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FLARE: A Flow Cytometry–Based Fluorescent Assay for Measuring HSV-1 Nuclear Egress

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Abstract

During herpesvirus replication, capsids are assembled inside the nucleus and translocated into the cytosol by a non-canonical nucleocytoplasmic export process termed *nuclear egress*. Traditional methods of measuring nuclear egress rely on imaging-based technologies such as confocal and electron microscopy. These techniques are labor-intensive, limited by the number of cells that can be examined, and may not accurately represent the entire population, generating a potential bias during data interpretation. To overcome these problems, we have developed a flow cytometry–based method to measure HSV-1 nuclear egress that we termed FLARE (FLow cytometry–based Assay of nucleaR Egress). This assay uses a double fluorescent reporter system, utilizing HSV-1-tdTomato to identify infected cells and an Alexa Fluor-488-conjugated, capsid-specific antibody to detect cytosolic capsids, thereby distinguishing infected cells with nuclear egress from those without it. This assay provides more quantitative results than traditional methods, enables large-scale high throughput, and can be adapted for use with other herpesviruses.

Key features

- Quantification of HSV-1 nuclear egress by flow cytometry using a double fluorescent reporter system.
- The assay is suitable for large-scale high-throughput screens, e.g., CRISPR/Cas9.
- The assay can be adapted for use with other herpesviruses, provided a mature capsid-specific antibody is available.

Keywords: HSV-1, Nuclear egress, Nucleocytoplasmic export, Capsid, Antibody, Fluorescence reporter, Double fluorescent reporter system, Flow cytometry

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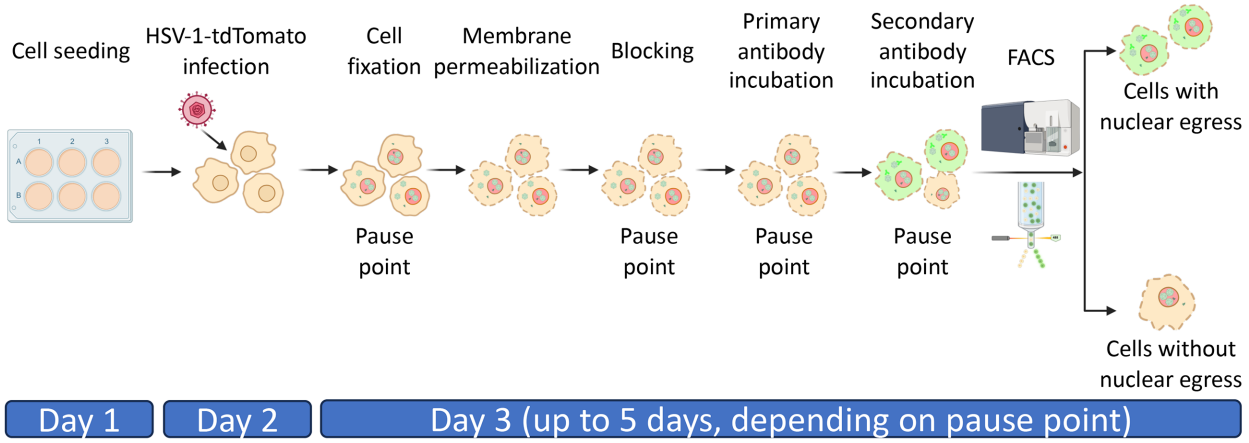
Graphical overview



Schematic overview of the FLow cytometry–based Assay of nucleaR Egress (FLARE). Workflow for quantifying nuclear egress in HSV-1-infected cells using the custom flow cytometry–based assay developed here. HeLa cells are infected with HSV-1 encoding a *tdTomato* transgene reporter. Twenty-four hours post-infection, cells are fixed with 4% paraformaldehyde, partially permeabilized with digitonin, and incubated with a capsid-specific primary antibody and Alexa Fluor-488-conjugated secondary antibody. Flow cytometry identifies cells positive for both tdTomato and Alexa Fluor-488 signal, which are HSV-1-infected cells undergoing nuclear egress.

Background

Herpesvirales is an order of enveloped double-stranded DNA viruses that can infect a variety of hosts, both invertebrate and vertebrate [1]. Nine herpesviruses infect humans, causing lifelong infections and diseases ranging from painful mucocutaneous sores to life-threatening encephalitis and cancers. Current therapeutic strategies are not curative and preventives are limited [2–6]. As such, in-depth studies of the herpesviral replication cycle will not only improve our understanding of the virus–host interactions but may lead to improved antivirals targeting herpesviruses.

During viral replication, herpesvirus genomes are packaged into capsids in the nucleus. These capsids then exit the nucleus using a non-canonical nucleocytoplasmic export, one that does not rely on the nuclear pore complex (NPC). Instead, viral capsids are recruited to the inner nuclear membrane (INM), where they bud into the perinuclear space, forming perinuclear enveloped vesicles (PEVs). These PEVs then fuse with the outer nuclear membrane (ONM), releasing the capsids into the cytoplasm. This process of capsid translocation from the nucleus to the cytoplasm is termed “herpesvirus nuclear egress” [7].

Traditional methods used by the field to study herpesvirus nuclear egress rely on observing phenotypes by confocal microscopy or transmission electron microscopy (TEM) [8–12]. However, these methods pose some limitations. Namely, the number of cells captured by the microscope is small, and the data analysis process is generally time-consuming and labor-intensive.

To address the need for a faster, easier, and more robust way of measuring nuclear egress, we have developed a flow cytometry–based method to directly measure HSV-1 nuclear egress across entire cell populations (Graphical overview). HSV-1-infected cells are first partially permeabilized using digitonin, a mild detergent, which disrupts the plasma membrane but leaves the nuclear envelope intact. Next, cells are incubated with a mature capsid-specific antibody that specifically binds viral capsids in the cytosol but not in the nucleus (due to intact nuclear envelope) and does not bind free capsid proteins synthesized in the cytosol. Cells are subsequently incubated with a fluorescently labeled secondary antibody. Finally, cells that emit fluorescence are sorted from those that do not by flow cytometry, which enables quantification of nuclear egress across the entire cell population.

This protocol uses a two-reporter system, in which the use of HSV-1 encoding a *tdTomato* transgene under the control of a CMV promoter [13] enables the detection of infected cells, and the use of a capsid-specific primary antibody [14] in conjunction with an Alexa-Fluor-488-conjugated secondary antibody enables the detection of cytoplasmic capsids. Thus, by flow cytometry, cells can be separated into three groups: uninfected cells (tdTomato-/488-), HSV-1-infected cells without

nuclear egress (tdTomato+/488-), and HSV-1-infected cells with nuclear egress (tdTomato+/488+). Although this protocol was optimized to measure nuclear egress in HeLa cells infected with HSV-1-tdTomato, it has also been used with Vero and Hep-2 cells, as well as with HeLa cells infected with HSV-1 lacking a fluorescent reporter. Our method, which we have termed FLARE (FLow cytometry-based Assay of nucleaR Egress), addresses the lack of robust measurements for herpesvirus nuclear egress and provides a faster route to identifying new factors controlling herpesvirus nuclear egress.

Materials and reagents

Biological materials

1. HeLa cells (ATCC, CCL2)
2. Hep-2 cells (ATCC, CCL23)
3. Vero Cells (ATCC, CCL-81)
4. HSV-1-tdTomato [strain HSV-1 F GS3217, a gift from Dr. Greg Smith, Northwestern University, encodes NLS-tdTomato under the control of a CMV immediate-early (IE) promoter in place of the envelope glycoprotein gJ] [13]
5. HSV-1 (strain HSV-1 F GS6000, a gift from Dr. Greg Smith, Northwestern University) [15]
6. HSV-1-tdTomato-d34 (strain HSV-1 F GS3217 containing a *UL34* gene knockout, generated by our lab using En Passant mutagenesis method [16]), serves as a no-nuclear-egress control
7. HSV-1-d34 (strain HSV-1 F GS6000 containing a *UL34* gene knockout, generated by our lab using En Passant mutagenesis method [16]), serves as a no-nuclear-egress control

