HIV Virions as Nanoscopic Test Tubes for Probing Oligomerization of the Integrase Enzyme

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ABSTRACT Employing viruses as nanoscopic lipid-enveloped test tubes allows the miniaturization of protein–protein interaction (PPI) assays while preserving the physiological environment necessary for particular biological processes. Applied to the study of the human immunodeficiency virus type 1 (HIV-1), viral biology and pathology can also be investigated in novel ways, both in vitro as well as in infected cells. In this work we report on an experimental strategy that makes use of engineered HIV-1 viral particles, to allow for probing PPIs of the HIV-1 integrase (IN) inside viruses with single-molecule Förster resonance energy transfer (FRET) using fluorescent proteins (FP). We show that infectious fluorescently labeled viruses can be obtained and that the quantity of labels can be accurately measured and controlled inside individual viral particles. We demonstrate, with proper control experiments, the formation of IN oligomers in single viral particles and inside viral complexes in infected cells. Finally, we show a clear effect on IN oligomerization of small molecule inhibitors of interactions of IN with its natural human cofactor LEDGF/p75, corroborating that IN oligomer enhancing drugs are active already at the level of the virus and strongly suggesting the presence of a dynamic, enhanceable equilibrium between the IN dimer and tetramer in viral particles. Although applied to the HIV-1 IN enzyme, our methodology for utilizing HIV virions as nanoscopic test tubes for probing PPIs is generic, i.e., other PPIs targeted into the HIV-1, or PPIs targeted into other viruses, can potentially be studied with a similar strategy.

KEYWORDS: single-molecule fluorescence microscopy · HIV-1 integrase · stoichiometry · Förster resonance energy transfer · protein–protein interactions · nanoscopy

The human immunodeficiency virus type 1 (HIV-1) is a ~145-nm lipid-enveloped globular lentivirus and is the causative agent of the acquired immunodeficiency syndrome (AIDS). Most, if not all, of the steps in its replication cycle have been studied via ensemble biochemistry and virology, which has resulted in 26 HIV-1 targeting drugs (U.S. Food and Drug Administration) available today. Redundancies in the replication cycle, such as the different possible pathways used by HIV-1 to enter the cell and frequent defects during replication caused by, for example, the low-fidelity reverse transcriptase enzyme, lead to heterogeneity between viral particles which only can be resolved by research methods that allow a single-particle type of analysis. Because of the current availability of sensitive single-molecule imaging systems, and their compatibility with complex biological systems, these methods are especially suited for HIV-1 research at the single-virus level. Single-molecule fluorescence microscopy (SMF), in particular, has been fundamental for a better understanding of the viral lifecycle of different viruses, including the HIV-1. For example, the ability to track individual viral particles in real-time with single-molecule sensitivity is a robust tool for characterizing the dynamic interaction between viruses and target cells and for investigating viral infection routes inside cells. Super-resolution fluorescence methods have offered the possibility for visualizing structural features of individual HIV-1 viral particles and for defining the distribution of the viral proteins inside a virus at the nanoscopic scale. Lacking in the field of single-virus research, however, are quantitative methods for probing protein–protein interactions (PPIs) at the level of a
single viral entity and/or of intracellular viral complexes during the infectious pathway. A clear niche for such methods exists in research on HIV-1 replication, but such methods would also constitute a valuable generic tool for studying other (enveloped) viruses. In the context of inhibitor screening, probing viral PPIs directly in viruses could reduce the overall difficulty and cost associated with setting up high-throughput assays, since protein purification steps, that often hamper in vitro protein investigations, would be eliminated. Finally, unlike lipid vesicle encapsulation, employing a viral targeting strategy would allow PPIs to be expressed in a native cellular environment, rather than having to produce purified protein that might suffer from low stability or solubility. Furthermore, at the nanoscopic virus level, interactions can be probed at concentrations exceeding the limit for complex detection, allowing PPIs with low interaction affinities to be studied.

Here, we have set up a strategy for probing PPIs of one of the three HIV-1 enzymes, integrase (IN), inside single HIV-1 derived virions. In infected cells, IN is the major constituent of the pre-integration complex (PIC), and catalyzes two temporally and spatially separated reactions with the viral cDNA genome as a substrate: (i) the 3′-processing reaction in the cytosol, in which a conserved dinucleotide is cleaved from the viral cDNA, and (ii) the strand transfer reaction in the nucleus, in which the viral cDNA is inserted into the target host cell chromatin.21,22 The enzyme also plays a pivotal role in the replication cycle of HIV-1 by interacting with different cellular cofactors important for nuclear import and chromatin tethering of the virus in infected cells.23–31 Tightly coupled with its different functions is the oligomerization state of IN. HIV-1 IN consists of three distinct domains, an N-terminal domain (NTD; residues 1–54), a catalytic core domain (CCD; residues 55–209), and a C-terminal domain (CTD; residues 210–288), which all three have been shown to crystallize into a dimer.32–39 The active and minimal guanternary structure of full-length IN for in vitro 3′-processing is a dimer, while at least a tetramer is needed for concerted in vitro strand transfer.40–44 Because of its very low intrinsic solubility in vitro, structural studies on full-length HIV-1 IN have been challenging. However, it has been shown that different oligomeric species of IN are present in viruses,45 a tetrameric IN is present in the IN–DNA complex (alias: the “intasome”) of prototype foamy virus (PFV), a distantly related retrovirus,46 and that an HIV-1 IN tetramer likely consists of two stacked reaching dimers that are stabilized by CCD–CCD interactions.47 Finally, the transcriptional coactivator LEDGF/p75 (Lens epithelium-derived growth factor), a cellular cofactor of IN that tethers the latter and the PIC to the host chromatin, is known to specifically stabilize a tetrameric structure of lentiviral IN,44,45,47 and very recently developed HIV-1 replication inhibitors targeting the LEDGF/p75-IN interaction (alias LEDGINS or ALLINIs) have also been shown to allosterically modulate IN multimerization.48–51 Although of crucial importance for the coordination of HIV-1 intracellular transport, nuclear import, and integration, the exact oligomerization state of IN in viruses and viral cellular complexes has been under debate for years, mostly due to a lack of methods to probe IN oligomerization in a spatiotemporally resolved manner during replication. Our strategy to solve this issue is to co-transfect a standard laboratory human cell line (human embryonic kidney, 293T cells) with DNA plasmids encoding the necessary viral structural and catalytic proteins and genomic RNA, as well as the IN enzyme fused to a fluorescent protein (FP; mTFP1 (monomeric teal fluorescent protein)152 or mVenus53). This “IN-FP” is incorporated into assembling viral particles through the Viral protein R (Vpr)-transincorporation technique.54,55 IN-FP is genetically fused with Vpr and a HIV-1 protease enzyme (PR) cleavage motif is introduced between Vpr and IN-FP (Figure 1A). During viral assembly, Vpr interacts with the p6-motif in the group-specific antigen (Gag), as such passively dragging IN-FP into the virus. Cleavage of the latter from Vpr during the viral maturation finally releases the IN-FP inside the newly synthesized virion. Next, we employ total internal reflection fluorescence microscopy (TIRFM) (Figure 1B, Supporting Information Figure S1) on clean solutions of immobilized fluorescent virions to demonstrate that the IN-FP content of viral particles can be accurately quantified. Finally, we employ Förster resonance energy transfer (FRET) via acceptor photobleaching (AP) to prove, with proper control experiments, that IN oligomerization can be probed at the level of a single virus in vitro and at the level of intracellular viral complexes inside infected cells (Figure 1C).

