medium (GMEM).
3. Baby hamster kidney cells BHK-21: cultured in GMEM supplemented with 5 % (v/v) fetal bovine serum (FBS), 10 % (w/v) tryptose phosphate broth (TPB), 1 % (v/v) penicillin/streptomycin.
4. Phosphate buffered saline (PBS).
5. Trypsin-ethylene(diamine)tetraacetic acid (EDTA).
6. UUKV S23: the prototype strain of UUKV that has been previously described [36,37].
7. Any classical 50-mL tubes.
8. Ultracentrifuge and a rotor with buckets equivalent to the SW28 or 32 (Beckman Coulter).
9. Ultracentrifuge tubes compatible with the SW28/32 buckets.
10. Sucrose.
11. 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES).
12. 10x stock solution of HNE buffer: water supplemented with 100 mM HEPES, 1.5 M NaCl, 10 mM EDTA, filtered through a 0.2 µm filtration membrane (can be stored at room temperature for months to years).

13. 25 % (w/v) sucrose solution: 25 g of sucrose is first diluted in 50 mL of deionized water, then supplemented with 10 mL of 10x HNE buffer, and finally completed to 100 mL with sterile water, then filtered through a 0.2 µm filtration membrane.

14. 1x HNE buffer: 10x stock solution diluted 1:10 in sterile water.

15. Pieces (about 3 cm x 3 cm) of Parafilm "M" (Laboratory Film).

2.2 Fluorescence labeling of viral particles

1. Precast 4-12 % Bis[Tris(hydroxymethyl)aminomethane] (Bis-Tris) 10-well gels (Thermo Fisher Scientific).

2. 3-(N-morpholino)propanesulfonic acid (MOPS) sodium dodecyl sulfate (SDS) running buffer (Thermo Fisher Scientific).

3. The protein blue loading buffer used here is the 4x lithium dodecyl sulfate (LDS) sample buffer from Thermo Fisher Scientific.

4. Gel fixative solution: 40 % (v/v) methanol and 10 % (v/v) acetic acid in water.

5. Coomassie blue staining solution: 50 % (v/v) methanol, 10 % (v/v) acetic acid and 0.25 % (w/v) Serva Blue G (Serva) in water.

6. Ultrapure bovine serum albumin (BSA).

7. AF and R18 dyes (Thermo Fisher Scientific): working stocks are resuspended in dimethylsulfoxide for AF dyes, and 100 % ethanol for R18 according to the manufacturer's recommendations. Aliquot and store at -80 °C.

8. OptiPrep (also named Iodixanol) (Axis-Shield): working solutions of OptiPrep are prepared at the desired concentration in sterile water supplemented with 1x HNE buffer and 8.6% sucrose, then filtered through a 0.2 µm filtration membrane.

9. Ultracentrifuge and a rotor with buckets equivalent to the SW60 (Beckman Coulter).

10. Ultracentrifuge tubes compatible with the SW60 buckets.

2.3 Characterization of fluorescently labeled viral particles

1. Any classical 24-well plates.

2. BHK-21 cells.

3. GMEM supplemented with 10 % (w/v) TPB, 1 % (v/v) penicillin/streptomycin.

4. Carboxymethyl cellulose (CMC) working solution: 3.2 % (w/v) CMC and 0.85 % (w/v) sodium chloride in water. The solution is continuously stirred overnight and then autoclaved.

5. PBS.

6. Formaldehyde (FA, 37 % pure).

7. PERM buffer: 2 % (v/v) FBS, 5 mM EDTA, 0.02 % (v/v) azide, 0.1 % (w/v) saponin in PBS, filtered through a 0.2 µm filtration membrane (can be stored at 4 °C for 3-4 months).

8. The polyclonal rabbit antibody U2: directed against all UUKV structural proteins (house-made, [15]).

9. Peroxidase-conjugated monoclonal antibody (Vector Laboratories).

10. Diaminobenzidine (DAB) solution kits (Vector Laboratories): DAB working solution is prepared according to the manufacturer's recommendations.

11. Precast 4-12 % Bis-Tris 10-well gels (Thermo Fisher Scientific).

12. 4x LDS sample buffer (Thermo Fisher Scientific).

13. A fluorescence imaging system such as the Odyssey imaging system.

14. Microscope slides (76 x 26 x 1 mm, Marienfeld).

15. 12-mm microscope cover glasses (Thermo Fisher Scientific).

16. A Leica SP8 confocal microscope or equivalent.

17. An Olympus IX81 wide-field microscope or equivalent.

2.4 Analysis of virus entry by fluorescence microscopy

1. Different models of Nunc Lab-Tek chambers (Thermo Fisher Scientific), with unique or multiple wells, can be used for fixed and live-cell microscopy analysis (*see* **Note 1**).

2. Phenol red-free Dulbecco's modified Eagle's medium (DMEM).

3. Binding buffer: phenol red-free DMEM supplemented with 0.2 % (w/v) BSA and 20 mM HEPES (pH ~7.4).

4. A Nikon TiE Eclipse microscope equipped with a PerkinElmer UltraVIEW VoX 3D module or any equivalent spinning disc or confocal microscope with live-cell imaging capacity.

5. A Nikon TiE Eclipse wide-field microscope equipped with a Perfect Focus System or any equivalent wide-field microscope with live-cell imaging capacity.

6. Human lung carcinoma cells A549: cultured in DMEM supplemented with 10 % (v/v) FBS and 1 % (v/v) MEM non-essential amino acids (Thermo Fischer Scientific).

7. Opti-MEM I (Thermo Fischer).

8. Lipofectamine 2000 (Thermo Fischer).

9. pcDNA3 plasmid coding for PH-PLCΔ1-EGFP, a fluorescent protein marker in the plasma membrane conjugated to the enhanced green fluorescent protein (EGFP), or any equivalent plasmid system encoding fluorescent plasma membrane marker.

10. EDTA.

11. Extracellular matrix (ECM) obtained by detaching cells with EDTA.

12. A Leica AM TIRF microscope or an equivalent microscope that allows the illumination of bottom surface or cytoplasm of live cells by adjusting the penetration depth between 90 and 200 nm.

13. Human cervix carcinoma cells HeLa: cultured in DMEM supplemented with 10 % (v/v) FBS.

14. FA.

2.5 Analysis of virus entry by flow cytometry and fluorimetry

1. U-bottom 96-well plates without cell culture treatment.

2. Binding buffer: phenol red-free DMEM supplemented with 0.2 % (w/v) BSA and 20 mM HEPES (pH ~7.4).

3. FA.

4. PBS.

5. A549 cells.

6. Trypan Blue.

7. A FACS Calibur cytometer (Becton Dickinson) or equivalent.

8. Any classical 1.5-mL tubes.

9. A Cary Eclipse spectrofluorometer (Agilent Technologies) or equivalent.

3. Methods

3.1 Virus production

1. Propagate BHK-21 cells to ~ 60-80 % confluency within 35 mL of complete growth medium in 175-cm² cell culture flasks (*see* **Note 2**). PBS is used for cell wash steps and Trypsin-EDTA solution for the detachment of cells as for cell passaging of adherent cells.

2. Wash cells once with serum-free media before exposition to UUKV in 15 mL of serum-free medium per flask at a multiplicity of infection (MOI) of 0.05 for 1 hour at 37 °C (*see* **Note 3**).

