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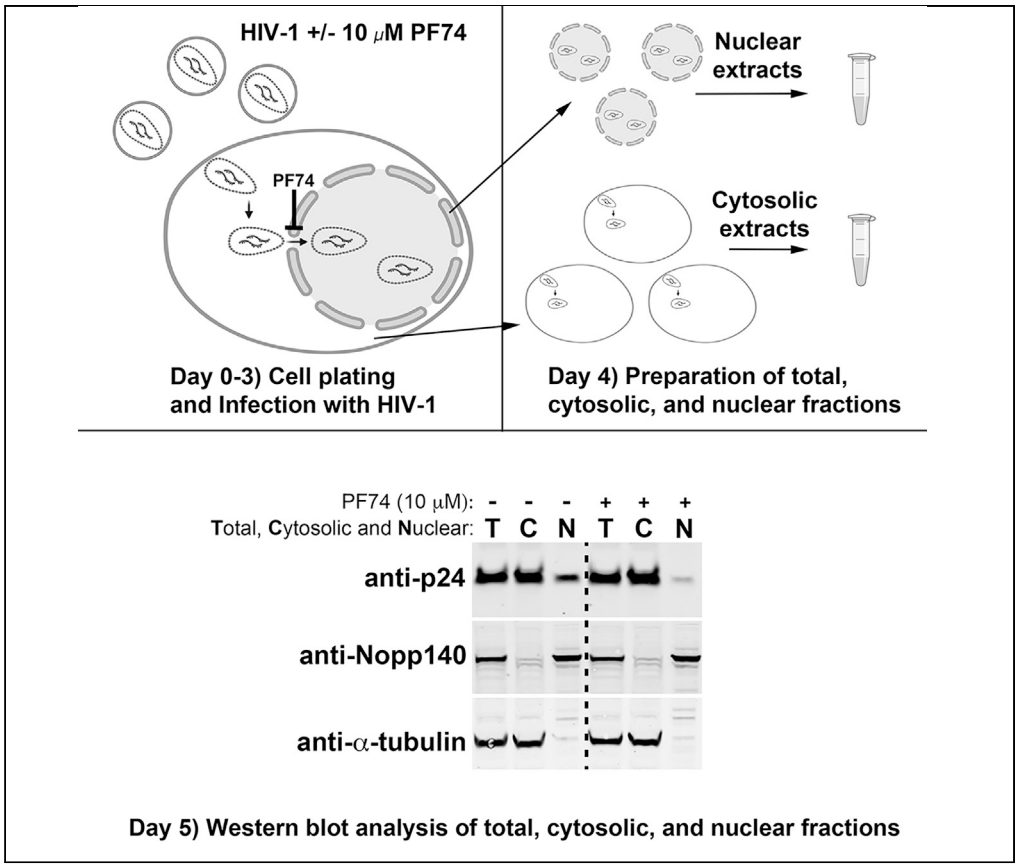
Biochemical detection of capsid in the nucleus during HIV-1 infection



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HIGHLIGHTS

Method to track the HIV-1 capsid in the nucleus during infection in human cell lines

Analysis of HIV-1 infected nuclear and cytosolic fractions

The small molecule PF74 prevents entry of HIV-1 capsid into the nucleus

This method can be used in cycling and non-cycling human cells

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Biochemical detection of capsid in the nucleus during HIV-1 infection

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SUMMARY

To understand the role of the HIV-1 capsid in viral replication, we developed a protocol to biochemically track capsid in the nucleus during infection. To this end, we separated HIV-1-infected cells into nuclear and cytosolic fractions. Fractions were analyzed by western blotting for HIV-1 capsid content as well as for nuclear and cytosolic markers to assess the bona fide origin of the fractions. This protocol can be applied in both cycling and non-cycling human cells.
For complete details on the use and execution of this protocol, please refer to Selyutina et al. (2020a).

BEFORE YOU BEGIN

Cell maintenance

Timing: 4-5 days

In our experiments we use HIV-1-based lentiviral vectors produced in HEK293T/17 cells (ATCC, CRL-11268). For infection we use human (MOLT-3, A549, HeLa, HT1080) or dog (Cf2Th) cell lines. We keep cells in liquid nitrogen tank and thaw them 4–5 days before the experiment. We grow cells in 10 cm cell culture plates at 37°C with 5% CO_2 for no longer than 4–6 weeks, then discard them and thaw fresh aliquots when required. For our experiments we use only healthy growing cells.

