Temporal Analysis of the Nuclear-to-cytoplasmic Translocation of a Herpes Simplex Virus 1 Protein by Immunofluorescent Confocal Microscopy

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Abstract

Infected cell protein 0 (ICP0) of herpes simplex virus 1 (HSV-1) is an immediate early protein containing a RING-type E3 ubiquitin ligase. It is responsible for the proteasomal degradation of host restrictive factors and the subsequent viral gene activation. ICP0 contains a canonical nuclear localization sequence (NLS). It enters the nucleus immediately after *de novo* synthesis and executes its anti-host defense functions mainly in the nucleus. However, later in infection, ICP0 is found solely in the cytoplasm, suggesting the occurrence of a nuclear-to-cytoplasmic translocation during HSV-1 infection. Presumably ICP0 translocation enables ICP0 to modulate its functions according to its subcellular locations at different infection phases. In order to delineate the biological function and regulatory mechanism of ICP0 nuclear-to-cytoplasmic translocation, we modified an immunofluorescent microscopy method to monitor ICP0 trafficking during HSV-1 infection. This protocol involves immunofluorescent staining, confocal microscope imaging, and nuclear vs. cytoplasmic distribution analysis. The goal of this protocol is to adapt the steady state confocal images taken in a time course into a quantitative documentation of ICP0 movement throughout the lytic infection. We propose that this method can be generalized to quantitatively analyze nuclear vs. cytoplasmic localization of other viral or cellular proteins without involving live imaging technology.

Keywords

Immunology and Infection; Issue 141; HSV-1; ICP0; E3 ubiquitin ligase; nuclear retention; nuclear-to-cytoplasmic translocation; virus- host interaction; immunofluorescent staining; confocal microscopy

Introduction

Herpes simplex virus 1 (HSV-1) causes a wide range of mild to severe herpetic diseases including herpes labialis, genital herpes, stromal keratitis, and encephalitis. Once infected,

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Disclosures

The authors have nothing to disclose.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58504/>

the virus establishes a lifelong latent infection in ganglia neurons. Occasionally, the virus can be reactivated by various reasons such as fever, stress, and immune suppression¹, leading to recurrent herpes infection. Infected cell protein 0 (ICP0) is a key viral regulator crucial for both lytic and latent HSV-1 infection. It transactivates downstream virus genes *via* counteracting the host intrinsic/innate antiviral defenses^{2,3}. ICP0 has an E3 ubiquitin ligase activity, which targets several cell factors for proteasome-dependent degradation³. It also interacts with various cell pathways to regulate their activities and subsequently to offset host antiviral restrictions³. ICP0 is known to locate at different subcellular compartments as the infection proceeds^{3,4,5}. The protein has a lysine/arginine-rich nuclear localization signal (NLS) located at residues 500 to 506. Upon *de novo* synthesis at early HSV-1 infection, ICP0 is immediately imported into the nucleus. It is first detected at a dynamic nuclear structure termed nuclear domain 10 (ND10)⁷. The E3 ubiquitin ligase activity of ICP0 triggers the degradation of ND10 organizer proteins, promyelocytic leukemia (PML) protein, and speckled protein 100 kDa (Sp100)^{8,9,10}. After the loss of organizer proteins, ND10 nuclear bodies are dispersed and ICP0 is diffused to fill the entire nucleus^{4,11}.

Interestingly, after the onset of viral DNA replication, ICP0 disappears from the nucleus. It is solely found in the cytoplasm, suggesting the occurrence of a nuclear-to-cytoplasmic translocation late in HSV-1 infection^{4,12}. The requirement of the DNA replication implies the potential involvement of a late viral protein(s) in facilitating the cytoplasmic translocation of HSV-1 ICP0^{4,12}. Apparently ICP0 trafficking among different compartments during infection empowers ICP0 to modulate its interactions to various cellular pathways in a spatial-temporal fashion, and therefore coordinate its multiple functions to fine tune the balance between the lytic and latent HSV-1 infection¹³. To better understand ICP0 multifunctionality and the coordination of ICP0 functional domains throughout the lytic infection, we carefully dissected the molecular basis of the dynamic ICP0 translocation¹². To conduct the mechanistic studies previously reported¹², we have applied an immunofluorescent staining method to visualize ICP0 subcellular localization at different infection status under confocal microscope. We have also developed a quantitative protocol to analyze the nuclear *vs.* cytoplasmic distribution of ICP0 using the confocal software. The population of HSV-1 infected cells was tabulated throughout the infection phases and the trends of ICP0 movement were analyzed, under different biochemical treatments¹². Here we describe the detailed protocol that documents ICP0 translocation in HSV-1 infection. We propose that this method can be adopted as a general method to study the nuclear *vs.* cytoplasmic translocation for other viral or cellular proteins, which can serve as an alternative to live imaging when the live imaging technique is inapplicable due to problems such as labeling method, signal intensity, or protein abundance.