RESULTS AND DISCUSSION

Characterization of HIVIN(D64E)/IN-FP viral Particles. To be able to generate different fluorescent viruses, we first constructed a set of plasmids encoding Vpr-fusions with a fluorescently labeled IN (Vpr-IN-mTFP1, Vpr-IN-mVenus) or with control proteins (Vpr-IN-mTFP1-Venus, Vpr-mTFP1, Vpr-mVenus). To produce the fluorescent viral particles, we expressed either of these proteins together in 293T cells with viral envelope proteins and with the major protein components of HIV-1: Gag precursor polyprotein, consisting of the structural proteins (Matrix, MA; Capsid, CA; p6 and Nucleocapsid, NC) and the Gag-Pol precursor polyprotein, additionally containing the three viral enzymes (protease, PR; reverse transcriptase, RT; and IN). As an internal control, gagpol-encoded IN was rendered catalytically dead by a D64E mutation.56 This ensures that if integration occurs after infection of cells with IN-FP containing viruses, it can only be due to a catalytically active IN-FP. Gag and Gag-Pol finally assemble at the
plasma membrane in a 20:1 ratio to form the shell of new viral particles, surrounded with the proteolipid envelope and containing one or more specific Vpr-fusion proteins (Figure 1A).

To determine whether viral particles containing Vpr-IN-FP can be generated, we performed Western blot analysis on lysates of viral productions with specific antibodies against IN, CA (24 kDa), and RT (51 and 66 kDa). In all conditions tested, RT, untagged IND64E (32 kDa), and CA were detected as single bands (Figure 2A, lanes 1-4). Unprocessed (72 kDa) and PR-processed (60 kDa) labeled IN were furthermore observed in HIVIN-mTFP1 and HIVIN-mTFP1 + IN-mVenus lysates (Figure 2A, lanes 3 and 4, respectively), proving that the Vpr-constructs were both incorporated and processed by PR upon viral maturation. The extra bands, detected in the lysates of HIVIN-mTFP1 and HIVIN-mTFP1 + IN-mVenus solutions, correspond to products of internal cleavage within mTFP1 (Supporting Information Figure S2), as observed before for other FPs having a similar chromophore.

Since we wished to employ the fluorescent viruses also for infecting cultured cells, we set out to compare the infectivity of the produced viruses with that of wild-type HIV-1. In the genome of the produced HIV viral particles, a firefly luciferase gene (fLuc) is also encoded as an internal control for infectivity of the viruses. To test this, we challenged cultured cells with different dilutions of the viral particles and evaluated the fLuc activity with a standard assay three days post-transduction. Viral particles containing a catalytically dead IND64E and no IN-FP (HIVD64E) exhibited 20-fold lower expression of the fLuc gene compared to wild-type HIV viral particles (HIVWT), as has been observed before (Figure 2B). HIVD64E viruses complemented with IN-FP (HIVIN-mTFP1) exhibited a much higher fLuc signal (30% of HIVWT), directly proving a partial rescue of single-round infectivity. These results are in good agreement with previously obtained results for HIVD64E complemented with IN-LexA transincorporation in a similar manner. Transincorporation of mTFP1 (HIVmTFP1) on the other hand, did not show any
increase of expression of the fLuc gene, proving the specificity of rescue by fluorescently labeled IN (Figure 2B). Noteworthy, the average infectivity is not necessarily representative of the infectivity of the fluorescent viral particle population, since only those viruses in the virus preparation that have an incorporated Vpr-IN-FP will exhibit both a significant infectivity rescue, as well as fluorescence. The observed single-round infectivity rescue is in accordance with combined immunostaining and single-molecule measurements, that showed that only ~23% of capsid (a marker for functional viruses) containing virions also exhibited detectable levels of IN-mTFP1 (data not shown). This rescue of infectivity, combined with the knowledge that catalytically active IN is an oligomer in vitro, implies that the IN complex in the virions would either consist of only IN-FP, or both IND64E and IN-FP. To clarify this we performed a coimmunoprecipitation experiment. Practically, when lysates from HIVmVenus and HIVIN-mVenus viral preparations were immunoprecipitated with an anti-GFP antibody, untagged IND64E (32 kDa) specifically co-precipitated with the FP-labeled IN in HIVIN-mVenus (Figure 2C, compare lanes 3 and 4), proving both proteins (IND64E and IN-FP) interact.

