Chapter 21

Determining the Importance of Peroxisomal Proteins for Viral Infections in Cultured Mammalian Cells

Mariana Marques, Ana Rita Ferreira, and Daniela Ribeiro

Abstract

Peroxisomes have recently been shown to play important roles in the context of viral infections. However, further and more detailed studies should be performed to unravel the specific mechanisms involved. The analysis of the relevance of particular peroxisomal components, such as peroxisomal proteins, for viral infections can be performed by comparing the production of new virus particles in the absence and presence of those specific components. Different methodologies are used to quantify the production of infectious virus particles, depending on the virus, cell type, and the specific characteristics of the viral infection to be analyzed. Here we provide a detailed protocol to study the importance of a putative peroxisomal protein on infection by viruses that induce the death of their host cells. We use the influenza A virus (IAV) infection in A549 cells as a model, and the quantification of the newly produced infectious virus particles is performed by a plaque assay.

Key words Peroxisomes, Viruses, Viral infection, Influenza A virus, Infectivity, Mammalian cells, Plaque Assay

1 Introduction

The importance of peroxisomes for the interplay between viruses and their host cells has been highlighted in multiple recent reports. Besides being established as critical platforms for the cellular antiviral response [1–3], peroxisomes have also been shown to be modulated by different viruses to favor virus particle formation and viral dissemination [4].

Upon infection of the host cell, viruses extensively manipulate and hijack cellular mechanisms to benefit translation of their own proteins, genome replication, and the efficient production of new infectious virus particles. Different viruses present distinct infection cycles, with particular kinetics and consequences for the infected cell.

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One of the most reliable approaches to specifically study the impact of peroxisomal mechanisms on viral infections involves the monitorization of the infection cycle in the absence and presence of specific peroxisomal components, e.g., upon silencing, knock-out, or overexpression of peroxisomal proteins. These analyses must be adapted not only to the virus and cell type under study but also to the features of the viral infection to be specifically analyzed in each case. The currently available methodologies to study the infection cycle and characterize the newly formed virus particles can be divided in two main categories: physical, which detect specific components of the viral infection and newly formed virus particles, and biological, which analyze the infectious properties of the produced virus particles [5, 6].

Physical methods include, among others, serologic assays, polymerase chain reaction and microscopy analyses. These methodologies allow the quantification of specific viral components, such as proteins or genomic material, at different stages of infection, and are normally used in parallel to biological methods. To evaluate the impact that any modulation of peroxisomal mechanisms may have on the virus' capacity to establish an effective infection and produce new viable and infectious particles, biological methods should be used. These methods allow the quantification of the newly formed virus particles which are able to infect new cells, by calculating viral titers. Quantification of viral titers involves the inoculation of serial dilutions of infected samples in new cultured cells and can be achieved by different methods such as plaque assay, end-point dilution assays and focus forming assay.

A plaque assay is the most used methodology to determine the concentration of infectious virus particles that induce the death of the infected cells. Serial dilutions of the medium containing the new virus particles to be quantified, resulting from a primary infection, are inoculated in a new cell monolayer that is then topped with a semi-solid media (e.g., agar or cellulose [7, 8]). After a certain time, depending on the virus and host cells used, the infected cells will lyse and infection spreads only to the immediate adjacent cells, due to the restricted diffusion imposed by the semi-solid media. The death of these surrounding cells allows the visualization of plaques (clear empty spots in a previously confluent monolayer) that can be observed by naked eye upon staining the remaining adherent living cells with crystal or toluidine blue [8]. Quantification of the number of plaques will allow the calculation of viral titers in plaque forming units per milliliter (PFU/ml), as explained below.

