Stimulated Emission Depletion Nanoscopy Reveals Time-Course of Human Immunodeficiency Virus Proteolytic Maturation

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ABSTRACT: Concomitant with human immunodeficiency virus type 1 (HIV-1) budding from a host cell, cleavage of the structural Gag polyproteins by the viral protease (PR) triggers complete remodeling of virion architecture. This maturation process is essential for virus infectivity. Electron tomography provided structures of immature and mature HIV-1 with a diameter of 120−140 nm, but information about the sequence and dynamics of structural rearrangements is lacking. Here, we employed super-resolution STED (stimulated emission depletion) fluorescence nanoscopy of HIV-1 carrying labeled Gag to visualize the virion architecture. The incomplete Gag lattice of immature virions was clearly distinguishable from the condensed distribution of mature protein subunits. Synchronized activation of PR within purified particles by photocleavage of a caged PR inhibitor enabled time-resolved in situ observation of the induction of proteolysis and maturation by super-resolution microscopy. This study shows the rearrangement of subviral structures in a super-resolution light microscope over time, outwitting phototoxicity and fluorophore bleaching through synchronization of a biological process by an optical switch.

KEYWORDS: HIV-1 maturation, STED nanoscopy, super-resolution microscopy, native virus imaging

Assembly of HIV-1 occurs at the plasma membrane and involves ≈2500 copies of Gag, consisting of the MA (matrix), CA (capsid), NC (nucleocapsid), and p6 domains, and ~125 copies of GagPol, comprising in addition the viral enzymes PR, reverse transcriptase (RT), and integrase (IN). PR is activated concomitant with virus release and cleaves Gag and GagPol in an ordered sequence of events, thereby releasing the functional proteins. In mature HIV-1, a homomultimer of CA forms the characteristic conical capsid around the viral genome.1,2 HIV-1 maturation is highly regulated; the timing of PR activation and processing kinetics at individual sites are important to render the virion infectious.3 The ratio of GagPol, which contains a monomeric PR subunit, to Gag and the obligate dimerization of PR regulate the timing of proteolysis, but the trigger for PR activation is currently unknown.

Increasing proteolytic activity by covalent PR dimerization4 or overexpression of GagPol5,6 leads to premature processing and abolished particle formation. It is thus conceivable that GagPol clustering within the nascent virus bud results in increased PR dimerization, thereby activating PR. However, both virion release and maturation are highly asynchronous in tissue culture, making analysis of post-assembly events difficult. Accordingly, current information is mostly based on static EM images of immature or mature particles and of variants with disabling mutations at specific Gag cleavage sites.7

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Controversial results have been reported concerning the dynamics of Gag processing and morphological maturation. Immature virions can be observed close to HIV-1-producing cells, suggesting that maturation may not be instantaneous. However, such images provide only static information, and the observed particles could be maturation defective. Conventional fluorescence imaging is unsuitable for studying maturation because substructures of HIV-1 particles (diameter ∼120−140 nm) are not resolved. An indirect, FRET-based approach suggested that proteolytic maturation of HIV-1 takes approximately 4 h, which is inconsistent with frequent detection of mature virions close to the cell and in vitro cleavage rates for peptides and recombinant Gag proteins. Recent PR inhibitor wash-out approaches allowed analysis of PR activation and Gag processing in native extracellular virions. The processing kinetics were largely dependent on inhibitor dissociation kinetics but provided an upper limit of 5 h for the half-time \( t_{1/2} \) of particles produced in the presence of the PR inhibitor amprenavir.

With a spatial resolution sufficient to visualize subviral details and the potential to image under live-cell conditions, recently developed nanoscopy techniques provide new options to investigate HIV-1 maturation. A previous study applied photoactivation localization microscopy to HIV-1 particles carrying IN fused to a tetracysteine tag to discriminate between the expected spherical arrangement of the IN domain in immature particles and a more elongated object interpreted as IN distributed within the mature core. However, this distinction required extensive image analysis, and time-resolved measurements were not possible. Determination of Env glycoprotein distribution on the surface of mature and immature particles by stimulated emission depletion (STED) microscopy revealed differences in Env clustering dependent on maturation, but the dynamics of these rearrangements could not be studied.