3. Discard the input virus and replace with 35 mL of serum-free medium per flask (*see* **Note 4**).

4. Incubate infected cells at 37 °C for 72 hours (*see* **Note 5**).

5. Harvest the 35 mL of virus-containing supernatant and collect in 50-mL tubes before clearing by centrifugation at 2,000 g for 20 min at 4 °C (*see* **Note 6**).

6. Pool virus-containing supernatants and distribute 33.5 mL per SW28/32 tube. Then carefully underlay the supernatant with 2.5 mL of 25 % (w/v) sucrose solution (*see* **Note 7**).

7. Concentrate the viral particles by ultracentrifugation at 100,000 g at 8 °C for 2 hours (acceleration and deceleration set to max) (*see* **Note 8**).

8. After ultracentrifugation, dispose the supernatant. Immediately add 300 µL of HNE buffer and cover each tube with parafilm to prevent the virus pellets from drying. Let the pellets dissolved on ice for at least 1 hour (*see* **Note 9**).

9. Gently resuspend the virus pellets by pipetting up and down several times on ice.

10. Pool the resuspended virus and make aliquots for long-term storage at -80 °C (*see* **Note 10**).

3.2 Fluorescence labeling of viral particles

1. The viral glycoproteins G_N and G_C in the virus stock are first semi-quantified following SDS-polyacrylamide gel electrophoresis (PAGE) separation and Coomassie blue staining. Mix different amounts of viral proteins (typically 15, 10, and 5 µL of the virus stock) with LDS sample buffer in a final volume of 20 µL and, separate by SDS-PAGE through a 10-well precast 4-12 % Bis-Tris gradient gel using a MOPS SDS running buffer.

2. Incubate gels in fixative solution for 1 hour before staining with Coomassie solution for up to 12 hours and extensive washing with water (*see* **Note 11**). An example is shown in Fig. 3A.

3. The glycoproteins G_N and G_C are normalized against BSA. The intensity of each band is measured with the software ImageJ [38]. One square is defined and used to measure each band, as illustrated in Fig. 3A (box1). An empty well (labeled "0" in Fig. 3A) is used to define the background and the value is subtracted from all other values. A standard curve correlating the relative unit (RU) and the quantity of protein is then obtained (Fig. 3B). A linear regression is applied to determine the quantity of G_N and G_C in each sample (Fig. 3C).

4. Add AF fluorescent dyes in a range of 1:1 - 1:5 molar ratio of viral envelope glycoproteins to dye while vortexing (*see* **Note 12**). Alternatively, 10^9 focus-forming units (ffu) of UUKV are labeled with the lipid dye R18 (20 µM) according to a similar protocol. Incubate at room temperature for 2 hours in the dark whilst gently mixing on a shaker.

5. Fluorescently labeled particles are banded in a density gradient composed of six steps prepared in ultracentrifuge SW60 tubes. For each step, 600 µL of OptiPrep with a final (v/v) concentration of 10 %, 20 %, 25 %, 30 %, 35 %, and 50 % are carefully pipetted on top of each other, starting with the highest concentration.

6. Overlay gently the AF-virus mix (from step 4) onto the gradient. Centrifuge at 100,000 g at 4 °C for 90 min (acceleration and deceleration set to max and min respectively), after which the viral particles with an identical density will typically reach the interface between the 25 % and 30 % OptiPrep layers. Unbound dyes remain at the top of the gradient (Fig. 4A) (*see* **Note 13**).

3.3 Characterization of fluorescently labeled viral particles

3.3.1 Virus titration by focus-forming assay

1. Seed BHK-21 cells in 24-well plates (10^5 cells per well) 18 hours before infection.

2. On the day of infection, cells should form confluent monolayers. Wash cells with serum-free medium and cover with 200 µL of serum-free medium.

3. Add 200 µL of 10-fold dilutions of viral particles in serum-free medium (from 10^{-2} to 10^{-8}) to the wells (i.e. final volume of 400 µL). Incubate at 37 °C for 1 hour.

4. Add 400 µL of medium containing 2.5 % (v/v) FBS and supplemented with 1.6 % (w/v) CMC to block virus cell-to-cell spread. Incubate cells at 37 °C for up to 72 hours (*see* **Note 14**).

5. Wash the cells twice with serum-free medium, and then once with PBS.

6. Fix cells with 4 % (v/v) FA diluted in PBS at room temperature for 20 min.

7. *As UUKV is not lytic, foci are detected by immunostaining.* Wash cells with PBS and then incubate with 300 µL of the antibody U2 (diluted 1:1,000 in PERM buffer) at room temperature for 1 hour (*see* **Note 15**).

8. Wash cells twice with PERM buffer, and incubate with 300 µL of secondary peroxidase-conjugated antibody (diluted 1:200 in PERM buffer) for 45 min at room temperature.

9. Wash cells once with PBS, incubate in DAB working solution for up to 10 min, and rinse twice with deionized water. The virus titer ($ffu.mL^{-1}$) is obtained by counting the number of ffu (n) at a factor of dilution (f) for which n is comprised between 3 and 30 using the equation 5n / f. In the example shown in Fig. 4B, one can count 22 ffu at the dilution 10^{-6}. Therefore the titer of this AF568-labeled UUKV stock is $5 \times 22 / 10^{-6} = 1.1 \times 10^{8}$ $ffu.mL^{-1}$.

3.3.2 Assessing integrity of labeled viruses

1. To control for the intactness of fluorescent viruses, viral particles are diluted in LDS sample buffer and analyzed by SDS-PAGE in 10-well precast gradient gels.

2. Expose gel to a fluorescence imaging system. Only G_N and G_C should be fluorescently labeled (Fig. 4C). *The absence of fluorescent signal from the nucleoprotein N indicates that the viral envelope is intact.*

3.3.3 Visualization of viral particles

Fluorescently labeled viral particles are analyzed by microscopy to confirm the homogeneity of the viral preparation and for the absence of aggregates. Add 5 µL of a fluorescently labeled virus preparation onto a microscopy slide and gently place a coverslip on top. Fig. 4D shows an illustration of AF568-labeled UUKV (UUKV-AF568) imaged by confocal microscopy. *However, wide-field microscopy with a magnification of 20x is usually sufficient to visualize the particles.*

3.4 Analysis of virus entry by fluorescence microscopy

3.4.1 Live-cell imaging using wide-field and confocal fluorescence microscopy

1. Seed cells in 8-well Nunc Lab-Tek chambers (2.5×10^{4} cells per well in 400 µL of complete medium) (*see* **Note 16**) and incubated at 37 °C overnight.

2. The next day, wash cells once with phenol red-free binding buffer and cover the wells with 400 µL of said pre-warmed binding buffer for 30 min at 37 °C.

3. Transfer the Nunc Lab-Tek chambers under the microscope (*see* **Note 17**).

4. Add the fluorescently labeled viral particles into the well when the confocal microscope is set up and ready for imaging (*see* **Note 18**). Fig. 5A is an illustration of virus motion monitored with a confocal spinning disc microscope. The time-lapse series shows UUKV-AF594 surfing on filopodia towards the cell body before being taken up into an A549 cell (human lung epithelium line). In this latter example, cells were reverse transfected with a plasmid carrying the gene for PH-PLCΔ1-EGFP, a protein associated with the plasma membrane. Deoxyribonucleic acid (DNA) transfection was performed using Opti-MEM I and Lipofectamine 2000 according to the manufacturer's recommendation.