Protocol

1. Cell Seeding and Virus Infection

1. At 20–24 h before the virus infection, seed 5×10^4 of human embryonic lung (HEL) fibroblast cells or other cells to be examined on a 4-well 11 mm staggered slide in growth medium (Dulbecco's Modified Eagle Medium (DMEM))

supplemented with 10% fetal bovine serum (FBS)). Incubate the cells at 37 °C with 5% carbon dioxide (CO₂).

NOTE: Each well should have 70–80% cell confluency at the time of infection.

2. On the next day, remove the growth medium and infect the cells with viruses in Medium-199 at a range of 4–10 pfu/cell. Incubate virus- infected cells for 1 h at 37 °C. Keep shaking the slide during the incubation period.

3. After the 1 h incubation, remove Medium-199 and supplement with growth medium.

NOTE: Drugs that interfere with different infection phases can be added at this step or prior to viral absorption.

4. Incubate the virus-infected cells at 37 °C with 5% CO₂ for various lengths of infection period.

2. Fixation and Permeabilization

1. At proper infection time, quickly wash the infected cells with phosphate-buffered saline (PBS) 3 times and add 200 µL of 4% paraformaldehyde freshly prepared in PBS. Incubate the cells with paraformaldehyde for 8–10 min at room temperature to fix the cells in each well.
2. Aspirate paraformaldehyde and wash the wells with 200 µL of PBS for 3 times. Completely aspirate PBS after the 3rd wash.
3. Add 100 µL of 0.2% non-ionic surfactant to each well to permeabilize the cells for 5–10 min.
4. Aspirate the non-ionic surfactant and wash the wells with 200 µL of PBS for 3 times.

3. Immunofluorescent Staining

1. Completely aspirate PBS and add 200 µL of blocking buffer (1% bovine serum albumin (BSA) and 5% horse serum in PBS) in each well and incubate at room temperature for 1 h or at 4 °C overnight.
2. Add experimentally determined concentration of primary antibody (rabbit anti-ICP0 polyclonal antibody¹²) in blocking buffer and incubate primary antibody at room temperature for 2 h or at 4 °C overnight.
3. Wash with blocking buffer 3 times with 10 min incubation. Add Alexa 594-conjugated goat anti-rabbit secondary antibody (1:400 diluted in blocking buffer) and incubate the slides at room temperature for 1 h. Then wash the slides 3 times with blocking buffer at 10 min interval.
4. Finally wash the slide once with PBS to remove residual BSA and horse serum.
5. Add one drop of antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) to mount the slide and seal it with coverslip using transparent nail polish.

4. Confocal Imaging

1. With a confocal microscope, set the wavelength at 590–650 nm for Alexa 594 and 410–520 nm for DAPI. Select image format at 1024×1024 and line average of 8 to acquire high resolution images.
2. Analyze each well on the 4-well slide under confocal microscope. Acquire representative cell images under the 100X objective, as shown in Figure 1 and Figure 2.
3. For counting large number of cells, take images of consecutive fields under the 40X objective.

NOTE: It requires 5–10 images to accumulate over 200 infected cells from each time point of each infection.

4. In each experiment, take pictures with constant confocal parameters for all samples need to be compared.

5. Analyzing Nuclear vs. Cytoplasmic Distribution

1. Open project with the confocal application software. Select an image from which cells need to be tabulated for nuclear vs. cytoplasmic distribution of ICP0.
2. Click the tab “Quantity” from top menu and select “sort ROIs” from tools menu.
3. Draw a longitudinal line across the cell to be analyzed by selecting “Draw line” from top menu.