Here, we performed STED nanoscopy of native, unfixed HIV-1 particles to visualize the arrangement of Gag in the immature virus and to discriminate between immature and mature phenotypes. Dynamic imaging of a small set of fluorophores in a super-resolution microscope is difficult because of fluorophore bleaching and phototoxic effects that have recently been quantified for STORM imaging. To circumvent these problems, we synchronized the highly asynchronous process of HIV-1 maturation by using a recently developed photodestructible PR inhibitor that allows rapid and synchronized induction of PR activity in situ. By combining STED imaging with this opto-chemical switch, we could directly observe the time-course of Gag processing and lattice rearrangement in situ.

RESULTS AND DISCUSSION

**Gag Lattice of Single HIV Particles Is Resolved as a Ring in STED Nanoscopy.** To evaluate whether STED nanoscopy can resolve the immature Gag lattice in purified HIV-1 particles, we prepared double-labeled particles based on the nonreplication competent proviral derivative pCHIV. Cells transfected with this construct express all viral proteins except Nef and release particles that are morphologically indistinguishable from those of wild-type HIV-1. The stainable protein tag CLIP was inserted between the MA and CA domains of the polyprotein, as previously described to be compatible with infectious virus production for other tags including the very similar SNAP-tag. To label the particle exterior, the stainable protein tag SNAP coupled to a...
transmembrane (TM) domain was incorporated into the particles upon exogenous expression from a cotransfected plasmid (Figure 1a and Supporting Information Figure S1a). The core-associated protein Vpr, fused to GFP, was used as a marker to identify viral particles, and proteolytic maturation was blocked by the presence of a specific PR inhibitor during virus production. The SNAP- and CLIP-tags in released virus-like particles were stained by incubation with silicon rhodamine (SiR) coupled substrates (SiR-SNAP or SiR-CLIP, respectively).

SiR-stained particles carrying Gag.CLIP and TM.SNAP were adhered to glass coverslips and visualized by STED nanoscopy. As shown in Figure 1b,c, ring-like structures, corresponding to two-dimensional projections of spherical structures, were easily resolved in both cases (note that the z resolution of the STED implementation is significantly lower than the HIV-1 particle diameter, preventing 3D representation). For comparative measurement of ring diameters, we used particles carrying both Gag.CLIP and TM.SNAP but stained individually with either SiR-SNAP or SiR-CLIP, respectively.

For comparative measurement of ring diameters, we used particles carrying both Gag.CLIP and TM.SNAP but stained individually with either SiR-SNAP or SiR-CLIP. Diameters were determined by fitting a two-dimensional ring function to each detected object. Modeling showed that spheres with diameters greater than 88 nm can be reliably detected as rings by STED nanoscopy at a resolution of approximately 40 nm, confirming that the obtained resolution is sufficient to analyze HIV-1 particles. We introduced a correction factor to compensate for the fact that convolution of the projected spheres results in smaller apparent ring diameters (Supporting Information Figure S1b). The corrected average diameter of TM.SNAP spheres, representing the outer layer of the particle, was calculated as $d_{\text{SNAP}} = 119.4 \pm 13.8$ nm ($n = 497$), and the average diameter of the inner Gag.CLIP layer was $d_{\text{CLIP}} = 97.4 \pm 8.5$ nm ($n = 370$) (Figure 1e). These results agree well with cryo-electron tomography studies reporting an outer diameter of HIV-1 of 120−140 nm. Single-particle analysis of all rings detected for either the Gag lattice or the membrane label (Supporting Information Figure S1c) revealed a size distribution consistent with the previously observed size heterogeneity of HIV-1. Radial alignment of TM.SNAP spheres showed a closed ring-like distribution, while radially aligned Gag.CLIP spheres indicated an incomplete Gag lattice, covering only ca. 70% of the inner membrane surface (Figure 1f). This nanoscopic observation indicates that the truncated Gag sphere model derived from previous cryo-electron tomography analyses of fixed and frozen immature particles holds true for unfixed intact HIV-1 particles imaged under live conditions.

