Visualizing Coronavirus Entry into Cells

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Abstract

Coronavirus entry encompasses the initial steps of infection, from virion attachment to genome release. Advances in fluorescent labeling of viral and cellular components and confocal imaging enable broad spectrum studies on this process. Here, we describe methods for visualization of coronavirus entry into immortalized cell lines and 3D tissue culture models.

Key words  Coronavirus, Entry, Endocytosis, Internalization, Confocal microscopy

1 Introduction

Coronavirus entry is initiated by interaction between the trimeric spike (S) protein and its receptor, which is expressed on the surface of the susceptible cell. During entry, the S protein undergoes structural rearrangement, which brings the cellular and viral membranes into proximity to mediate fusion. Such a structural switch may be triggered by different stimuli, including receptor binding, proteolytic cleavage of the S protein, and/or acidification of the microenvironment. The requirement for specific stimuli is species-dependent, and consequently, different coronaviruses enter cells at various subcellular sites. Some coronaviruses fuse at the plasma membrane, whereas others are believed to enter the cell through receptor-mediated endocytosis, followed by fusion within the endosomal compartments. Furthermore, recent reports show that the entry portal may vary depending on the tissue and the cell. These differences may affect the host range, pathogenicity, and cell/tissue specificity [1].

To visualize coronavirus entry into cells, we developed a confocal microscopy-based analysis method and single-virus particle-tracking tools. We believe that coronavirus entry is highly dependent on the in vitro model used, and therefore, we study coronavirus entry using susceptible cell lines, but we also confirm
our observations using the complex ex vivo model of human airway epithelium (HAE). HAE cultures are formed by the multilayered, fully differentiated primary human airway epithelial cells grown at the air/liquid interface built on collagen-coated plastic supports. HAE cultures mimic the natural conductive airway epithelium and serve as the most reliable model to study coronavirus infection [2–6].

To study coronavirus entry, we first prepared concentrated stocks of coronaviruses. We found the iodixanol-based medium to be optimal for coronavirus concentration and purification, as in other media (e.g., sucrose gradient), virions rapidly lose infectivity (Fig. 1). Single-step iodixanol purification was sufficient for the successful visualization of single-virus particles in the cell (Fig. 2). We visualized single coronaviral particles using immunodetection, and we decided that staining specifically for the N protein was superior due to the high availability of antibodies and abundance of the protein itself [7, 8].

In our research, we tracked virus entry from virus attachment to the cell. First, using a variety of techniques, we studied the attachment itself and made an effort to identify attachment receptors, which are usually broadly distributed molecules, e.g., sugar moieties [8–11]. Using confocal microscopy-based analysis complemented with flow cytometry appeared to be the optimal means to investigate these processes [11–13]. Further, we aimed to identify entry receptors and virus–receptor interactions. Except for proteomic analyses, which are not covered in this chapter, confocal analysis of virus–receptor interaction allowed us to delineate the process of coronavirus entry [14, 15].
Next, we checked whether the virus undergoes fusion on the surface of the cell or first requires endocytic internalization. For this we employed chemical inhibitors hampering alteration of the endosomal microenvironment (e.g., ammonium chloride, bafilomycin A), which should affect endocytic entry, but not fusion on the cell surface. Further, we tested whether the virus colocalizes with the early endosome antigen 1 (EEA-1) early after internalization. EEA-1 is a hydrophilic protein, which in cells is found exclusively in early endosomes [16] (see Fig. 3 for examples) [17, 18].

Subsequently, we found that identification of the endocytosis route for a given coronavirus should first rely on determining the colocalization of virions with proteins specific for endosomes
entering by a given pathway. For this, we incubated permissive cells with a purified coronavirus stock at 4 °C to enable virus adhesion to the cell surface and block its internalization, as at this temperature intracellular transport is inhibited. Consequently, we increased virus density on the cell surface, and by increasing the temperature, we were able to synchronize virus entry. Cultures were incubated at higher temperatures and fixed at specific time points. Samples were immunostained for coronaviral and host proteins. The colocalization rate between viral and cellular proteins was estimated using Pearson’s and Manders’ correlation coefficients. As controls, we used protein cargos, such as transferrin, which is endocytosed via a clathrin-mediated pathway or cholera toxin or albumin, which enter cells via caveosomes [19, 20] (Fig. 4). We verified virion colocalization with markers for specific entry routes in cells pretreated with chemical inhibitors or cells with silenced expression of proteins required for specific endocytic pathways. In these experiments, endocytosis of the virus or reference cargo was blocked, and internalization was analyzed with confocal imaging. For example, for all coronaviruses studied, we observed efficient inhibition of internalization using dynamin-2 inhibitors. On the other hand, chemicals blocking clathrin-mediated endocytosis affected only human coronavirus (HCoV)-NL63 entry, and caveolin inhibitors hampered canine respiratory coronavirus (CRCoV) and HCoV-OC43 internalization. Furthermore, as entry via endocytosis usually requires re-arrangement of the cytoskeleton, we also tested virus internalization in the presence of inhibitors affecting actin filaments and microtubules [11, 12, 21–27] (Fig. 5).