NOTE: Histogram will appear showing the fluorescence intensity along the line for both ICP0 and DAPI. In the histogram, blue line represents DAPI pixels and marks the boundary of the nucleus whereas the red line represents ICP0 pixels.

4. **Based on background staining, set up a constant threshold for ICP0 intensity to analyze ICP0 subcellular distribution in each experiment.**
 1. As exemplified in Figure 2, if the red signal on average is below the threshold in the nuclear region but is above the threshold beyond the blue boundary, categorize the red signal as predominantly located in the cytoplasm.
 2. If the red signal is above the threshold throughout the nucleus and beyond the boundary of blue signal, group the red signal as nucleus plus cytoplasmic localization.
 3. If the red signal is above the threshold in the nucleus but on average is below it outside the boundary of blue signal, group the red signal as nuclear localization.
5. Tabulate more than 200 infected cells from each sample at different infection time and plot in bar graph to illustrate ICP0 movement according to time (Figure 3).

Representative Results

To understand the molecular basis and biological functions of ICP0 trafficking during HSV-1 infection, we use an immunofluorescent microscopy method to analyze ICP0 subcellular distribution at different infection phases. Figure 1 shows the representative cells with distinctive ICP0 localization as the infection progresses. To quantify the nuclear-to-cytoplasmic translocation of ICP0, we analyze ICP0 distribution relative to the nucleus by categorizing infected cells into three groups: nuclear localization, cytoplasmic localization, and nuclear plus cytoplasmic localization (Figure 2). To understand elements required for ICP0 trafficking during infection, we track ICP0 movements in wild type or mutant HSV-1 at different infection phases. Figure 3 shows an example of tabulation results for subcellular distribution of ICP0 at different time point of infection.

Discussion

This protocol has been used to study the nuclear-to-cytoplasmic translocation of HSV-1 ICP0. ICP0 undergoes subcellular trafficking during HSV-1 infection (Figure 1). Likely, ICP0 interacts with various cell pathways to carry out different functions at different locations. This enables ICP0 to fine tune its multiple functions in the tug-of-war with human host¹. However, how ICP0 coordinates the multiple functions in a spatial- temporal manner has not been well studied. With the fluorescent microscopy protocol described above, we started to analyze the molecular basis of ICP0 nuclear-to-cytoplasmic translocation. As of now, we have identified the ICP0 C-terminal 35 amino acids as a required element important for this translocation. In the absence of C-terminus, ICP0 is restrained within the nucleus throughout infection (Figure 3). We have also found that an ICP0 E3 ligase-dependent nuclear retention force delays the nuclear-to-cytoplasmic translocation in U2OS cells¹². Furthermore, we have discovered that ICP0 C-terminus and the expression of late viral proteins cooperate to overcome the nuclear retention and facilitate cytoplasmic translocation¹². Currently, we are using this protocol to screen for the late viral proteins involved in the ICP0 nuclear-to-cytoplasmic translocation.

The protocol was initially developed to study the dynamic trafficking of ICP0 in HSV-1 infection. As shown in Figure 1, early in HSV-1 infection, ICP0 is colocalized with ND10, where several key components of cellular restrictive factors and ND10 components such as PML and Sp100⁸, are degraded. After degrading ND10 key constituents, ICP0 diffuses throughout the nucleus and late in infection, ICP0 is translocated to the cytoplasm. Because ICP0 undergoes *de novo* synthesis upon infection, the initial protein abundance is very low and then a robust viral synthesis will quickly obscure the movement of any individual molecules, which makes it difficult to track a single molecule using live imaging technology. Therefore, we deliberately chose not to use live imaging. Instead, we adopted the above protocol to study the steady state ICP0 localization at different infection points, which served us well in tracking ICP0 temporal movement in a population of HSV-1 infected cells.