Thus, dual-color STED nanoscopy of labeled HIV-1 derivatives permitted super-resolution analysis of the arrangement of the main structural protein Gag in immature HIV-1 particles in situ. The variable size of HIV-1 particles, the average

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**Figure 2.** Mature and immature HIV-1 virions are distinguishable by STED nanoscopy. (a,b) Schematic representation (top right panels) and STED images of immature (a) or mature (b) HIV-1 particles carrying a CLIP-tag flanked by two PR cleavage sites within Gag and stained with SiR-CLIP. Immature particles were prepared from cells grown in the presence of a PR inhibitor. Arrowheads indicate particles shown in the enlargements. Scale bars: 500 nm in overviews, 100 nm in enlargements. (c) Immunoblot analysis of particles used for imaging in (a) and (b), using polyclonal sheep antiserum raised against HIV-1 CA. (d,e) Histograms (bin size $h = 0.05$) showing the distribution of hole depths ($h$) in immature (d) or mature (e) particle preparations. Ring depths were calculated as described in Materials and Methods. Dashed lines indicate the threshold used for classification as ring-like (<0.7) or condensed (>0.7) structures. (f) Bar graph showing the proportion of ring-like structures (i.e., $h < 0.7$) compared to all structures found in immature and mature images. Mean values and SD for seven different images are shown.
particle diameter, and the incompleteness of the Gag shell that we observed are all in accordance with previous electron tomography studies of fixed and frozen particles, confirming that these are inherent properties of the native virion and not induced by fixation and preparation for EM. The difference between the determined average radii of the inner and outer layers (11 nm) corresponds well to the combined distance between the determined average radii of the inner and outer layers (11 nm), the thickness of the lipid bilayer (4–6 nm), and the size of the SNAP- and CLIP-tags from the fusion site to the fluorophore position (approximately 2 nm each), highlighting the spatial accuracy of the labeling and imaging approach.

Immature and Mature HIV-1 Particles Can Be Discerned by STED Nanoscopy. A central aim of our study was to observe the transition from immature to mature HIV-1 particles under native conditions. To determine whether STED nanoscopy allows a robust distinction between these structures, we prepared immature and mature particles based on a construct carrying CLIP flanked by two PR recognition sites within Gag (pCHV-iCLIP). Addition of a specific PR inhibitor during production resulted in immature particles carrying the CLIP domain in the Gag lattice, while the released CLIP-tag is expected to distribute throughout the liquid volume of the particle in the absence of a PR inhibitor. The Gag-iCLIP processing status was confirmed by immunoblot of particle samples (Figure 2c). STED analysis of particles stained with SiR-CLIP demonstrated that immature and mature HIV-1 particles could be clearly discerned. Particles produced in the presence of an HIV PR inhibitor displayed the described ring-like phenotype (Figure 2a), while particles with active PR exhibited a condensed appearance of the SiR-CLIP signal (Figure 2b).

For classification, deconvolved particles were fitted to a two-dimensional ring function from which the relative depth (h) of the dark zone in the center of the ring was determined. For h = 0, the intensity in the center is zero. For h = 1, an even distribution of molecules within the sphere results in the center being filled and the function resembling a Gaussian distribution. The parameter h was determined for approximately 1500 individual particles from each sample. Figure 2d,e shows histograms for the distribution of h values in immature and mature particles, respectively. We observed a large proportion of particles with h close to 0 in the immature particle sample, which was absent in the mature sample. For binary classification, a threshold of h = 0.7 was set to define the phenotype as either ring-like (h ≤ 0.7) or condensed (h > 0.7). This classification yielded a total percentage of 55% ring-like structures for the immature particle preparation compared to only 11% for the mature particle preparation (Figure 2f). The proportion of structures not classified as rings in immature samples may be explained by a combination of dye aggregates on the coverslip, incompletely stained particles, and incomplete inhibition of Gag processing.