Fig. 3 Colocalization of viruses with early endosome marker EEA1. (a) HCoV-OC43 colocalizing with EEA1 in HCT-8 cells, (b) CRCoV colocalizing with EEA1 in HRT-18G cells. Viral nucleocapsids are presented in green (Alexa Fluor 488), EEA1 in red (Alexa Fluor 546). Cell nuclei are shown in blue (DAPI). Scale bar 10 μm.
Fig. 4 Colocalization of reference cargoes with entry pathway markers. (a) Transferrin colocalizing with clathrin. Clathrin HC is presented in red (Alexa Fluor 546), transferrin conjugated to Alexa Fluor 488 in green, and cell nuclei are shown in blue (DAPI). (b) Cholera toxin colocalizing with caveolin. Caveolin 1 is presented in red, cholera toxin subunit B conjugated to FITC in green. Cell nuclei are shown in blue (DAPI). Scale bar 10 μm

Fig. 5 Inhibition of virus entry. Compounds with a known inhibitory effect on different internalization pathways are indicated at their respective sites of action. The direct fusion of viruses with cell surface may be blocked by the application of virus-specific inhibitors.
Confocal imaging of cells allows us to visualize not only the process but also to quantify it. The efficiency of entry can be estimated based on the ratio between internalized viruses and viruses attached to the surface, e.g., in the presence and absence of an inhibitor.

Virus internalization is not always linked with infectious entry. As we have shown in Owczarek et al [22], the re-direction of the virus into the micropinocytosis pathway allows effective entry but does not allow for fusion and infectious entry. For that reason, it is of importance to verify whether effective virus entry relies on specific pathways. We therefore always complement experiments described above with studies on virus replication in the presence or absence of chemical inhibitors of specific pathways [21, 22].

2 Materials

2.1 Purification of a Coronavirus Stock

1. Cell lines: LLC-Mk2 (ATCC: CCL-7), HCT-8 (ATCC: CCL-244), HRT-18G (ATCC: CRL-11663).
2. Antibiotics (1×): penicillin (100 U/ml), streptomycin (100 µg/ml), ciprofloxacin (5 µg/ml).
3. DMEM: Dulbecco’s modified Eagle’s medium, supplemented with 3% heat-inactivated fetal bovine serum (FBS) and antibiotics (1×).
4. MEM: two parts Hanks’ MEM, one part Earle’s MEM, supplemented with 3% heat-inactivated fetal bovine serum (FBS), and antibiotics (1×).
5. RPMI: RPMI-1640, supplemented with 3% heat-inactivated fetal bovine serum (FBS) and antibiotics (1×).
6. T75 tissue culture flasks.
7. Cell culture incubator set at 32 or 37 °C and with 5% CO2.
8. Virus stock (HCoV-NL63, HCoV-OC43 or CRCoV).
9. 50 ml conical centrifuge tubes.
10. Benchtop centrifuge.
11. Syringe fitted with a 0.45 µm filter.
12. Centrifugal filters (10,000 kDa cut off).
13. Phosphate-buffered saline (PBS).
14. Iodixanol solution (Optiprep medium).
15. Ultracentrifuge vials.
16. Ultracentrifuge capable of reaching 170,000 × g.
17. Denaturing sample buffer for SDS–PAGE.
2.2 Cell Culture Experiments

2.2.1 In Vitro Cell Cultures

1. Cell lines (see Subheading 2.1, item 1).
2. Media (see Subheading 2.1, items 3–5).
3. Coverslips.
4. Six-well plates.
5. Purified virus stock or mock stock.
6. Wet chamber (wet paper towels in a box).
7. PBS.
8. 4% paraformaldehyde (PFA) (see Note 1).

2.2.2 Ex Vivo HAE Cultures

1. 0.4 μm ThinCert (Greiner Bio-one) or Transwell (Costar) inserts.
2. Human tracheobronchial epithelial cells obtained from airway specimens—refer to the approved protocols.
3. Bronchial epithelial growth medium (BEGM).
4. Air–liquid interface medium (ALI), as described by Fulcher et al. [4].
5. Antibiotics: penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (10 μg/ml), amphotericin B (250 μg/ml), ciprofloxacin (5 μg/ml), Fungin (10 mg/ml).
6. Purified virus stock or mock stock preparation.
7. PBS.
8. 4% PFA (see Note 1).