1. Take a vial with cells from the nitrogen tank. Thaw it fast by putting the vial on a water bath. Transfer cells into a 15 mL Falcon with 10 mL complete cell culture medium and centrifuge for 5 min, 200 × *g* at 25°C. Remove supernatant. Resuspend cell pellet in 10 mL of complete cell culture medium and culture them on 10 cm plate.
2. Maintain HEK293T/17 (ATCC, CRL-11268), A549 (ATCC, CCL-185), HeLa (ATCC, CCL-2), HT1080 (ATCC, CCL-1430), and Cf2Th (ATCC, CRL-1552) cells in Dulbecco's Modified Eagle Medium (DMEM) with 2 mM L-glutamine, 4.5 g/L glucose and sodium pyruvate supplemented with 10% FBS (heat-inactivated) and 100 μg/mL streptomycin and 100 units/mL penicillin.
3. Maintain MOLT-3 cells (ATCC, CRL-1552) in Roswell Park Memorial Institute (RPMI) medium with 2 mM L-glutamine supplemented with 10% FBS (heat-inactivated) and 100 μg/mL streptomycin and 100 units/mL penicillin.
4. Passage cells 1–2 times before using them for viral production or viral infection experiments. We routinely passage cells every 2–3 days (at confluency <90%).

Production of HIV-1-based lentiviral particles

Timing: 1 week

1. Day 0: Prepare cells for transfection: seed 6 × 10^6 HEK293T/17 cells in 10 mL of complete cell growth medium in 10 cm cell culture plate. Incubate for 16 h at 37°C with 5% CO_2.
2. Day 1: In the evening transfect cells with plasmids for viral production. Per plate, prepare two 15 mL Falcon tubes with 1.5 mL of DMEM. To the first Falcon tube add plasmids: 1 μg Tat, 1 μg Rev, 1.5 μg VSV-G, 1 μg HIV-1 Gag-pol, 7.5 μg LTR-GFP-LTR. To the second Falcon tube add 50 μg of polyethylenimine (PEI). Combine the content of two Falcon tubes and mix it by gentle vortexing. Collect any droplets by centrifugation at 200 × *g* for 1 min at 25°C. Incubate the mixture 20 min at 25°C. Add 2 mL of DMEM and gently mix with the pipette. Remove cell growth medium from HEK293T/17 cell plate and very carefully add the transfection mixture (lean the pipette tip against the wall of the plate and very slowly eject the transfection mixture). HEK293T/17 cells detach from the plate very easily that is why all the steps involving media change must be executed with additional care. Incubate at 37°C with 5% CO_2 for 16 h.
3. Day 2: In the morning replace the transfection media with 10 mL of complete growth medium.
4. Day 3: Collect cell medium containing lentiviral particles into 15 mL Falcon tube. Centrifuge 5 min, 3,200 × *g*, 4°C. Transfer supernatant into a fresh tube, discard the pellet (cell debris). Concentrate your lentiviral particles using ultracentrifugation method as described (Kutner et al., 2009)

Titration of HIV-1-based lentiviral particle stocks

Timing: 3 days

The titration of viral stock is performed essentially as described (Drayman and Oppenheim, 2011).

1. Day 0: Seed 3 × 10^4 human A549 cells in 500 μL of the complete growth media per well on a 24-well plate. Prepare the amount of wells you need for infection plus extra wells that will be used to count the amount of cells at the moment of infection (N). Incubate cells at 37°C with 5% CO_2 for 16 h.
2. Day 1: In the morning determine (N), which is the amount of cells per well at the moment of infection. For this detach the cells from your extra wells by trypsin treatment and count the cells. Cell Number (N) per well = total amount of cells at the time of infection.
3. In 1.5 mL tubes prepare serial dilution of your virus. Prepare 10 tubes and put 1.2 mL of the complete growth media in the first one and 0.6 mL of the complete growth media into the rest of the tubes. Make 1,000-fold dilution of your viral stock in the first tube: add 1.2 μL of the viral stock to 1.2 mL of the complete growth media. Mix by gentle vortexing. Next, prepare 2-fold serial dilutions: transfer 0.6 mL of 1,000-fold dilution into the next tube and mix with 0.6 mL of the complete growth media. As a result you will have 10 serial dilutions: 1,000-fold, 2,000-fold, 4,000-fold and so on.
4. Completely remove growth media from the well with A549 cells seeded at Day 0. Add 0.25 mL of your viral dilution (or complete growth media without virus for mock-infected control) per well. Note V – the volume of the original viral stock (in mL) used for infection. For example, for 1,000-fold dilution V = 0.25 × 10^{-3} mL, for 2,000-fold dilution V = 0.125 × 10^{-3} mL and so on. Incubate at 37°C with 5% CO_2 for 8 h. After that add 0.25 mL of fresh complete growth media and incubate at 37°C with 5% CO_2 for 24 h.
5. Day 2: Remove media from 24-well plate. Wash wells with PBS. Add 75 μL of 0.25% Trypsin per well. Incubate at 37°C with 5% CO_2 for 5 min until cells start to detach. Add 125 μL of complete growth medium per well and transfer the cells into the FACS tube.Perform FACS analysis. Determine the percentage of GFP-expressing cells for each viral dilution.
6. To calculate the viral titer use at least 3 dilutions with 30% or less GFP-positive cells. Points with 30% or lower infection are expected to be within the linear range of infectivity. To determine virus titer as infecting units per mL use the following formula:

$$\text{Titer (IU/mL)} = (N \times P)/V$$

N = Cell Number in each well used for infection on Day 1 (determined at step 2);