Use of a Photodegradable PR Inhibitor Enables Dynamic Analysis of HIV-1 Proteolytic Maturation in Situ. The ability to discriminate between mature and immature HIV-1 by STED nanoscopy offers the potential to visualize proteolytic maturation on a single-particle level. To test whether dynamic changes could be observed, we used a recently described photodegradable PR inhibitor ("compound 1" in ref 19, referred to here as PDI), in which a coumarin moiety can be cleaved by UV irradiation, resulting in PR activation in situ. Particles were prepared in the presence of PDI, adhered to glass chamber slides, and illuminated with a 400 nm LED source directly in the STED microscope setup to achieve synchronous PR activation (Figure 3a). Particles produced in the presence of a photostable HIV-1 PR inhibitor were used as control. Immunoblot analysis verified that particles produced in the presence of PDI contained largely uncleaved Gag-iCLIP, which was almost completely processed following photoinactivation of PDI (Figure 3b). Consistent with the observed polyprotein processing, illuminated of the PDI-inhibited particles yielded a change in morphology in

Figure 3. Induction of morphological transition in situ. (a) Diagram of the experimental workflow. (b) Immunoblot analysis of SiR-CLIP-stained HIV-1iCLIP particles produced in the presence of 2 μM PDI or 2 μM LPV as control. Particles adhered to LabTek cover slides in PR buffer were either illuminated (+) or not illuminated (−) and incubated for 2 h at room temperature. Particles were recovered from the glass surface using SDS sample buffer and analyzed by quantitative immunoblot using anti-SNAP antibody. (c) STED images of SiR-CLIP-stained HIV-1iCLIP particles produced in the presence of the indicated inhibitor without illumination or 2 h after illumination. Top and bottom images show different fields of view from the same sample. (d) STED images of HIV-1iCLIP particles stained with SiR-CLIP produced in the presence of PDI without illumination and 2 h after illumination. Top and bottom images show the same field of view. Scale bars in panels (c) and (d) represent 300 nm.
Figure 4. Dynamics of the morphological transition analyzed by STED microscopy. (a) HIV<sup>CLIP</sup> particles produced in the presence of 2 μM PDI were adhered to a LabTek chamber slide and illuminated at t = 0 in PR buffer lacking inhibitor. Samples were imaged by STED nanoscopy at the indicated time points, with a new field of view imaged at every time point. Images shown represent overlays of particles detected at the respective time (n ~ 500). Scale bar: 300 nm. (b) Time-course analysis showing the proportion of rings compared to all particles detected in the respective image. Numbers were normalized to the mean value of the proportion of rings detected at the time points before illumination. The time point of illumination is indicated by the purple line. The graphs show combined data from five independent experiments, indicated by differently colored symbols; the inset is a log-transformed representation of the same data set. Dashed curves represent an exponential fit to the data set. (c) Control time-course experiment using particles prepared in the presence of a stable PR inhibitor (three independent experiments). Data collection and representation was performed as in panel (b). (d) In-gel fluorescence analysis of HIV<sup>CLIP</sup> particles stained with Sf9-CLIP incubated in PR buffer supplemented with the indicated inhibitor for 2 h following illumination (+) or without illumination (−).

STED nanoscopy from the immature to the mature phenotype (Figure 3c), demonstrating that we were able to induce and visualize Gag proteolytic processing and consequent structural changes in situ.