2.2.3 Entry Inhibitors

The chemical inhibitors used in the studies together with the approximate concentrations in different cells are listed in Table 1, and a graphical illustration of their mechanism of action is presented in Fig. 5 (see Note 2).

2.2.4 Endocytosis Cargo Proteins

1. Clathrin-dependent cargo protein: Alexa Fluor 488-labeled transferrin (100 μg/ml).
2. Caveolin-dependent cargo protein: FITC-labeled albumin (500 μg/ml), FITC-labeled cholera toxin (40 μg/ml).
3. Macropinocytosis: dextran derivatives.

2.3 Immunostaining to Visualize Viral Entry into Cells

1. Washing buffer: 0.5% Tween 20 in PBS.
2. Blocking buffer: 5% BSA in PBS.
3. Dilution buffer: 1% BSA and 0.5% Tween 20 in PBS.
4. Primary and secondary antibodies (Table 2).
5. 0.1% Triton X-100 in PBS.
6. 0.1 μg/ml 4’, 6’-diamidino-2-phenylindole dihydrochloride (DAPI).
| Inhibitor            | Process affected                              | Direct effect                                                                 | Working concentration | References (PMID) |
|----------------------|-----------------------------------------------|-------------------------------------------------------------------------------|-----------------------|-------------------|
| Bafilomycin A1       | pH-dependent endocytosis                      | Inhibition of vacuolar-type H⁺-ATPase; prevents acidification of endosomes    | 2.5 nM 10 nM 100 nM  | [28, 29]          |
| NH₄Cl                |                                               | Acts as a proton sink, thereby inhibiting acidification of the endosome       | 50 mM 50 mM 50 mM    | [29]              |
| EIPA                 | Macropinocytosis                              | Inhibition of Na⁺/H⁺ antiport → change of submembranous pH → deactivation of the GTPases that promote actin remodeling | 20 μM 10 μM 40 μM   | [30, 31]          |
| Wortmannin           |                                               | Inhibition of phosphoinositide-3-kinase (PI3K)                                | 5 μM 5 μM 5 μM       | [32]              |
| Dynasore             | Dynamin-dependent entry                      | Inhibition of GTPase activity of dynamin 1, dynamin 2, and Drp1 (mitochondrial dynamin) | 40 μM 40 μM 80 μM   | [33]              |
| Iminodyn 22          |                                               | Binding of the GTPase domain at an allosteric site; uncompetitive antagonist to GTP; broad-spectrum dynamin inhibitor | 40 μM 15–25 μM 20 μM | [34]              |
| MitMab               |                                               | Blocking of pleckstrin homology lipid-binding domain of the dynamin 1 and dynamin 2 | 10 μM 5 μM 10 μM    | [35]              |
| CT04                 | Actin-dependent entry                         | Inhibition of RhoA, RhoB, and RhoC GTPases                                   | 0.5–2 μg/ml 1 μg/ml ND | [36]              |
| Cytochalasin D       |                                               | Disrupting actin polymerization                                              | 2 μM 10 μM 10 μM    | [37]              |
| IPA-3                |                                               | Inhibition of group I p21-activated kinase (PAK1)                            | 20 μM 20 μM          | [38]              |
| Jasplakinolide       |                                               | Stabilization of actin microfilaments                                         | 150 nM 150 nM 1.5 μM | [39]              |
| NSC23766 trihydrochloride |                                               | Inhibition of Rac1, a Rho-family GTPase                                      | 50 μM 100 μM ND     | [40]              |
| Y27632               |                                               | Inhibition of Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) | 10 μM 10 μM 10 μM   | [41, 42]          |
| Compound            | Route                        | Effect                                                                 | Concentration       | Ref. |
|---------------------|------------------------------|------------------------------------------------------------------------|---------------------|------|
| Nocodazole          | Microtubule-dependent entry  | Depolymerization of microtubules                                       | 0.5 μM 0.5 μM 400 nM | [43] |
| Amantadine          | Clathrin-dependent route     | Stabilization of clathrin-coated pits, probably due to the interactions of the drug with the clathrin cage or the membrane structure of the vesicle | 750 μM 500 μM 500 μM | [44] |
| Chlorpromazine      | Clathrin-dependent route     | Stabilization of clathrin-coated pits, probably due to the interactions of the drug with the clathrin cage or the membrane structure of the vesicle | 5 μM 5 μM 5 μM | [45] |
| Pitstop 2           | Clathrin-dependent route     | Competitively inhibits clathrin terminal domain (TD)                   | 15 μM 5 μM 10 μM | [46] |
| Filipin III         | Caveolin-dependent route     | Alteration of membrane permeability and associated functions; binding to membrane sterols | 1–10 μg/ml 2 μg/ml 2–100 μg/ml | [47] |
| Methyl-β-cyclodextrin| Sterol-binding agent         | Removing cholesterol from lipid membranes                              | 100 μM–5 mM 2 mM 5 mM | [48] |
| Nystatin            | Sterol-binding agent         | Removing cholesterol from lipid membranes                              | 100 μM–5 mM 2 mM 5 mM | [49] |