As a final control experiment, we wished to directly visualize the infectivity of the fluorescent viruses. To that extent, we infected HeLaP4 cells with the fluorescent viruses, stained the cells three hours post-infection with antibodies recognizing the CA protein and performed confocal imaging. To position the intracellular PICs relative to the nucleus, we also immunostained the nuclear lamina (lamin A/C). In cells infected with different viral particles, mTFP1 or mVenus fluorescence was indeed detected (Figure 2D–G, green), indicating complexes in the cell that contained the mTFP1 or mVenus protein, respectively. Colocalization (Figure 2D–G, yellow) of CA containing complexes (Figure 2D–G, red) with IN-FP or FP was also observed (HIVIN-mTFP1, 56 ± 6%; HIVIN-mVenus, 29 ± 5%; HIVmTFP1, 11 ± 8%; HIVmVenus, 31 ± 5%). Intracellular complexes in which CA and (IN-) FP colocalize suggest the latter is present inside an intact CA core, i.e., that CA dissociation (uncoating) has not occurred yet, as has been suggested before for IN-FP. Synchronization of infection might provide a more homogeneous phenotype of intracellular complexes.

Taken together, at the ensemble level, Vpr-labeled fluorescent IN is incorporated into viruses, maturates, efficiently trans-complements the catalytically inactive
IN and IN-FP engages in a stable hetero-oligomeric complex with the latter. These results, thus, suggest the catalytically active IN species in the fluorescent viruses is a labeled/unlabeled IN hetero-oligomer, as illustrated in Figure 1. Finally, intracellular viral particles contain the IN-FP (or FP) as well as a marker (CA) for specific viral complexes.

**Accurate Control of the Viral Particle Content.** To probe PPIs in single viruses at protein concentrations exhibiting significant complex formation but avoiding false-positive FRET, one needs accurate control of the concentration of active FRET donor and acceptor in the produced viral particles. To show that this can be achieved by Vpr transincorporation, we performed TIRFM experiments on coverslips coated with solutions containing HIVIN⁻mTFP1 or HIVIN⁻mVenus at concentrations at which single particles could be discerned. For both samples, brightly fluorescent spots were observed with a diffraction-limited size of \( \sim 200–300 \text{ nm} \) after excitation at 445 nm (mTFP1) or 514 nm (mVenus) (Figure 3A). To investigate whether these particles indeed represent HIV-1 virions, we performed a counterstaining with an antibody recognizing the capsid protein (Supporting Information Figure S3). From single-particle intensity analyses, we could conclude that the intensity histogram of the HIVIN⁻mVenus particles containing detectable levels of IN-mVenus completely overlaps with the intensity histogram of the HIVIN⁻mVenus particles that colocalize with CA, while for HIVIN⁻mTFP1 particles containing detectable levels of IN-mTFP1, the intensity histogram is slightly lower compared to the intensity histogram of HIVIN⁻mTFP1 particles that colocalize with CA (Supporting Information Figure S3). It is possible that some particles in HIVIN⁻mTFP1 samples are not immunostained well, or might even constitute VSV-G envelope-induced microvesicles containing only IN-FP, an effect that could be related to a possible less efficient folding and/or maturation of the mTFP1 fluorophore. Additionally, the mTFP1 exhibits a lower brightness under our experimental conditions (see Supporting Information Figure S6 and Figure 4E), increasing the likelihood of spurious single-particle localization. With respect to misfolding or incomplete maturation, the mEGFP has around an 80% probability of being fluorescent in distinct fusion proteins. For mVenus, a variant of the yellow fluorescent protein (YFP) that contains multiple mutations that accelerate the rate of protein folding and increase maturation at 37 °C, we expect this probability to be the same or higher. For mTFP1, unfortunately only little is known about the

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**Figure 3.** Accurate control of the fluorescent content of HIV viral particles. (A) TIRFM image at (top) 445-nm or (bottom) 514-nm excitation (9 W/cm²) of viral particles produced with 5 μg Vpr-IN-FP-plasmid. Particles selected for further analysis are indicated with a red arrow. Scale bar is 1 μm. (B) Histograms of the single-viral particle intensity \( F \) as a function of Vpr-plasmid concentration. The black arrow indicates the increase of the amount of Vpr-plasmid from 2.5 to 12.5 μg in steps of 2.5 μg. The single-molecule intensity histograms of purified (top) mTFP1 and (bottom) mVenus are displayed as a reference (black). The solid lines are a b-spline interpolation of the data. (C) Concentration dependence of the average intensity \( F \) (left axis), and the fold increase of \( F \) relative to the single-molecule FP intensity (right axis). The error bars represent the standard deviation \( \sigma \) on \( F \), expressed in camera counts on the left axis and in single-FP number units on the right axis. The different colors in panels B and C represent different Vpr-plasmid amounts.
folding and maturation properties.\textsuperscript{52} Determination of the percentage of fluorescent FPs would be useful for more accurate analysis of the number of labeled INs incorporated in the viruses. Nevertheless, these results suggest that experiments of mTFP1-only containing samples need to be interpreted with care. When analyzing FRET experiments, however, only those viral particles that exhibited mVenus (and mTFP1) fluorescence were taken into further analysis, ensuring that the intensity of the particles taken into account likely represent functional virions.