P = fraction of GFP-positive cells (to get this number you should divide the percentage of infected cells (determined at step 5) to 100);

V = The Volume of the original viral stock (in mL) used for infection in each well (see step 4);

IU = Infecting Units

For example, at moment of infection you had 4×10^4 cells (N). 16,000-fold dilution (V = 0.015625×10^{-3} mL) resulted in 14% GFP-positive cells (P = 14/100=0.14). So the titer of the virus will be:

$$\text{Titer (IU/mL)} = (N \times P)/V=(4 \times 10^4) \times (0.14)/ 0.015625 \times 10^{-3}=3.584 \times 10^8 \text{ IU/mL}$$

Determine viral titer for at least 3 data points. Data points in the linear range should give similar titers. The average of these titers is the titer of your viral stock.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-HIV-1 p24 (183-H12-5C)	NIH AIDS Reagent Program	Cat# 3537
Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Invitrogen (Thermo Fisher Scientific)	Cat# AM4300
Rabbit polyclonal anti-alpha tubulin	Invitrogen	Cat# PA5-29444
Rabbit polyclonal anti-Nopp140 (human) (RS8 serum)	(Kittur et al., 2007)	Gift of Thomas Meier, Albert Einstein College of Medicine
Goat anti-mouse IRDye 680LT	LI-COR	Cat# 925-68020
Goat anti-rabbit IRDye 680LT	LI-COR	Cat# 926-68021
Goat anti-mouse IRDye 800CW	LI-COR	Cat# 926-32210
Goat anti-rabbit IRDye 800CW	LI-COR	Cat# 925-32211
Bacterial and virus strains		
E. Coli DH5α competent cells	Zymo Research	Cat# T3007
HIV-1_{NL4-3}	(Selyutina et al., 2020b)	N/A
Chemicals, peptides, and recombinant proteins		
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#: D2438; CAS: 67-68-5
Difco LB Broth, Miller (Luria-Bertani)	Fisher Scientific	Cat#: BD244610
Tris (tris(hydroxymethyl)aminomethane)	Crystalgen	Cat#: 300-844-5000; CAS: 77-86-1
cOmplete EDTA-free protease inhibitor cocktail	Millipore Sigma (Roche)	Cat#: 11873580001
Sodium chloride (NaCl)	Crystalgen	Cat# 300-747-5000; CAS: 767-14-5
β-Mercaptoethanol (BME)	Acros organics	Cat# 125470010; CAS: 60-24-2
2-(N-morpholino) ethanesulfonic acid (MES)	Calbiochem	Cat# 475893; CAS: 4432-31-9
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich	Cat# M2670; CAS: 7791-18-6
Potassium chloride (KCl)	Fisher Scientific	Cat# BP366-1; CAS: 7447-40-7
Dithiothreitol (DTT)	VWR	Cat# 97061-340; CAS: 3483-12-3
D-(+)-Sucrose	VWR	Cat# 97061-432; CAS: 57-50-1
Dulbecco's phosphate-buffered salt (PBS) solution 1×	Corning	21031CV
EDTA, pH 8.0, 0.5 M	Corning	46034CI
PF74	Sigma-Aldrich	Cat# SML0835
Experimental models: cell lines		
Human: MOLT-3	ATCC	CRL-1552
Human: 293T/17	ATCC	CRL-11268

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human: A549	ATCC	CCL-185
Human: HeLa	ATCC	CCL-2
Human: HT1080	ATCC	CCL-121
Canine: Cf2Th	ATCC	CRL-1430
Recombinant DNA		
Plasmid: Tat	(Stremlau et al., 2006)	N/A
Plasmid: Rev	(Stremlau et al., 2006)	N/A
HIV-1 NL4-3 Δenv		
pVPack-VSV-G	Agilent	Cat #: 217567
Other		
Fetal bovine serum (FBS, heat-inactivated)	Gibco	16140-071
RPMI-1640 (high glucose)	Corning	MT10-017-CV
Penicillin–streptomycin (5 mg/mL)	Corning	MT10-040-CV
Sodium pyruvate (100×)	Corning	MT25-000-CI
HEPES (100×)	Fisher/HyClone	SH3023701
DMEM (high glucose)	Corning	MT10-017-CV
NuPAGE 10% Bis-Tris gel	Invitrogen	NP0315BOX
Precision Plus protein dual color standards	Bio-Rad	1610374
Instant nonfat dry milk	Quality Biological, Inc	A614-1000
Nitrocellulose membranes, 0.45 μm	Bio-Rad	1620115

MATERIALS AND EQUIPMENT

Stock solutions

PF74 stock

You can prepare PF74 stock in advance, make 100 μL aliquots, and keep frozen at −20°C for at least 6 month.