For a high signal-to-background ratio in confocal analysis, two critical steps are noteworthy in the wet-bench part of this protocol. First, the 4- well staggered slides allow multiple samples to be handled on one single slide. It greatly saves the usage of precious reagents like

viruses and antibodies. However, because the volume held in each well is so small, residual buffer not completely cleared during buffer changes can interfere with the subsequent reagent. Therefore, in each buffer switch, a thorough aspiration is needed before adding the new buffer. Second, based on our experiences, the extent of cell crosslinking and membrane permeability is important for the clarity of fluorescence signals. We have set an empirical number of 10 min for both paraformaldehyde and nonionic surfactant treatments. We found that time much longer or shorter than 10 min can decrease the signal-to-background ratio. As shown in Figure 1 and Figure 2, as well as in a previous study¹², images obtained in our experiments are crystal clear. The prominent blue signal that clearly outlines the nuclear boundary is key to determining the subcellular distribution of ICP0. In the computational part of this protocol, one crucial step is to set a constant threshold to eliminate the background. A successful staining with high signal-to-background ratio is the key to a lower threshold line and better signal contrast. Keeping a constant threshold for all samples in the same experiment, however, is the foundation for the quantitative documentation of ICP0 (Figure 2 and Figure 3).

The protocol can also serve as a general tool to study subcellular trafficking for other viral or cellular proteins when a suitable live imaging method is lacking. In live imaging technique, cells are kept at optimal physiological environment to maintain cell metabolic status^{14,15}. A basic requirement for live cell imaging is to fluorescently label the target protein, which can be achieved by fusing the target protein with a fluorescent tag¹⁶, or to deliver a fluorophore conjugated molecule specific for the target protein¹⁷. In either case, problems may rise if the fusion of fluorescent tag changes target protein property or fluorophore conjugated molecule has difficulty to cross cell membrane. Photobleaching that causes cell damage in the process is an additional concern in live imaging¹⁸. Therefore, new strategies to overcome the limitation of live imaging continue to be the frontier of technology development. The protocol we described here provides temporal analysis of the steady state confocal images, which can serve as an alternative tool when a proper live imaging method is unavailable. The method is easy and reliable. It provides clear detection of protein subcellular localization with minimum background. Using confocal software, we are able to quantitatively analyze the percentage of cells with different distributions of the target protein in a cell population and document the movement of target protein at different cell phases.

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Figure 1: Dynamic trafficking of ICP0 during HSV-1 infection.

HEL cells grown on 4-well slides were infected with prototype HSV-1 (strain F) at 10 pfu/cell. At 1, 5, and 9 h post infection (hpi), cells were fixed, permeabilized, and reacted to rabbit anti-ICP0 and mouse anti-PML primary antibodies, and then reacted to Alexa 594-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse secondary antibodies for imaging under 100X objectives. Promyelocytic leukemia (PML) protein serves as a marker protein for ND10 nuclear bodies, which disappears at 5 and 9 hpi due to PML degradation in infection. Scale bar = 10 μm. Please click [here](#) to view a larger version of this figure.

Figure 2: Analysis of ICP0 subcellular distribution.

Left panel: With a confocal microscope, representative cells were enlarged to show the longitudinal line drawn across the cell that defines the region of interest (ROI). Right panel: Fluorescence pixel intensities in ROI were quantified for both ICP0 and DAPI in individual cells and illustrated as histograms by the confocal application software. An arbitrary threshold (green line) was set to reflect the background staining. Scale bar = 10 μm. Please [click here to view a larger version of this figure](#).

Figure 3: Percentage of subcellular distribution for wild-type and C-terminal truncated ICP0. HEL cells were infected by recombinant viruses containing wild-type ICP0 (ICP0 WT) or C-terminal truncated ICP0 (ICP0 C-truncation) at 4 pfu/cell. At indicated time points, cells were stained and analyzed as described above. Over 200 cells were tabulated for ICP0 location. Percentage of cells containing nuclear, cytoplasmic, or nuclear+cytoplasmic ICP0 were plotted with a spreadsheet computation software. This is an exemplary experiment to show that using this method, we have identified ICP0 C-terminus as a domain required for ICP0 nuclear-to-cytoplasmic translocation. Please click [here](#) to view a larger version of this figure.