Different methodologies must be used to monitor infection by viruses that do not induce the death of the infected cells [9]. One of the most used methods to study infectivity of viruses that can induce a visible cytopathic effect on their host cells is the endpoint dilution assay, where serial dilutions of medium containing newly

Table 1
Most commonly used methodologies to quantify infectious particles produced upon infection by a selected group of medically relevant human viruses. Name abbreviations and genome type are indicated between brackets

Virus	Most commonly used infectivity assays	References
Influenza A virus (IAV, -ssRNA)	Plaque assay Endpoint dilution assay	[12–14]
Respiratory syncytial virus (RSV, -ssRNA)	Plaque assay	[15–17]
Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2, +ssRNA)	Plaque assay	[18–21]
Hepatitis C virus (HCV, +ssRNA)	Focus-forming assay	[22, 23]
Zika virus (ZIKV, +ssRNA)	Plaque assay Focus-forming assay	[24, 25]
Dengue virus (DENV, +ssRNA)	Plaque assay Focus-forming assay	[26, 27]
Kaposi's sarcoma-associated herpesvirus (KSHV, dsDNA)	Endpoint dilution assay	[28]
Human cytomegalovirus (HCMV, dsDNA)	Endpoint dilution assay	[29]

formed virus particles are inoculated in replicated test units (typically 8–10) which, after a predetermined inoculation period, are analyzed to detect the presence or absence of infection. In cell culture, the viral titer is reported as tissue culture infectious doses 50% (TCID50) and represents the dilution in which 50% of the replicated test units were infected. Another methodology used to study virus infectivity is the focus-forming assay, which involves the quantification of fluorescent foci in an infected sample. Similarly to plaque assays, cell monolayers are inoculated with serial dilutions of medium containing newly formed virus particles and incubated under a semi-solid overlay medium. After incubation, cells are subsequently probed with an antibody against a specific viral antigen and analyzed by fluorescence microscopy to quantify the focus-forming units per milliliter (FFU/ml).

Different methodologies are commonly used for different viruses and, often, a combination of two or more physical and biological approaches is chosen to further substantiate the obtained results. Table 1 presents the most common methodologies used for the study of different human viruses of medical relevance.

Here we present a detailed protocol to study the influence of a peroxisomal protein on viral infection by determination of viral titers by plaque assay. As a model, we will use the influenza A virus (IAV) and infect A549 cells, IAV-permissive alveolar basal epithelial cells, knocked-out for a putative peroxisomal protein. All procedures in this protocol using infectious virus particles

must be performed under biosafety level 2 conditions, using appropriate personal protection material and following the guidelines enforced at each institute. Depending on the scientific question and the target virus, the cell line to be used and biosafety conditions to be applied should be carefully accessed.

2 Materials

2.1 Infection of Cultured Mammalian Cells with Influenza A Virus

1. Human lung carcinoma epithelial cells (A549, ATCC CCL-185TM).
2. 1x Phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}).
3. 0.05% Trypsin-EDTA.
4. 7% Bovine serum albumin (BSA) solution in double distilled H_2O (ddH_2O) (sterilized using a 0.2 μm filter).
5. Growth medium: Dulbecco's Modified Eagle Medium (DMEM), high glucose (4.5 g/L) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin.
6. Infection medium: DMEM, high glucose (4.5 g/L) with pyruvate and without glutamine, supplemented with 1% glutamine and 100 units/ml penicillin and 100 μg/ml streptomycin.
7. Reverse-genetics-derived influenza A virus (Puerto Rico/8/1934, H1N1).
8. Acid wash buffer: 135 mM sodium chloride, 10 mM potassium chloride, 40 mM citric acid (pH 3).
9. Clean and sterile glass coverslips (12 mm ∅).
10. 24-well plate for cell culture.

2.2 Immunofluorescence Analysis

1. 1x PBS.
2. 4% Paraformaldehyde (PFA) in PBS.
3. 0.2% Triton X-100 in PBS.
4. 2% BSA in PBS.
5. Mowiol Mounting Medium (3 parts Mowiol for 1 part n-propyl-galate).

2.3 Plaque Assay

1. Madin-Darby Canine Kidney cells (MDCK™, ATCC CRL2936).
2. 1x PBS (without Ca^{2+} and Mg^{2+}).
3. 0.05% Trypsin-EDTA.
4. Growth medium: DMEM, high glucose (4.5 g/L) supplemented with 10% (v/v) FBS and 100 units/ml penicillin and 100 μg/ml streptomycin.