Reagent	Final concentration	Amount
PF74	10 mM	5 mg
DMSO (100%)	n/a	1.17 mL
Total	**n/a**	**1.17 mL**

Cell fractionation

The day of the fractionation prepare the following buffers. Keep buffers on ice during the fractionation experiment as they must be ice-cold when used. Add components in the order they listed.

Lysis buffer		
Reagent	Final concentration	Amount
Tris, pH 6.8 (1 M)	10 mM	0.1 mL
DTT (1 M)	1 mM	0.01 mL
$MgCl_2$ (1 M)	1 mM	0.01 mL
Sucrose (50%)	10%	2 mL
NaCl (5 M)	100 mM	0.2 mL
NP-40 (10%)	0.5%	0.5 mL
Protease inhibitors cocktail	1×	1 tablet
ddH_2O	n/a	7.18 mL
Total	**n/a**	**10 mL**

Wash buffer		
Reagent	Final concentration	Amount
Tris, pH 6.8 (1 M)	10 mM	0.4 mL
DTT (1 M)	1 mM	0.04 mL
$MgCl_2$ (1 M)	1 mM	0.04 mL
Sucrose (50%)	10%	8 mL
NaCl (5 M)	100 mM	0.8 mL
Protease inhibitors cocktail	1×	4 tablets
ddH_2O	n/a	30.72 mL
Total	**n/a**	**40 mL**

Extraction buffer (this buffer is required for nuclear lysis)		
Reagent	Final concentration	Amount
Tris, pH 6.8 (1 M)	10 mM	0.1 mL
DTT (1 M)	1 mM	0.01 mL
$MgCl_2$ (1 M)	1 mM	0.01 mL
Sucrose (50%)	10%	2 mL
NaCl (5 M)	400 mM	0.8 mL
Protease inhibitors cocktail	1×	1 tablet
ddH_2O	n/a	7.08 mL
Total	**n/a**	**10 mL**

Whole cell extract (WCE) buffer		
Reagent	Final concentration	Amount
Tris, pH 8.0 (1 M)	50 mM	0.5 mL
NaCl (5 M)	280 mM	0.56 mL
Glycerol (100%)	10%	1 mL
NP-40 (100%)	0.5%	0.05 mL
$MgCl_2$ (1 M)	5 mM	0.05 mL
Benzonase nuclease (250,000 U/mL)	250 U/mL	0.01 mL
Ethidium bromide (10 mg/mL)	10 μg/mL	0.001 mL
Protease inhibitors cocktail	1×	1 tablet
ddH_2O	n/a	7.83 mL
Total	**n/a**	**10 mL**

Protein PAGE gel electrophoresis

Samples after fractionation mixed with Laemmli buffer can be stored at −20°C for at least 1 month. Before running protein PAGE gel electrophoresis take samples out of the freezer, unfreeze them at 25°C, and mix by gentle vortexing. Heat samples 100°C for 5 min (either water bath or dry bath can be used), then cool samples down to 25°C.

Laemmli sample buffer

You can prepare 50× Laemmli sample buffer in advance, make 1 mL aliquots, and keep frozen at −20°C for at least 6 month.

Reagent	Final concentration	Amount
Tris, pH 6.8 (1 M)	250 mM	12.5 mL
SDS	10%	5 g
Glycerol (100 %)	50%	25 mL
β-Mercaptoethanol (100%)	20%	12.5 mL
Bromophenol blue	0.2%	0.1 g
Total	**n/a**	**50 mL**

20× PAGE running buffer

You can prepare 20× PAGE running buffer in advance and keep at 25°C for at least 2 month. The pH of the buffer must be 7.7.

Reagent	Final concentration	Amount
MOPS	1 M	209.2 g
Tris	1 M	121.14 g
SDS	2%	20 g
ddH_2O	n/a	To make 1,000 mL total
Total	**n/a**	**1,000 mL**

1× PAGE running buffer

Before running the gel electrophoresis dilute the 20× PAGE running buffer to make 1× PAGE Running buffer.

Reagent	Final concentration	Amount
20× PAGE running buffer	1×	50 mL
ddH_2O	n/a	950 mL
Total	**n/a**	**1,000 mL**

Western blot

10× Tris-glycine western blot transfer buffer

You can prepare 10× Tris-Glycine western blot transfer buffer in advance and keep at 25°C for at least 2 month.

Reagent	Final concentration	Amount
Tris	250 mM	30.3 g
Glycine	2.2 M	164.4 g
ddH_2O	n/a	To make 1,000 mL total
Total	**n/a**	**1,000 mL**

1× western blot transfer buffer

Before doing the transfer prepare 1× western blot transfer buffer. Add components in the order listed.

