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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58504R1
Full Title:	Temporal analysis of the nuclear-to-cytoplasmic translocation of a herpes simplex virus1 protein by immunofluorescent confocal microscopy
Keywords:	HSV-1, ICP0, E3 ubiquitin ligase, nuclear retention, nuclear-to-cytoplasmic translocation, virus-host interaction, immunofluorescent staining, confocal microscopy
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Additional Information:	
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TITLE:

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

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KEYWORDS:
HSV-1, ICP0, E3 ubiquitin ligase, nuclear retention, nuclear-to-cytoplasmic translocation, virus-host interaction, immunofluorescent staining, confocal microscopy

SUMMARY:
ICP0 undergoes nuclear-to-cytoplasmic translocation during HSV-1 infection. The molecular mechanism of this event is not known. Here we describe the use of confocal microscope as a tool to quantify ICP0 movement in HSV-1 infection, which lays the groundwork for quantitatively analyzing ICP0 translocation in future mechanistic studies.

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.

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, 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 suppression[1], 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 degradation[3]. It also interacts with various cell pathways to regulate their activities and subsequently to offset host antiviral restrictions[3]. ICP0 is known to locate at different subcellular compartments as the infection proceeds[3-5]. The protein has a lysine/arginine-rich nuclear localization signal (NLS) located at residues 500 to 506[6]. 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)[8-10]. 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 infection[13]. 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 translocation[12]. To conduct the mechanistic studies previously reported[12], 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 treatments[12]. 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

1.1. 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 (CO_2).

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

1.2. 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.

1.3. 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.

1.4. Incubate the virus-infected cells at 37 °C with 5% CO_2 for various lengths of infection period.

2. **Fixation and Permeabilization**

2.1. 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.

2.2. Aspirate paraformaldehyde and wash the wells with 200 µL of PBS for 3 times. Completely aspirate PBS after the 3rd wash.

2.3. Add 100 µL of 0.2% non-ionic surfactant to each well to permeabilize the cells for 5-10 minutes.

2.4. Aspirate the non-ionic surfactant and wash the wells with 200 µL of PBS for 3 times.

3. **Immunofluorescent Staining**

3.1. 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.

3.2. Add experimentally determined concentration of primary antibody (rabbit anti-ICP0 polyclonal antibody[12]) in blocking buffer and incubate primary antibody at room temperature for 2 h or at 4 °C overnight.

3.3. 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.

3.4. Finally wash the slide once with PBS to remove residual BSA and horse serum.

3.5. 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

4.1 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.

4.2 Analyze each well on the 4-well slide under confocal microscope. Acquire representative cell images under the 100 X objective, as shown in **Figures 1** and **2**.

4.3 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.

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

5. Analyzing Nuclear *vs.* Cytoplasmic Distribution

5.1. Open project with the confocal application software. Select an image from which cells need to be tabulated for nuclear *vs.* cytoplasmic distribution of ICP0.

5.2. Click the tab "Quantity" from top menu and select "sort ROIs" from tools menu.

5.3. 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.

5.4. Based on background staining, set up a constant threshold for ICP0 intensity to analyze ICP0 subcellular distribution in each experiment.

5.4.1. 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.

5.4.2. 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.

5.4.3. 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.

5.5. 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.

FIGURE AND TABLE LEGENDS:

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 hours 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-cojugated anti-mouse secondary antibodies for imaging under 100 X 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. The scale bar represents 10 μm in length.

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. The scale bar represents 10 μm in length.

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.

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 host[13]. 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 cells[12]. Furthermore, we have discovered that ICP0 C-terminus and the expression of late viral proteins cooperate to overcome the nuclear retention and facilitate cytoplasmic translocation[12]. 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 Sp100[8], 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 abundancy 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 **Figures 1 and 2**, as well as in a previous study[12], 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 (**Figures 2 and 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 tag[16], or to deliver a fluorophore conjugated molecule specific for the target protein[17]. 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 imaging[18]. 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.

ACKNOWLEDGMENTS:

We thank financial support from an NIH grant (RO1AI118992) awarded to Haidong Gu. We thank the Microscopy, Imaging & Cytometry Resources (MICR) Core facility at Wayne State University for technical support.

DISCLOSURES:

The authors have nothing to disclose.