3.4.2 Live-cell imaging by TIRF microscopy

1. For the tracking of fluorescent viral particles using TIRF microscopy, two methods can be applied to localize virions between the cells and coverslip (*see* **Note 19**).

a. In the first case, cells are detached from the culture plate with EDTA, and exposed to the virus in suspension in phenol red-free binding buffer on ice. Virus-bound cells are then seeded on coverslips and immediately imaged (*see* **Note 20**).

b. The second case involves pre-binding of virions to coverslips coated with ECM and seeding of cells on top of the bound virions (*see* **Note 21**).

2. A TIRF microscope is used to illuminate the bottom surface or cytoplasm of live cells by adjusting the penetration depth at 90 and 200 nm, respectively. Trajectories and fluorescence intensity of virus spots are analyzed by computational analysis with the Image J-based plugin software Particle Tracker (http://imagej.net/Particle_Tracker) [39]. An example of UUKV trajectory on HeLa cells expressing the virus receptor DC-SIGN [15] is shown in Fig. 5B.

3.4.3 Fixed-cell imaging to monitor virus binding and internalization

1. Seed cells in 8-well Nunc Lab-Tek chamber (2.5×10^4 cells per well in 400 µL of complete medium) and incubated at 37 °C overnight (*see* **Note 22**).

2. The following day, wash cells once with binding buffer and expose the cells to the desired MOI of fluorescently labeled viral particles diluted in 200 µL binding buffer on ice for 2 hours.

3. Wash cells with pre-chilled binding buffer. Samples remain on ice or are rapidly warmed to 37 °C by adding 400 µL pre-warmed complete medium and incubation at 37 °C for up to 40 min (*see* **Note 23**).

4. Discard supernatant and fix infected cells in 200 µL of 4 % (w/v) FA in PBS on ice for 30 min prior confocal microscopy analysis (*see* **Note 24**). Fig. 5C shows UUKV-AF594 either bound to the surface of DCs or internalized inside the cells.

3.5 Analysis of virus entry by flow cytometry and fluorimetry

3.5.1 Virus binding

1. Binding assays are performed in 96-well plates with U-bottom using 2 x 10^5 cells in 200 µL of binding buffer (*see* **Note 25**).

2. Pellet cells by centrifugation (300 g, 4 °C, 5 min) and replace the binding buffer by fluorescently labeled viral particles at desired concentrations in 100 µL of binding buffer. Binding is carried out at 4 °C with gentle agitation for 2 hours.

3. Remove unbound viral particles by a single wash with 200 µL of binding buffer and resupsend cell pellets in 100 µL of 4 % (w/v) FA in PBS for 20 min at 4 °C.

4. Wash cells once with PBS before quantification by flow cytometry. The binding of AF647-labeled UUKV particles (UUKV-AF647) bound to A549 cells is shown in Fig. 6A.

3.5.2 Virus internalization

1. For internalization assays, bind AF488-labeled viral particles to cells as described above.
2. After 2 hours on ice, wash cells with pre-chilled binding buffer to remove unbound particles.

3. Rapidly warm infected cells at 37 °C by resuspension in pre-warmed binding buffer supplemented with 5 % (v/v) FBS to allow virus internalization and kept the cells at 37 °C for the required time period (*see* **Note 26**).

4. To stop internalization, put cells back on ice until flow cytometry analysis.

5. To distinguish between internalized and surface-bound particles, add Trypan Blue to the cells at a final concentration of 0.04 % (w/v) for 15 sec and immediately process the sample in the flow cytometer [31]. *Trypan Blue is membrane impermeable, and thus only able to quench the fluorescence of UUKV-AF488 particles still exposed on the cell surface.* Fig. 6B and 6C are an example of analysis showing the internalization of AF488-conjugated viral particles (UUKV-AF488) into DC-SIGN-expressing HeLa cells.

3.5.3 Viral fusion

1. Our approach to analyze acid-activated virus membrane fusion from endosomes depends on the auto-quenching properties of the lipid dye R18 [28]. *This method allows for accurate quantification of virus fusion with endosomal membranes in living cells* (*see* **Note 27**).

2. Expose cells to R18-labeled UUKV (UUKV-R18) at a MOI of 5 in 1.5-mL tubes containing 500 µL of binding buffer on ice for 1 hour (*see* **Note 28**).

3. After binding, pellet and wash the cells once with pre-chilled binding buffer at 4 °C.

4. Resuspend the cells in pre-warmed complete phenol red-free medium at 37 °C, *to trigger virus internalization*, and immediately transfer the cells inside the spectrofluorometer at 37 °C while measuring the fluorescence in live (*see* **Note 29**). Fig. 7 illustrates the monitoring of UUKV-R18 penetration into BHK-21 cells by measuring the dequenching of the dye R18 with a spectrofluorometer.

4. Notes

1. Alternative cell chambers to Nunc Lab-Tek exist on the market, e.g. Ibidi cell culture dishes with or without culture inserts and MatTek culture slides and dishes.

2. Usually, about 20 millions of cells are seeded to reach a confluence of 70-80 % the next day (roughly 24 hours). To get a homogenous distribution of cells over the whole flask, keep the flasks on a flat surface; i.e. bench, for 5-10 min before incubation at 37 °C. Very often shelves in incubators are not perfectly flat. It is recommended to use 7 flasks for each virus production. One is used as a mock-infected control and help to detect the apparition of cytopathogenic effect (CPE) by comparison with infected flasks. The total volume of 6 flasks corresponds to the maximal volume that is possible to load into SW28/32 buckets for one round of ultracentrifugation.

3. Move the flasks back and forth every 10 min. This will minimize the risk that cells get dry because of the low volume used for infection.

4. BHK-21 cells support the absence of serum for several days. This represents the advantage to facilitate the subsequent purification of the viral particles required for the labeling procedure.

5. To preserve the newly produced viral particles, it is important to harvest the supernatant before infected cells die. Typically CPE appears in cells infected by UUKV at MOI of 0.1 after 60-70 hours exposure to the virus. During this period, cells must be carefully monitored to collect the virus at the most appropriate moment, i.e. just when cells start to detach from the

plastic. The timing can vary at large from one virus production to another and must be seriously taken into consideration to establish an efficient protocol of production.

6. UUKV supports several freezing – thawing cycles without altering the infectivity of the virions. It is therefore possible to stop the protocol at this step by adding 10 mM final concentration of HEPES and store the supernatant either at 4 °C overnight or -80 °C for a longer period. In the latter case, the thawing step must be done slowly at room temperature. HEPES helps to buffer the supernatant at neutral pH and protect viral particles against the important drop in pH during the freezing – thawing steps.

7. It is recommended to aspirate a higher volume of 25 % (w/v)-sucrose solution with a 5-mL pipette than required to underlay the supernatant in the ultracentrifuge tube. This helps to avoid refluxes of supernatant in the pipette during the procedure, and in turn, facilitates the formation of a well-defined sucrose cushion. In addition, the sucrose cushion is made in the same buffer as used to resuspend the virus pellet.

8. The velocity of ultracentrifugation depends on the biophysical properties of the virus studied (density, volume etc.).