*ND* no data. Working concentration optimized for the stated cell lines in our laboratory.
Table 2
Antibodies used in these studies

| Target                              | Antibody                          | Dilution | Conjugate | Supplier information                  |
|-------------------------------------|------------------------------------|----------|-----------|----------------------------------------|
| Endocytosis                         | Anti human early endosome antigen 1 | 2 μg/ml  | x         | Santa Cruz Biotechnology; AB_2277714    |
|                                     | Rabbit anti-Rab 7                  | 2 μg/ml  | x         | Santa Cruz Biotechnology; AB_2175483    |
|                                     | Rabbit anti-Rab11A                 | 2.5 μg/ml| x         | Proteintech; AB_2173458                 |
|                                     | Rabbit anti-LAMP1                  | 10 μg/ml | x         | Thermofisher Scientific; AB_2134611     |
| Clathrin-dependent route            | Anti-human clathrin heavy chain    | 10 μg/ml | x         | Santa Cruz Biotechnology; AB_2083170    |
| Caveolin-dependent route            | Anti-human caveolin                | 2 μg/ml  | x         | Santa Cruz Biotechnology; AB_2072042    |
| Coronavirus proteins                | Anti-HCoV-NL63 N IgG               | 0.25 μg/ml| x         | Ingenansa; M.30.HCo.I2D4                |
|                                     | Anti-HCoV-OC43 N IgG               | 1 μg/ml  | x         | Merck Millpore; AB_95424                |
| Secondary antibodies               | Goat anti-mouse                    | 10 μg/ml | Alexa Fluor® 488 | Thermofisher Scientific                |
|                                     | Rabbit anti-mouse                  | 10 μg/ml | Alexa Fluor® 488 | Thermofisher Scientific                |
|                                     | Goat anti-rabbit                   | 10 μg/ml | Alexa Fluor® 546 | Thermofisher Scientific                |
|                                     | Donkey anti-goat                   | 10 μg/ml | Alexa Fluor® 546 | Thermofisher Scientific                |
| Actin                               | Phalloidin                         | 0.2 U/ml | Alexa Fluor® 633 | Thermofisher Scientific                |

These antibodies were used successfully in our laboratory. Alternative antibodies can be used after validation.

7. 0.2 U/ml Phalloidin labeled with Alexa Fluor 633.
8. Antifade medium (e.g., ProLong Gold antifade medium).
9. Immersion oil (e.g., Immersol 518 F),
10. Rotary shaker.
11. Fine tipped forceps.
12. Scalpel.
13. Glass slides.
14. Nail polish.
2.4 Image Acquisition, Processing, and Presentation

1. Confocal microscope, e.g., Zen 2.3 SP1, Carl Zeiss Microscopy GmbH.
2. Image analysis software capable of colocalization analysis and counting three dimensional objects, e.g., ImageJ1.52i [50, 51].

3 Methods

All procedures involving infectious material should be performed inside a biological safety cabinet until the fixing step.