Reagents

1. DMEM high glucose, with sodium pyruvate, without L-glutamine (Cytiva, catalog number: SH30285.02), store at 4 °C
2. Opti-MEM reduced serum medium (Thermo Fisher Scientific, catalog number: 31985070), store at 4 °C
3. Fetal bovine serum (FBS), heat-inactivated (Biowest, USDA-approved, catalog number: S1680-500, or Thermo Fisher Scientific, catalog number: 10438026), store at -20 °C
Note: Catalog numbers may vary depending on the manufacturer, source, and batch. Researchers are encouraged to contact the company prior to placing the order.
4. L-Glutamine, 200 mM (Corning, catalog number: 25-005-CI), store at 4 °C
5. Penicillin-Streptomycin (100×), penicillin 10,000 U/mL, streptomycin: 10,000 µg/mL (Cytiva, catalog number: SV30010), store at 4 °C
6. Phosphate-buffered saline (PBS), 1× (Cytiva, catalog number: SH30256.FS), store at room temperature
7. Trypsin, 0.05% (w/v), 1× (Cytiva, catalog number: SH30236.01), store at 4 °C
8. Trypan blue in PBS, 0.4% (w/v) (Cytiva, catalog number: SV30084.01), store at room temperature
9. Paraformaldehyde (PFA) in PBS, 4% (w/v) (Thermo Scientific, catalog number: J19943K2), store at 4 °C
10. Digitonin, 5% (w/v) (Thermo Fisher Scientific, catalog number: BN2006), store at 4 °C
11. Triton X-100 (Sigma-Aldrich, catalog number: T8787-100ML), store at room temperature
12. Bovine serum albumin (BSA) (Fisher Scientific, BP1600100), store at 4 °C
13. 8F5 mouse anti-VP5 capsid-specific antibody [14], 1.3 mg/mL (hybridoma generated by Dr. Jay Brown, University of Virginia, and purchased from the University of Virginia Stem Core Facility, single cell clone 8F5-D12-A6; purified IgG was produced from the hybridoma by Cell Essentials, Inc. and can be ordered by referencing 8F5-D12-A6), long-term storage at -80 °C for up to 5 years, short-term storage at 4 °C for up to 1 year. Additional details are provided in the Validation of Protocol section, under the subsection “Testing the efficacy of an anti-VP5 antibody.”
14. Alexa-Fluor-488-conjugated goat anti-mouse antibody (Thermo Scientific, catalog number: A28175), store at 4 °C
15. Bleach (Clorox, catalog number: 30966), store at room temperature

Solutions

1. Complete cell culture growth medium (see Recipes)
2. Stock digitonin solution (see Recipes)
3. Partial (plasma membrane) permeabilization solution, 2× (see Recipes)
4. Full membrane permeabilization solution, 2× (see Recipes)

5. Blocking buffer (see Recipes)
6. Primary antibody solution, 5× (see Recipes)
7. Secondary antibody solution, 1× (see Recipes)
8. Bleach, 20% (v/v) (see Recipes)

Recipes

1. Complete cell culture growth medium

Reagent	Final concentration	Quantity or volume
DMEM	1×	1 L
FBS	~10% (v/v)	100 mL
L-Glutamine, 200 mM	~2 mM	11 mL
Penicillin-Streptomycin (100×)	~1× (penicillin 100 U/mL and streptomycin 100 µg/mL)	11 mL

Store at 4 °C for up to 1 year.

2. Stock digitonin solution

Reagent	Final concentration	Quantity or volume
Digitonin, 5% (w/v)	5 mg/mL	1 mL
PBS	1×	9 mL

Heat the 5% digitonin solution at 95 °C for 5 min and allow to fully dissolve before use. Store in 1 mL aliquots at -20 °C for up to 3 years or at 4 °C for up to 1 year. Avoid freeze and thaw cycles.

3. Partial (plasma membrane) permeabilization solution, 2×

Reagent	Final concentration	Quantity or volume
Stock digitonin solution (5 mg/mL)	80 µg/mL	16 µL
PBS	1×	1 mL

If the stock digitonin solution precipitates, heat at 95 °C for 3 min to fully dissolve before use. Prepare fresh for each experiment.

4. Full membrane permeabilization solution, 2×

Reagent	Final concentration	Quantity or volume
Triton X-100	0.4% (v/v)	4 µL
PBS	1×	1 mL

Prepare fresh for each experiment.

5. Blocking buffer, 2×

Reagent	Final concentration	Quantity or volume
BSA	1% (w/v)	0.5 g
PBS	1×	50 mL

Store at 4 °C for up to 1 month.

6. Primary antibody solution, 5×

Reagent	Final concentration	Quantity or volume
8F5 mouse anti-VP5 antibody (1.3 mg/mL)	3.25 µg/mL	2.5 µL
Blocking buffer	2×	1 mL

Prepare fresh for each experiment.

7. Secondary antibody solution, 1×

Reagent	Final concentration	Quantity or volume
Alexa Fluor-488 goat anti-mouse antibody	2 µg/mL	2 µL
Blocking buffer	2×	1 mL

Prepare fresh for each experiment.

8. Bleach 20% (v/v)

Reagent	Final concentration	Quantity or volume
Bleach	20% (v/v)	200 mL
Water	1×	800 mL

Store at room temperature for up to 1 year.

Laboratory supplies

1. 10-cm tissue culture dish (CELLTREAT, catalog number: 229620)
2. 6-well tissue culture plates (CELLTREAT, catalog number: 229106)
3. Conical tubes, 50 mL (CELLTREAT, catalog number: 229421)
4. Conical tubes, 15 mL (CELLTREAT, catalog number: 229411)
5. Centrifuge tubes, 1.5 mL (Bio Plas, catalog number: 4030)
6. Pipette set (Eppendorf, catalog number: 2231300004)
7. Pipet controller (Drummond Scientific, catalog number: 4000501)
8. Filter pipette tips (USA Scientific, catalog numbers: 1122-1830, 1120-8810, 1120-3810)
9. Serological pipettes (CELLTREAT, catalog numbers: 229005B, 229010B, 229025B)
10. Cell counter slides (Thermo Scientific, catalog number: C10228)

Equipment

1. CO_2 cell culture incubator (Thermo Scientific, model: Heracell VIOS 160i)
2. Biosafety cabinet (Nuaire, model: NU-425-400)
3. Countess II cell counter (Thermo Fisher Scientific, model: AMQAX1000)
4. Centrifuge for 15/50 mL tubes (Thermo Fisher Scientific, model: 75009381)
5. Centrifuge for 1.5 mL tubes (Eppendorf, model: 5424R)
6. Plate rocker (VWR, model: 75832-308)
7. Benchtop vortex (Fisher Scientific, model: 02215365)
8. Flow cytometer (Thermo Fisher Scientific, model: Attune Cytpix)

Software and datasets

1. Attune cytometric software (Thermo Scientific, Version 6.21)
2. FlowJo (Becton, Dickinson & Company, Version 10.10.0)
3. Excel (Microsoft, Version 16.96.1)
4. Prism (GraphPad, Version 10.4.1)

Procedure

A. Before experiments (BSL-2)

1. Maintain HeLa cells in 10-cm culture dishes at 37 °C with 5% CO_2. Keep cell density between 1 million/dish and 15 million/dish. Prepare and keep the stock of HSV-1 virus at -80 °C.

B. Day 1: Cell seeding (BSL-2)

1. Retrieve HeLa cells from the incubator. Aspirate the supernatant and wash cells once with 5 mL of PBS.
2. Aspirate the PBS and add 2 mL of 0.05% trypsin. Incubate cells for ~3 min at 37 °C with 5% CO_2 to allow cells to detach.
3. Resuspend cells in 8 mL of complete cell culture growth medium (see Recipe 1).
4. Pipette 25 µL of cell suspension into a centrifuge tube and mix with 25 µL of 0.4% trypan blue. Pipette 10 µL of this

mixture into a cell counting slide. Measure the cell density with a cell counter.
5. Seed HeLa cells in a 6-well plate at a cell density of 200,000 cells/mL, with 2 mL of total volume per well. Incubate overnight at 37 °C with 5% CO_2.
6. Label one well as “counting,” one well as “UC” (uninfected control), two wells as “HSV-1-tdTomato,” and two wells as “HSV-1-tdTomato-d34” (a control for the defect in nuclear egress) (see General notes 1 and 2). For any additional conditions, prepare 2 wells for each condition as desired.

C. Day 2: Virus infection (BSL-2)

1. Retrieve the 6-well plate from the incubator. Aspirate the growth medium from the well labeled “counting” and wash once with 2 mL of PBS.
2. Aspirate the PBS and add 300 µL of 0.05% trypsin. Incubate cells for ~3 min at 37 °C with 5% CO_2 until all cells have detached.
3. Resuspend cells with 700 µL of growth medium.
4. Pipette 25 µL of resuspended cells into a centrifuge tube and mix with 25 µL of 0.4% trypan blue. Pipette 10 µL of the mixture into a cell counting slide and measure the cell density with a cell counter. Discard cells after counting.
a. Target cell density should be between 4 and 8 × 10^5 cells/mL.

5. Calculate the amount of virus to use for the experiment based on the determined cell density. Virus should be made up in 1 mL of Opti-MEM media.
a. For HSV-1-tdTomato, use a multiplicity of infection (MOI) of 5.
b. For HSV-1-tdTomato-d34, use an MOI of 10 (see General note 3).

6. Aspirate the supernatant from wells labeled “UC,” “HSV-1-tdTomato,” and “HSV-1-tdTomato-d34.” Infect cells with 300 µL of their respective viruses. For the UC, mock-infect cells using 300 µL of Opti-MEM media. Return the plate to the incubator.
7. Incubate cells at 37 °C with 5% CO_2 for 1 h, gently agitating the plate by hand every 15 min to distribute the virus evenly.
8. Following the 1 h infection, aspirate the supernatant and add 2 mL of growth medium into each well. Return the plate to the incubator.