Reagent	Final concentration	Amount
ddH_2O	n/a	696 mL
10× Tris-glycine Western blot transfer buffer	1×	100 mL
Methanol (100%)	20%	200 mL
SDS (10%)	0.04%	4 mL
Total	**n/a**	**1,000 mL**

Western blot wash buffer

Western blot wash buffer you can prepare in advance and keep it at 25°C for at least 1 month.

Reagent	Final concentration	Amount
PBS (10×)	1×	100 mL
Tween 20 (100%)	0.1%	1 mL
ddH_2O	n/a	900 mL
Total	**n/a**	**1,000 mL**

Western blot blocking buffer

Western blot blocking buffer you can prepare in advance and keep it at 4°C for no longer than 4 days.

Reagent	Final concentration	Amount
Dry nonfat milk	5%	5 g
Western blot wash buffer	n/a	To make 100 mL total
Total	**n/a**	**100 mL**

The Odyssey infrared imager

The Odyssey infrared imager is required to scan the signal from your western blot antibodies at the last step of your experiment.

> ***Alternatives:*** Although we prefer the Odyssey infrared imager and corresponding IRDye 680LT or IRDye 800CW secondary antibodies, this is not the only way to detect the signal from the western blot. Secondary antibodies conjugated with horse radish peroxidase (HRP) or alkaline phosphatase (AP) can also be used and the signal can be developed using film by chemiluminescence.

STEP-BY-STEP METHOD DETAILS

To understand the role of HIV-1 capsid in the nucleus, we designed a simple assay to biochemically monitor the amount of HIV-1 capsid in the cytosol and nucleus during infection. To this end, we challenge human cells with HIV-1 (Figure 1), and collected cells at different time points post-infection. After collections, cells are separated into total, cytosolic and nuclear fractions (Figure 1). The different fractions are analyzed by western blotting using anti-HIV-1-capsid, anti-Nopp140, and anti-GAPDH/α-tubulin antibodies (Figure 1).

This assay has been tested in several different mammalian cell lines: human HT1080, human A549, human U937, human MOLT3, and dog Cf2Th cells.

It takes approximately 4 h to collect the cells and separate them into fractions. The timing depends a lot on the amount of points you process. We do not recommend processing more than 8 points at the same time. Depending on the goal of your experiment and time of incubation you want to do, we would suggest the following time frame for the experiment:

Variant A – Seed cells on plates in the morning (step 1), infect in the evening the same day and incubate with the virus for 16 h (step 2), and next morning collect the cells and perform the fractionation (step 3 and all steps after that).

Variant B – Seed cells on plates in the evening (step 1), infect in the morning next day (step 2), and collect the cells and perform the fractionation during the day at desired time points.

Seeding cells for infection

Timing: 30 min and at least 6 h of incubation

1. Seed 5×10^6 mammalian cells on two 10 cm^2 plates per every point you have in your experiment. For this detach the cells by trypsin treatment, determine the amount of cells you have, resuspend the cells to concentration 2.5×10^6 cells in 10 mL of complete growth media and put 10 mL per each 10 cm plate (you need 2 plates and 5×10^6 cells for point). Incubate cells at 37°C with 5% CO_2 for at least 6 h to allow cells to attach.