We subsequently explored whether the transition from immature to mature morphology could be followed directly by STED nanoscopy of individual particles. This proved to be challenging since illumination with the STED laser for determination of the initial morphology affected subsequent PR activation and/or structural rearrangements (Supporting Information Figure S2). Addition of 4-hydroxy-TEMPO to the imaging buffer resulted in partial rescue of in situ maturation following illumination with the STED laser and allowed acquisition of STED images of the same field of view before and after photodegradation of PDI in situ. Under these experimental conditions, roughly 50% of particles underwent structural transition from the immature to the mature phenotype following PR activation (Figure 3d).

To circumvent interference of prior STED illumination with subsequent UV-induced morphological conversion, we performed quantitative time-lapse experiments by selecting a new field of view within the same sample for each time point following PR activation and quantified the proportion of ring-like and condensed structures in each image (Supporting Information Figure S3a). Initial images of particles produced in the presence of PDI comprised mostly rings as described above. Following PR activation, the proportion of rings decreased over time, whereas the population of bright condensed structures, resulting from proteolytic release of CLIP, increased (Figure 4a, corresponding to the green curve in Figure 4b; see Supporting Information Movie S1). Control particles prepared in the presence of a stable PR inhibitor retained the ring-like, immature morphology, confirming that the morphological transition of PDI-inhibited particles upon photoactivation was PR-specific (Figure 4c). Wash-out of PDI from inhibited particles without UV illumination also yielded conversion of rings to the mature-like structures (Supporting Information Figure S3b) despite very limited polyprotein processing (Figure 4d and Figure S3c). This result indicates that the morphological transition is observed upon cleavage of a limited number of Gag polyproteins, and our readout thus reflects the initial stages of maturation. Time-course analyses of the conversion of ring-like to condensed structures following photoactivation of PDI allowed us to determine the half-time of the in situ morphological transition caused by Gag polyprotein processing. Data averaged from five independent experiments were used to calculate an estimated t<sub>1/2</sub> of 29 ± 8 min (Figure 4b).

Our data are consistent with either slow gradual Gag<i>i</i>CLIP processing and CLIP redistribution or with slow activation of PR followed by rapid processing and morphological transition. To discriminate between these possibilities, we analyzed the size of the Gaussian spots over time (Supporting Information Figure S4). Control particles made in the presence of a stable PR inhibitor did not show a significant difference in spot sizes over time (Figure S4b). In contrast, the population of 2D Gaussian spots detected in the PDI-treated samples displayed a time-dependent increase in apparent mean diameter following photoactivation due to rearrangement of rings into virion-sized spots (Figure S4a,c). At later times, the apparent particle sizes decreased again, indicating redistribution of fluorophores inside the particles. This biphasic time-course is consistent with a gradual release and redistribution of the CLIP-tag, with a half-time in the range of tens of minutes.
In summary, combining dual-color STED nanoscopy of labeled HIV-1 derivatives with a photodestructible HIV PR inhibitor permitted super-resolution analysis of the time-course of morphological alterations within individual particles in situ. STED nanoscopy achieved a clear distinction between immature and mature HIV-1 BeClIP particles, revealing morphological differences consistent with the transition from an incomplete spherical shell to a more condensed distribution of the tag. Rapid in situ PR activation allowed us to synchronize HIV maturation and investigate the time-course of subviral rearrangements within assembled native virus particles by STED nanoscopy. This approach detected Gag processing and consequent structural rearrangements of the Gag lattice following proteolytic release of the ClIP-tag and appeared to be very sensitive to production of free ClIP moieties. Accordingly, the observed conversion to a condensed morphology is likely to reflect an early stage of Gag proteolysis preceding conversion to the mature capsid structure. The observed half-time of 29 ± 8 min is in excellent agreement with the half-time of Gag processing derived from immunoblot analysis of particles that underwent induced maturation based on the same photodestructible inhibitor strategy,19 suggesting that proteolysis directly induces morphological conversion without further delay. These results support the model that Gag proteolysis and redistribution of cleavage products are rate-limiting steps in HIV-1 maturation.