3.1 Purification of a Coronavirus Stock

1. Obtain a stock of a given coronavirus by infecting monolayers of permissive cells growing in T75 flasks. Prepare a second T75 flask for mock-infection (negative control). Maintain the cells at 32 °C or 37 °C under 5% CO₂ for 4–5 days, depending on the virus species (see Note 3).
2. Lyse the cells by freeze-thawing at −80 °C and collect the solutions in conical 50 ml tubes (see Note 4).
3. Centrifuge at 4500 × g for 15 min at 4 °C.
4. Filter the supernatants using sterile syringe 0.45 μm filters.
5. Concentrate the supernatants using centrifugal filters to 8 ml each.
6. Prepare iodixanol solutions: 10%, 12.5%, 15%, 17.5%, 20% in 1× PBS, and store at 4 °C.
7. Add 2 ml of 15% iodixanol solution in the ultracentrifuge vial and overlay gently with 8 ml of mock/virus concentrate. Centrifuge at 170,000 × g for 3 h at 4 °C.
8. Collect ten fractions of 1 ml from the top to the bottom of the vial. The virus should be present in the fraction at the bottom of the tube, but this needs to be verified by western blot. Combine the virus-containing fraction (and corresponding mock fraction) with 1 ml of PBS and mix thoroughly.
9. In an ultracentrifuge vial, prepare gradient medium by overlaying 1.6 ml of each iodixanol solution (20% to 10%). Add 2 ml of virus/mock sample obtained in the previous step to the top of the gradient. Centrifuge at 170,000 × g for 18 h at 4 °C.
10. Collect ten fractions from the top to the bottom (1 ml each). Mix 15 μl of each fraction with a denaturing sample buffer for SDS–PAGE electrophoresis and perform western blot analysis to identify fractions containing the virus. Aliquot each fraction and store at −80 °C (see Note 5).
3.2 Cell Culture Experiments

3.2.1 Viral Entry In Vitro into Cell Lines

1. Seed the cells on coverslips in six-well plates and culture for 2 days at 32 or 37 °C with 5% CO₂, when they should reach 70–80% confluence. LLC-Mk2 cells are cultured in MEM, HCT-8 cells in RPMI and HRT-18G cells in DMEM.

2. Where required, incubate the cells with a given inhibitor (dissolved in cell culture medium; see Table 1) for 30 min at 32 or 37 °C (see Note 6).

3. Discard the supernatant and overlay the cells with 50 μl of purified virus or mock sample and incubate for 1 h at virus-specific temperature (i.e., 32 or 37 °C). To avoid evaporation put the plate in a wet chamber. For control experiments with reference proteins (cargo), incubate the cells with a given cargo dissolved in a culture medium for 1 h at virus-specific temperature (see Note 7).

4. Wash the cells three times with PBS and fix with 4% PFA diluted in PBS. Incubate the plate at RT for at least 15 min before further analysis (see Note 8).

3.2.2 Viral Entry into Ex Vivo HAE Cultures

1. Culture primary epithelial cells on cell culture inserts in multi-well plates in BEGM medium supplemented with antibiotics until they reach 100% confluence. For further culture and differentiation, maintain the cells in ALI supplemented with antibiotics for 4–8 weeks at 37 °C under 5% CO₂.

2. Maintain HAE cultures until fully differentiated. It is essential to verify the differentiation using marker proteins and phenotypic changes of cultures, e.g., mucus production, synchronized cilia movements.

3. Wash the cultures three times with 200 μl of PBS to remove the mucus. For each washing incubate the cultures for 5 min at 37 °C.

4. Where required, incubate the cultures with a given inhibitor (diluted in PBS, see Table 1) for 60 min at 37 °C.

5. Discard the supernatant and add 100 μl of purified virus or mock sample per insert and incubate for 2 h at 32 °C. For entry experiments of control cargo proteins, incubate the cultures with a given protein (diluted in PBS).

6. Wash the cells three times with PBS and fix with 4% PFA diluted in PBS. Incubate the plate at RT for at least 30 min before further analysis (see Note 8).

3.2.3 Synchronized Viral Entry into Cell Lines

1. Seed the cells on coverslips in six-well plates and culture for 2 days at 37 °C until they reach 70–80% confluence.

2. Cool the plate to 4 °C on ice. Washing is not necessary.
3. Overlay the cells with 50 μl of pre-cooled (4 °C) purified virus or mock preparation and incubate for 1 h at 4 °C on ice to synchronize the virions at the cell surface.

4. To avoid evaporation of the liquid, place the plate in a wet chamber, then warm up the plate to 32 or 37 °C, depending on the optimal temperature for the particular virus. Remove samples from the incubator at set time intervals ranging between 0 and 180 min to get a clear picture of the route of entry (see Note 9). For control, cargo proteins, incubate the cells with a given protein (diluted in a culture medium) at the same temperature for a given time.

5. Wash the cells three times with PBS and fix with 4% PFA in PBS. Incubate the plate at RT for at least 30 min before further analysis (see Note 8).

3.3 Immunostaining to Visualize Viral Entry into Cells

3.3.1 Immunostaining Cell Lines

1. Incubate the fixed cells on coverslips in six-well plates with 0.5 ml 0.1% Triton X-100 in PBS for 5 min at room temperature (RT) on a rotary shaker.

2. Replace Triton X-100 with 1 ml blocking buffer and incubate overnight at 4 °C. Alternatively, incubate 2 h at RT or 30 min at 37 °C.