D. Day 3: Sample preparation for flow cytometry (before step 6: BSL-2; after step 6: BSL-1)

1. Twenty-four hours post-infection (hpi) (see General note 4), take the plate out of the incubator and aspirate the growth medium from each well. Wash cells once with 2 mL of PBS.
2. Aspirate the PBS and add 300 µL of 0.05% trypsin into each well. Incubate cells at 37 °C with 5% CO_2 for ~3 min until all cells detach.
3. Resuspend cells with 300 µL of growth medium.
4. Pool and transfer cells infected with the same virus into 1.5 mL centrifuge tubes. Centrifuge cells at 500× *g* for 5 min at room temperature. Bleach the 6-well plates with 20% bleach.
5. Aspirate the supernatant, leaving the pellet intact. Fix cells by resuspending the cell pellet in 200 µL of 4% PFA for the UC-labeled tube (containing a 200 µL cell aliquot) and 400 µL of 4% PFA for the HSV-1-tdTomato- and HSV-1-tdTomato-d34-labeled tubes (containing 400 µL cell aliquots in each tube). Rock cells in PFA for 20 min at room temperature on a rocker set to low-to-medium speed.
Pause point: (Optional) Fix cells overnight at 4 °C on a rocker set to low speed (see General note 5).
6. Prepare samples for membrane permeabilization. For the uninfected cell sample, transfer 100 µL of fixed cells into a new centrifuge tube labeled “UC.” For HSV-1-tdTomato and HSV-1-tdTomato-d34-infected cell samples, transfer 100 µL of fixed, infected cells into centrifuge tubes labeled as “2^{ry} only,” “Digitonin,” and “Triton X-100” for each virus.
7. Permeabilize cells for 30 min at room temperature by mixing 100 µL of cells with 100 µL of detergent by pipetting cells up and down 5–10 times without introducing any bubbles.
a. For “UC” and “2^{ry} only” samples, permeabilize cells with 100 µL of 2× full membrane permeabilization solution (see Recipe 4).
b. For “Digitonin” samples, permeabilize cells with 100 µL of 2× partial (plasma membrane) permeabilization solution (see Recipes 2 and 3).
c. For “Triton X-100” samples, permeabilize cells with 100 µL of 2× full membrane permeabilization solution (see Recipe 4).

Critical: The extent of permeabilization will influence the signal read out by flow cytometry (see General note 6). We do not recommend vortexing samples during permeabilization, as the use of greater mechanical force may over-permeabilize the cells, especially for partial permeabilization.

8. Block cells (200 µL) for 1 h at room temperature by adding 200 µL of 2× blocking buffer (see Recipe 5 and General note 7).
9. Add 100 µL of 5× primary antibody solution (see Recipe 6) into "UC," "Digitonin," and "Triton X-100" tubes for each virus. (Working concentration of the 8F5 mouse anti-VP5 antibody is 0.65 µg/mL.) Add 100 µL of blocking buffer into "2^{ry} only."
10. Incubate cells in primary antibody for 1 h at room temperature on a rocker set to low speed to ensure uniform antibody binding.
Pause point: (Optional) Incubate cells in primary antibody overnight at 4 °C on a rocker set to low speed (see General note 8).
11. Following incubation with primary antibody, add 1,000 µL of PBS to each sample, bringing up the total volume to 1.5 mL.
12. Centrifuge samples at 500× *g* for 5 min at room temperature and aspirate the supernatant. Make sure not to disturb the pellet (see General note 9).
13. Resuspend cell pellets with 200 µL of secondary antibody solution (see Recipe 7).
14. Incubate samples at room temperature for 1 h on a rocker set to low speed to ensure uniform antibody binding.
Pause point: (Optional) Incubate samples in secondary antibody overnight at 4 °C on a rocker set to low speed.
15. Process samples with flow cytometry.

Data analysis

Singlet selection

The singlet populations are selected based on the FSC-A and SSC-A plot to remove doublets and cell debris (Figure 1) (see General note 10).

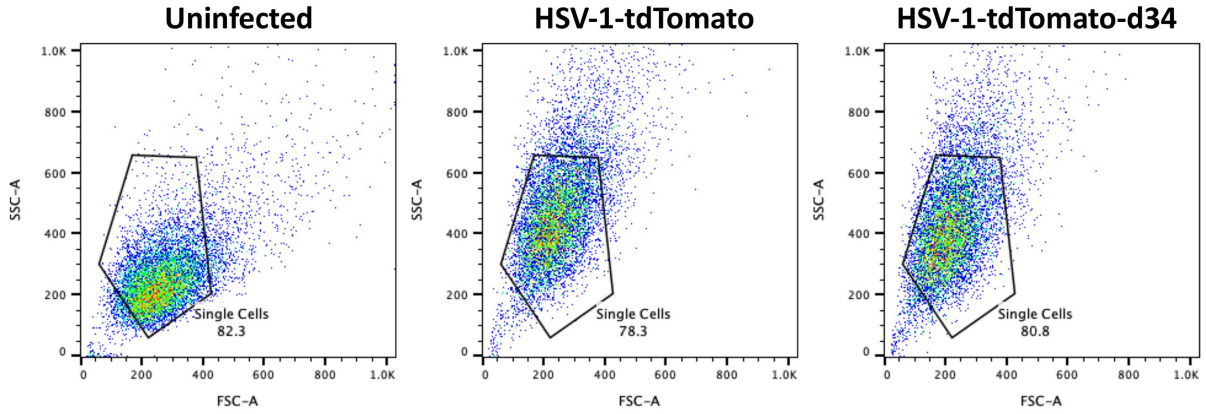


Figure 1. Singlet selection for uninfected and HSV-1-infected HeLa cells. HeLa cells were either left uninfected, infected with HSV-1-tdTomato (MOI 5), or infected with HSV-1-tdTomato-d34 (MOI 10) virus. Twenty-four hours post-infection, cells were fixed, partially (digitonin) or fully (Triton X-100) permeabilized, and incubated with the capsid-specific (85F) antibody and Alexa Fluor-488-conjugated secondary antibody. Detergent treatment does not influence the singlet selection process. Here, only the data for the fully permeabilized (Triton X-100) group are shown.

Gating strategy for measuring nuclear egress

Singlets are further divided into four populations based on Alexa Fluor-488 (X-axis) and tdTomato (Y-axis) expression (Figure 2). The "UC" sample, in which most cells are double-negative (tdTomato-/488-), serves as a gating control for uninfected cells with no nuclear egress. The HSV-1-tdTomato-d34 "Digitonin" group, in which most cells are single positive

(tdTomato+/488-), serves as a gating control for HSV-1 infected cells with no nuclear egress. The HSV-1-tdTomato and HSV-1-tdTomato-d34 "Triton X-100" groups, in which most cells are double positive (tdTomato+/488+), serve as normalization controls to confirm successful capsid assembly (Figure 2).