As is clear from Figure 3A, intensity variations between particles presumably represent different amounts of internalized IN-FP. To investigate this, we analyzed the dependence of the single-virus intensity on the Vpr-plasmid concentration during production. After localizing 1000–5000 single particles, we determined the single-particle intensity \( F_{\text{HIV-IN-FP}} \) by 2D Gaussian fitting and plotted the data in a histogram (Figure 3B). From the raw data, the average fluorescence intensity per virus, \( F_{\text{HIV-IN-FP}} \) was calculated (Figure 3C). Clearly, the higher the amount of Vpr-IN-mTFP1/mVenus plasmid used for viral production (from 2.5 to 12.5 \( \mu \)g), the higher the average intensity of the viral particles. To count the number of fluorescent IN-FPs in a single viral particle, we performed a comparative analysis with single FP molecules, obtained by \textit{in vitro} purification, and immobilized onto the coverslip (Figure 3B,C). This way we could deduce that the average intensity of HIV\textsubscript{IN}-mTFP1 viral particles was between 4 ± 3 and 7.5 ± 7 times higher than that of the single mTFP1 protein, depending on the amount of Vpr-IN-mTFP1 plasmid (2.5–12.5 \( \mu \)g) used to produce the virus. It needs to be stressed again that these numbers might be biased (see above). For HIV\textsubscript{IN}-mVenus viral particles, the average number of fluorescing mVenus varied between 3 ± 2.5 and 5.5 ± 5, with increasing amounts of Vpr-IN-mVenus plasmid (2.5–12.5 \( \mu \)g). As a comparison, considering a \( \sim 145\)-nm viral diameter and 5 incorporated fluorescent IN-FP molecules, this amounts to a concentration of \( \sim 5 \text{ nM} \) in the virus. A significant spread (standard deviation \( \sigma \)) on the single-virus FP content is also obvious (error bars in Figure 3C). This spread, which increased with Vpr-plasmid concentration, arises due to a varying per-cell concentration of Vpr\textsuperscript{65}

![Figure 4. Imaging FRET in single viral particles. (A) Average normalized photobleaching curves of 10 bright HIV\textsubscript{IN}-mVenus particles as a function of the laser power. From top to bottom, 0.22, 1.1, and 15.4 W/cm\textsuperscript{2} excitation power. Black lines, average data; gray lines, standard deviation; green lines, exponential fit. (B) Pre- and post-brightness histogram of HIV\textsubscript{IN}-mTFP1 in black and gray, respectively. Squares: mean virus fluorescence intensity \( F \). The solid line is a b-spline interpolation of the data. (C) HIV\textsubscript{IN}-mTFP1-Venus viral particles are imaged in the blue (D,pre) and yellow (A,pre) detection channel at low laser power, after which the acceptor is photobleached at high laser power and imaged again at low laser power (A,post). Finally, the donor is imaged again in the blue channel at low laser power (D,post). (D) FRET histograms for HIV\textsubscript{IN}-mTFP1-Venus (solid) and HIV\textsubscript{IN}-mTFP1 (dash) viral particles, each fit with a Gaussian distribution. (E) Post-bleaching versus pre-bleaching intensity of mTFP1 fluorescence in HIV\textsubscript{IN}-mTFP1 (cyan) and HIV\textsubscript{IN}-mTFP1-Venus (magenta). Post-bleaching intensity of mTFP1 fluorescence versus pre-bleaching intensity of Venus fluorescence in HIV\textsubscript{IN}-mTFP1-Venus (black). (F) The apparent FRET efficiency \( E \) calculated for HIV\textsubscript{IN}-mTFP1-Venus viral particles.](image-url)
Because of the proclaimed low average FP numbers in single viruses, we also verified whether single-FP bleaching steps could be observed for HIV\textsubscript{IN-mTFP1} and HIV\textsubscript{IN-mVenus} viral particles, by prolonged TIRFM imaging at high excitation intensity (8.8 W/cm\textsuperscript{2} excitation power). Indeed, for low-intensity particles, single and twofold bleaching steps could be observed, corroborating our previous observations (Supporting Information Figure S4). Taken together, observed per-virus intensity variations could be traced back to a different (low) number of fluorescent IN incorporated by the Vpr transincorporation. Although being absolute numbers, these IN numbers are not representative for the "endogenous" IN count in HIV, since IN-FP had to be targeted in a synthetic manner into viral particles. Suffice it to state here, that direct fusion of IN to an FP in the genome impacts viral production and therefore cannot be used for counting endogenous IN molecules.\textsuperscript{54} Importantly, this low IN average copy number explains why a subpopulation of viral particles is left without any IN-FP molecule (data not shown), which in turn explains the modest, yet significant infectivity rescue of viruses containing the catalytically inactive IN\textsubscript{D324A} when measured at the ensemble level (Figure 2B). Finally, future studies might be performed with more recently engineered teal- or cyan-FPs, exhibiting superior signal-to-noise levels, or even with a more red-shifted FRET pair such as the recently developed mClover-mRuby2.\textsuperscript{66}

FRET Analysis in Individual Viral Particles. To quantify FRET, we employ direct AP, in which the FRET donor (D) fluorescence intensity is measured before and after photobleaching of the FRET acceptor (A) (Figure 1C). To show that mVenus is a suitable probe for the AP method, we measured the excitation intensity dependence of the fluorescence of individual bright (containing multiple FPs) HIV\textsubscript{IN-mVenus} viral particles under TIR excitation (Figure 1B). Particles were imaged at three 514-nm laser intensities: low (0.22 W/cm\textsuperscript{2}), intermediate (1.1 W/cm\textsuperscript{2}), and high (15.4 W/cm\textsuperscript{2}). At low 514-nm laser intensity, individual bright viral particles exhibited a moderate decrease in intensity over a time-span of 100 s, while at intermediate and high excitation intensities, the largest proportion of the fluorescent particles photobleached (Figure 4A). When illuminated for at least 30 s at 15.4 W/cm\textsuperscript{2}, the probability of completely photobleaching virus-incorporated mVenus approaches unity, proving that the mVenus is a suitable FRET acceptor probe for the AP method. We performed a similar experiment using 445-nm excitation, to optimize the imaging conditions for HIV\textsubscript{IN-mTFP1} viral particles (Supporting Information Figure S5). In brief, at 0.45 W/cm\textsuperscript{2} mTFP1-bearing viral particles can be imaged, during the 2-s time interval needed to quantify FRET, without significant photobleaching. We also tested the effect of high 514-nm laser intensities on the mTFP1 fluorescence. As can be seen in Figure 4B, the average viral particle intensity before and after illumination was not altered for a sample containing HIV\textsubscript{IN-mTFP1} for 30 s with 15.4 W/cm\textsuperscript{2} 514-nm light, proving the absence of a negative effect of 514 nm illumination on mTFP1, and thus a highly specific excitation of mVenus.