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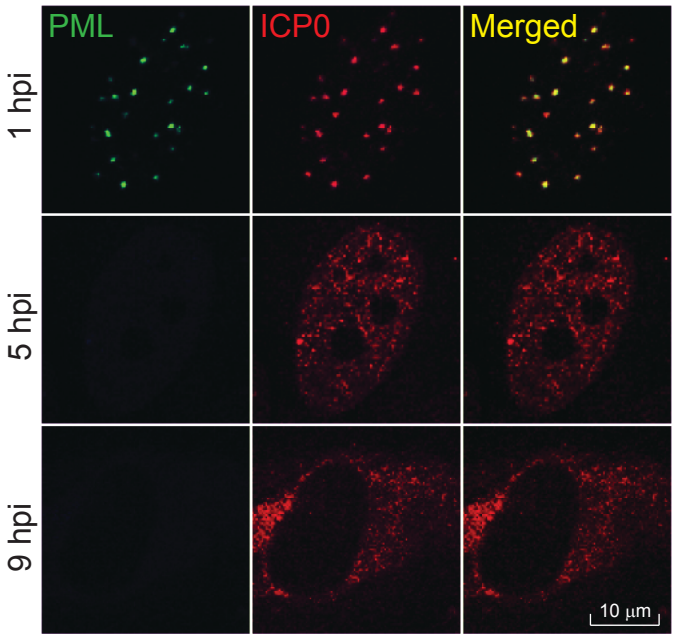