CONCLUSION

Our results demonstrate that morphological rearrangements following triggered PR activation can be directly monitored in situ under live conditions and on a single-particle basis. We directly observed changes of subviral HIV-1 structures in a fluorescent super-resolution microscope over time. Our study thus not only sets the stage for simultaneous imaging of Gag assembly and processing at the plasma membrane of living cells but also highlights the potential of STED nanoscopy in combination with the synchronization of biological processes by opto-chemical switches.

MATERIALS AND METHODS

Plasmids. All HIV-1 plasmids used in this study were based on the nonreplication competent derivative pHIV, which lacks the viral long terminal repeat regions and expresses all proteins from HIV-1 NL4-3, except Nef under the control of a CMV promoter.20 pCHIV-CLIP and pCHIV-ClIP variants are derivatives of the previously reported pCHIVSNAP and pCHIVSNAP, respectively,23 in which the SNAP-tag coding sequence was replaced with the coding sequence of the closely related ClIP-tag.24 derived by PCR from the plasmid pCLIPi (New England Biolabs). PR-deficient derivatives of pHIV and pCHIV-ClIP were generated by exchange of an ApaI restriction fragment to a corresponding fragment encoding a D25N mutation in the PR active site. The plasmid pTMSNAP was cloned by ligating pDisplay (Invitrogen) cleaved with BglII with a SNAP-tag encoding an NcoI/NotI fragment from the plasmid p26m after generating blunt ends by Klenow polymerase fill-in. The plasmid pEGFP.Vpr25 was kindly provided by T. Hope (Chicago, USA).

Virus Particle Preparation. HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS; Biochrom), penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37 °C with 5% CO₂. Cells (3.5 × 10⁶ per 10 cm culture dish) were seeded 1 day prior to transfection. Ten micrograms of HIV-1-derived plasmid (an equimolar mixture of SNAP or ClIP-tagged variants and the respective untagged counterpart) and 1.5 μg of pTMSNAP and/or 0.5 μg of pEGFP.Vpr, where applicable, were transfected in a 10 cm dish.

Transfections were performed using polyethylenimine according to standard procedures. For inhibitor experiments, 2 μM lopinavir (LPV) or 2 μM PDI was added to the medium at the time of transfection. At 40–44 h post-transfection, the tissue culture supernatant was filtered through a 0.45 μm nitrocellulose filter. Particles were concentrated from the supernatant by ultracentrifugation through a sucrose cushion (20% w/v in PBS) and carefully resuspended in PBS. Particles were either stored at −80 °C or processed directly for time-lapse experiments.

Immunoblot Analysis. Particle samples were resuspended in SDS sample buffer and separated by SDS-PAGE (17.5%; acrylamide/bis(acrylamide), 200:1). Proteins were transferred to a nitrocellulose membrane by semidy blotting. Proteins of interest were detected by staining with primary antibodies [polyclonal sheep anti-CA (in house) or rabbit anti-SNAP-tag (polyclonal, NEB)] and secondary antibodies [donkey-anti-sheep800, donkey-anti-rabbit700 (both LI-COR Biosciences)]. A LiCor Odyssey detection system (LI-COR Biosciences) and ImageStudioLite5.0 software (LiCor) were used for detection and signal quantification. Direct detection of SiR labeling was performed either by imaging of acrylamide gels on a Typhoon scanner (GE Healthcare Life Sciences) before blotting or by readout of the nitrocellulose membrane after blotting in channel 700 of the LiCor system.