In a next step, we employed viral particles transincorporating a Vpr-IN-mTFP1-Venus fusion construct (HIV\textsubscript{IN-mTFP1-Venus}). On coverslips coated with these particles, we imaged mTFP1 at low laser power in the blue detection channel (Figure 4C, “D,pre”) and subsequently imaged mVenus at low laser power in the yellow detection channel (Figure 4C, “A,pre”). Next, mVenus was photobleached for 30 s at high power and imaged again at low power (Figure 4C, “A,post”). Finally, mTFP1 was imaged again at low power (Figure 4C, “D,post”). As can be seen in Figure 4C, after photobleaching, only background signal was detected for mVenus, while a careful comparison of the mTFP1 images already reveals an increased brightness of the mTFP1 molecules. Next, spots were localized and fitted with a 2D-Gaussian function in the D,pre; D,post; and A,pre fluorescence images, and those particles located within 160 nm (2 pixels) distance in all three images and lacking neighboring particles within 400 nm (5 pixels) were considered to be individual viral particles containing both the fluorescing FRET donor and fluorescing FRET acceptor. From this population (~5000 viral particles), the per-viral particle FRET ratio, \( r_{\text{FRET}} \), was calculated and plotted in a histogram (eq 1). If FRET occurred, the average FRET ratio, \( r_{\text{FRET}} \), should be larger than unity. Indeed, relative to the negative control (HIV\textsubscript{IN-mTFP1}), the HIV\textsubscript{IN-mTFP1-Venus} sample exhibited a right-shifted (and wider) \( r_{\text{FRET}} \) distribution (\( p < 0.01 \)). Histograms were fitted with a Gaussian distribution, with a mean value \( r_{\text{FRET}} \) for HIV\textsubscript{IN-mTFP1-Venus} viral particles of 1.87 ± 0.07 (Figure 4D, solid line). Without photobleaching of the acceptor, the calculated \( r_{\text{FRET}} \) was 1.06 ± 0.02, indicating that the increase in FRET donor intensity is indeed due to photobleaching of the acceptor (Supporting Information Table S1). For the HIV\textsubscript{IN-mTFP1} viral particles, where no FRET could occur due to the absence of the acceptor, the \( r_{\text{FRET}} \) was 1.05 ± 0.06 (Figure 4D, dashed line).

Another way to analyze FRET is by plotting the post-vs pre-bleaching mTFP1 intensity for individual particles and performing a linear fit (Figure 4E). The slope of this fit showed similar \( r_{\text{FRET}} \) values as in Figure 4D (Figure 4E, cyan/magenta and Supporting Information Table S1). The advantage of representing the data this way is that \( F_{\text{mTFP1,post}} \) can also be plotted against \( F_{\text{Venus,pre}} \) for HIV\textsubscript{IN-mTFP1-Venus} (Figure 4E, black). The slope then indicated that, when measured at the same excitation intensity (0.45 W/cm\textsuperscript{2}), the per-particle intensity in the Venus imaging channel is about 8-fold higher than that in the mTFP1 channel. Since we calculated that Venus should theoretically be detected only ~2.4-fold more efficient than mTFP1 in our
microscopic setup (Supporting Information Figure S6), we can therefore conclude that the mTFP1 fusion folds and/or matures less efficiently than Venus in the context of IN-mTFP1-Venus, giving rise to viral particles with, on average, an excess of fluorescent Venus protein. Finally, from the mean squared deviation of the scatter plots in Figure 4E, we could conclude that there is a larger variation on the intensity of mTFP1 in HIV\textsubscript{IN-mTFP1-Venus} (SD = 2402, plotted in magenta) than on the intensity of mTFP1 in HIV\textsubscript{IN-mTFP1} (SD = 141, plotted in cyan), which can partly be explained by a relatively larger effect of Poissonian shot noise induced localization errors in the \textsubscript{FmTFP1-pre} image of HIV\textsubscript{IN-mTFP1-Venus}, where per-particle intensities were considerably lower due to mTFP1-quenching by FRET. The additional spread of the black data (SD = 9055; Venus vs mTFP1 in HIV\textsubscript{IN-mTFP1-Venus}), on the other hand, is not a FRET related effect, but a direct proof of the variable per-particle concentration ratio of fluorescent Venus vs mTFP1, even when the proteins are expressed as part of the same construct.

Finally, we also calculated the apparent FRET efficiency, \(E\), with \(E = 1 - \frac{(F_{D,pre}/F_{D,post})}{(F_{D,pre}/F_{D,post})}\) (Figure 4F). The average \(E\) (~0.44) for HIV\textsubscript{IN-mTFP1-Venus} was in good agreement with reported values for constructs having a similar distance between the same D and A, but measured with time-domain fluorescence lifetime imaging microscopy (FRET-FLIM).\(^{67}\) This result shows that we can accurately measure energy transfer with our AP approach in individual viral particles.

In summary, on the basis of excitation intensity dependence studies, we conclude that both donor and acceptor probes are suitable for the AP-FRET approach, although we did point out that the mTFP1 likely exhibits suboptimal folding and/or maturation. Additionally, the observation of FRET inside these viral particles provides a basic proof-of-principle that viral particles can be used as nanoscopic test tubes for probing PPIs. Next, we will employ the developed method to investigate actual PPIs inside individual viral particles.