9. After pouring out the supernatant, excess liquid can be absorbed from the upper rim of the centrifugation tubes with paper. Avoid touching the virus pellet during this step. Optionally, but also depending on the virus produced, the pellets can be dissolved on ice overnight. Finally, virus pellets can also be resuspended in other buffers than HNE, e.g. Tris-based buffer. However, HNE is preferred for labeling with AF dyes. These dyes react with free amines, which are present in large amounts in the Tris buffer itself. This can result in a lower efficiency of the labeling step.

10. It can happen that white aggregates float in suspension after resuspension of the virus pellets, depending on the cell type and culture media products used. These aggregates can be easily removed by an additional centrifugation step at 3,000 g for 15 min at 4 °C.

11. A washing solution of 10 % (v/v) acetic acid can also be used for a more efficient destaining. Note that UUKV glycoproteins appear with a better focus after SDS-PAGE and Coomassie blue staining when they are not reduced.

12. Cautionary steps should be taken to ensure that viral infectivity is not impaired by fluorescent labeling, which typically happens when a high molar ratio of viral envelope glycoproteins to dyes is used (>1:10). This ratio usually depends on the number of amine groups on the viral envelope glycoproteins that are free to react with the dye molecules. For

instance, with a high number of free amine groups in G_N and G_C, it is possible to get bright fluorescent UUKV particles with a relatively low quantity of dyes (ratio 1:1) and nearly no impact on viral infectivity. Furthermore, the choice of AF dye, i.e. the choice of the dye color, depends on the purpose and constraint/restrictions of each experiment involving fluorescent viral particles.

13. The OptiPrep step gradients are freshly prepared. To see a virus band in gradients by eye, a minimal amount of 100 µg of UUKV G_N/G_C must be loaded. Below this quantity, it is extremely difficult to see the virus band. In this latter case, collect fractions from the gradient and assess each of them for the presence of fluorescently labeled virions (e.g. by western blot analysis against viral proteins). Furthermore, the percentage in OptiPrep for each step as well as the velocity of ultracentrifugation must be adjusted according to the biophysical properties of the virus studied.

14. Incubation can be reduced to 48 hours. The size of foci will consequently appear smaller.

15. Alternatively, the incubation with the primary antibody can be done overnight at 4 °C. For lytic viruses, substitute the immunostaining steps for a crystal violet staining [20 % (v/v) ethanol, 10 % (v/v) FA, 0.2 % (w/v) crystal violet in water (Sigma)].

16. For tracking viral particles in cells, imaging of isolated, single cells is in general easier. For this reason, a relatively low number of cells is seeded the day before the experimentation.

17. For live-cell imaging, it is recommended to use a microscope equipped with a chamber that allows for the control of temperature, carbon dioxide, and humidity.

18. Usually the volume of fluorescently labeled viral particles is negligible in comparison with the volume of medium in the well. Depending on the virus analyzed, it can take up to several minutes before the viral particles sediment and attach to the cells. Alternatively, it is possible to synchronize the binding on ice before warming and imaging. The disadvantage of this latter method is that (1) a thermal shock can result in difficulties to get the right focus and (2) it requires to set up the microscope very rapidly to not miss the early steps of virus internalization.

19. For the visualization of cell-bound particles by TIRF microscopy, the virions have to be localized between the cell and the coverslip. Small viruses such as murine polyomavirus can diffuse into this narrow space, but larger viruses such as UUKV do not. Therefore for small viruses, it is possible to seed the cells the day prior infection and microscopy analysis. The viral particles can be added directly in the well at 37 °C inside the environmental chamber of the

microscope. This has the advantage to enable the presetting of the microscope for imaging before adding the virus.

20. Imaging starts when cells come into contact with the glass surface, which can take a few minutes. Furthermore, one technical problem is that it takes a minimum of 5 min to set up the TIRF microscope and start recording. This means that reactions at 37 °C are already well on their way before recording starts. To slow down the process, imaging can be performed at room temperature.

21. To attach viruses on coverslips prior TIRF microscopy, coverslips are coated with ECM [32]. This simply consists in seeding cells on the coverslips and, in detaching them with EDTA few days later. The ECM remains on the coverslips. Alternatively, fibronectin or polylysine can be used to pre-coat coverslips and attach viral particles.

22. For the same reasons as exposed in Note 16, only a low number of cells is seeded the day before infection and imaging.

23. The timing for penetration depends on the virus studied. It can vary at large, from a few minutes (e.g. Semliki forest virus) to hours (e.g. human papillomavirus).

24. The choice of a classical or ultrafast confocal microscope (i.e. spinning disc microscope) depends on the purpose of the experiment. An ultrafast confocal microscope is particularly appropriate for live-cell imaging. Such a microscope is also substantially faster than a normal scanning confocal microscope for the analysis of fixed samples, when large series of z-stacks and many fields must be imaged.

25. Both none- and adherent cells can be employed in this assay. When adherent cells are used, they are first detached from their plastic culture flasks by treatment with 0.5 mM EDTA PBS for 10 min at 37 °C. Cells are then extensively washed to get rid of EDTA and incubated in complete medium at 37 °C for 30 min. This step helps cells to recover from EDTA treatment. Wash cells at least twice with binding buffer before distribution into 96-well plates.

26. The first 10 min of warming have to be performed in a water bath. Thermal conduction in water is much more efficient than in the air, and therefore enables faster warming of the samples. Results can significantly vary if warming is done in a regular incubator. Depending of the virus studied, the presence or absence of serum during the warming step must be carefully considered since it can impact at large the virus internalization process.

27. This approach can also be applied to study fusion with liposomes.

28. This assay can involve the use of both none- and adherent cells. For studies with adherent cells, see Note 25. Moreover, infection of cells with a lower MOI is possible. In this latter case, it is recommended to increase the number of cells to keep a level of total fluorescence sufficiently high to be measured by fluorimetry.

29. For live-cell measurements, it is important to stir the cells with a magnet. Otherwise cells will sediment on the bottom of the fluorimeter cuvette. As explained in Note 26, the use of serum for the virus internalization should be carefully considered. Alternatively, virus internalization can be performed in a water bath. Cells can be then kept on ice to block endocytosis. This approach presents the advantage to allow measuring all samples at the same time. Finally, as explained in Note 23, the timing of internalization depends on the virus that is investigated.

Acknowledgments

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Figure legends

Figure 1 – The different ways for viruses to enter cells. Viruses employ two main strategies to enter the cytosol of host cells, either through endocytosis or by direct penetration from the plasma membrane. Here enveloped viruses are illustrated; however non-enveloped viruses have evolved similar strategies. Adapted from [40].

Figure 2 – The different fluoresecent dyes used to label UUKV. A. Schematic representation of UUKV. The three viral genomic segments are designated according to their size: S (small), M (medium), and L (large). Adapted from [12]. **B.** Two types of chemical compounds have been used to fluorescently label UUKV particles, i.e. AF molecules that react with free primary amines on the viral glycoproteins G_N and G_C (left column) and, the lipohilic dye R18 that inserts into the viral lipid bilayer envelope (right column). **C.** Principle of the R18-based UUKV fusion assay.