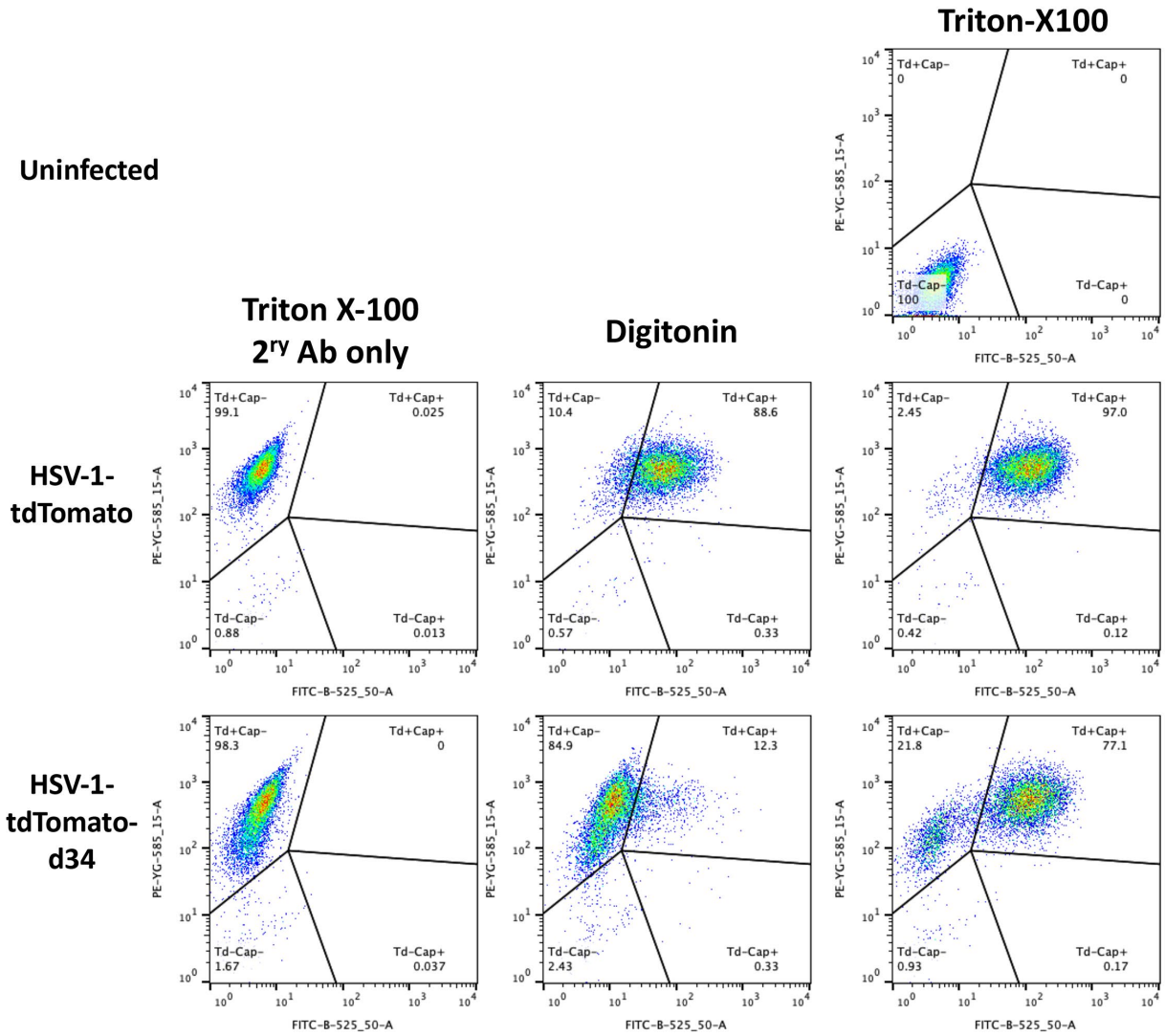


Figure 2. Gating strategy for measuring nuclear egress. HeLa cells were uninfected or infected with either HSV-1-tdTomato (MOI 5) or HSV-1-tdTomato-d34 (MOI 10). Twenty-four hours post-infection, cells were fixed in 4% PFA and permeabilized with either cell membrane permeabilization solution (digitonin) or full membrane permeabilization solution (Triton X-100). Cells were incubated with the capsid-specific (8F5) primary antibody, except for secondary antibody–only samples (2^{ry} Ab only), and with Alexa Fluor-488 conjugated secondary antibody. The Triton X-100 group was the same as that of Figure 1. From the selected singlets, cells were further divided by the green channel (Alexa-Fluor-488, FITC-B-525_50-A) and red channel (tdTomato, PE-YC-585_15A). Uninfected cells and HSV-1-tdTomato-d34-infected cells treated with digitonin served as gating controls.

Nuclear egress quantification

After gating, nuclear egress was calculated based on the value of the top right (double-positive) quadrant in the digitonin-treated group (percent cells with cytosolic capsids) over the Triton X-100-treated group (percent cells with capsids throughout the entire cell). For example, based on Figure 2, the percent of nuclear egress for the HSV-1-tdTomato sample is 91.3% (88.6%/97%), whereas the percent of nuclear egress for the HSV-1-tdTomato-d34 sample is 16.0% (12.3%/77.1%). Based on three biological replicates, the quantification of nuclear egress of HSV-1-tdTomato (GS3217) and HSV-1-tdTomato-d34 (GS3217-d34) infected cells is shown in Figure 3.

Statistical analysis

Student's t-test (two groups) or a one-way ANOVA (more than two groups) can be applied to the percent of nuclear egress as done in our prior report (see reference [17] in Validation of Protocol section).

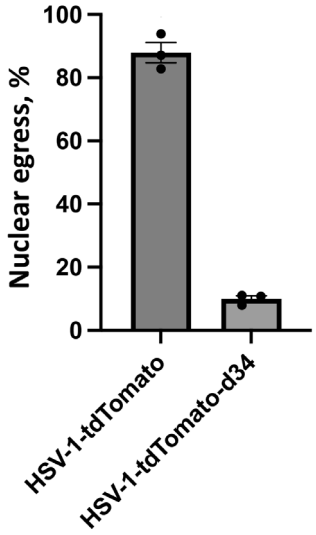


Figure 3. Quantification of nuclear egress in HeLa cells infected with HSV-1-tdTomato or HSV-1-tdTomato-d34. Population rates of nuclear egress observed from three biological replicates of HSV-1-tdTomato and HSV-1-tdTomato-d34-infected HeLa cells. Percent nuclear egress was calculated by dividing the percent of TdTomato+/Alexa-Fluor-488+ (double-positive) cells from the digitonin-treated group by the percent of double-positive cells from the Triton X-100-treated group. Error bars indicate standard error of the mean.

Validation of protocol

This protocol has been used and validated in the following research article:

- Dai et al. [17]. ER protein CLCC1 promotes nuclear envelope fusion in herpesviral and host processes. *Nat Commun* (Figure 1A and Extended Data Figure 1A–C).

The same images were used for Figures 2 and 4 of this paper, alongside additional information provided in this protocol.

Measuring nuclear egress infected with an HSV-1 mutant with a nuclear egress defect [17]

To confirm the phenotype of our assay, we generated an HSV-1 mutant virus defective in nuclear egress, HSV-1-tdTomato-d34. In this mutant virus, the *UL34* gene encoding one of the nuclear egress complex components is replaced by an ATGTCC sequence. Nuclear egress in HeLa cells infected with HSV-1-tdTomato-d34 is measured using the same procedure described for infection with HSV-1-tdTomato (Figures 2 and 3). In tandem with flow cytometry experiments, confocal microscopy was performed in non-infected, HSV-1-tdTomato-infected, and HSV-1-tdTomato-d34-infected HeLa cells (Figure 4). HSV-1-tdTomato-d34-infected cells exhibited ~10% nuclear egress compared to HSV-1-tdTomato-infected cells, where ~90% nuclear egress was observed (Figure 3). Accordingly, by confocal microscopy, nuclear egress was observed in HSV-1-tdTomato- but not HSV-1-tdTomato-d34-infected cells under partial permeabilization conditions (digitonin treatment). As a control, cells were treated with Triton X-100 to confirm the presence of capsids in both HSV-1-tdTomato- and HSV-1-tdTomato-d34-infected cells.