IN Forms Stable Oligomers in Viral Particles. To investigate whether IN oligomers can be detected in HIV-1 viral particles, we produced viral particles containing IN-mTFP1 and IN-mVenus by transfecting the producer cells with the corresponding Vpr-plasmids in a 1:1 stoichiometry, which empirically worked best. For viral particles containing both mTFP1 and mVenus fluorescence (HIV\textsubscript{IN-mTFP1-IN-mVenus}), an \(\tau_{\text{FRET}}\) (eq 1) value of 1.27 ± 0.09 was measured, suggesting the presence of IN-IN oligomers capable of FRET (Figure 5A,B, solid black line and Supporting Information Table S2). To prove that overexpression of the Vpr-plasmids in the producer cells did not result in an aspecific FRET signal, we repeated the experiment for viral particles produced in cells expressing Vpr-FP plasmids instead of Vpr-IN-FP. No increased \(\tau_{\text{FRET}}\) was observed in these control viral particles (Figure 5A,B, Supporting Information Table S2), suggesting that the observed increase in \(\tau_{\text{FRET}}\) is due to oligomerization of fluorescently labeled IN.

To investigate IN oligomerization in viral particles in more detail and to be able to corroborate our initial observations, we sought to reduce the dimerization affinity of IN. As shown before in crude lysates,\(^{68}\) W108G is crucial for IN multimerization (Figure 5C). Combined \textit{in vitro} energy calculations on the IN catalytic core domain dimer (PDB ID: 2ITG) and predictions from the Robetta Computational Interface Alanine Scanning Server (http://robetta.bakerlab.org/alascansubmit.jsp),\(^{69}\) suggest that a W108G substitution can create an energetically unfavorable cavity at the interface, reducing dimerization and higher order oligomerization. The effect of W108G on oligomerization was first investigated with an \textit{in vitro} AlphaScreen interaction assay (Figure 5D) using purified recombinant IN. W108G IN mutant exhibited a 4-fold lower AlphaScreen signal compared to wild-type IN, corroborating the predicted negative effect of the mutation on IN dimerization in solution. Next, we produced viral particles with a W108G mutation either in one Vpr-IN-FP (mTFP1 or mVenus) or in both Vpr-IN-FP (mTFP1 and mVenus) proteins and measured their \(\tau_{\text{FRET}}\) value. For all viral particles containing at least one IN\textsubscript{W108G-FP} (mTFP1 and/or mVenus), the obtained FRET ratios dropped to unity within experimental error (Figure 5E and Supporting Information Table S2), indicating that the W108G substitution efficiently disrupts IN oligomers inside viral particles. This result further corroborates the specificity of the intravirus IN oligomerization measurements.

To gain more insight in the absolute stoichiometry of IN inside viral particles, we set out to test the effect of a class of integrase inhibitors, LEDGINs,\(^{49}\) on the observed FRET ratio. LEDGINs are small-molecule inhibitors of the interactions of HIV-1 IN with LEDGF/p75, a human transcriptional coactivator that protects IN against proteasomal degradation,\(^{70}\) stabilizes IN tetramers,\(^{24,31}\) locks the PIC onto the chromatin,\(^{25}\) and targets integration to transcriptionally active regions in the genome.\(^{30}\) LEDGINs are designed to mimic the unstructured loop of LEDGF/p75 that binds to the dimer interface of IN. It has recently been suggested, from \textit{in vitro} experiments on recombinant IN, that LEDGINs enhance the oligomerization state of IN.\(^{50,51}\) To prove this proposed property of LEDGINs, we previously produced dual-color viral particles (HIV\textsubscript{IN-mTFP1-IN-mVenus}) in the presence of the control DMSO, Raltegravir (an IN strand transfer catalytic inhibitor not expected to have an influence on the IN oligomerization), and the CX05045 LEDGIN and performed FRET experiments.\(^{48}\) No increase in \(\tau_{\text{FRET}}\) could be observed in the presence of Raltegravir or DMSO, while on the contrary, the FRET ratio was significantly larger for viral particles produced in the presence of the
Here, we now corroborated this finding and additionally show that an increase in FRET also occurs in the presence of CX14442. This strongly suggests IN oligomerization enhancement is indeed a general mechanism of LEDGINs, and not a unique property of the CX05045 compound (Figure 5F).

In summary, these experiments allowed us to prove, directly inside HIV-1 derived virions, that IN is part of an oligomer, and corroborate previously performed in vitro experiments involving cross-linking, lysis, and Western blotting of whole-population preparations. Our findings involving a particular set of LEDGINs clearly prove that IN multimerization, most likely the dimer to tetramer equilibrium, is indeed enhanced inside viral particles by allosteric IN inhibitors. Finally, our observation of IN oligomers inside virions would be compatible with a suggested dimeric IN structure bound to viral LTR ends inside virions already containing RT-transcribed viral RNA.