3. To visualize coronavirus particles or endocytosis-related proteins, incubate the cells for 2 h at RT with specific antibodies diluted in dilution buffer. To limit the antibody usage, place a 50 μl drop of antibody solution on the plate cover and overlay with the inverted coverslip with cells (see Note 10).

4. Wash the coverslips three times with 1 ml washing buffer on a rotary shaker for 5 min each wash.

5. Incubate for 1 h at RT with secondary antibodies labeled with fluorophores in dilution buffer.

6. Wash three times with 1 ml washing buffer on a rotary shaker for 5 min each wash.

7. To stain actin filaments, incubate the coverslips for 60 min at RT with fluorescently labeled phalloidin in PBS (50 μl drop, as in step 3). Refrain from using detergents after this step.

8. To stain nuclear DNA stain the coverslips for 10 min with 0.5 ml of DAPI in PBS.

9. Wash twice in 1 ml PBS to remove residual Tween 20. Mount the coverslips onto antifade medium on glass slides. Seal the coverslips with nail polish and leave to dry overnight at RT.

3.3.2 HAE Cultures

1. Incubate the fixed cultures on inserts with 200 μl of 0.1% Triton X-100 in PBS for 15 min at RT on a rotary shaker (see Note 11).
2. Replace Triton X-100 with 0.5 ml of blocking buffer and incubate overnight at 4 °C. Alternatively, incubate 2 h at RT or 30 min at 37 °C.

3. To visualize coronavirus particles or endocytosis-related proteins, incubate the cultures for 2 h at RT with 100 μl of specific antibodies in dilution buffer (see Note 10).

4. Wash the cultures three times with 300 μl of washing buffer on a rotary shaker for 10 min per wash.

5. Incubate for 1 h at RT with 100 μl of secondary antibodies labeled with fluorescent dyes in dilution buffer.

6. Wash the cultures three times with 300 μl of washing buffer on a rotary shaker for 10 min each wash.

7. To stain actin filaments, incubate the cultures for 20 min at RT with 100 μl fluorescently labeled phalloidin in PBS.

8. To stain nuclear DNA, incubate the cultures for 15 min with 200 μl DAPI in PBS. Wash twice with 300 μl PBS to remove residual Tween 20.

9. Cut out the membrane from the insert with a scalpel or push out using a tube. Mount on a glass slide with the antifade medium. Cover the membrane with a coverslip and seal with nail polish. Let it dry overnight at RT.

3.4 Image Acquisition

3.4.1 Selection of Fluorescent Dyes

1. Check the confocal microscope to determine which light sources are available (emission wavelengths).

2. Choose fluorophores. Fluorescent dyes not only differ in the maximal excitation and emission wavelengths but also exhibit different spectrum width and quantum yield (which is a measure of the efficiency of photon emission compared to the number of photons absorbed). We have successfully visualized up to four fluorophores at the same time (cells’ nuclei stained with DAPI + coronaviral proteins stained with Alexa Fluor 488 + host entry molecules stained with Alexa Fluor 546 + actin cytoskeleton stained with Atto 647 or Atto 633), but a higher number of fluorophores is possible.

To analyze the excitation and emission spectra and model compensation matrixes for evaluation of the spectral compatibility of fluorescent dyes and probes, online tools, e.g., Fluorescence SpectraViewer (www.lifetechnologies.com/handbook/spectraviewer) may be used.

3.4.2 Scanning Settings

1. Define tracks by specifying the fluorophore to be used. Set the spectrum that should be acquired. If the excitation and emission spectra are not overlapping, it is possible to detect up to three fluorophores in a single track. The configuration of a track consists of a laser line, excitation dichroic, and detector (see Note 12).
2. Choose the scanning mode. Simultaneous scanning of different wavelengths in samples labeled with multiple fluorophores is faster, but often falsifies the signal (cross talk between channels). Therefore, sequential scanning with a single laser line and a single detector activated (single fluorophore) at a time is the method of choice. The region of interest may be scanned by switching tracks line-by-line or frame-by-frame. In the first case, lasers and detectors are changed for each line, so the multicolor image is acquired at once. In the latter case, a single color is captured for the entire frame, and frames representing different fluorophores are superimposed. Scanning line-by-line while faster and favored allows modifying only channel and laser intensities between different fluorophores. In turn, frame-by-frame scanning allows also for changing beam splitters, gain, offset, and pinhole setting between the acquisition of single-color tracks.

3. Set the optical and digital zoom. To ensure adequate spatial sampling, the pixel size should be half of the microscope resolution to ensure Nyquist sampling conditions for all measurements.