Sample Preparation. Particles were prepared as described above. For time-lapse experiments, filtered supernatants were treated with 1 μM SiR-CLIP16 (kindly provided by K. Johnsson) for 50 min at 37 °C before ultracentrifugation. Purified particles were stained with 1 μM SiR-CLIP, SiR-SNAP (kindly provided by K. Johnsson) or 80R-SNAP27,28 (kindly provided by V. Below) for 50 min at 37 °C. Particles were adhered for 10 min to poly-1-lysine-coated LabTek 8-well chamber slides (for time-lapse experiments) or on coverslips. Coverslips were mounted in Mowiol containing DABCO17 and sealed with nail polish. LabTek chamber slides were transferred to the microscope, and the buffer was changed to 400 μL of protease buffer (50 mM MES, 150 mM NaCl, 2 mM DTT, 1 mM EDTA), supplemented with 300 μM 4-hydroxy-TEMPO (Sigma-Aldrich) with or without 2 μM LPV and immediately imaged.

Maturation Induction. Maturation of HIV-1 particles produced in the presence of 2 μM PDI was induced by illumination with an LED (λ = 400 nm, LED Engin, Inc.), run at 15 V for 3 min. Illumination was performed either directly on the LabTek chamber slide surface by placing the LED ~2.5 cm above the specimen or by pointing the light source toward the opening of an Eppendorf tube containing the sample. The light intensity imposed on the specimen was I = 91 mW/cm².

Imaging. Imaging was performed mainly at a λ = 775 nm STED system (Abberior Instruments GmbH, Göttingen, Germany) with λ = 590 and 640 nm excitation laser lines at room temperature. Nominal STED laser power was set to ~60% of the maximal power of 1250 mW (corresponding to about 220 mW in the focus, repetition rate 40 MHz) with 20–100 μs dwell time and 20 nm pixel size. For time-lapse experiments, images were acquired continuously for approximately 2 h, changing the region of interest for every image. Data shown in Figures 1 and 2 and Supporting Information Figure S1 were acquired with a previously described custom-built STED system.34 Thirty to 150 counts were maximally detected for each STED image. For particles labeled with both tags (SNAP-tag and ClIP-tag), no cross-reactivity from SiR-ClIP to the SNAP-tag was observed.

Image Analysis. Before further processing, images were linearly deconvolved with a Lorentzian function (fwhm 50 nm) using the software Inspector (Abberior Instruments GmbH).

Ring Quantification. After background subtraction, images were convolved with a Gaussian and local maxima defined to detect single bright spots. Each spot was fitted with a two-dimensional ring function.

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with fit parameters $p_1$ to $p_5$. The quality of the fit was determined by cross-correlation of the fitted and the original images. Only fits above the threshold of 0.7 were considered particles (and not clusters). The depth of the zero in the middle of ring $h = \frac{p_2 \times p_5}{\max(f)}$ was calculated from the fit parameters. If $h \leq 0.7$, the object in the image was defined as a ring; if $h > 0.7$, the object was defined as a condensed structure. From this, the relative number of rings per image was calculated and normalized. If not stated otherwise, image analysis was performed with MATLAB 2007b (The Mathworks, Inc.).

**Modeling of Ring Diameter Measuring Error.** To quantitatively measure the ring diameter, a 3D sphere with different diameters and a 5 nm thick shell was modeled. The sphere was projected on 2D and convoluted with a 40 nm Gaussian to simulate the STED resolution. Images were further processed as done for HIV-1 images (deconvolution in Impactor, ring function fitting). The measured diameter was plotted against the actual diameter, and the curve was used for correction of the measured ring diameters.

**Single-Particle Averaging.** The position of the determined ring-like or condensed distribution was revealed by the two-dimensional ring used for correction of the measured ring diameters.

**Kinetics Fit Curve.** To fit the time-lapse data, Michaelis–Menten kinetics

$$\frac{d[S]}{dt} = v = \frac{v_{\text{max}} [S]}{[S] + K_m}$$

were assumed. The proteolysis was approximated as a first-order reaction, simplifying $S(t)$ to $S(t) = S_0 \times e^{-t/\tau} + S_{\text{residual}}$. Fitting was performed in Matlab.

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