SINGLE VIRUS IMAGING OF HIV-1 WITH FLUORESCENTLY LABELED CA

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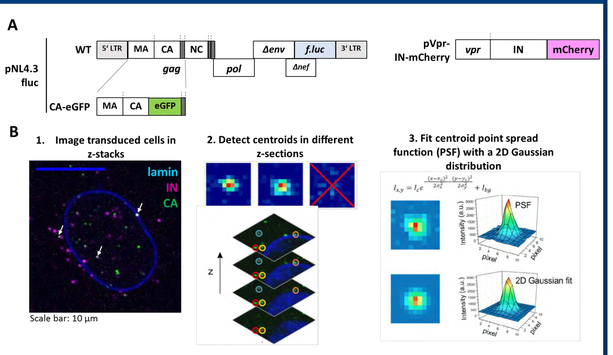
BACKGROUND

HIV-1 capsid uncoating is a crucial step in HIV-1 replication. It is tightly regulated in a spatiotemporal fashion. Both accelerated and delayed uncoating hinder productive infection, and require a complex interplay between reverse transcription, uncoating and nuclear import. Although different available *in vitro* and *in vivo* uncoating assays already revealed valuable information, a consensus uncoating model is lacking. To date, uncoating is studied mostly indirectly in a heterogeneous pool of viruses during asynchronous infection, lacking the ability to discern the transient stages of uncoating. Therefore, conflicting results on the dynamics of this process have been obtained. Recently, single virus studies with imunolabelled CA suggested the presence of CA in the nucleus. Resolving the dynamics of capsid uncoating during infection awaits the development of a method to image functional virus containing labeled CA.

METHODS

We fluorescently labeled CA within the molecular clone at different selected positions, and evaluated the production efficiency and single round infectivity of these labeled viruses. We generated dual labeled VSV-G pseudotyped particles containing CA-eGFP and Vpr-transincorporated IN-mCherry. Using confocal microscopy, we evaluated the potential to study HIV-1 uncoating at a single virus level. Hereto we investigated the cellular distribution of CA and IN at a single virus level in fixed HeLaP4 cells. Validation of the model was provided through the use of inhibitors blocking HIV replication at discrete steps.

Figure 1: Experimental setup of fluorescent particle production and analysis

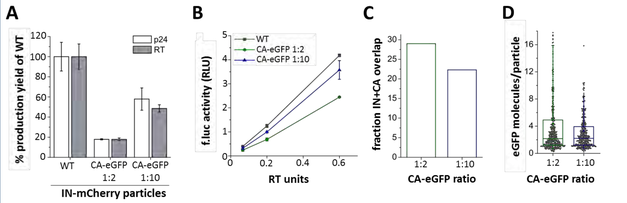


(A) Packaging constructs used for VSV-G-enveloped, fluorescent HIV-1 particle production. Dashed lines indicate protease cleavage sites. **(B)** Imaging methodology for fluorescent virus particle detection in transduced cells. 3D images of cells are acquired in 0.3 µm z-stack scans and viral particles are detected by a home-made Matlab routine as centroids appearing in ≥3 z-sections. The intensity measured in each section is fitted to a 2D Gaussian distribution to measure particle fluorescence intensity.

RESULTS

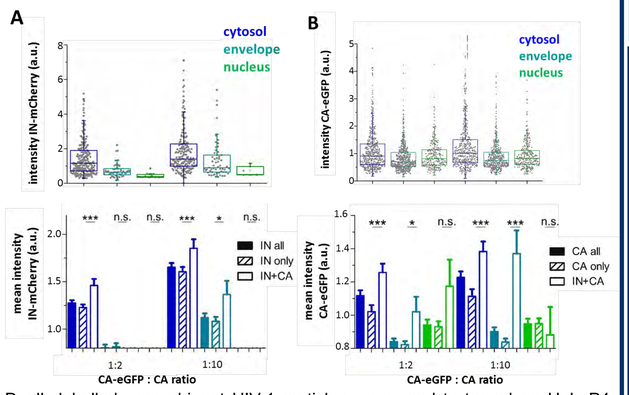
A specific (1:10) mixture of labeled and unlabeled CA was required to produce viral particles containing both labeled CA and IN yet maintaining infectivity in single round experiments. After entry, colocalisation of CA and IN was observed. When reaching the nuclear membrane, CA containing complexes accumulated in the perinuclear area and were depleted for IN. Using both eGFP-labeled CA and immunocytochemistry, we confirmed the presence of CA in the nucleus, although the role and nature of this nuclear CA remains unclear. Still, complexes with a high CA-eGFP content were mainly found in the cytoplasm and not in the nucleus. Most CA complexes in the nucleus did not contain IN.