Figure 3 – Quantification of viral glycoproteins in virus stocks. A. UUKV was first pelleted through a 25 % (w/v)-sucrose cushion. Next, different volumes of the virus stock and known amounts of BSA were separated by non-reducing SDS-PAGE and stained with Coomassie blue. **B.** The intensity of each band of BSA analyzed in (A) was then determined using the gate 'box1' with ImageJ software (values in relative units, RU) and plotted against the corresponding known amount of BSA. The lane termed "0" served as background and this value was subtracted from all others. The equation obtained from the linear trendline of the BSA standard enabled the determination of the UUKV glycoprotein quantity. **C.** Similarly, the intensity of each volume of virus stock analyzed in (A) was defined with ImageJ software (column "RU"). The background was subtracted from these values (column "RU – BG"). Using the equation defined in (B), it was possible to determine the amount of UUKV G_N and G_C (x = y / 24) and finally the concentration of the two glycoproteins (x / v). In this example, G_N and G_C are considered as a single glycoprotein and an average molecular weight of 63.4 kDa is used to calculate the molarity of both glycoproteins in the virus stock (i.e. 0.194 $g.L^{-1}$ / 63'400 $g.mol^{-1}$ = 3.1 x 10^{-6} $mol.L^{-1}$) [16]. The left column gives the average concentration ($\mu g.mL^{-1}$) and molarity of UUKV glycoproteins G_N and G_C in the virus stock.

Figure 4 – Characterization of fluorescently labeled viral particles. A. The picture shows an OptiPrep step gradient after ultracentrifugation with unbound AF568 dye floating on the top and a band that corresponds to UUKV-AF568 with an identical density (between the 25 % and 30 % OptiPrep steps). **B.** Focus-forming assay used for the titration of UUKV-AF568. After 3 days of incubation with different dilutions of the labeled viral particles at 37 °C, BHK-21 cells were immunostained with a rabbit polyclonal antibody (U2) against UUKV structural proteins. The foci were then revealed with an anti-rabbit horseradish peroxidase-conjugated secondary antibody and a DAB solution kit. **C.** The unlabeled (UUKV) and fluorescent particles (UUKV-AF568) were analyzed by non-reducing SDS-PAGE with Coomassie blue staining or fluorography. The envelope proteins G_N and G_C of UUKV-AF568 were the only fluorescently labeled proteins. **D.** A confocal microscopy picture of individual fluorescent UUKV-AF568 particles.

Figure 5 – Fluorescence microscopy to analyze virus motion. A. Live imaging of A549 cells with a spinning-disc microscope was performed in the continuous presence of UUKV-AF594. A time-lapse series is shown for one UUKV-AF594 particle surfing on filopodia towards the plasma membrane before entering the host cell. **B.** UUKV-AF647 was bound to HeLa cells expressing the virus receptor DC-SIGN, in suspension, and on ice. Cells were then seeded on

coverslips before warming to room temperature and imaging of UUKV-AF647 on the bottom cell surface by TIRF microscopy. UUKV trajectory was recorded at 1/10 Hz and analyzed by computational analysis with the Image J-based plugin software Particle Tracker [39] (green line). **C.** Human monocyte-derived DCs were exposed to UUKV-AF594 on ice for 1 hour, and then, either maintained on ice or warmed for 30 min before fixation and imaging by confocal microscopy. White spots are cell-associated viral particles in one focal plane.

Figure 6 – Flow cytometry to measure virus binding and internalization. A. Various amounts of UUKV-AF647 particles were bound to A549 cells on ice and samples were analyzed by flow cytometry. **B.** UUKV-AF488 (MOI ~1) was bound to DC-SIGN-expressing HeLa cells on ice before warming to 37 °C for 15 min. Internalization was analyzed by flow cytometry after cell fixation and trypan blue treatment to quench fluorescence due to cell-surface bound viral particles. RFI stands for relative fluorescence intensity. **C.** Shows the same data as in (B) but expressed as the percentage of fluorescence measured in samples not treated with trypan blue (% of control). The fluorescence observed for cells not exposed to UUKV-AF488 was considered as background and subtracted from other values (data not shown). Error bars represent the standard deviation.

Figure 7 – Fluorimetry to quantify virus fusion in cells. UUKV-R18 binding was synchronized on ice, and internalization into cells was triggered by rapid warming. The R18 diffusion was quantified by measuring R18 dequenching in living cells (blue curve). In the presence of ammonium chloride (NH_4Cl, red curve), intracellular pH is neutralized, and therefore, virus fusion is completely blocked. Only free R18 diffusion is measured. The grey curve shows the virus fusion-specific R18 release, i.e. blue (fusion + free diffusion) – red (free diffusion). RFI stands for relative fluorescence intensity.