Oligomerization of IN inside Infected Cells. Because our strategy for creating viruses containing labeled IN preserves viral infectivity up until integration, we next investigated IN-IN oligomerization at the level of the viral complex inside infected cells using quasi-TIR illumination for a larger axial penetration depth of the excitation light. We produced viral particles containing IN-mTFP1 and IN-mVenus (HIVIN‑mTFP1þmVenus) and used these dual-color viral particles to infect HeLaP4 cells. Three hours post-infection, cells were immunostained with antibodies recognizing the epidermal growth factor receptor (EGFR), which are present on the cell surface, as well as with antibodies recognizing the nuclear lamina to delineate the cell/nucleus boundaries (Figure 6A). After localizing and fitting the viral complexes (∼50–500) in the cytoplasm of the cells, we calculated an FRET value of 1.30 ± 0.09 for dual-color viral particles containing both mTFP1 and mVenus fluorescence (Figure 6B,C and Supporting Information Table S3). We also produced viral particles in cells expressing Vpr-FP instead of Vpr-IN-FP and used these particles to infect HeLaP4 cells (∼50–200). In agreement with the results obtained from viral particles in solution, no increased FRET was observed, proving the specificity of the observed FRET (Supporting Information Table S3). Logically, considerably less dual-color viral complexes were observed in cells infected...
HIVIN inside HIV-1 virions and for the interaction-competent HIV-1 derived viral particles. With our methodology, we visualized IN oligomerization directly inside HIV-1 virions and for the first time provided direct experimental proof for the persistence of IN dimers inside complexes within HIV-1 infected cells. We observed a similar FRET ratio in solution and in cells, suggesting that already in the mature virion, the IN is stably associated as an oligomeric complex with the viral content that eventually forms the PIC. Future studies might provide an even more detailed insight into the function of IN oligomers throughout viral replication, and the involvement of human cofactors in this. FRET measurements inside cells might also be combined with single-particle tracking fluorescence lifetime imaging for more robust FRET quantifications, or advanced multicolor fluorescence fluctuation microscopy for monitoring multiple interactions simultaneously. More generally, our work should serve as an experimental proof that PPIs can be quantitatively studied, by TIRFM and acceptor photobleaching mediated FRET with fluorescent proteins as markers, at the level of single viruses. Therefore, we feel our strategy has the potential for being widely applicable for studying other PPIs of HIV, but potentially also PPIs of other viruses, in the miniaturized context of a single viral entity in solution, but also in infected cells.

**MATERIAL AND METHODS**

**Plasmids.** The HIV-1 molecular clone pNL4-3.Luc.R.E- (Cat.3418, NIH AIDS Research and Reference Reagent Program) was used for the production of HIV_WT, and pD64E (Cat.3418, NIH AIDS Research and Reference Reagent Program) encoding a catalytically inactive IND64E, a useful for studying other PPIs of HIV, but potentially also PPIs of other viruses, in the miniaturized context of a single viral entity in solution, but also in infected cells.

**Figure 6. IN forms oligomers inside viral complexes in infected cells:** (A) Quasi-TIR excitation wide field detection image of HeLaP4 cells infected with HIVIN-mTFP1 viral particles, imaged for mTFP1 (green) and immunostained for nuclear lamina (A/C) (red) and EGFR (red) to show the nuclear and cell membrane, respectively. (B) FRET histograms for HeLaP4 cells infected with HIVIN-mTFP1: m-Venus (black), HIVIN-mTFP1: m-Venus (red), HIVIN-mTFP1: m-Venus (green), HIVIN-mTFP1: mVenus (blue), or HIVIN(W108G)-mTFP1: m-Venus (magenta) viral particles. (C) FRET ratios of viral particles (HIVIN-mTFP1: m-Venus (black), HIVIN(W108G)-mTFP1: m-Venus (red), HIVIN-mTFP1: mVenus (green), HIVIN-mTFP1: mVenus (blue), or HIVIN(W108G)-mTFP1: m-Venus (magenta)) showed in a bar graph; p-value <0.01 obtained from a Student's t-test with unequal variance of the data compared to HIVIN-mTFP1: m-mVenus. Error bars on the bar graphs represent the standard deviation of T_FRET between different experiments.

with HIVIN-mTFP1: m-mVenus or HIVIN-mTFP1: mVenus viral particles (data not shown), because of the unstable association of free FP with the viral complexes inside cells. When infecting HeLaP4 cells with viral particles containing the W108G substitution in both IN (HIVIN(W108G)-mTFP1: IN(W108G)-mVenus), the obtained FRET ratios dropped to unity within experimental error, which is again in agreement with the results obtained from viral particles in solution.

Taken together, these experiments proved that PPIs can be studied inside cells in the context of a viral complex, and more specifically, that IN also takes part in and stays part of an oligomeric complex.

In conclusion of this work, we have developed, characterized, and applied an experimental methodology for assessing PPIs of IN via single-molecule FRET inside infection-competent HIV-1 derived viral particles. With our methodology, we visualized IN oligomerization directly inside HIV-1 virions and for the first time provided direct experimental proof for the persistence of IN dimers inside complexes within HIV-1 infected cells. We observed...
the following primer 5'-TGGCCGCGCAGGGGCGCGCGTG-3'. The original template was digested with DpnI, and the complementary strand was synthesized using the GFP-Nr primer.

**In Vitro Purification of Fluorescent Proteins.** For protein purification, pVSV-G and mTFP1 were transformed into JM109-(DE3) *Escherichia coli* cells (Promega, Leiden, The Netherlands), and a single colony was picked to inoculate 350 mL of LB medium supplemented with ampicillin (100 mg/L in 50% ethanol, Carl Roth GmbH, Karlsruhe, Germany). The culture was incubated at 20 °C for 48–72 h. The bacterial cells were harvested by centrifugation, cells were transfected with a transfection mix (1 μL Tris-Cl (pH 7.5, 300 mM NaCl), 20% (v/v) glycerol, 1 mM MgCl2, 1% (v/v) Triton X-100, 4 mM dithiothreitol (DTT), Complete protease inhibitors (1/100 dilutions, Roche)). Five microliters of protein was diluted directly into SDS-PAGE loading buffer (200 mM Tris-HCl pH 6.8, 400 mM DTT, 8% (w/v) SDS, 40% (v/v) glycerol, 0.5% bromophenol blue). Viral lysates were incubated with anti-GFP antibody (in house) for 4 h, followed by 3 h incubation with 40 μL of protein G-agarose beads (Roche Molecular Biochemicals). After this, the agarose beads were washed 5 times with a 1× lysis buffer containing 300 mM NaCl and the immunoprecipitated proteins were eluted in 30 μL of SDS-PAGE loading buffer. The crude lysates (as loading control) and immunoprecipitates were mixed onto a protein gel (4–12% NuPAGE Novex Bis-Tris Gel, Life Technologies) and electroblotted onto nitrocellulose (C:1N0G0, The Netherlands). Elution of the protein was done using Tris/NaCl (100 mM/300 mM, pH 7.2) supplemented with 200 mM imidazole (Sigma-Aldrich, pH 7.4), followed by buffer exchange on a PD-10 desalting column (GE Healthcare, Londen, UK). Elution was performed with Tris/NaCl (50 mM/150 mM).