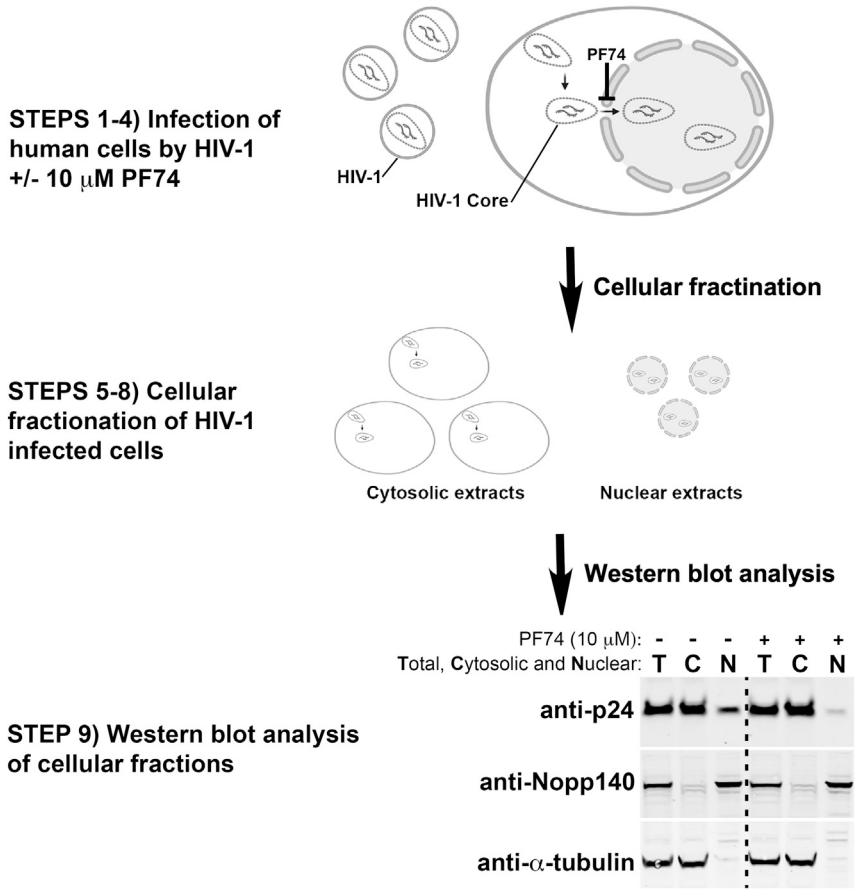

Figure 1. Biochemical detection of capsid in the nucleus during HIV-1 infection

Human cells are infected with HIV-1 viruses using an MOI = 2 for different times ranging from 1–48 h in the presence or absence of 10 μM PF74 (steps 1–4). PF74 blocks the entry of the HIV-1 core into the nucleus. Subsequently cells are separated into cytosolic and nuclear fractions (steps 5–8). Total **(T)**, cytosolic **(C)** and nuclear extracts **(N)** are analyzed by western blotting using anti-HIV-1 capsid antibodies (anti-p24). To identify the bona fide origin of cellular extracts, cytosolic and nuclear extracts are analyzed by western blotting using antibodies against cytosolic(anti-α-tubulin) and nuclear proteins (anti-Nopp140).

Infection of the cells

Timing: 30 min

2. Cells are challenged using HIV-1 viruses at a MOI = 2 for the indicated times (1–48 h). Take 15 mL tube per every point you have. Put 1 mL of complete growth media into the tube. Add 10 μL of DMSO or 10 μL of 10 mM PF74 (to achieve the final concentration 10 μM PF74). Gently vortex the tube. Add complete growth media and the virus (final volume should be 10 mL). Mix by pipetting. Remove the old media from the plates with cells. Gently put 5 mL of your viral mixture per plate. Incubate at 37°C with 5% CO_2 for desired time. The assay works using replication competent HIV-$1_{NL\text{-}4\text{-}3}$, or HIV-$1_{NL\text{-}4\text{-}3}$ Δenv pseudotyped with the envelope of vesicular stomatitis virus (VSV-G). Virus challenges have been performed from 1 h to 48 h. Capsid can be detected in the nuclear fraction 1 h post-infection (Selyutina et al., 2020a).

Cell harvest

Timing: 30 min

3. Harvest the cells (10–30 min). Aspirate medium from both plates. Add 1 mL of 0.25% Trypsin per plate and incubate 2–3 min at 25°C until cells are completely detached from the plate. You can shake the plates to ensure the complete detachment of the cells. Transfer all the cells to a 50 mL Falcon tube. To achieve this, add to the first plate 7 mL of DMEM medium, collect the cells, transfer the whole mixture into the second plate, collect the cells, and transfer the whole mixture into the 50 mL Falcon on ice. To ensure the complete transfer of cells repeat this 2 more times, so the final volume in the tube would be 23 mL (2 mL of trypsin and 21 mL of DMEM).

⚠ CRITICAL: It is crucial to collect ALL the cells from the plate, as it has a great impact on point-to-point variation.

⚠ CRITICAL: It is crucial not to damage the cells during trypsin treatment. For this incubate cells with trypsin minimum time required for cells to detach from the plate.

Wash the cells

Timing: 30 min to 1 h

4. Wash the cells (30 min to 1 h). Centrifuge cells at 200 × *g* for 7 min at 4°C. Discard the supernatant and keep the cell pellet. Whenever the cells are not in the centrifuge you should keep them on ice. Harvested cells are washed twice with 1× cold PBS. For this resuspend cell pellet in 10 mL of ice-cold 1× PBS and add 40 mL of ice-cold PBS. Centrifuge at 200 × *g* for 7 min at 4°C. Repeat twice. Supernatant is discarded and cell pellet is resuspended in 1 mL of PBS.

⚠ CRITICAL: It is important to completely remove any traces of virus and trypsin from your cell mixture, as failure to do that will influence the result of your experiment.