4. Adjust the speed of scanning. If the sample is not prone to photobleaching, decreasing the speed will yield higher image quality; however, this may also increase the noise signal, so this step should be optimized. Otherwise, averaging may be applied while the speed of scanning remains high—such settings should improve the signal-to-noise ratio. The low scanning speed and averaging will impact the acquisition time.

5. Set image resolution at 16 bit (65,536 levels of gray, optimal for quantitative measurements).

6. Set laser power, gain, and offset values. To ensure that the signal is within the dynamic range of the detector, live mode along with range indicator should be used. It will also show under- and overexposed pixels. If there is a need to increase the fluorescence signal, it is better to modify the power of the laser or the detector’s gain than to apply the digital gain boost. This step demands optimization because too high laser intensity will photobleach the sample, while too high gain will generate noisy images (see Note 13).

3.4.3 3D Imaging

To acquire a 3D image of the region of interest, activate the z-stack mode.

1. Set the top and the bottom limits of the cell of interest. It is good practice to mark it based on the fluorescence signal of the actin cytoskeleton. For HAE cultures, it is easy to set the bottom border at the ThinCert insert membrane and the top in the distant part of immunostained cilia (see Note 14).
2. Adjust the step size. It is appropriate to set a value that fits the Nyquist sampling criteria; however, for some purposes, over-sampling may be recommended, e.g., it may help to separate high-frequency noise from the signal during image deconvolution. Our standard setting is 150 nm because it helps us to distinguish viruses by increasing signal to noise ratio in the z-axis.

3.5 Image Analysis

A number of image analysis software packages are available. In our research, we use the free software package ImageJ.

3.5.1 Image Processing and Presentation

It is easy to misrepresent data when adjusting the images. Therefore, it is essential to understand the difference between acceptable and unacceptable image adjustment. This is especially important for confocal microscopy or superresolution microscopy, where the presented image is always an interpretation of the signal imposed by the operator.

1. Limit adjustments as much as possible. Linear adjustments of brightness and contrast are generally considered safe and do not require an explanation as long as they are applied uniformly to all pictures in the set. Changes that do not affect each pixel in the same way (e.g., gamma) or more radical changes while allowed should be clearly described and justified in the Methods section and mentioned in the figure caption (see Note 15).

2. Even basic manipulations, as contrast adjustments, may remove some or introduce new elements into image, falsifying the message. When selecting parameters, always refer to mock-treated cells and suitable isotype controls.

3. The reader assumes that the picture presented in a figure represents a single microscope field. Therefore, always make clear divisions between elements from different images. A comparison of different images or their fragments may require resizing to obtain matching resolutions across the complete figure. While downsizing requires averaging of adjacent pixels and is acceptable, upsizing of the image introduces artificial pixels to the image and should be avoided.

4. Often, when imaging single virus particles, the number of events is low in a single field of view. To cope with this issue, imageJ Z functions allow for a combination of multiple slices into one using one of six different projection methods.

5. It is not possible to determine whether the virus entered the cell or is retained on the surface using only xy images, but it is necessary to analyze orthogonal views. The reslice option allows for the generation of xz and yz planes and also allows for downstream analyses and adjustments.
6. Never mix different color models. Doing so splits the signal into two channels, adding some of it to pre-existing channels (e.g., yellow added to RGB is split to red and green channels) and may yield false colocalization.

### 3.5.2 Colocalization

1. The ImageJ JACoP plugin is used for the calculation of colocalization. To use it, images should be split into single channels and loaded in pairs to be compared.

2. Select the method. There are a number of parameters that describe the colocalization of objects, and we find Pearson’s and Mander’s coefficients most convenient. While Pearson’s correlation is easy and fast, Mander’s coefficient allows to determine how object A colocalizes with object B, and how object B colocalizes with object A. To illustrate the importance of such distinction, one may imagine that while all virions (A) colocalize with clathrin (B) while entering the cell, not all clathrin-coated vesicles (B) carry a virus (A), and some clathrin is dispersed in the cell.

3. When calculating Mander’s coefficient, it is essential to adjust the threshold for both channels—in contrast to Pearson’s, it is necessary to define background signal before analysis (see Note 16).

### 3.5.3 Statistics: Particle Counting

1. Split the image into separate channels.

2. Calculate the total number of viruses using, e.g., 3D Objects Counter tool (Fiji ImageJ)—adjust the threshold value using mock-treated cells and remove noise and aggregates using minimum and maximum size filters.