Figure 2: Replication features of singly- and dually-labelled (IN-mCherry +/- CA-eGFP) HIV-1 particles



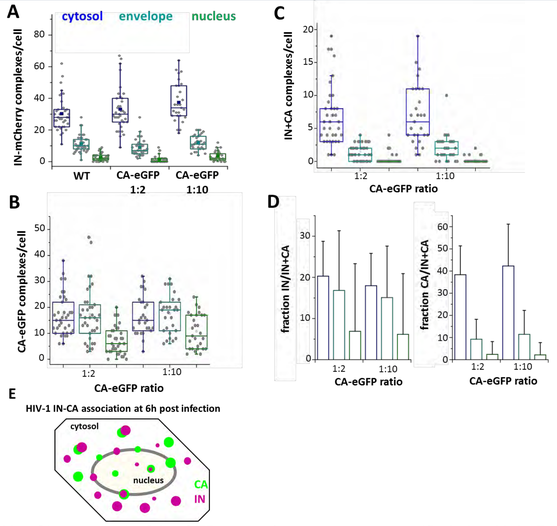
Recombinant HIV-1 particles were produced by transient transfection of the vector system (shown in Figure 1) into 293T cells. The supernatants were harvested 48h post transfection and their replication competence was assayed as follows: **(A)** Quantification of recombinant particle production by p24 levels and reverse transcriptase (RT) activity in harvested supernatants. For both tests, the values obtained for IN-mCherry-labelled (WT) particles are normalized to 100%. **(B)** Transduction efficiency of the annotated vectors was measured 3 days post infection by firefly luciferase (f.luc) reporter expression (RLU) in infected HeLa P4 cells. The obtained values are expressed relative to the RT units of the corresponding supernatants (as determined in A). **(C)** Purified and concentrated viral particles were spotted onto microscopy slides and mCherry and eGFP-fluorescent spots were imaged by confocal laser scanning microscopy (CLSM). Fraction of overlap between the two fluorophores was measured for a population of particles and describes the percentage of eGFP centroids colocalizing with mCherry fluorophores (all of which represent 100%). **(D)** The number of eGFP molecules in each virus particle (indicative of the number of incorporated CA-eGFP monomers) was measured on spotted particles (as in C) excited with an 800W 488nm laser power (indicated on the x-axis) by TIRF microscopy.

Figure 3: Fluorescence intensities of labelled IN and CA proteins change with different dynamics upon HIV-1 nuclear entry



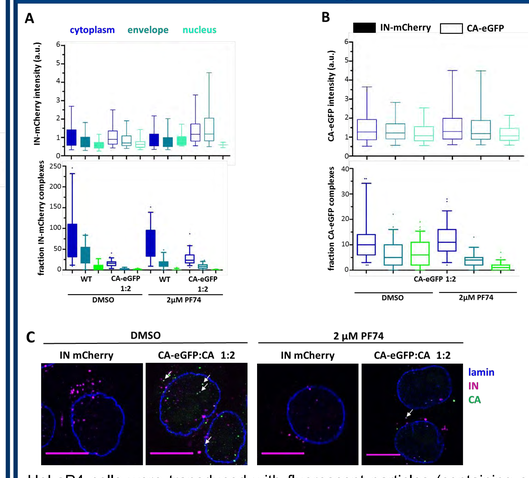
Dually-labelled, recombinant HIV-1 particles were used to transduce HeLaP4 cells. The cells were fixed 6h post infection and fluorescence intensity and cellular localization of viral complexes were determined. **(A)** Fluorescence intensity measurements of IN-mCherry complexes in cells transduced with dually-labelled viruses in different intracellular compartments. **Upper panel:** intensity distributions of all IN-mCherry complexes change upon nuclear entry. **Lower panel:** Intensity of cytosolic IN-mCherry complexes increases upon association with the CA-eGFP protein. The cutoff was set to illustrate the IN-mCherry complexes of the highest intensity. **(B)** Fluorescence intensity measurements of CA-eGFP complexes in cells transduced with dually-labelled viruses in different intracellular compartments. **Upper panel:** CA-eGFP intensity in all viral complexes depending on the cellular compartment. **Lower panel:** Fluorescence intensity of high-content CA-eGFP centroids increases upon association with IN-mCherry, in the cytosol and at the nuclear envelope (but not the nucleus).