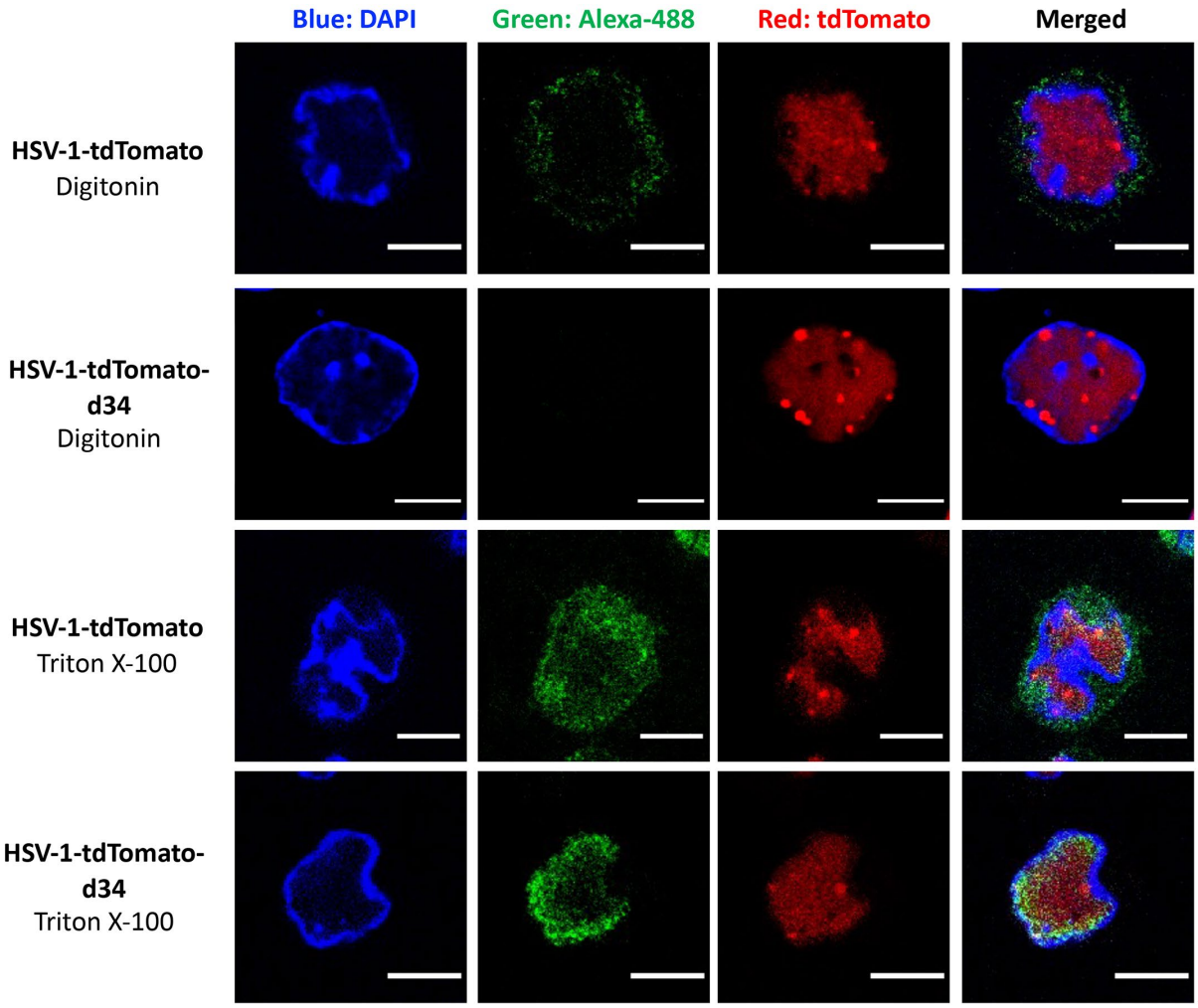


Figure 4. Confocal images validate the FLow cytometry–based Assay of nucleaR Egress (FLARE). Immunofluorescence images of cells permeabilized using the same conditions as in the FLARE assay. Cells were seeded into 24-well plates containing coverslips. The next day, cells were infected with either HSV-1-tdTomato or HSV-1-tdTomato-d34 mutant virus. Twenty-four hours post-infection, cells were either partially permeabilized with digitonin or fully permeabilized with Triton X-100 and then incubated with the capsid-specific (8F5) primary antibody and Alexa Fluor-488-conjugated secondary antibody. Conditions were the same as in Figure 2. Green = capsids, blue = nucleus, red = infection. Cells infected with HSV-1-tdTomato have only the cytoplasmic capsid signal (green) when permeabilized with digitonin (first row), but both nuclear and cytoplasmic signals when permeabilized with Triton X-100 (third row). Cells infected with HSV-1-tdTomato-d34 mutant, which has a nuclear egress defect, have no cytoplasmic capsid signal when permeabilized with digitonin (second row), but have nuclear signal when permeabilized with Triton X-100 (fourth row). Scale bar = 10 μm. Each image is representative of at least three biological replicates.

Testing the efficacy of an anti-VP5 antibody

As viral capsid proteins are first synthesized in ribosomes in the cytoplasm, we tested how much background signal was detected when using an antibody bound to free capsid proteins but not specific to the mature capsid. To address this, we used a 6F10 antibody, which binds its epitope on the major capsid protein VP5 (residues A862-H880) [18], in free or capsid-bound form. During infection with the HSV-1-tdTomato-d34 strain, under partial permeabilization conditions (digitonin), we observed a high percentage of double-positive cells in the 6F10-stained sample (Figure 5). This confirmed that the free VP5 protein can be detected in the cytoplasm regardless of the nuclear egress. Therefore, the use of a capsid-specific antibody, i.e., an antibody that only binds a fully formed capsid but not a free capsid protein, is essential for the detection of nuclear egress in our assay.

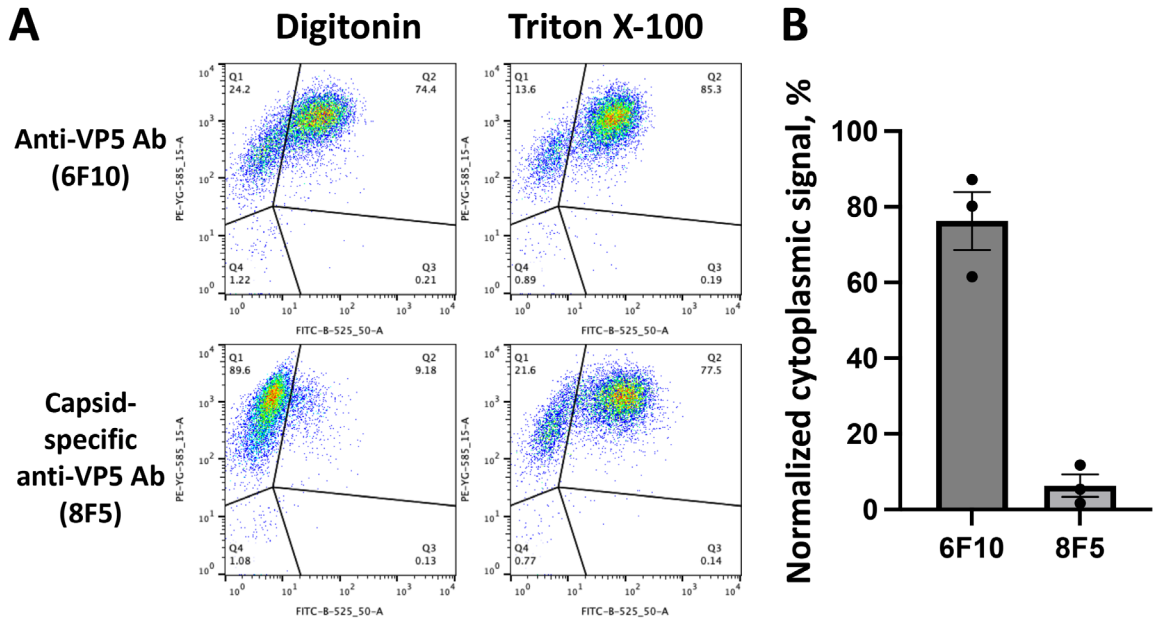


Figure 5. A capsid-specific antibody is required for the nuclear egress assay. (A) HeLa cells were infected with HSV-1-tdTomato-d34 (impaired nuclear egress) at an MOI of 10. Twenty-four hours post-infection, cells were fixed in 4% PFA and either partially (digitonin) or fully permeabilized (Triton X-100). Subsequently, cells were incubated with either an anti-VP5 (6F10) or the capsid-specific anti-VP5 (8F5) primary antibody and Alexa Fluor-488-conjugated secondary antibody. Signal was measured by flow cytometry, with cells plotted against Td-Tomato (Y-axis) and Alexa Fluor-488 (X-axis). Cells were gated as previously described. (B) Quantification of antibody specificity (TdTomato+/Alexa Fluor-488+, percent double-positive in digitonin over percent double-positive in Triton X-100) in HSV-1-tdTomato-d34-infected cells from three individual experiments. Error bars indicate standard error of the mean.

Testing nuclear egress in other cell lines

To broaden the applicability of our assay, we also performed it on Vero and Hep-2 cells, which are commonly used to study HSV-1 infection (Figure 6). When comparing nuclear egress results during HSV-1-tdTomato and HSV-1-tdTomato-d34 infection, there is a significant difference observed in both Vero and Hep-2 infected cells. We conclude that our assay works for a variety of cell lines and would serve as a great tool to better study HSV-1 nuclear egress. Nonetheless, optimization may be required for other cell lines.

General notes and troubleshooting

General notes

1. If using a single-reporter system (WT HSV-1 without a fluorescent transgene), process samples prior to flow cytometry the same way as described in the protocol for a two-reporter system. For the flow cytometry, select singlets similarly to what was described in the protocol for a two-reporter system. After singlet selection, the gating strategy should use only the 488 channel instead of both tdTomato and 488 channels (Figure 7). This protocol has also been validated using HSV-1 strain F US3-K220A, containing an inactivating mutation (K220A) in the *US3* kinase gene (which has a moderate defect in nuclear egress), a gift from Dr. Richard Roller (University of Iowa) [19] (Figure 7).
2. This plate setup best accommodates the 6-well plate layout and ensures enough cells for flow cytometry. Exercise caution when handling samples from different conditions.