5. Infection medium: DMEM, high glucose (4.5 g/L) with pyruvate and without glutamine, supplemented with 1% glutamine and 100 units/ml penicillin and 100 μg/ml streptomycin.
6. 7% BSA solution in ddH_2O (sterilized using 0.2 μm filter).
7. 1 mg/ml Trypsin from bovine pancreas, TPCK-treated, in ddH_2O (sterilized using 0.2 μm filter).
8. 2.4% Microcrystalline cellulose (Sigma-Aldrich, #435244) in ddH_2O (sterilized by autoclaving).
9. Plaque assay overlay medium: 50% microcrystalline cellulose, 50% infection medium, 0.14% BSA, 1 μg/ml TPCK-trypsin.
10. 4% PFA in PBS with toluidine blue.
11. 25 cm^2 and 75 cm^2 flasks for cell culture.
12. 12-well plate for cell culture.

3 Methods

3.1 Infection of Cultured Mammalian Cells with Influenza A Virus

Here, we describe a methodology to study influenza A virus (IAV) infection in A549 cells, knocked-out for a putative peroxisomal protein (PEX) of interest (A549 KO PEX) (Fig. 1).

1. Seed 6×10^4 A549 KO PEX cells per well of a 24-well plate (*see* **Note 1**) and let them grow overnight in 500 μl growth medium. Enough wells for controls, such as non-infected samples (mock), and biological duplicates, should also be included. Cells should also be seeded on extra wells containing coverslips to monitor the infection cycle by immunofluorescence analysis (*see* Subheading 3.2).
2. Wash cells with 200 μl PBS or serum-free media to remove the serum (*see* **Note 2**).
3. Infect cells with IAV [10, 11], with a multiplicity of infection (MOI) of 3-10, in infection medium (*see* **Notes 3** and **4**). The volume of virus stock needed to prepare virus dilutions should be determined considering the MOI required, the number of cells at the moment of the infection and the virus stock titer, as in:

Fig. 1 Schematic overview of the IAV infection protocol. (Created with BioRender.com)

$$\text{virus stock (ml)} = \frac{\text{MOI (PFU/cell)} \times \text{number of cells}}{\text{virus titer (PFU/ml)}}$$

4. Incubate cells for 10–15 min on a rocker to allow even distribution, and afterwards up to 45 min in an incubator at 37 °C with 5% CO_2 and 95% humidity.
5. Aspirate the medium and incubate the cells with 200 μl acid wash buffer for 1 min at room temperature.
6. Remove the buffer and rinse the cells with 200 μl PBS.
7. Add 500 μl infection medium supplemented with 0.14% BSA and incubate for 16 h (*see* **Note 5**).
8. Harvest the medium into sterile microtubes and freeze it immediately at −80 °C. Cells can be harvested for further complementary analysis.

3.2 Immunofluorescence Analysis

Before moving forward with the quantification of the newly formed infectious virus particles, confirmation on whether the viral infection cycle occurred as expected should be obtained. To do so, cells should be harvested at (at least) two time points post infection, representative of different infection cycle stages (*see* **Note 6**). Here we describe a protocol for an indirect immunofluorescence assay that can be used to analyze IAV-infected cells.

The volumes indicated in the next steps are adequate for processing a 12 mm ∅ coverslip in a 24-well plate and must be adjusted if needed. A triple wash with PBS should be performed between each step of this procedure.

1. Aspirate the medium from the wells containing the coverslips.
2. Fix the cells using 200 μl 4% PFA for 20 min at room temperature.
3. Permeabilize the cells using 200 μl 0.2% Triton X-100 for 10 min at room temperature.
4. Block unspecific binding using 200 μl 1% BSA in PBS for 10 min at room temperature.
5. Drop 20 μl of the primary antibody (anti-NP, Abcam ab20343, 1:2000 dilution) on top of each coverslip and incubate 1 h at room temperature in a box wrapped in wet paper.
6. Pipette 20 μl of the preferred secondary antibody on top of each coverslip and incubate 1 h at room temperature, wrapped in wet paper and protected from the light.
7. Stain cell nuclei with 20 μl of Hoechst (Polysciences, #09460, 1:2000) for 3 min at room temperature and protected from light.
8. Dip the coverslip in dH_2O and mount on a drop of Mowiol mounting media on top of the microscopy slide.