Figure 1

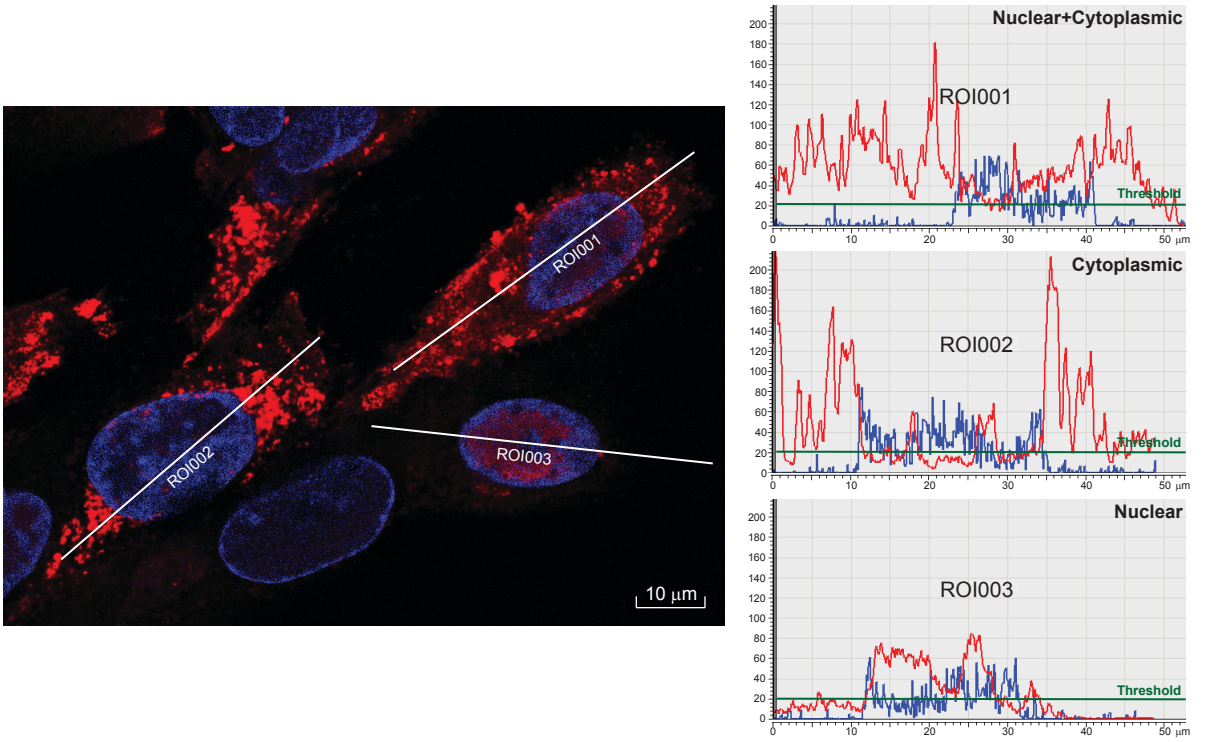

Figure 2

Figure 3

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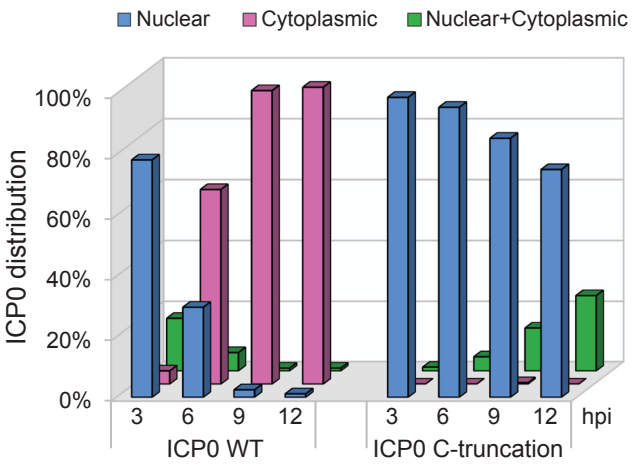

Figure 3

Table of Materials

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	Name of Material/ Equipment
Cells and viruses	Human Embryonic Lung fibroblasts (HEL Cells)
	HSV-1 viral Stock (Strain F)
Medium	Dulbecco's modified Eagle's medium (DMEM)
	Fetal Bovine Serum (FBS)
	Medium-199 (10X)
Reagents	4- well 11 mm staggered slide
	16% Paraformaldehyde solution(w/v) Methanol free
	Triton X-100
	Bovine Serum Abumin (BSA)
	Horse Serum
	Phosphate Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH7.4)
	NaCl
	KH_2PO_4
	KCl
	Na_2HPO_4
	Blocking buffer (PBS with 1% BSA and 5% Horse serum)
	Rabiit Anti-ICP0 antibody
	PML (PG-M3)-Mouse monoclonal IgG
	Alexa Fluor 594-goat anti-rabbit IgG
	Alexa Fluor 488-goat anti-mouse IgG
	Vectashield Mouting medium with DAPI
	Pasteur pipette
	Nail Polish
Equipment	Confocal Microscope
	Confocal Software
	Excel software
	HERAcell 150i CO_2 incubator

Company	Catalog Number	Comments/Description
Dr. Thomas E. Shenk (Princeton University) Dr. Bernard Roizman Lab		HEL cells were grown in DMEM supplemented with 10% FBS
Invitrogen	11965-092	
Sigma	F0926-500ml	
Gibco	11825-015	
Cel-Line/Thermofisher Scientific	30-149H-BLACK	
Thermo Scientific	28908	
Fisher reagents	BP151-1C0	
Calbiochem	CAS 9048-46-8	
Sigma	H1270	
Dr. Haidong Gu lab		
Fisher Bioreagent	BP358-212	
Fisher Bioreagent	BP362-500	
Fisher Scientific	BP366-500	
Fisher Bioreagent	BP332-500	
Dr. Haidong Gu lab		
Dr. Haidong Gu lab		
santa Cruz Biotechnology	SC-966	
invitrogen	A11012	
invitrogen	A11001	
Vector laboratories	H-1200	
Fisher Brand	13-678-20D	
Sally Hansen		
Leica SP8		
Leica LAS X Application suite		
Microsoft Excel		
Thermo Scientific	Order code 51026282	

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We thank the constructive suggestions from reviewers. Below are the point-to-point responses to reviewers' comments.

Reviewer #1:

Manuscript Summary:

This manuscript describes a protocol for visualizing the cellular localization of HSV-1 immediate early viral protein, ICP0 at various times post infection. Although the protocol is a general method commonly used for staining proteins for confocal analysis, this journal does not require it to be novel. The authors have also described that the movement of ICP0 from the nucleus to the cytoplasm requires its C-terminal 35 amino acids. This protocol has been suggested as an alternative to live-cell imaging.

Major Concerns:

Line 78-79: 'under different biochemical treatments'.....I don't see these described throughout the paper.

We have described different drug treatment and various infection conditions in reference 12 (*J Virol.* 92 (2), e01673-17 (2018)). As this is a methodology paper, we did not detail any particular experiment in the *Protocol* section. Instead, we have described it as a general method without a particular treatment but mentioned where drugs can be added in section 1.3.