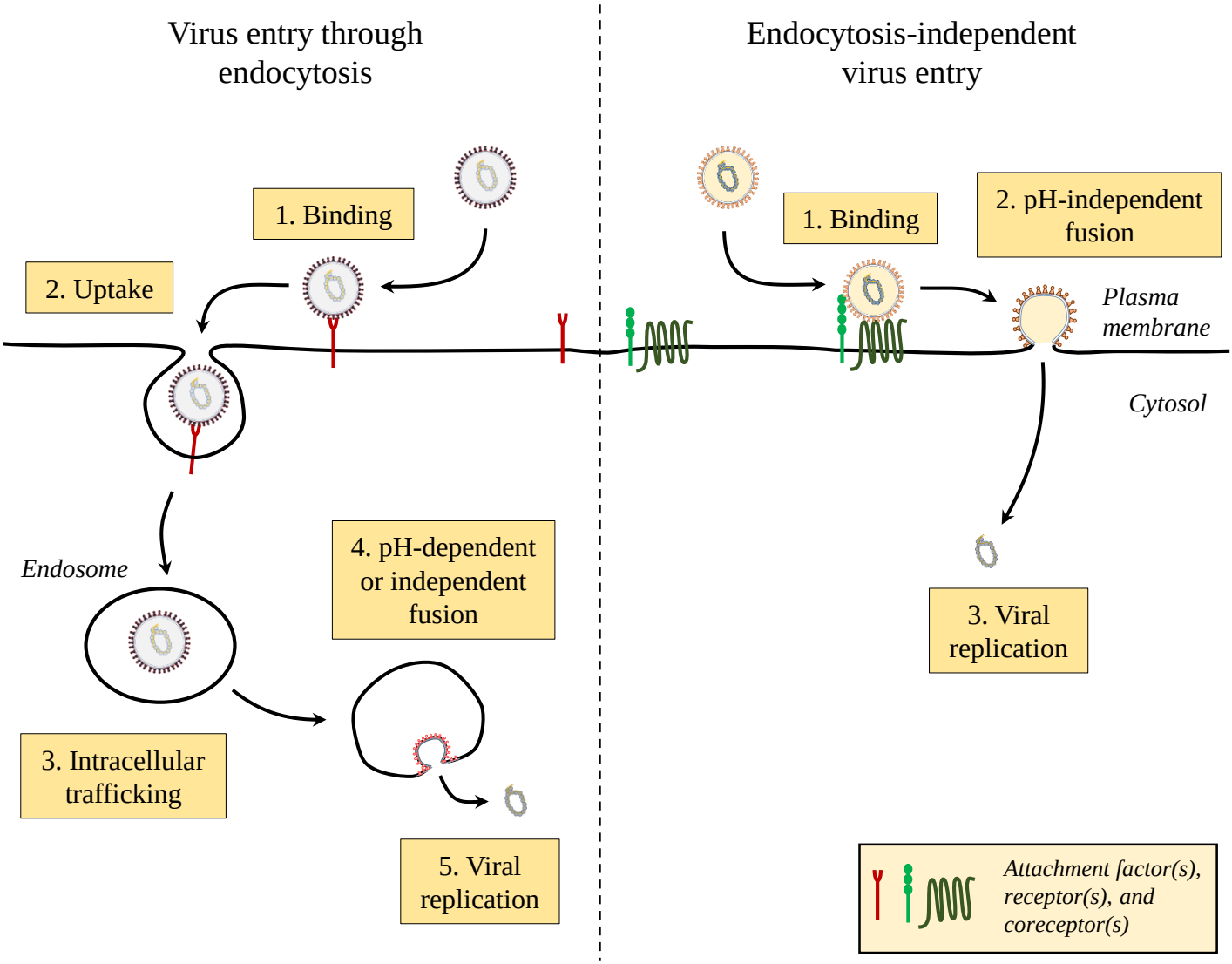
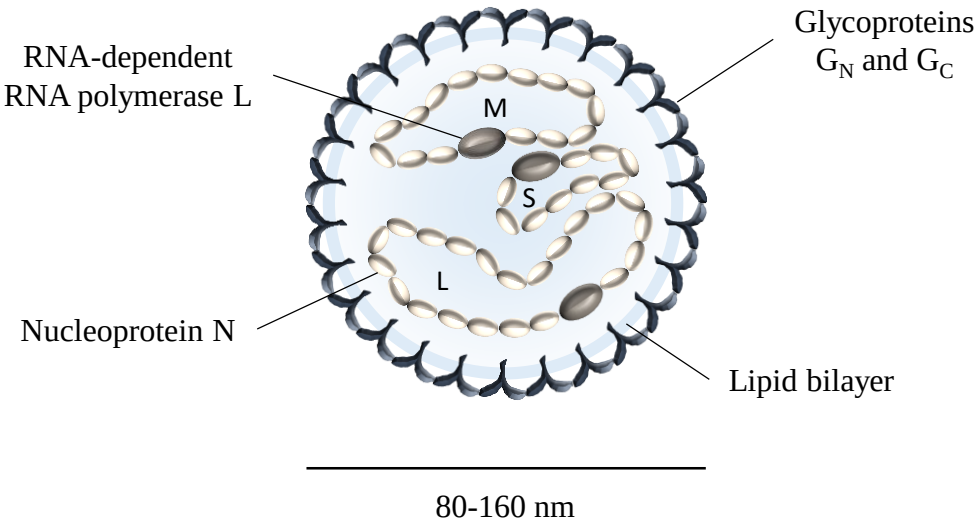
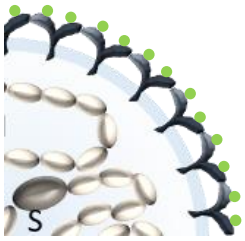
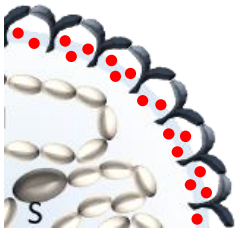

Virus entry through endocytosis
Endocytosis-independent virus entry
1. Binding
2. Uptake
Endosome
4. pH-dependent or independent fusion
3. Intracellular trafficking
5. Viral replication
1. Binding
2. pH-independent fusion
Plasma membrane
Cytosol
3. Viral replication
Attachment factor(s), receptor(s), and coreceptor(s)

Fig. 1

A.



B.

Dye (s)	Alexa Fluor (AF) succinimidyl esters	Octadecyl rhodamine B chloride (R18)
		
Target	Viral envelope glycoproteins	Viral envelope
React with	Primary amine groups	Lipophilic
Emission	From ultraviolet to far red	Yellow

C.

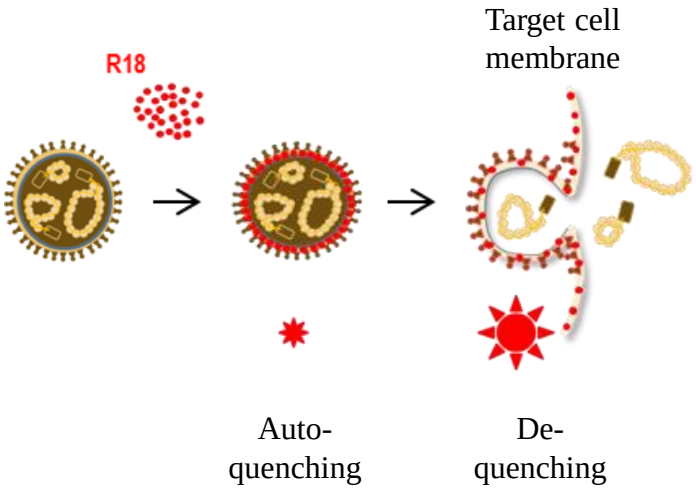
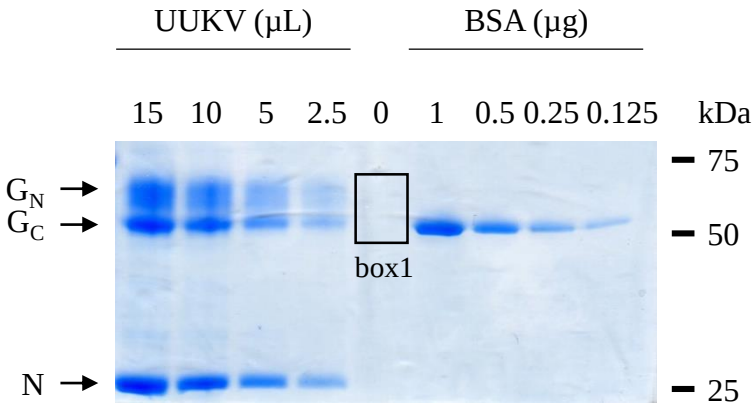
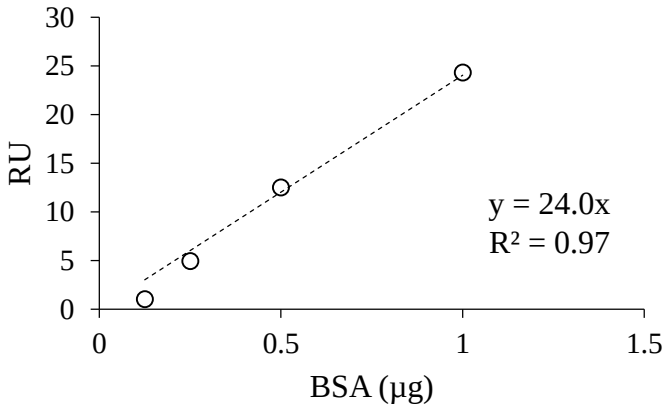


Fig. 2

A.



B.