Figure4: Intracellular localization patterns of IN-mCherry and CA-eGFP particles



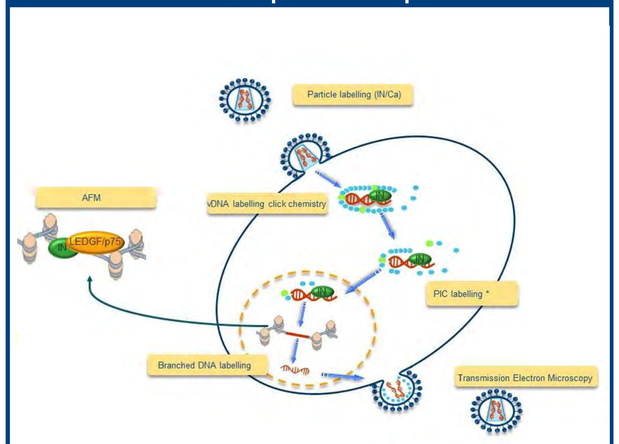
IN-mCherry and CA-eGFP complexes were quantified in HeLaP4 cells transduced with recombinant virus particles (indicated in the graphs' x-axes) at 6h post infection. **(A)** and **(B)** Measurement of mCherry- or eGFP-containing complexes in denoted intracellular locations. **(C)** Quantification of viral complexes containing both mCherry and eGFP fluorophores, indicating the level of IN-CA colocalization, in the transduced cells. **(D)** Fraction of IN-mCherry and CA-eGFP–derived signals in the colocalizing complexes (containing both fluorescent proteins) was quantified with reference to all IN- and CA-containing complexes, respectively. **(E)** Schematic representation of the data in A-D. The circle sizes correspond to the intensity of the IN-mCherry and CA-complexes (as in Figure 3).

Figure 5: PF74 a capsid modulator alters fluorescent HIV-1 complex rearrangement patterns



HeLaP4 cells were transduced with fluorescent particles (containing only IN-mCherry or both IN-mCherry and CA-eGFP), in the presence of either DMSO (vehicle control) or 2 µM PF74. Shown are intensities and average numbers of IN-mCherry **(A)** and CA-eGFP **(B)** complexes in different intracellular locations. **(C)** Confocal fluorescence images of representative cells, transduced with indicated viruses under control (DMSO) or inhibitor (PF74) conditions. Colocalizing complexes (containing both IN-mCherry and CA-eGFP) are indicated by white arrows.

Figure 6: Outlook on a imaging platform to study early HIV-1 replication steps



Outlook for our integrated HIV-1 imaging platform:
Entry of HIV-1 particles will be studied by (simultaneous) labelling of viral components, integrase (IN), capsid (CA; p24 protein) and viral DNA. By observing both labelling intensity and location of each of these components separately and in combination, we aim to unambiguously identify functional viral particles. Branched DNA technology allows to identify integrated proviral DNA as well as active transcription. High resolution technologies like TEM (transmission Electron Microscopy) and AFM (Atomic Force Microscopy) allow us to visualize viral particles and integration complexes to molecular detail. Combination of the techniques provides a clear view on the fate of viral replication in the host cell.

CONCLUSIONS

- Directly labeled CA allows single virus imaging of early replication steps and provides novel insights in the cellular distribution of CA. In agreement with others we detect CA in the nucleus, yet rarely associated with IN. Ongoing research will evaluate the significance of nuclear CA.
- Partial incorporation of CA-eGFP into HIV-1 particles has limited adverse effects on viral replication and does not interfere with virus nuclear import pathways. Incorporation is compatible with previously reported incorporation of mCherry-IN.
- IN-mCherry dissociates from CA-eGFP complexes at the nuclear envelope. The role of residual nuclear CA remains unclear.
- PF74 treatment leads to exclusion of viral particles from the nucleus demonstrating that eGFP-CA containing particles are likely conical and functional viral complexes. These manage to shed CA-eGFP molecules at the nuclear envelope concurrently with dissociation of IN and CA from each other.

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