The protocol states 4 pfu/ml is used for infections (line 91), however the figure legend for Figure 1 states 10 pfu/ml (line 160)

Multiplicity of infection used in individual experiment determines the speed of viral progression. We have used both 4 pfu/ml and 10 pfu/ml in different experiments. To write the manuscript as a general protocol, we have changed the text to "at a range of 4-10 pfu/cell" in section 1.2.

Line 198: Author claims that the protocol they describe is a good alternative to live cell imaging as it can track a single molecule. This is not true.

We apologize for the confusing language. The method described here cannot track a single molecule. It can serve as an alternative to document the process of protein movement in an infected cell population. To avoid ambiguity, we have changed the text to "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 HSV-1 infection".

Minor Concerns:
ICP0 has been previously described to be present in the virion and therefore will be present prior to replication of the virus. The author should mention this in the introduction when describing what is known about ICP0.

ICP0 is a multi-functional protein that has many interesting biochemical properties. For example, ICP0 is found to be dimerized and post-translationally modified. It has also been found to interact with many cellular partners, such as USP7, RNF8 and CoREST. The incorporation of ICP0 into the virion is another unique property of ICP0, of which the function has not been well understood. In this paper, we wanted to focus on the nuclear-to-cytoplasmic translocation process of ICP0. To avoid distraction and lengthy explanation of the complex biochemical properties and the multifunctionality for ICP0, we chose to omit many aspects of ICP0, for which evidence linking them to ICP0 translocation is still lacking.

State what is 'normal growth medium' (line 93)

We revised section 1 by adding “growth medium (Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS))” in section 1.1 and using growth medium in rest of the *Protocol* section.

Figure 1 says HSV-1 (F) was used for infections. what is HSV-1 (F)? Is it a wt strain of HSV-1? (line 160)

We have changed the text to “prototype HSV-1 (strain F).

Figure 1 includes staining of PML. It was not clear why this was done? (line 161)

PML is a key constituent of ND10 macromolecular structure. PML staining was done to visualize ND10. We have previously stated “Promyelocytic leukemia (PML) protein serves as a marker protein for ND10 nuclear bodies”. We now added “which disappears at 5 and 9 hpi due to PML degradation in infection” for more clarity.

Figure 2: how was the threshold calculated? This should be mentioned in the manuscript.

Threshold was set arbitrarily to remove the background staining. It was set at same number for individual histograms in the same experiment. We added “An arbitrary

threshold (green line) was set to reflect the background staining." In the legend for figure 2.

Figure 2: The figure has ROI numbers on it. Please describe what these represent in the figure legend

ROI is short form region of interest. We have revised the legend for figure 2.

Figure 3 (starting line 172): does not describe the whole image. There is no mention here of the ICPO c-truncation and why it was included

We have revised the legend for figure 3 as the following: "**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."

Line 178: sentence states 'likely', I think this has been shown on a number of occasions to be in fact true.

Although ICP0 has been shown to interact with various cell pathways, and it has been shown to traffic through different cellular compartments, the key evidence to connect a particular ICP0 interaction/function to a specific subcellular domain is still missing. For example, ICP0 colocalizes with ND10 is believed to result in the degradation of ND10 organizer, PML. However, we have found that an ICP0 mutant that was able to dock at ND10 but lost the ability to merge with ND10 can still ubiquitinate and degrade PML isoform I, but not the isoform II (*J Virol*. **89** (8), 4214-4226 (2015); *J Virol.* **90** (23), 10875-10885 (2016)), suggesting that ICP0 ubiquitinates similar substrates, PML isoforms I and II, in different subcellular domains. Why a similar reaction is carried out in different locations and how ICP0 functions are fine-tuned for differential regulation remains unknown. The hypothesis of "ICP0 interacts with various cell pathways to carry out different functions at different locations" still needs large amounts of work to fill in the blanks.

Line 185: sentence stating that E3 ligase activity was required for sequestering ICP0 in the nucleus. Is this 'data not shown' if not then this needs a reference as this manuscript does not show this result.

We have added the reference.

Sentence starting on line 216 continuing until line 222 seems out of place and needs to be move to the previous paragraph with the other discussion of results before stating the pro's and con's of the protocol.

We have added one paragraph to discuss technical issues of the method and revised this part as suggested.

Figure 3: graph lists 'ICP0 wt' and 'ICP0 c-truncation'. It is not clear from this if this is an infection study or not just ICP0 expressed alone. ICP0 wt and ICP0 c-truncation should be described in the figure legend or relabeled on the figure to reflect this.