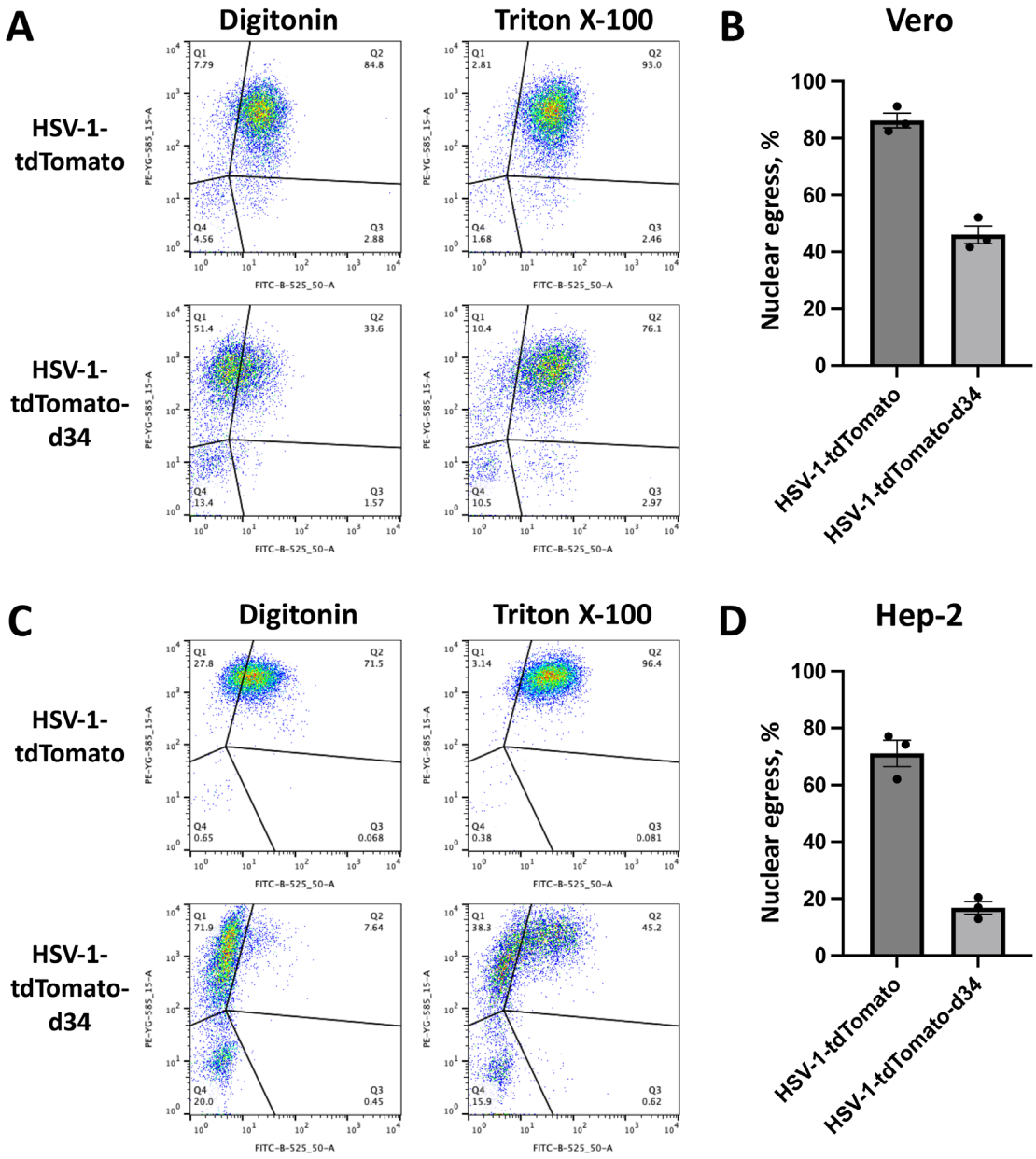


Figure 6. Measuring nuclear egress in Vero and Hep-2 cells. (A, C). Vero (A) or Hep-2 (C) cells were either infected with HSV1-tdTomato (WT virus strain with nuclear egress) or HSV-1-tdTomato-d34 (virus strain without nuclear egress). Twenty-four hours post-infection, cells were fixed in 4% PFA and partially permeabilized (digitonin) or fully permeabilized (Triton X-100). Cells were incubated with the capsid-specific (8F5) primary antibody and an Alexa Fluor-488-conjugated secondary antibody and analyzed by flow cytometry. Cells were plotted against Td-Tomato (T-axis) and Alexa-Fluor-488 (X-axis). (B, D) Quantification of percent nuclear egress in Vero (B) and Hep-2 (D) cells across three experiments. Error bars indicate standard error of the mean.

3. For the MOI determination, HSV-1-tdTomato virus was titrated on HeLa cells using a plaque assay [20], with the titer measured in plaque-forming units (PFUs)/mL. The effect of different MOIs on flow cytometry signal was tested using HeLa cells (Figure 8). The use of an MOI of 5 maximized the number of cells with nuclear egress while preserving optimal cell viability. HSV-1-tdTomato-d34 virus was titrated on HeLa cells by counting the tdTomato-positive cells 24 h post-infection, with the viral titer measured in infectious units (IUs)/mL. The use of an MOI of 10 maximized double-positive cells in the Triton X-100-treated group (data not shown). For infections in Vero or Hep-2 cells, the viral titer was measured on the respective cell lines using the plaque assay for the HSV-1-tdTomato strain or counting tdTomato-positive cells for the HSV-1-tdTomato-d34 strain.

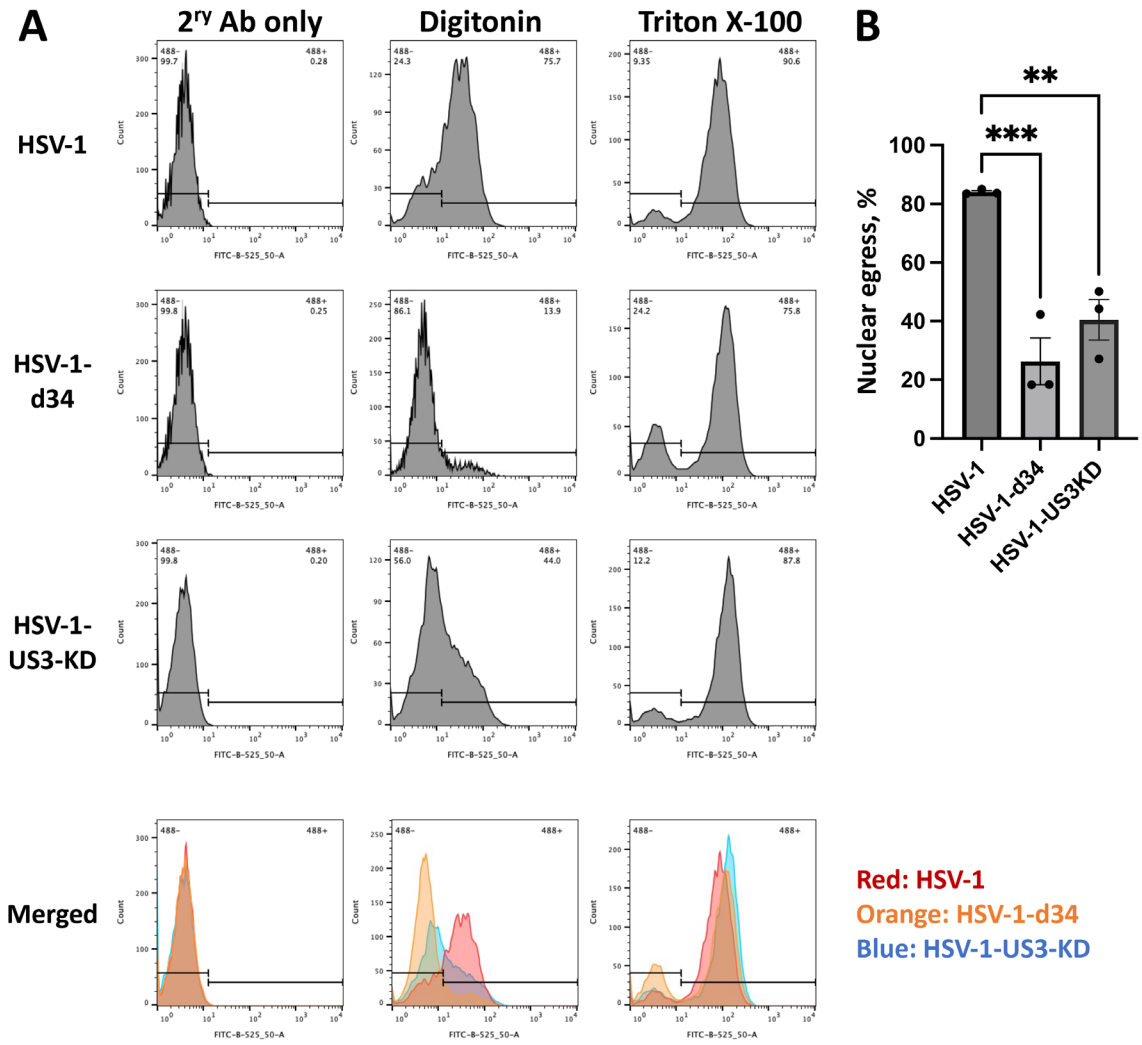


Figure 7. Nuclear egress assay in a single-reporter system (HSV-1 without tdTomato). (A) HeLa cells were infected with HSV-1 (WT) at an MOI of 5, HSV-1-d34 (no nuclear egress) at an MOI of 10, or HSV-1-US3-KD (impaired nuclear egress) at an MOI of 10 (titrated by plaque assay on HeLa cells). Twenty-four hours post-infection, cells were fixed in 4% PFA and permeabilized using either the partial (plasma membrane) permeabilization solution (digitonin) or full membrane permeabilization solution (Triton X-100). Cells were incubated with the capsid-specific primary antibody (8F5) and Alexa Fluor-488-conjugated secondary antibody and analyzed by flow cytometry. Cells were plotted using event count (Y-axis) and Alexa Fluor-488 signal (X-axis), with gates based on the HSV-1-d34. (B) Nuclear egress was measured by dividing the percentage of Alexa Fluor-488-positive cells in the digitonin-treated group by the percentage of Alexa Fluor-488-positive cells in the Triton X-100-treated group. Results were from three individual experiments. Unpaired one-way ANOVA was performed, with a multiple comparison to HSV-1 (WT). ***p-value = 0.001, **p-value = 0.0041. Error bars indicate standard error of the mean.

