Identifying Discrepancies in HIV Infectivity and Virion Maturation Using FRET-

Based Detection and Quantification

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Abstract

The maturation of HIV-1 virions is a crucial process in viral replication. Although T cells are a primary source of virus production, much of our understanding of virion maturation comes from studies using the HEK293T human embryonic kidney cell line. Notably, there is a lack of comparative analyses between T cells and HEK293T cells in terms of virion maturation efficiency in existing literature. We previously developed an advanced virion visualization system based on the FRET principle, enabling the effective distinction between immature and mature virions via fluorescence microscopy. In this study, fully infectious FRET-labeled viruses (HIV-1 Gag-iFRET) from Jurkat (a human T lymphocyte cell line) and HEK293T cells were used to assess their virion maturation rates. Our results showed that viruses originating from Jurkat cells had an infectivity rate approximately tenfold higher than that from HEK293T cells. Concerning virion maturity, HEK293T-derived virions demonstrated a maturity rate of 81.79%, consistent with other studies and our previous findings. However, virions from Jurkat cells exhibited a notably lower maturity rate of 68.67% (p <0.0001), indicating an interesting deviation from the expected positive correlation with infectivity. These findings suggest that the initiation of virion maturation does not directly correlate with viral infectivity. Instead, these observations suggest that the rate of conical core formation following the initiation of virion maturation plays a more pivotal role in determining viral infectivity, even when the overall level of maturation is relatively low.

Keywords:

Human Immunodeficiency Virus (HIV), Forster Resonance Energy Transfer (FRET), Virion Maturation, Comparative Viral Analysis, Fluorescent Microscopic Imaging,

1. Introduction

Human Immunodeficiency Virus-1 (HIV-1) continues to pose a significant global health challenge. The advancement of combination antivirals has dramatically improved the management of HIV, targeting various stages of the virus lifecycle, including entry, reverse transcription, integration, and protease activity [1-6]. Recently, the development of capsid inhibitors has emerged as a promising strategy for long-acting antiviral treatments against HIV-1 [7-12]. These novel inhibitors, targeting critical steps in both the early and late stages of the virus lifecycle, notably disrupt the formation of infectious particles. This innovation underscores the need for a comprehensive understanding of the virion maturation process, a key phase in the HIV life cycle where non-infectious particles are transformed into infectious virions.

Our previous research developed a unique tool for detecting virus maturation utilizing a fluorescence resonance energy transfer (FRET)-based technique [13]. This approach enables the semi-automatic evaluation of virion maturation through fluorescent microscopy, effectively minimizing human bias. It offers crucial insights into the structural changes that are vital for virus infectivity. Building upon this, our current study explores virion maturation in different cellular environments. Although T cells are recognized as the primary site for HIV-1 replication [14-20], most existing research assessing viral maturation predominantly utilizes the HEK293T human embryonic kidney cell line [21-24]. Utilizing the FRET-based system, we generated infectious FRET-labeled viruses in both HEK293T and Jurkat cells, an immortal human T-cell leukemia cell line. Surprisingly, we observed that viruses produced by Jurkat cells exhibited a significantly lower maturation efficiency (approximately 69%) compared to those from HEK293T cells (approximately 82%). Contrasting with this, the infectivity of viruses from Jurkat cells was around ten times higher than those

from HEK293T cells. These findings call into question the previously accepted belief

of a direct correlation between virion maturation and virus infectivity, thereby

deepening our understanding of the intricate balance between these two crucial

aspects, virion maturation and virus infectivity of HIV-1 pathology. This research

represents the first to specifically compare the virion maturation in different cell lines.

2. Materials and Methods

2.1 Plasmid DNA Construction

The plasmid DNA utilized for HIV-1 Gag-iFRET labeling virus production incorporates

an efficient intramolecular FRET pair, ECFPΔC11 and cp173Venus [25]. These

genes are positioned between the MA and CA regions of the HIV-1 Gag protein

based on the HIV-1 Gag-iGFP construct [26]. The FRET pair genes, along with the

junction with the MA and CA regions, are flanked by HIV-1 protease cleavage sites

(SQNYPIVQ). This design enables the HIV-1 Protease to specifically cleave at these

sites, resulting in a modification of the FRET signal during the maturation of the

virion. Additional details are provided in our previous publication [13].