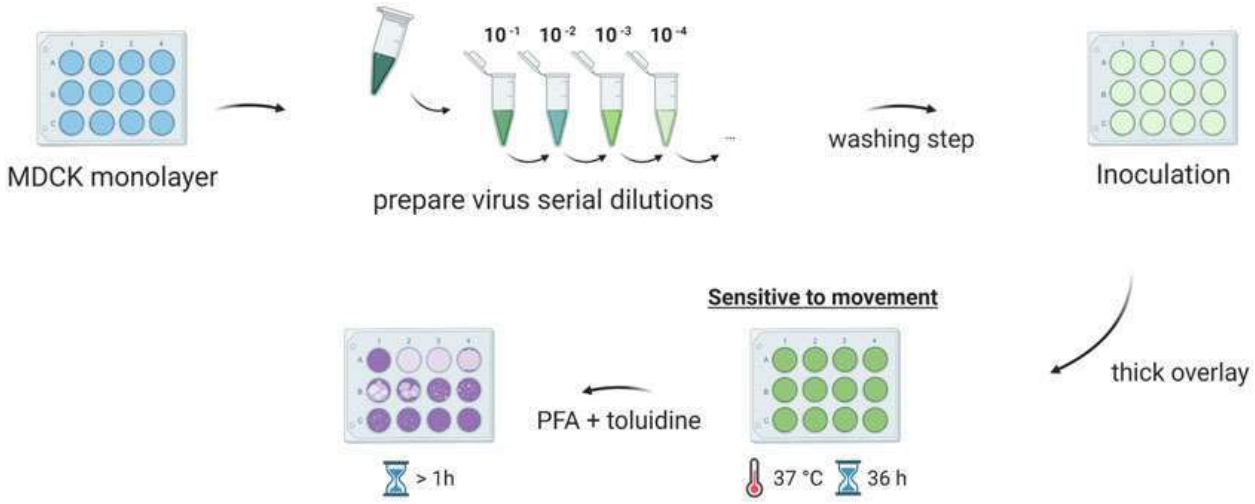

Fig. 2 Schematic overview of the plaque assay protocol to determine IAV viral titers

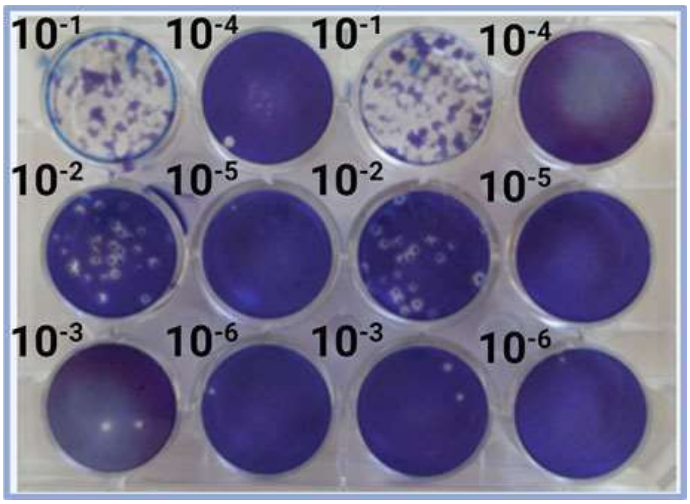

Fig. 3 Example of plaques observed in MDCK cells inoculated with different dilutions of newly formed virus particles. (Created with BioRender.com)

3.3 Plaque Assay

This method encompasses a quantitative measure of the number of infectious particles in a given sample volume. In theory, one plaque of dead cells is formed by a single infectious virus particle, allowing the quantification of plaque forming units (PFUs) (Figs. 2 and 3).