4. Based on this protocol, we observed nuclear egress as early as 9 h post-infection (Figure 9), which is in line with other reports [21]. To maximize the number of cells with nuclear egress, 16–24 h post-infection is a more appropriate timing for our assay. For this protocol, we chose 24 h post-infection as the endpoint to maximize the signal differences in nuclear egress between the HSV-1-tdTomato and nuclear-egress-defective HSV-1-tdTomato-d34 mutant while avoiding excessive cell death.

5. If you are planning to extract genomic DNA from cells, centrifuge cells at 500× *g* for 5 min following the fixation step and resuspend the pellet in PBS. Keeping cells in PFA for longer than 1 h could reduce the DNA extraction yields and inhibit PCR reactions. The current protocol is for analyzing nuclear egress by flow cytometry, wherein the PFA is maintained in the solution until later steps. This does not influence the detection of nuclear egress by flow cytometry.

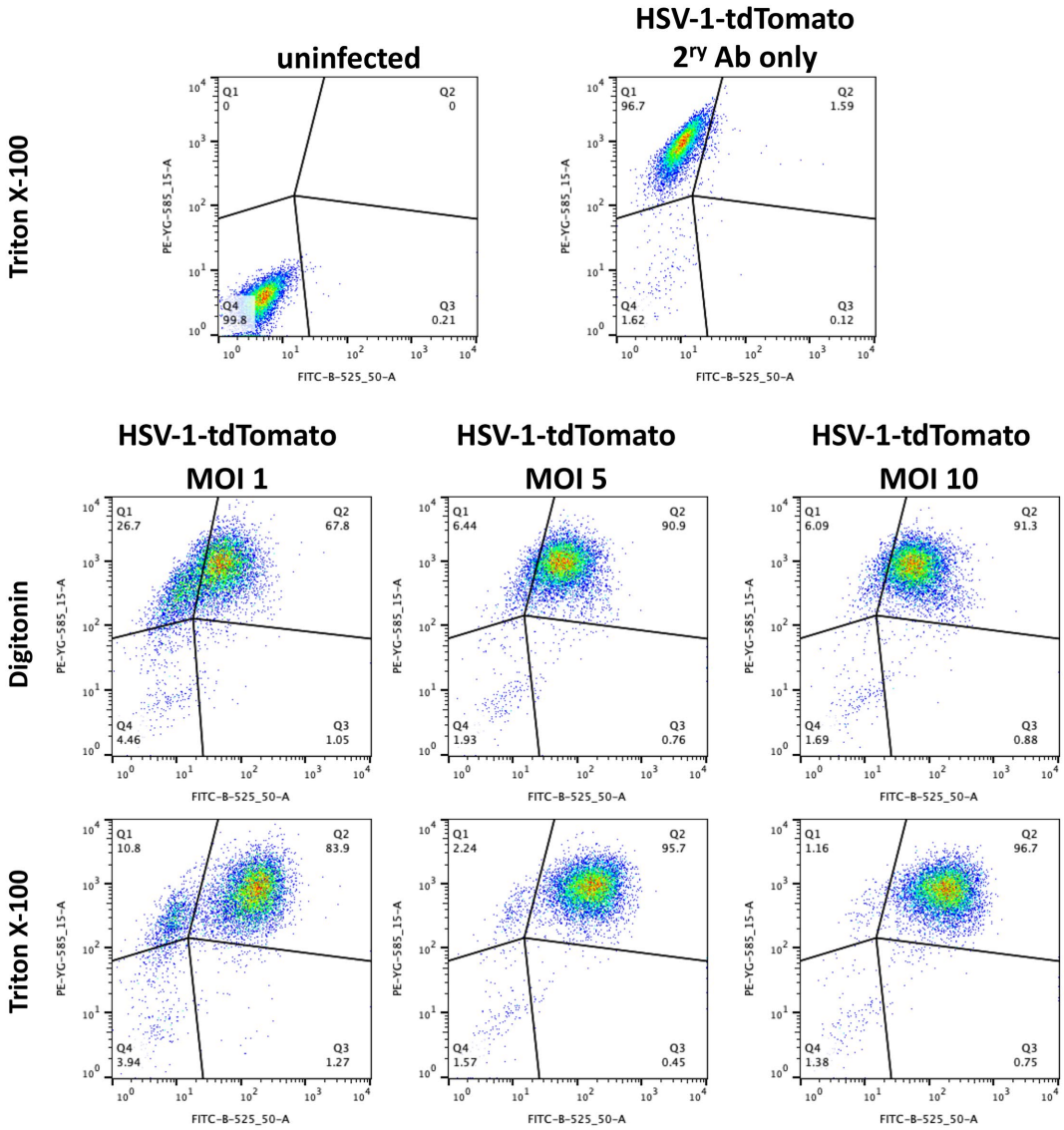


Figure 8. Optimization of the MOI for nuclear egress assay. HeLa cells were either infected with HSV-1-tdTomato at an MOI of 1, 5, or 10. Twenty-four hours post-infection, cells were fixed in 4% PFA and permeabilized using the partial (plasma membrane) permeabilization solution (digitonin) or full membrane permeabilization solution (Triton X-100). Cells were incubated with the capsid-specific primary antibody (8F5), except for secondary antibody–only samples (2ry Ab only), and with Alexa Fluor-488-conjugated secondary antibody and analyzed by flow cytometry. Cells were plotted against Td-Tomato (Y-axis) and Alexa Fluor-488 (X-axis). Representative results were from one of the two individual experiments.

6. For large-scale experiments, the cell density should be below 3×10^6 cells/mL because high cell density may lead to inefficient membrane permeabilization and antibody staining. In general, we do not recommend vortexing samples during permeabilization because the use of greater mechanical force may over-permeabilize the cells, especially for partial permeabilization. The membrane permeabilization solutions should be diluted in PBS. Preparing the digitonin solution in a BSA-containing buffer will decrease the permeabilization efficacy. By testing the effects of different digitonin concentrations during HeLa plasma membrane permeabilization experiments, we have determined the working concentration to be between 40 and 140 μg/mL. At the highest tested concentration of digitonin (320 μg/mL), the percentage of double-positive cells in HSV1-tdTomato-d34-infected cells was the highest. However, it never reached the signal observed in the Triton X-100-treated group. This implies that even at 320 μg/mL, digitonin cannot fully permeabilize the nuclear envelope (data not shown). This concentration may need to be optimized for different cell lines (Figure 10). The ratio of double-positive cells in cells infected with HSV-1-tdTomato and HSV-1-tdTomato-d34 can be used to determine the optimal digitonin concentration.