2.2 Virus Production and Cell Culture

HIV-1 Gag-iFRET plasmid DNA was transfected into HEK293T cells using a PEI

transfection method (Polysciences) and into Jurkat cells using the Neon NxT

Electroporation System (Invitrogen). HEK293T and TZM-bl cells were cultured in

Dulbecco's Modified Eagle's Medium (Cytiva) supplemented with 10% Fetal Bovine

Serum (Gibco), 1% Penicillin-Streptomycin-Glutamine (Invitrogen), and 1%

GlutaMax (Gibco)(D10) at 37°C in a 5% CO2 environment. Suspended Jurkat cells

were cultured in RPMI 1640 media (Cytiva) with the same supplements (R10). For

producing FRET-labelled virions, HEK293T cells (3.5×10^6 cells/10 cm dish) or

Jurkat cells (2×10^6 cells/24 well plate) were co-transfected with pHIV-1 Gag-iFRET

or iFRET∆Pro and the parental plasmid pNL4-3 or pNL43∆Pro at a 1:10 ratio. Post-

transfection, the culture medium was replaced with fresh D10 medium after 3.5

hours for PEI transfection. Virus-containing supernatants were collected 24 hours

post-medium change from HEK293T cells or 72 hours post-electroporation from

Jurkat cells. The supernatants were then filtered through a 0.45 μm sterile

polyvinylidene difluoride (PVDF, Millipore) membrane and concentrated up to 20-fold

using Lenti-X Concentrator (TaKaRa). Finally, the virus pellet was resuspended in

500 μL of Hank's Balanced Salt Solution (HBSS) without phenol red (Cytiva).

2.3 FRET-Based Virion Visualization

To visualize HIV-1 Gag-iFRET/iFRET∆Pro labeled virions, the concentrated virus

supernatant was diluted 800-fold in 0.22 μm PVDF filtered Hank's Balanced Salt

Solution (HBSS) without Phenol Red (Cytiva) and loaded (350 μL) onto non-coated

30mm x 10mm glass-bottom Tissue Culture Treated Dish (CELLTREAT). The Dish

was then incubated overnight at 4°C. Single-virion images were captured using an

A1R MP+ Multiphoton Confocal Microscope (Nikon). Two sets of 21 images were

automatically acquired for each sample under optimal focus conditions. The first set

employed a 457.9 nm laser for CFP excitation, capturing emissions through 482

nm/35 nm and 540 nm/30 nm filter cubes for CFP and YFP signals, respectively

(FRET images). The second set used a 514.5 nm laser for Venus excitation, with

emissions read through a 540 nm/30 nm filter cube to detect the YFP signal. The

maturation status was quantified as FRET efficiency relative to signals detected in

HIV-1 Gag-iFRETΔ1Pro labeled virions. Images were captured in RAW ND2 format

and converted to TIFF files using Fiji, an image processing package based on

ImageJ2. Binary images, generated based on the YFP signal, provided XY

coordinates for each particle. Using these coordinates, the FRET signal intensity of

each virion was extracted from the raw data, and the FRET ratio (YFP/CFP) was

calculated for each particle. Histograms displaying the distribution of FRET ratio

values were generated, with 100 bins division, and kernel density estimation curves

were plotted alongside. The proportion of the total Gaussian distribution or kernel

density estimation area overlapping with the HIV-1 Gag-iFRETΔPro area was used

to determine the proportion of immature virions. Image data analysis and subsequent

calculations of mature and immature virion proportions were performed using an

updated in-house MATLAB program [13].

2.4 Infectivity Assays

Pseudotyped parental NL4-3 viruses were produced in HEK293T or Jurkat cells

using the transfection methods described in the above FRET-based Virion

Visualization section. The cells were transfected with pNL4-3ΔEnv parental plasmid

along with the HIV-1 envelope expression plasmid pSVIII-92HT593.1, kindly

provided by Dr. Viviana Simon at Mount Sinai. Viral titers were determined using the

HIV-1 Gag p24 DuoSet ELISA (R&D Systems). After seeding TZM-bl cells at 5×10^3

in 96-well plates, they were exposed to an equivalent amount of virus (5 ng of HIV-1

p24 total) the following day. The cells were incubated at 37°C for 48 hours in a CO_2

incubator. Subsequently, luciferase activity in the infected cells was measured with

the Luciferase Assay System (Promega) using a Molecular Devices SpectraMax

Microplate Reader.