C.

v = UUKV from (A)	RU	RU - BG	x = y / 24.0 [from (B)]	x / v (µg.mL^{-1})	UUKV (µg.mL^{-1} / µM)
15 µL	98.8	71.6	3.0 µg	199.1	194.4 / 3.1
10 µL	78.9	51.7	2.2 µg	215.8	
5 µL	50.7	23.5	1.0 µg	195.9	
2.5 µL	37.2	10.0	0.4 µg	166.7	
0 (BG)	27.2				

Fig. 3

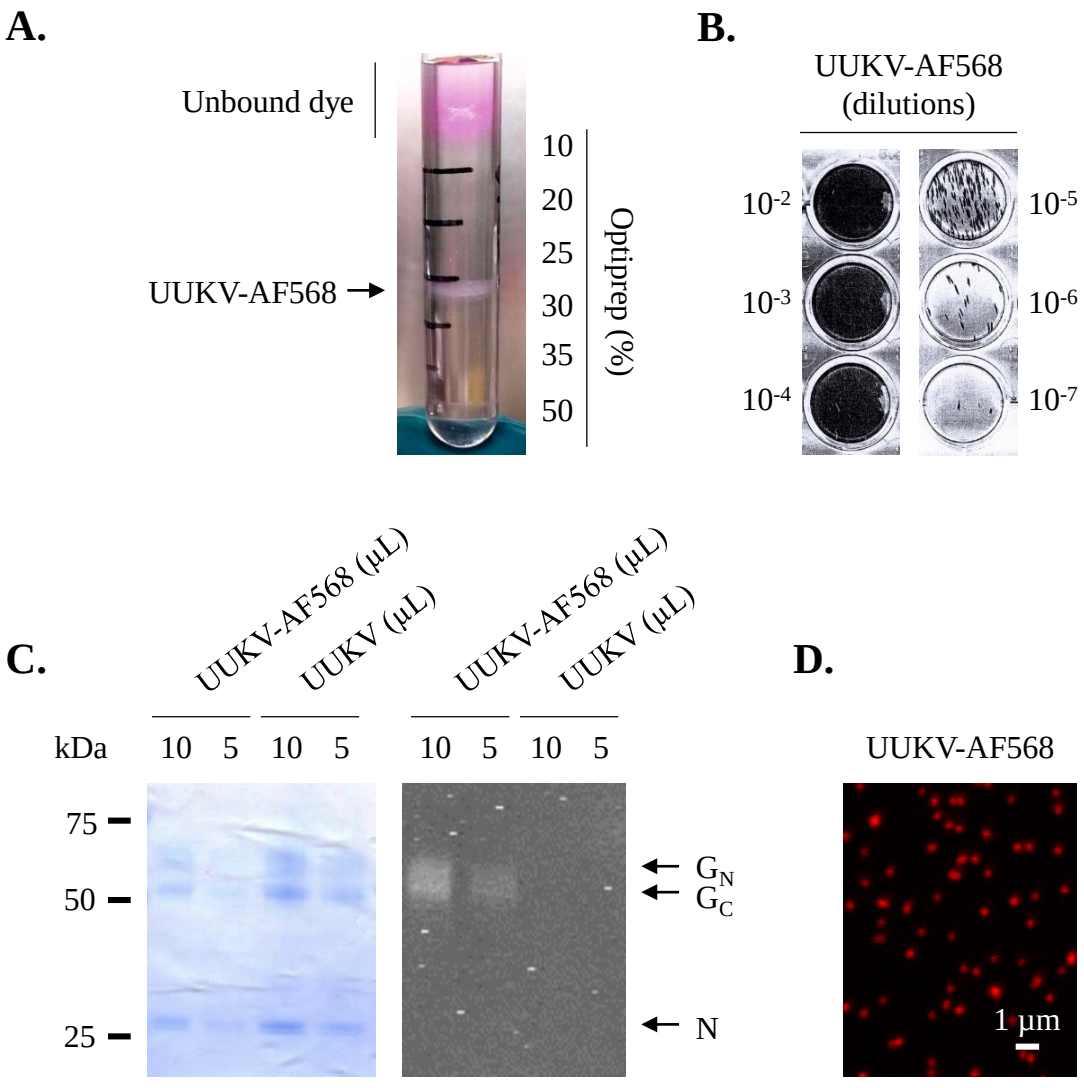

A.
Unbound dye
UUKV-AF568
10
20
25
30
35
50
Optiprep (%)
B.
UUKV-AF568
(dilutions)
10-2
10-3
10-4
10-5
10-6
10-7
C.
UUKV-AF568 (µL)
UUKV (µL)
UUKV-AF568 (µL)
UUKV (µL)
kDa
10 5 10 5
10 5 10 5
75
50
25
GN
GC
N
D.
UUKV-AF568
1 µm