As responded above, we have revised the legend for figure 3

Reviewer #2:
Manuscript Summary:
The HSV-1 immediate early (IE) protein ICP0 plays multiple roles during the course of lytic infection and establishment of latency. As an extension of their recent manuscript published in J. Virology, Samarat and Gu describe the methodology for visualizing the dynamic kinetics of ICP0 subcellular localization. Overall, the methods are clear and concise while a sufficient level of information is provided in regards to the purpose of this technique in relationship with ICP0.

As detailed below, comments are provided to address the need for clarification of specific areas in the protocol sections while also potentially modifying the image acquisition step of confocal microscopy.

Major Concerns:
-Lines 116-126, Figs 1-3: To quantify the localization of a specific protein, the authors should really consider collecting multiple confocal images along the Z-axis ("Z-Stack") paired with deconvolution (Huygens software, etc.). Representing the localization of protein in a 3-D space is unbiased and more accurate than collecting only one image of a cell in a single plane. Furthermore, analyzing cells in a 3-D space is one of the powerful application of confocal microscopy. Based on how the localization is mapped from one single image/plane using a standard fluorescence microscope would have been sufficient.
Also, Z-stacks with deconvolution may help to resolve the Nuclear+Cytoplasmic localization of ICP0 (Fig 2, ROI001).

We agree with the reviewer. We will further improve our quantification details in future studies. The current manuscript is based on our published data (*J Virol.* 92 (2), e01673-17 (2018)) to introduce a methodology concept of using steady-state confocal to document protein translocation when live imaging is inappropriate for studies.

-Fig 2: When completing ROI analysis on cells in which ICP0 is only nuclear (ROI003), how is it known that the "draw line" feature is through the cytoplasm and not just the background level of the slide? Without a cytoplasmic marker, cell membrane stain, or differential interference contrast (DIC) an accurate assessment of the cytoplasmic signal is difficult. As an example, there is ICP0 signal on the lower left corner of ROI003, should this be considered in the "Nuclear+Cytoplasmic" category and not "Nuclear"?

First, human embryotic lung fibroblasts (HEL) are very thin and flat and they seldom craw over each other. In our past experiences, we found that most cells captured in a single focal plane have similar morphology, and the localization analysis was done based on large amounts of cells tabulated via these confocal images. The chance that a few nuclei are captured without scanning through cytoplasm might be there but it will not influence the overall results due to the low number.

Second, we recognize that background staining such as the lower left corner of ROI003 does exist. We set up arbitrary threshold line (Figure 2) for each experiment. Within a particular experiment, all cells were analysed with the same threshold line to exclude background staining.

-Line 135: Provide further detail on how a "consistent threshold for ICP0 intensity" was selected. Was the threshold value arbitrary selected or was there a specific reason? May need to describe the "thought process" or method of selecting a threshold.

See above response and response to the minor concern #5 from reviewer #1.

Minor Concerns:
-Lines 70-72: To be clear may want to emphasis that this method only evaluates ICP0 localization during lytic infection (not latency) rather than stating "throughout infection". Specifically, line 71 references ICP0 multiple functions during "lytic and latent HSV-1 infection," while line 72 references the method to evaluate "ICP0 functional domains throughout infection."

Latent HSV-1 infection cannot be recapitulated in cultured cell lines. Therefore most of the biochemical studies for HSV-1 viral proteins can only be performed with lytic infection in cell culture. Here “throughout infection” refers the entire lytic cycle that includes the entry, uncoating, early gene expression, genome replication, late gene

expression and virion assembly processes, which reflects the progression of infection in one single cell. We have changed line 72 into “throughout the lytic infection” to avoid confusion.

-Line 94: Would suggest rephrasing "drugs that interfere with different infection phases can be added at this step [or prior to viral absorption]." Depending on the kinetics of some inhibitors, a longer treatment may be required.

We rephrased to “Drugs that interfere with different infection phases can be added at this step or prior to viral absorption”.

-Line 97: Given this a methods paper, authors should consider describing "proper infection time." May want to state the duration of infection used to evaluate ICP0 localization (Figure 1) and possibly provide an estimate time for the kinetics for IE, E, and L protein expression.