2.5 Statistical Analysis

Data were analyzed using standard statistical methods. The significance of

differences in virion maturation and infectivity between the two cell types was

determined using the Mann-Whitney U test and an unpaired *t*-test with Welch's

correction, respectively, with a p-value of less than 0.05 considered statistically

significant.

3. Results

3.1 Virion Maturation Efficiency in Different Producer Cells

In this study, we quantified the proportion of mature and immature virions in FRET-

labeled viruses produced by HEK293T (kidney cell lines) and Jurkat (T-cell leukemia

cell lines). In each sample, we captured a total of 21 images, from which we created

binary images based on the emitted and excited images of YFP (Fig. 1A). We then

calculated the FRET ratio for each virion by dividing the intensity of YFP (the FRET

acceptor) by the total CFP intensity, which serves as the FRET donor. After

calculating the FRET ratio for each virion, based on the extracted signal intensities of

the FRET donor and acceptor, we applied kernel density estimation to the

histograms of FRET efficiencies. The area where the HIV-1 Gag-iFRET curve

overlapped with the HIV-1 Gag-iFRETΔPro curve was identified as the proportion of

immature virions, as per our established methodology (Fig. 1B) [13]. In our analysis,

we examined 14,021 and 22,779 particles, as well as 20,457 and 23,181 particles of

HIV-1 Gag-iFRET and HIV-1 Gag-iFRETΔPro labeled virions produced by HEK293T

and Jurkat cells, respectively, across three independent experiments. The mature

virion proportion in the HIV-1 Gag-iFRET population from HEK293T cells was

81.79% ± 0.06 (Fig. 1C), consistent with our previous findings and electron

microscopic counts reported in other studies [13, 21-24]. In contrast, Jurkat cells

produced mature virions at a lower rate of 68.67% ± 0.04 (Fig. 1C), which is

significantly different from that of HEK293T cells (p-value < 0.0001). Furthermore, we

quantified the Mean Fluorescent Intensity (MFI) of the YFP signal in both mature and

immature virions. The results confirmed that the average incorporation of fluorescent

proteins, indicated by the MFI of YFP (Fig. 1D), was similar in both mature and

immature virions from HEK293T and Jurkat cells (26,691.31 ± 2,733.73 vs.

23,309.51 ± 766.93 in mature virions and 24,619.37 ± 2,857.71 vs. 24,536.10 ±

2,046.71 in immature virions, respectively, and 24,619.37 ± 2,857.71 vs. 24,536.10 ±

2,036.70 in ΔPro). These findings indicate that the number of incorporated

fluorescent proteins is comparable, ensuring that the FRET energy transfer efficiency

is neither under nor overestimated in virions produced by either HEK293T or Jurkat

cells.

3.2 Virus Infectivity in Different Producer Cells

To further examine the characteristic differences between viruses produced by

HEK293T and Jurkat cells, we performed a single-round infection assay using TZM-

bl cells to assess the infectivity of FRET-labeled viruses originating from these cell

lines. Notably, viruses produced in Jurkat cells exhibited relative infectivity

approximately tenfold higher than those from HEK293T cells (Fig. 2). This marked

difference in infectivity was consistently observed in three independent experiments.

4.1 Discussion

The findings of this study provide groundbreaking insights into the maturation

process of HIV-1 virions and its impact on viral infectivity, challenging several

established notions in the field. Our utilization of an advanced FRET-based

visualization system has allowed for a detailed comparative analysis of virion

maturation in two distinct cellular environments, HEK293T and Jurkat cell lines. One

of the most striking observations from our research is the inverse relationship

between virion maturation efficiency and infectivity. While HEK293T cells exhibited a

higher rate of virion maturation (81.79%), virions from Jurkat cells, with a lower

maturation rate of 68.67%, demonstrated significantly higher infectivity,

approximately tenfold greater than the HEK293T counterparts. This finding is in stark

contrast to the traditional view that higher virion maturation correlates directly with

increased infectivity. It suggests that factors other than maturation efficiency, possibly

inherent to the host cell environment or the virion composition, play a crucial role in

determining the infectivity of HIV-1. Recent studies have provided deeper insights

into the role of Inositol hexakisphosphate (IP6) in HIV-1 capsid assembly, a critical

factor in virus infectivity [27]. These studies reveal that the immature Gag lattice of