1. Seed 6×10^5 MDCK cells per well in a 24-well plate and allow overnight growth in 1 ml growth medium. Prior to virus inoculation, confirm that cells have formed a confluent monolayer (*see* **Note 7**).
2. Thaw and vortex the samples collected in Subheading 3.1 **step 8**.
3. Prepare a serial dilution set for every sample. As control, use an undiluted or one-time diluted mock sample. We recommend

diluting 55 μl of each sample of the previous dilution in 500 μl of infection medium. Change the pipette tip between dilutions and vortex the prepared solutions (*see* **Note 8**).

4. Aspirate the medium from the plated MDCK cells and rinse with 1 ml PBS or serum-free media (*see* **Note 2**).
5. Inoculate cells with 300 μL of the prepared dilutions.
6. Incubate cells at room temperature on a rocker for 10–15 min and afterwards for 35–45 min in an incubator at 37 °C with 5% CO_2 and 95% humidity.
7. Add 1 ml of overlay medium to each well (*see* **Note 9**) and incubate for the appropriate time (*see* **Note 10**).
8. Carefully aspirate the overlay media (*see* **Note 11**) and rinse twice with 1 ml of PBS.
9. Add 500 μl of 4% paraformaldehyde with 0.1% toluidine blue and incubate for at least one hour on a rocker, or until cells are stained in blue and plaques are visible with naked eye.
10. Discard fixation solution into an appropriate waste container, wash wells with tap water, and air-dry plates before counting the plaques (*see* **Note 12**).
11. To determine viral titers as plaque forming units per ml (PFU/ml), multiply the average plaque number of your condition by the respective well dilution where plaques were counted, considering the initial volume used for the virus inoculation step (in this case 300 μl).

$$\text{average number of plaques} \times \text{dilution factor} \div \text{infection volume} = \text{PFU/ml}$$

At the end of this procedure, the viral titers obtained upon infection of A549 KO PEX should be compared to the ones obtained upon infection of A549 wild-type control cells. These analyses will determine the importance of the putative PEX protein for the IAV infection cycle and for the formation of new infectious virus particles.

4 Notes

1. We recommend an 80–90% cell confluence to reach higher infection rates.
2. Serum can impede infection by the influenza A virus.
3. Pyruvate is required in the medium for an efficient infectious cycle.
4. Harvest samples 16 h post infection with a high multiplicity of infection (MOI) of 3-10 when studying a single infectious

cycle. If multiple infectious cycles are required for the analysis, use a low MOI (e.g. 0.01-0.1) and add trypsin-TPCK to the media formulation. Also, make sure every sample has the same volume, so the viral titers are comparable in the end.

5. 16 h is the suggested incubation time for plaque assay analyses as, at this time point, the medium should contain a high number of newly produced virus particles that have not yet infected other cells. For infection cycle monitorization, earlier time points should be chosen, as explained in **Note 6**.
6. We recommend staining the IAV nucleoprotein (NP), a protein that encapsulates the viral RNA and can be used to follow and determine the stage of the infection. In A549 cells infected with IAV PR8, NP can be detected in the nucleus at 4 h post infection, and in the cytosol at 8 h post infection.
7. It is extremely important to confirm the confluence of MDCK cells prior to inoculation, to ensure that cells are homogeneously distributed and form a complete monolayer.
8. It is important to choose the appropriate range of dilutions for the specific samples. We recommend dilutions between −1 and −6.
9. Do not refreeze trypsin-TPCK, as it loses its biological activity when exposed to multiple freeze–thaw cycles.
10. The incubator should be perfectly leveled and stable, and the plates should not be moved during the overlay media incubation, as the movement can induce the diffusion of viral particles to more distant cells.
11. Carefully aspirate the cell media without touching the bottom of the well to avoid cell detachment and the formation of unintended holes, which may be mistaken as plaques and affect plaque counting and viral titer determination.
12. The best dilution to use to determine viral titers must be the one with more countable and well-defined plaques, usually containing between 10 and 100 plaques.

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