**Escherichia Cell Culture.** HEK293T cells and HeLaP4 cells were maintained at 37 °C in 5% CO2 humidified atmosphere in Dulbecco's modified Eagle medium (Life Technologies Europe, Merelbeke, Belgium) supplemented with 8% (v/v) fetal bovine serum (FBS, Life Technologies, Belgium) and 50 μg/mL gentamicin (Life Technologies).

**Virus Production.** Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 derived viral particles containing fluorescent IN were generated by a Vpr-mediated transincorporation strategy. For the in vitro single-virus experiments, 7 × 105 HEK293T producer cells were seeded per well in a 6-well plate in DMEM supplemented with 2% FBS. At a cell density of 90%, cells were transfected with 1.25 μg of VSV-G, 3.75 μg of p64E, and 12.5 μg of each Vpr plasmid per well using branched polyethyleneimine (bPEI, 20 μL, 10 μg/mL, Sigma-Aldrich).

Six hours post-transfection, the medium was replaced with pre-warmed OptiMEM (Life Technologies) supplemented with 50 μg/mL gentamicin. At 48 h post-transfection, the cell supernatant was filtered through a 0.45-μm pore-size syringe filter (Sartorius), and concentrated by ultracentrifugation sedimentation (S28 rotor, Beckman Coulter, Ireland). Next, the iodixanol was removed by ultrafiltration (Vivaspin, NMWL = 50 kDa, Sartorius). For the immunoprecipitation, the ultrafiltrate was recovered in 150 mM NaCl, for all other experiments in OptiMEM. For the production of viruses in presence of inhibitors, the transfection medium was replaced with gentamicin supplemented OptiMEM with Haltegravis (0.03 μM), CX05045 (5 μM), or CX14442 (0.3 μM).

**Western Blotting and Coimmunoprecipitation.** Protein concentrations of supernatants from virus-producing cells were first determined using a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA). The crude lysates (as loading control) and immunoprecipitates were mixed onto a protein gel (4–12% NuPAGE Novex Bis-Tris Gel, Life Technologies) and electroblotted onto a PVDF membrane (Bio-Rad). The following antibodies were used: anti-HIV-1 IN (mouse monoclonal, 1/5000, Santa Cruz Biotechnology, Heidelberg, Germany), as well as HIV-1 CA (1/5000, mouse monoclonal, #24-2, NIH AIDS reagent Program, Division of AIDS, NIAID). Blots were subsequently stained with 440 or 514 nm. The image resolution was 512 × 512 pixels, with a pixel size of 160 nm. For the dual or three-color imaging, different channels were combined for each Z-plane and three consecutive Z-planes were then assembled using ImageJ software (NIH).

**Immunofluorescence Imaging.** For immunofluorescence imaging, 3 × 105 HeLaP4 cells were seeded per well in poly-L-lysine coated 8-well chambered coverglasses and infected the next day with a volume (equivalent to 1 μg of p24 antigen) of filtered supernatant from virus-producing cells. Three hours post-infection, cells were briefly incubated with trypsin (0.25% (w/v), 30 s, Life Technologies), fixed with 4% formaldehyde, and permeabilized with 0.1% (w/v) (in PBS) Triton X-100 (Sigma-Aldrich) prior to overnight immunostaining of the nuclear lamina with lamin AC antibody (1/2000 dilution, sc7293, Santa Cruz Biotechnology, Heidelberg, Germany), as well as HIV-1 CA (1/500, mouse monoclonal, #24-2, NIH AIDS reagent Program, Division of AIDS, NIAID, NIH). After staining with a secondary goat anti-mouse antibody labeled with Alexa Fluor 647 (Promega GmbH, Mannheim, Germany), each virus sample was tested in triplicate with a serial 3-fold dilution of virus. Data are presented as relative infectivity compared to viral particles lacking Vpr-incorporation.

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immobilized in 0.1% (w/v) poly-o-lysine (Sigma-Aldrich) coated wells in a #1 Lab-Tek chambered coverslip (WWR international, Leuven, Belgium) for 4 h at 37 °C, washed with PBS buffer (Life Technologies), fixed with 4% (v/v) parafomaldehyde (Sigma-Aldrich) in PBS, washed twice with PBS, and kept at room temperature in PBS. Imaging was subsequently performed on an inverted microscope (Olympus IX-71, Olympus NV, Aartselaar, Belgium) by objective-type total internal reflection (TIR) excitation and wide field detection. The mTFP1 was excited at 445 nm (Cube 445-40c, Coherent, Utrecht, The Netherlands) and mVenuses at 514 nm (Sapphire 514-100 CW CDRH, 100 mW, Coherent) (Supporting Information Figure S1). The two different laser lines were combined using a dichroic mirror (z445/514/633 Plano-Convex lens, Newport). Emission was collected by the same objective and split by a polychroic mirror (z445/514/633 Plano-Convex lens, Newport). This laser line was combined with the previous laser lines using a dichroic mirror (z532rdc, Chroma Technology GmbH). Emission was collected at 644 nm (Excelsior, Newport Spectra Physics BV, Utrecht, The Netherlands) for a Ph.D. grant (SFR/BD/2726/2006). Z.D. acknowledges the FWO (Grants G0607.09, G0962.13, Methusalem funding CASAS METH/08/04), the KU Leuven Concerted Research Action (GOA 2011/03), and the Hercules foundation (HER/08/21).

Supporting Information Available: FRET ratios in table format, supporting experiments containing a TIRFM setup scheme, a SDS-PAGE of mTFP1, colocalization and photobleaching experiments, the laser power dependence of mTFP1 and the optical properties of the fluorescent probes and optical elements. This material is available of charge via the Internet at http://pubs.acs.org.

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