HIV-1 virions enriches IP6, facilitating capsid maturation. This process is

characterized by the proteolysis of the Gag polyprotein by the viral protease,

inducing vital conformational changes in the capsid protein (CA). These changes

lead to the formation of a conical, cone-shaped mature capsid, consisting of over

1,000 CA copies and forming approximately 200 hexamers and 12 pentamers,

known collectively as capsomers. In the context of our FRET-based assay, which

specifically detects the cleavage of the Gag polyprotein between MA-CA, research

by Sowd et al. has shown that IP6-depleted T-cell lines produce virion particles with

incompletely cleaved Gag proteins [28]. This study indicated that virions from IP6-

deficient cells exhibited lower quantities of mature MA and CA cleavage products

and higher proportions of MA-CA-SP1/MA-CA cleavage intermediates compared to

virions from wild-type cells. Therefore, our FRET detection system could potentially

monitor the IP6-dependent efficiency of Gag polyprotein cleavage. Furthermore, they

characterized virus particle morphology using electron microscopy and calculated

that mature conical core particles in another T-cell leukemia cell line, MT-4 cells,

comprised 62% of the total virion, which aligns with the result from our FRET

detection system in Jurkat cells (Fig. 1B [II] and 1C).

Another study employing a similar concept of FRET-based assay with different FRET

pair proteins reveals that the activation of the HIV-1 viral protease occurs during

assembly and budding before the release of particles in HEK293T cells [29]. Since

the recruitment of IP6 requires the immature Gag lattice, understanding the extent of

viral protease activation during assembly and its role in initiating Gag polyprotein

cleavage before particle release must be crucial. This early activation potentially

influences the formation of the infectious core through IP6 recruitment. Our results,

combined with previous reports, lead to the hypothesis that virions produced by

HEK293T cells may undergo partial Gag-polyprotein cleavage prior to release from

the assembly site. Moreover, several researchers have shown by electron

microscopic analyses that about 70% of viruses produced by HEK293T cells contain

closed conical cores, while the remaining 30% display eccentric or aberrant forms

among the mature virions [21-23]. This suggests that only around 56% of total virions

from HEK293T cells have a closed conical core, a proportion likely lower than that

observed in viruses produced by T-cell lines, which is about 62% [28]. The early

maturation observed in HEK293T cells may result in decreased recruitment of IP6

within the virions, which could affect the formation of the infectious conical core. This

phenomenon likely contributes to the higher maturation ratio observed in HEK293T

cells in our study and in other research (Fig. 1) [29]. However, this early maturation

might also lead to a reduction in viral infectivity when compared to virions produced

in Jurkat cells (Fig. 2). Mathematical modeling suggests that the transport of the pre-

integration complex into the nucleus, along with DNA integration and reverse

transcription, is one of the major factors that significantly influence HIV-1 replication

[30]. The formation of a conical core is essential for both steps of reverse

transcription and the subsequent transport of viral DNA into the nucleus for

integration into the host genome [31, 32]. Consequently, the formation of an

infectious conical core following Gag polyprotein cleavage is a critical determinant of

virus infectivity. This implies that viruses produced from T-cell lines may be more

efficient in forming appropriate conical cores, thereby enhancing their infectivity.