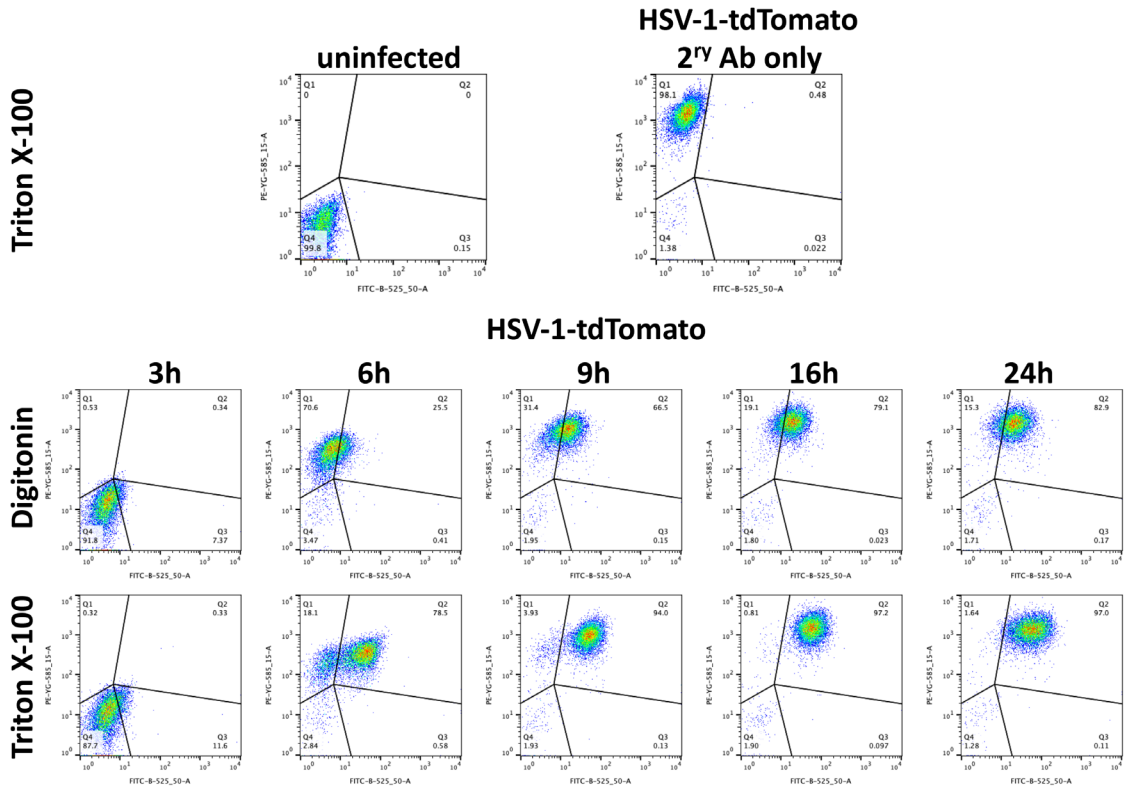


Figure 9. Optimization of the time-course of HSV-1 infection for nuclear egress assay. HeLa cells were either uninfected or infected with HSV1-tdTomato at an MOI of 5. At 3, 6, 9, 16, and 24 h post-infection, cells were fixed in 4% PFA and permeabilized using the partial (plasma membrane) permeabilization solution (digitonin) or full membrane permeabilization solution (Triton X-100). Cells were then incubated with the capsid-specific (8F5) primary antibody, except for secondary antibody–only samples (2^{ry} Ab only), and an Alexa Fluor-488-conjugated secondary antibody, and analyzed by flow cytometry. Cells were plotted against Td-Tomato (Y-axis) and Alexa Fluor-488 (X-axis). Representative results are from one of the three individual experiments.

7. The working (1×) concentration of BSA during the blocking step is 0.5%. During the primary Ab incubation (step D9) and secondary Ab incubation (step D13), the concentration of BSA will be 0.6% and 1% in solution, respectively. However, this difference in BSA concentration during sample preparation does not influence the detection of nuclear egress. The same with the residual detergent: digitonin or Triton X-100 will not influence the downstream steps.
8. We recommend an overnight incubation at 4 °C with a primary antibody rather than a 1-h incubation at room temperature. In our experience, an overnight incubation makes gating easier when comparing the digitonin-treated group with the secondary antibody–only group.
9. Aspirating the supernatant in its entirety can be difficult. We have found that leaving <10 µL of supernatant above the pellet does not influence the result signal when compared to washing samples with blocking solution (data not shown).
10. FSC-A vs. FSC-H and SSC-A vs. SSC-H gating strategy has been initially used after this step but was ultimately eliminated because it excluded only a small number of cells. This singlet gating strategy has been confirmed with Attune Cytpix Flow cytometry brightfield function.

Troubleshooting

Problem 1: No cells collected during flow cytometry.
Possible cause: Cells aspirated during the washing step.
Solution 1: When aspirating liquid, leave some liquid above the pellet to avoid disturbing it.
Solution 2: Prep more wells during the initial experiment setup.

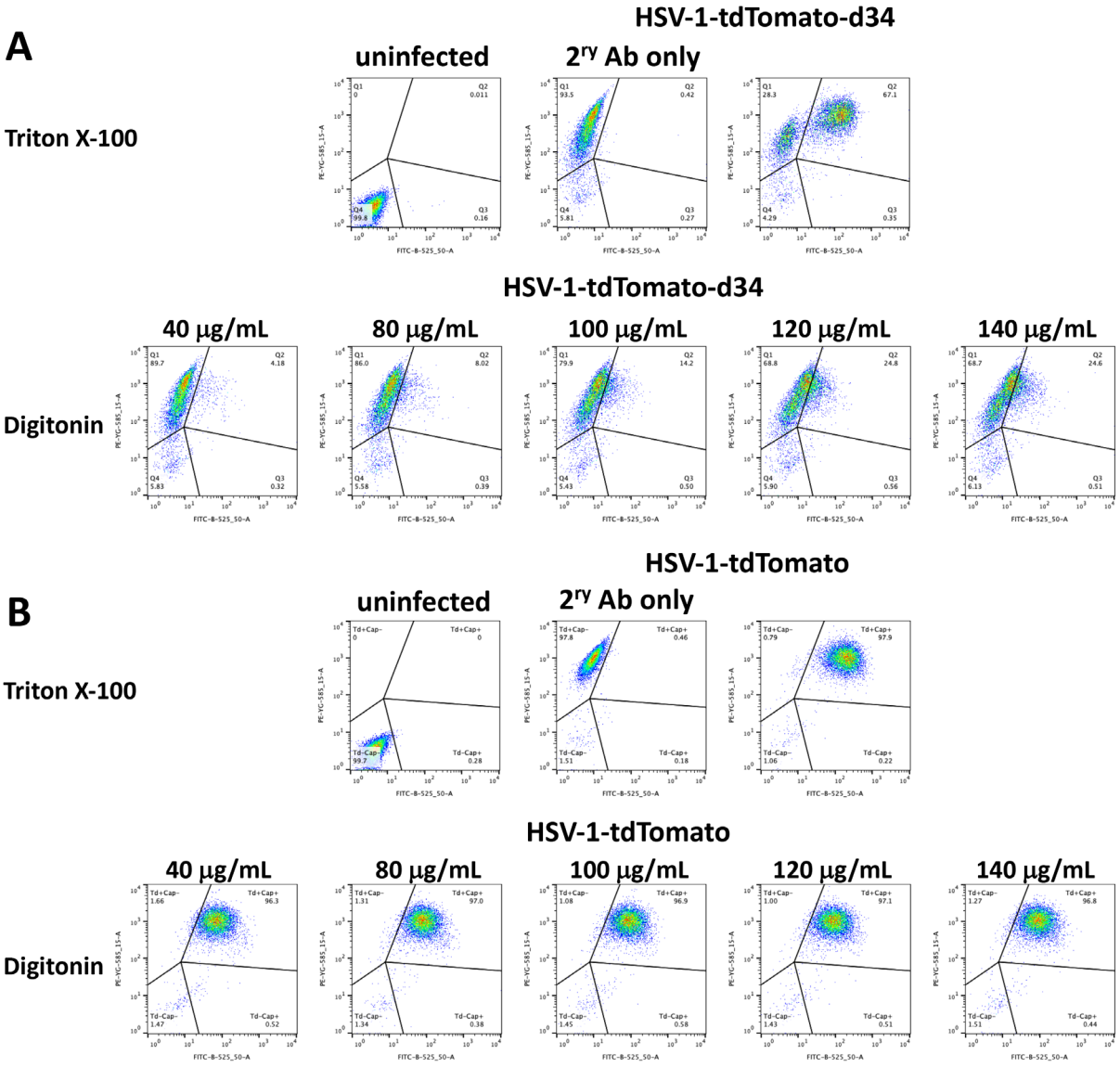


Figure 10. Optimization of the digitonin concentration for nuclear egress assay. HeLa cells were either left uninfected, infected with HSV1-tdTomato-d34 at an MOI of 10 (A), or infected with HSV-1-tdTomato at an MOI of 5 (B). Twenty-four hours post-infection, cells were fixed with 4% PFA and permeabilized with either increasing concentrations of digitonin (40, 80, 100, 120, and 140 µg/mL) or with a full membrane permeabilization solution (Triton X-100). Cells were then incubated with a capsid-specific (8F5) primary antibody, except for secondary antibody only samples (2^{ry} Ab only), and an Alexa Fluor-488-conjugated secondary antibody, and analyzed by flow cytometry. Cells were plotted against Td-Tomato (Y-axis) and Alexa Fluor-488 (X-axis), with gates based on the secondary–only control. Representative results were from one of two individual experiments. A and B are from different biological replicates.

Problem 2: The Alexa Fluor-488 signal is low, or it is hard to separate Alexa Fluor-488-positive cells compared with secondary-only/nuclear egress–defective control.
Possible cause 1: Cell density is high during membrane permeabilization.
Solution 1: Decrease cell density before permeabilization by adding PBS.
Possible cause 2: Cells were permeabilized using blocking buffer instead of PBS.
Solution 2: Check buffer conditions prior to membrane permeabilization.

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The Graphical Overview was created in BioRender. Dai, B. (2025) https://BioRender.com/2l0nwl7.
The acronym FLARE was developed with assistance from ChatGPT (OpenAI).

Competing interests

The authors declare no conflicts of interest or competing interests.

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