Multiplicity of infection used in each experiment determines the speed of infection progression, which is usually reflected by monitoring protein expression cascade with Western or Northern blotting. For some mutant viruses, infection is delayed, so infection time points for individual experiments vary. Within the same experiment, it is important to follow the same infection points for different viruses used in comparison.

-Line 98, 102, 105: Was the paraformaldehyde, Triton X-100, and blocking buffer diluted in PBS? If yes, these should be included in the text.

4% paraformaldehyde is diluted in PBS. Triton X-100 is dissolved in water. Blocking buffer is prepared with PBS. These are now described in the text.

-Line 107: Consider changing "add properly diluted primary antibody" to "experimentally determined concentration of primary antibody" for clarity.

We changed the text as suggested.

-Line 112: How long (time) is the wash step?

“3 times with 10 min incubation”, as described in section 3.3

-Line 123 and 143: Why collect images from 200 cells? It's clear that 200 cells were

evaluated in their recent J. Virology paper, but is there a statistical reason or just arbitrary?

It is a number commonly accepted in virology field to study a population of infected cells. Although we use high multiplicity of infection (4-10 pfu/cell) and try to synchronize viral infection, it is still very common that individual cells receive different amount of virus and infection progresses at different speed. Usually we tabulate large amounts of cells in an infected population to document the infection process when comparing different viruses.

-Lines 172-174, Fig 3: Specify viruses used (ICP0 WT and ICP0 C-truncation) within the figure legend.

We have revised the legend for figure 3, see responses to reviewer #1.

-Materials table: Information is missing for PBS, HSV-1 virus stocks (strain), nail polish, and blocking buffer. Ensure all reagents are listed.

We have included the information in materials table.

Reviewer #3:

Manuscript Summary:

The authors appear to be expert researchers in the area of herpes simplex virus 1 molecular biology, particularly the well-studied viral ICP0 protein. They describe the use of immunofluorescence and confocal imaging to analyze the nuclear to cytoplasmic trafficking of this protein during viral infection. The paper, including the protocol portion of it are clearly written.

Major Concerns:

My major concern is that the techniques of immunofluorescence and confocal microscopy are very long established (decades old) and extremely standard and as such this paper is unlikely to be of much interest to the biomedical community. Quantitation of nuclear versus cytoplasmic signals is a little less common, but the authors' technique in this regard is not very novel either, essentially taking advantage of the analysis software provided by makers of the confocal microscope. It does not seem to me that this paper falls within the scope of JOVE, which is defined on the website as pertaining to "novel techniques, innovative applications of existing techniques, and gold standard protocols".

In this manuscript, we introduced a new concept of adapting the steady-state confocal imaging to document protein trafficking at different infection phases. This fits with the scope of “innovative application of existing techniques”.

Another serious concern is that in the manuscript, including the title, the authors state that their protocol is designed to study the movement of ICP0 within the cell. However, immunofluorescence of fixed cells merely provides a snap-shot of steady-state protein levels in various compartments. Steady-state protein levels in various compartments are affected by protein synthesis, protein trafficking, and protein degradation. Thus, although IF is quite a valuable technique, it can't be used to measure protein movement, unless we are sure that protein synthesis and degradation are not factoring in to what is seen. For example, in regard to the present work, could the appearance of ICP0 in the cytoplasm at late times after infection be explained by continued synthesis of ICP0 late in infection coupled with a block to its nuclear import?

The reviewer has a good point. In fact, trying to determine whether the appearance of ICP0 in the cytoplasm is related to a block to its nuclear impart is one of the objectives in the whole study. Due to the complex and robust changes occur in viral infection, it is very difficult to study a single protein trafficking with live imaging. Here we introduce a new methodology concept that by using steady state confocal imaging at different infection time points and by documenting the percentage of cells in an infected population, we can connect viral protein movements to other infection events, such as viral DNA replication and late protein expression. This gives us one more tool to understand viral protein functions in different cell compartments.

Minor Concerns:
Line 87 - HEL cells should be defined. Presumably these aren't essential for the protocol so perhaps the protocol should be written a bit more generally.

We have changed the text to “Seed $5x10^4$ of human embryonic lung fibroblast (HEL) cells or other cells to be examined”.

Line 117 and following - The protocol involves using Leica microscope software to carry out the analysis, but presumably other confocal microscopes would come with software that would be able to do the analysis. This could be clarified.

We have revised the entire protocol in general terms as requested by the editor.