Preparation of the total fraction

Timing: 15 min and 2.5 h to prepare the lysate

5. Preparation of the total fraction (15 min to collect the cells and 2.5 h to prepare the lysate). 1/10 aliquot of the cell suspension (100 µL) is transferred to 1.5 mL tube and centrifuged (200 × *g* for 7 min at 4°C), the supernatant is discarded and the cell pellet can be processed immediately or can be frozen and kept at −20°C for 24 h or −80°C for a longer time. To prepare the total fraction resuspend the cell pellet in 38 µL of WCE (50 mM Tris pH=8.0, 280 mM NaCl, 10% glycerol, 0.5% NP-40, 5 mM MgCl2, 250 units/mL Benzonase, 10 µg/mL ethidium bromide, 1× protease inhibitor), incubate for 1 h on ice, centrifuge at 20,000 × *g* for 1 h at 4°C, then 34.2 µL of the resulting supernatant mix with 10.8 µL of 5× Laemmli buffer and use 40 µL of this mixture to measure the **Total** amount of capsid by western blotting (see step 8). Ethidium bromide is used to unwind genomic DNA an improve lysis.

Pause point: after you have collected the cells for total fraction you can freeze it and keep at −20°C for 24 h or −80°C for a longer time.

Preparation of the cytosolic fraction

Timing: 10–20 min

6. Preparation of the cytosolic fraction (10–20 min). The rest of the cell suspension 9/10 (900 µL) is centrifuged (800 × *g* for 7 min at 4°C), supernatant is discarded and cell pellet is resuspended in

315 μL of lysis buffer (10 mM Tris pH = 6.8, 1 mM DTT, 1 mM MgCl2, 10% sucrose, 100 mM NaCl, 0.5% NP-40, 1× protease inhibitor) and incubated for 5 min on ice. Subsequently, the sample is centrifuged at 664 × *g* for 2 min at 4°C. The resulting supernatant and pellet correspond to cytosolic and nuclear fractions, respectively. 1/9 aliquot of the cytosolic fraction (35 μL) is mixed with 15 μL of 5× Laemmli buffer and 40 μL of this mixture is used as **Cytosolic** fraction for detection by western blotting (see step 8).

⚠ CRITICAL: Make sure to that the amount of lysis buffer you use is enough to lyse your cell pellet (see Troubleshooting). Incomplete lysis will result in contamination of your nuclear fraction with unlysed cells. We recommend using 5× cell pellet volumes of the lysis buffer. To determine the correct volume of lysis buffer you can make a pilot experiment testing several volumes of lysis buffer and checking by western blot your fractions for nuclear and cytoplasm markers content.

Wash nuclear pellet

Timing: 10 min

7. Wash the nuclear pellet (10 min). The nuclear pellet is washed twice using 1 mL of washing buffer (10 mM Tris, pH = 6.8, 1 mM DTT, 1 mM MgCl2, 10% sucrose, 100 mM NaCl, 1× protease inhibitor) by gently inverting the tube 2–3 times. The sample is then centrifuged at 664 × *g* for 2 min at 4°C, and the supernatant is discarded.

Preparation of the nuclear fraction

Timing: 15–30 min

8. Preparation of the nuclear fraction (15–30 min). The nuclear pellet is resuspended in 315 μL of extraction buffer (10 mM Tris, pH = 6.8, 1 mM DTT, 1 mM MgCl2, 10% sucrose, 400 mM NaCl, 1× protease inhibitor), and incubated on ice for 10 min. Subsequently, the sample is centrifuged at 5,200 × *g* for 2 min at 4°C. 1/9 aliquot of the supernatant (35 μL) is mixed with 15 μL of 5× Laemmli buffer and 40 μL of this mixture are used as nuclear fraction (see step 8).

Pause point: Fractions mixed with the Laemmli buffer can be frozen and kept at −20°C for at least 1 month.

Western blot

Timing: 3 days

9. By loading 40 μl in the western blot as specified above approximately 8% of the total, cytosolic, and nuclear fractions are analyzed using anti-p24, anti-Nopp140, anti-α-tubulin, or anti-GAPDH antibodies. Detection of Nopp140 is used as a nuclear marker, but other nuclear markers can be used (including but not limited to Lamin A/C, Lamin B1, Histone H1, Histone H3). α-tubulin and GAPDH are used as a cytosolic markers.

After total, cytosolic, and nuclear fractions are prepared as described (see steps 5, 6, and 8 of this protocol), they are stable at −20°C for at least a month. The day you perform the western blot completely thaw the samples and boil them at 100°C for 3 min. For electrophoresis we use NuPAGE 10% Bis-Tris gels. Load 40 μL of each fraction per well and 2 μL of Protein Standard on a separate well of the same gel. Run the gel in 1× PAGE running buffer at 25 mA per gel for