3. Calculate the total number of cells using the DAPI channel and the 3D Objects Counter tool—adjust threshold value and minimum size filter to remove the signal from cell debris. As this analysis is prone to error while analyzing pictures of dense cultures (e.g., HAEs), select for the option to create a surface map, and revise obtained results.

4. Divide the number of virus particles by the number of cells to obtain an average number of particles per cell.

5. To calculate the number of particles in the nuclei of the cell, one may create a mask using the DAPI channel and subtract parts of other channels using an image calculator tool before counting. Unfortunately, masks created using the signal for actin are not sufficient to distinguish internalized and surface viral particles and they should be counted using other methods (e.g., manually). To reduce uncertainty, use gamma on the actin channel to obtain surface without gaps.
4 Notes

1. PFA preparation procedure, purity, and supplier may affect the efficacy and specificity of the immunostaining. This step requires optimization.

2. Cytotoxic activity of chemical inhibitors may affect coronavirus entry into cells in a nonspecific way. A cell viability assay for each compound should be carried out in the experimental setting before the actual experiment.

3. Obtain HCoV-NL63 stock by infecting monolayers of LLC-Mk2 cells in T75 flasks and maintaining at 32°C. Obtain a HCoV-OC43 stock by infecting monolayers of HCT-8 cells in T75 flasks and maintaining at 32°C. Obtain a stock of CRCoV by infecting monolayers of HRT-18G cells in T75 flasks and maintaining at 37°C. To assess virus yield, titrate on fully confluent permissive cells, according to the method of Reed and Muench [52]. For CRCoV, additional staining is required as the virus does not produce cytopathic effect on HRT-18G cells [12].

4. To obtain HCoV stocks, lyse cells by two freeze-thaw cycles and aliquot at −80°C. To obtain CRCoV stock, remove the supernatant, scrape the cells, and freeze-thaw them in a low volume of liquid. Mix with previously removed supernatant and aliquot at −80°C.

5. For some coronaviral strains (e.g., clinical isolates), the stock titer may be low and insufficient for immunostaining. In such cases, virus stock should not be frozen but processed immediately after collection. It is also possible to scale up the culture and concentrate the media to 8 ml.

6. We observed that some inhibitors are active in some cells while showing no activity in others. It is essential to include reference compounds in each study.

7. The fusogenic activity of some viruses can be inhibited by acidic buffer (0.1 M NaCl, 0.1 M glycine, pH = 3; “acid wash”). This may be useful if one is willing to test which virions were on the cell surface at the time of exposition. For this, cells are washed three times with cold acidic buffer, followed by washing with PBS (pH = 7.4).

8. Importantly, coronavirus-infected samples will be treated as non-infectious after incubation in PFA. For that reason, it is important to transfer the cells on coverslips to new non-contaminated plates for fixation. For ease, fixative can be added to wells prior to the transfer of coverslips.

9. The optimal time for each route to be tested is best optimized for each cell type by testing various time points. However, a good starting point is a timecourse study, taking samples every
20–30 min from 0 to 180 min, perhaps longer depending on the endocytic marker being used. Early events are often best studied in 5-min increments. If seeking to understand whether the virus is colocalising with a marker of an endosome (e.g., EEA1), colocalisation might be expected a bit later in this timecourse.

10. Sequential immunostaining of different epitopes while lengthening the procedure allows for optimization of conditions for each antibody and allows for some atypical staining combinations (e.g., first staining with rabbit antibody specific to protein X and a secondary goat anti-rabbit antibody followed by blocking and second staining with mouse antibody specific to protein Y and secondary rabbit anti-mouse antibody).

11. Alternatively, insert membranes may be cut out after fixation, cut into 2–4 pieces and perform subsequent steps to stain for different antigens in microcentrifuge tubes.

12. Stabilize the temperature of the sample and the microscope’s environment before image acquisition. All materials should be left to equilibrate the temperature for at least 15 min. This will help to avoid image deformations caused by temperature drift.

13. If slides are imaged during different sessions, acquisition settings should be identical (tip: they are usually saved as metadata in the image file). It is also essential to verify the values set by manual knobs, e.g., laser power regulation. Often they function independently of the software and will not be adjusted automatically.

14. When counting particles from cultures propagated on Thin-Cert insert membranes, exclude few bottom slices as high membrane autofluorescence tends to impair 3D Objects Counter algorithm function.

15. ImageJ scale bars during generation delete the fragment of the picture they cover. This process is irreversible, so it is good practice to store original versions of photos for future use.

16. The antibody size is in a range of 10–15 nm, which may severely affect the results, especially in superresolution microscopy; to improve the localization analysis, smaller labels, such as aptamers, nanobodies, or quantum-dots, may be used.

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