Our research fills a crucial gap in the existing literature by providing a comparative

analysis of virion maturation in kidney cells (HEK293T) and T cells (Jurkat). This

comparison is vital, considering T cells are the primary source of HIV-1 replication *in*

vivo. The observed differences in maturation efficiencies and infectivity rates

between HEK293T and Jurkat cells highlight the importance of considering the

cellular context in HIV-1 biology and pathogenesis studies. Furthermore, our findings

have significant implications for the evaluation of antiviral effects, particularly in the

context of long-acting treatments with maturation and capsid inhibitors. These

inhibitors target various stages of virus infection, including viral budding and virion

maturation [33-35]. The substantial disparities in viral maturation rates between T-

cells and HEK293T cells, the latter of which is a standard cellular resource for *in-vitro*

virus production, suggest that relying solely on HEK293T cells could lead to

misleading assessments of antiviral efficacy. Therefore, our study indicates the

necessity of including T-cell-based assessments to more accurately determine the

antiviral efficacy of maturation and capsid inhibitors in virus maturation. In addition,

by pinpointing the stages at which these inhibitors can be most effective, our findings

could contribute to more efficient and effective HIV treatment regimens.

In conclusion, the results of our study not only enhance our understanding of HIV-1

pathogenesis but also emphasize the complexity of the virus life cycle. The unique

insights gained into the relationship between virion maturation and infectivity open

new pathways for future research, potentially leading to novel therapeutic

approaches in the fight against HIV.

Author's contributions

TI conceptualized and designed the study. Experiments were carried out by JC, AM,

SG, HT, LMB, CA, and TI. The development of the in-house MATLAB script for

automated image data analysis was a collaborative effort between KN and TI. Data

analysis was conducted by KN, SG, and TI. The manuscript was written by TI. TI

provides financial support for the research. All authors have made substantial

contributions to the work and have given their approval for the final version of the

article to be published.

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Figure Legend

Figure 1. Quantitative Analysis of Mature Virions from HEK293T and Jurkat Cells.

(A) Representative FRET-labeled HIV-1 Gag-iFRET virion images from Jurkat cells displayed in a single field. The left panel shows an image captured through the YFP excitation (515 nm) and emission (540 nm) channels (YFP channel). The second image shows the binary image, derived from the YFP channel, identifying virus particle locations as regions of interest (ROI). The third and fourth images are captured using CFP (FRET donor) excitation (454 nm), with CFP emission (483 nm, third) and YFP emission (540 nm, fourth) channels. The right panel shows the FRET efficiency ratio view, computationally constructed from FRET donor (CFP excitation/CFP emission) and acceptor (CFP excitation/YFP emission) images. The

color spectrum indicates FRET signal strength, with high FRET appearing in red

(yellow arrows) and decreasing FRET shifting toward blue (white arrows). (B) Kernel

Density Estimation Curves of FRET Intensity in HIV-1 Gag-iFRET Virions Produced

by HEK293T and Jurkat Cells. The curves depict the distribution histograms of FRET

intensity for virions labeled with HIV-1 Gag-iFRET from (I) HEK293T and (II) Jurkat

cells. The x-axis represents the range of FRET intensity. The y-axis shows the

adjusted density of curves for both HIV-1 Gag-iFRET and HIV-1 Gag-iFRETΔPro

virions. The area under the curve for HIV-1 Gag-iFRET overlapping with that of HIV-1

Gag-iFRETΔPro is calculated and considered indicative of the proportion of

immature virions within the total population of HIV-1 Gag-iFRET virions. (C) Scatter

Plot of Mature Virion Quantification displays the median percentage of mature virions

across 21 images derived from three independent experiments, culminating in a total

of 63 data points. Statistical significance was assessed using the Mann-Whitney U

test. (D) Comparison of Mean Fluorescent Intensity (MFI) of YFP Signals in Mature

and Immature HIV-1 Gag-iFRET labeling Virions. The levels of fluorescent protein

incorporation, as indicated by the YFP signal, were not significantly different between

mature and immature virions in either the wild-type or ΔPro viruses. The error bars

represent the standard error from three independent experiments. Statistical

significance was assessed using the Wilcoxon matched-pairs signed rank test.

Figure 2. Comparing Virus Infectivity Between HEK293T and Jurkat Cells.

Single-round infectivity assays using TZM-bl cells were performed to evaluate the

differences in virus infectivity produced by HEK293T and Jurkat cells. The bar plot

illustrates the relative infectivity (%) of the NL4-3 virus produced by Jurkat cells in

327   comparison to its infectivity when produced by HEK293T cells. Error bars represent

328   the standard deviation from three independent experiments. Statistical significance

329   was determined using an unpaired t-test with Welch's correction.

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Figure 1

Figure 2