2–3 h at 25°C or until the bromophenol blue dye riches the end of the gel. Transfer the proteins to a Nitrocellulose membrane in 1× western blot transfer buffer at 100 Volts for 1 h at 4°C or on ice. After the transfer block the membrane in western blot blocking buffer for 0.5–1 h at 25°C or at 4°C for 16 h. Incubate with antibody against p24 (1:1,000 dilution in western blot blocking buffer) for 1 h at 25°C or at 4°C for 16 h. Wash the membrane in western blot wash buffer 3 times for 10 min. Incubate with secondary antibody (Goat anti-Mouse IRDye 680LT, 1:10,000 dilution in western blot blocking buffer) for 20–30 min at 25°C. Wash the membrane in western blot wash buffer 3 times for 10 min. Dry the membrane with a paper towel and scan using the Odyssey Infrared Imager. After that re-hydrate the membrane in western blot wash buffer for at least 30 min at 25°C and incubate with the nuclear marker antibody (anti-Nopp140, 1:5,000 dilution in western blot blocking buffer). As a secondary antibody we use Goat anti-Rabbit IRDye 680LT, 1:10,000 dilution in western blot blocking buffer. After scanning the membrane, re-hydrate the membrane again and incubate with antibody against α-tubulin (1:8,000 dilution in western blot blocking buffer). As a secondary antibody we use Goat anti-Rabbit IRDye 800CW (1:10,000 dilution in western blot blocking buffer). As alternative anti-GAPDH antibodies can be used (1:5,000 dilution in western blot blocking buffer and Goat anti-Mouse IRDye 800CW 1:10,000 dilution in western blot blocking buffer).

Pause point: After you detected the signal from your western blot you can keep the membrane dry at 25°C for no longer than 2 weeks before proceeding with the next antibody.

EXPECTED OUTCOMES

This methodology provides information about the amount of HIV-1 capsid that enters the nucleus during infection. The expected outcome is that the small molecule PF74 prevents the entry of capsid into the nucleus, as we have previously shown (Selyutina et al 2020). it is also expected that there is no cross-contamination between nuclear and cytosolic fractions meaning that we will not observe a positive signal for GAPDH or α-tubulin in the nuclear fraction when performing western blot analysis. This methodology could be used to study the entry of HIV-1 capsid in the nucleus of dividing and in non-dividing cells, which is relevant to the infection of lentiviruses. This could be also used to study the entry capsid into the nucleus during infection of other lentiviruses/retroviruses such as Simian immunodeficiency virus (SIVmac), murine leukemia virus (MLV) and others.

QUANTIFICATION AND STATISTICAL ANALYSIS

To quantify the amount of capsid in the nucleus, we calculate the ratio of nuclear to cytosolic HIV-1 capsid for three independent experiments, and perform statistical analysis by applying the unpaired t test.

LIMITATIONS

One important limitation of this methodology is that our analysis does not provide information regarding the oligomerization state of capsid. After HIV-1 infected cells are separated into cytosolic and nuclear fractions, proteins are extracted with a lysate buffer containing detergent, which disrupts the oligomerization state of the HIV-1 capsid rendering the assay unable to state the oligomeric state of capsid during infection. Although this assay provides the total amount of capsid in each subcellular compartment during infection, it does not provide information regarding the oligomerization state of the HIV-1 capsid.

Determination of the bona fide origin of cytosolic and nuclear fractions, by western blotting using the appropriate cytosolic and nuclear protein markers (GAPDH and Nopp140), is essential for validating the results.

TROUBLESHOOTING

Problem 1

A crucial initial experiment is the verification that the assay is separating the nuclear from the cytosolic proteins. To verify whether the assay is separating cytosolic from nuclear fractions, it is essential that nuclear fraction does not contain cytosolic markers (GAPDH and *α-tubulin*) and *vice versa*. Although we have performed this assay in several mammalian cell lines (Selyutina et al., 2020a), the investigator will need to test whether the separation of cellular fractions works in the desire cells. If the nuclear fraction is contaminated with cytosolic protein, the investigator can further wash the nuclear fraction before lysis (see step 7 of Step-by-step method details).

Potential solution

Increase the number of washing steps before lysis of the nuclear fraction.

Problem 2

Although rare, If the cytosolic fraction is contaminated with nuclear proteins, these results would imply that the initial lysis (see step 5) is breaking also the nuclear envelope releasing nuclear proteins. To solve this issue, we recommend decreasing the amount of NP-40 until the cytosolic fraction does not show nuclear markers. i.e., instead of using 0.5% NP-40, the experiment could be done with 0.4, 0.3 or 0.2% NP-40.

A second reason that may cause cross-contamination of nuclear with cytosolic fractions is the incomplete lysis of cells on step 5. To verify for complete lysis, cells should be stained using Trypan Blue after the lysis procedure to ensure ~95% of cells have been disrupted (5% viability). If the lysis is affecting a lower percentage of cells, the amount of lysis buffer should be increased to ensure that ~95% cells are lysed.

Potential solution

Decrease deconcentration of the detergent NP-40 in the lysis buffer, and/or use trypan blue to ensure 5% cellular viablity after lysis, which indicates that the cells have not been over-lysed preventing cross-contamination of fractions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Felipe Díaz-Griffero (Felipe.diaz-griffero@einsteinmed.org).

Materials availability

The development of this method did not generated any new reagents.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

A.S. and F.D.-G. designed and set up the method. A.S. and F.D.-G wrote the method.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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