Fig. 4

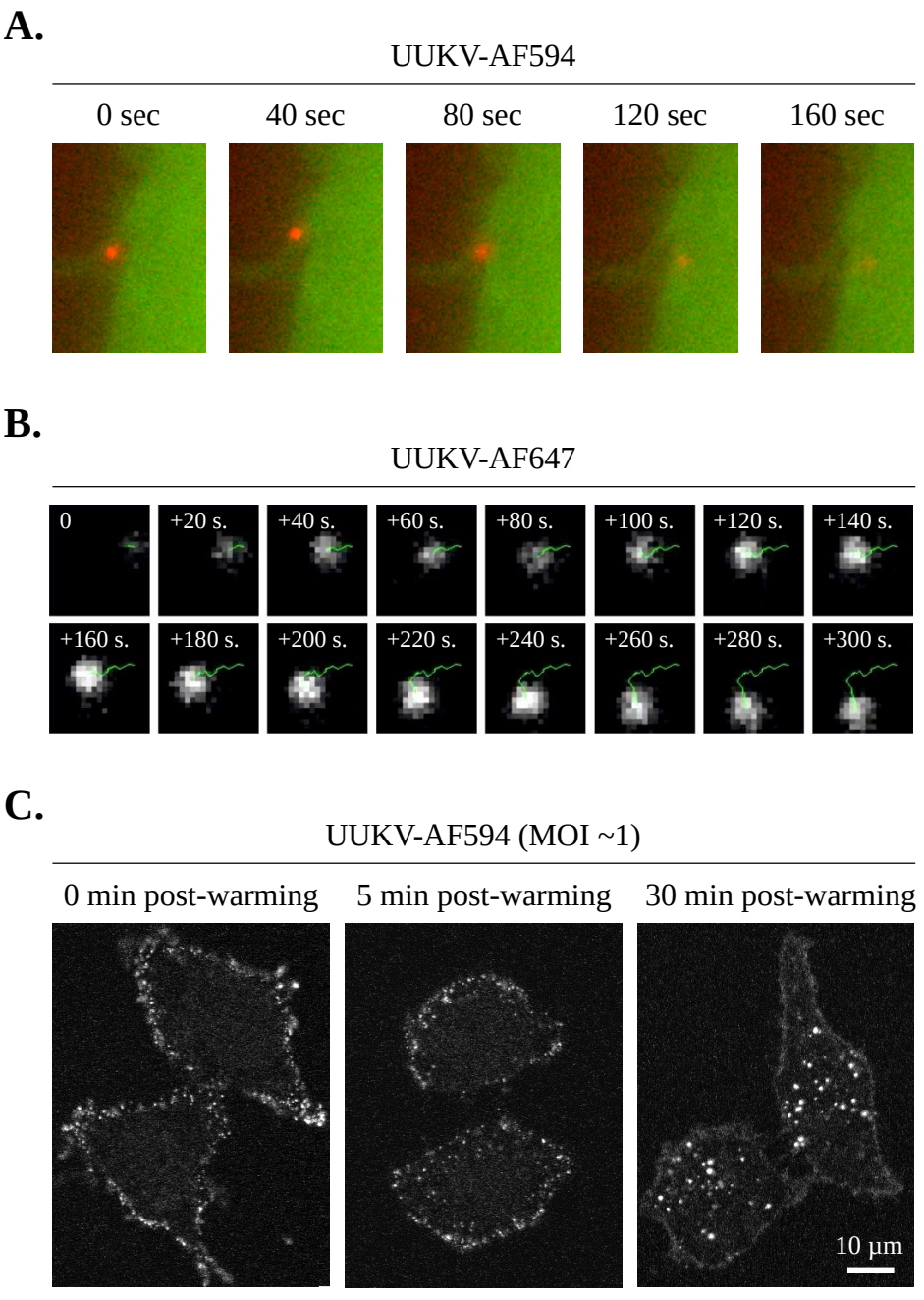

A.
UUKV-AF594
0 sec
40 sec
80 sec
120 sec
160 sec
B.
UUKV-AF647
0
+20 s.
+40 s.
+60 s.
+80 s.
+100 s.
+120 s.
+140 s.
+160 s.
+180 s.
+200 s.
+220 s.
+240 s.
+260 s.
+280 s.
+300 s.
C.
UUKV-AF594 (MOI ~1)
0 min post-warming
5 min post-warming
30 min post-warming
10 µm

Fig. 5

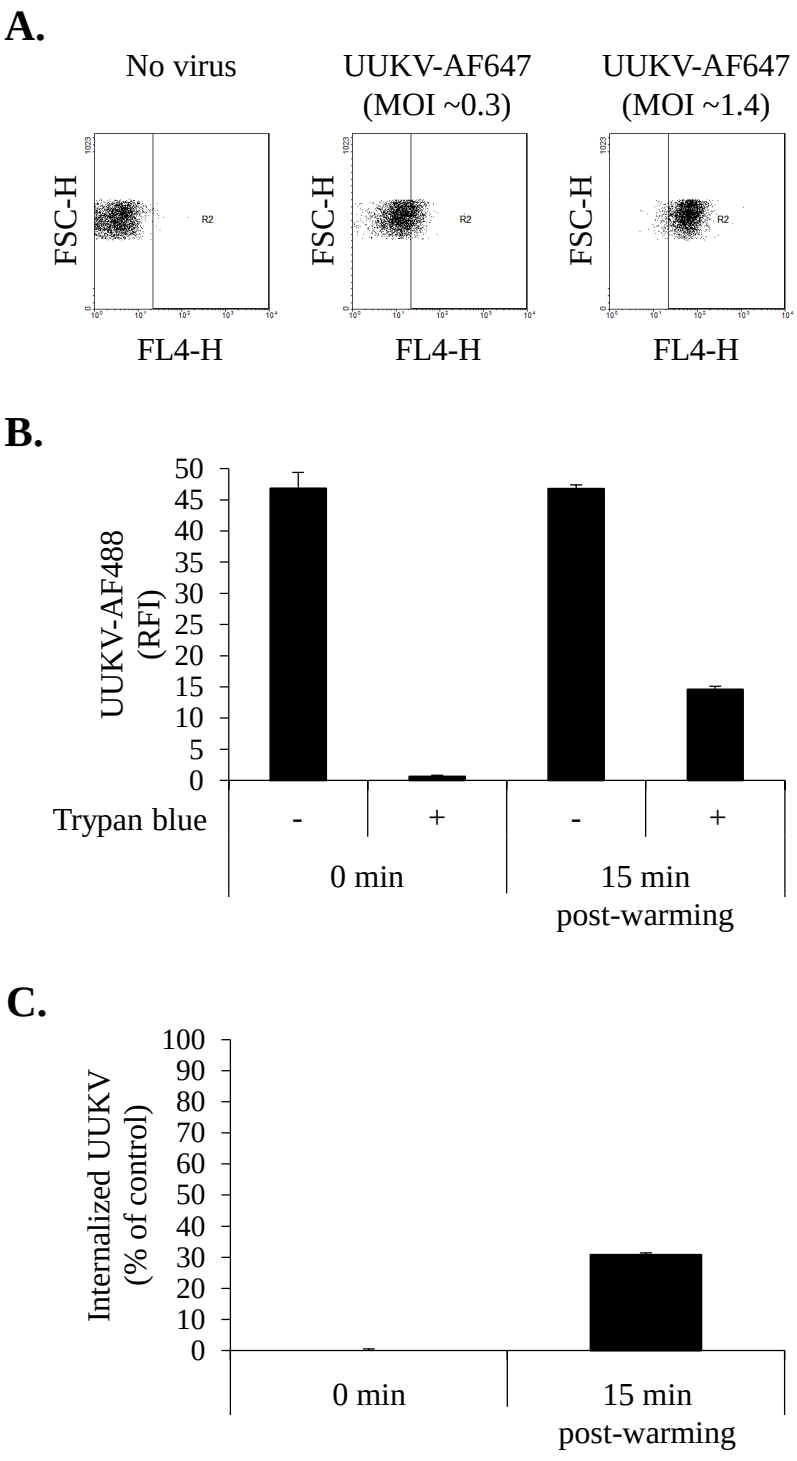

A.
No virus
UUKV-AF647 (MOI ~0.3)
UUKV-AF647 (MOI ~1.4)
FSC-H
FL4-H
R2
B.
UUKV-AF488 (RFI)
50
45
40
35
30
25
20
15
10
5
0
Trypan blue
-
+
-
+
0 min
15 min post-warming
C.
Internalized UUKV (% of control)
100
90
80
70
60
50
40
30
20
10
0
0 min
15 min post-warming

Fig. 6

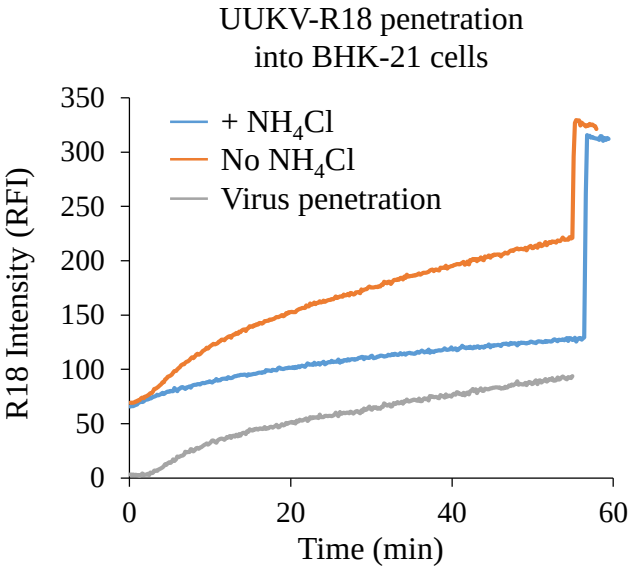

UUKV-R18 penetration
into BHK-21 cells
R18 Intensity (RFI)
350
300
250
200
150
100
50
0
0
20
40
60
Time (min)
+ NH4Cl
No NH4Cl
Virus penetration

Fig. 7