doi:10.1016/j.jmb.2011.04.062 J. Mol. Biol. (2011) 410, 501–511

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

REVIEW

Visualizing HIV-1 Assembly

Nolwenn Jouvenet[1], Sanford M. Simon[2] and Paul D. Bieniasz[1,3]*

[1]*Laboratory of Retrovirology, The Rockefeller University, 455 First Avenue, New York, NY 10016, USA*
[2]*Laboratory of Cellular Biophysics, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA*
[3]*Howard Hughes Medical Institute, Aaron Diamond AIDS Research Center, The Rockefeller University, 455 First Avenue, New York, NY 10016, USA*

Received 15 February 2011; received in revised form 25 April 2011; accepted 25 April 2011

Edited by M. F. Summers

Keywords:
HIV-1;
assembly;
RNA;
ESCRT;
imaging

The assembly of an HIV-1 particle is a complex, multistep process involving several viral and cellular proteins, RNAs and lipids. While many macroscopic and fixed-cell microscopic techniques have provided important insights into the structure of HIV-1 particles and the mechanisms by which they assemble, analysis of individual particles and their assembly in living cells offers the potential of surmounting many of the limitations inherent in other approaches. In this review, we discuss how the recent application of live-cell microscopic imaging techniques has increased our understanding of the process of HIV-1 particle assembly. In particular, we focus on recent studies that have employed total internal reflection fluorescence microscopy and other single-virion imaging techniques in live cells. These approaches have illuminated the dynamics of Gag protein assembly, viral RNA packaging and ESCRT (*e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport) protein recruitment at the level of individual viral particles. Overall, the particular advantages of individual particle imaging in living cells have yielded findings that would have been difficult or impossible to obtain using macroscopic or fixed-cell microscopic techniques.

Introduction

The assembly of an HIV-1 particle is a complex, multistep process involving several viral and cellular proteins, RNAs and lipids. Each of the components of a virus particle needs to move to the site of virion assembly, via diffusion or transport, and then interact with other virion components in specific ways as the particle is assembled.[1,2] The application of a variety of techniques from the disciplines of biochemistry, spectroscopy and structural biology, as well as various forms of microscopy, has resulted in a fairly good understanding of the molecular details of viral and cellular protein recruitment, that is, how the various components of virions bind to one another. However, some parameters of HIV-1 assembly have been challenging to define, and some have been controversial. These include the location in the cell at which particle assembly is initiated, how and when the various virion components and required cellular cofactors move to sites of particle assembly, and the overall dynamics of virion genesis.

In this review, we discuss how the recent application of live-cell microscopic imaging techniques has increased our understanding of the process of HIV-1 particle assembly. In particular,

*Corresponding author. Aaron Diamond AIDS Research Center, 455 First Avenue, New York, NY 10016, USA. E-mail address: pbienias@adarc.org.

Abbreviations used: TIR-FM, total internal reflection fluorescence microscopy; GFP, green fluorescent protein; MA, matrix; FRAP, fluorescence recovery after photobleaching; EIAV, equine infectious anemia virus; MLV, murine leukemia virus; FRET, fluorescence resonance energy transfer.

we focus on recent studies that have employed total internal reflection fluorescence microscopy (TIR-FM). These and other imaging approaches, including confocal microscopy and epifluorescence microscopy, have allowed new insights into the behavior of viral proteins in living cells and the dynamics with which HIV-1 particles are constructed in a physiologically relevant setting, that is, a living cell. The application of these techniques has allowed, for the first time, the visualization of the assembly of individual virus particles in living cells, from the initiation of particle assembly to virion release, as well as quantitative analysis of the dynamics of particle assembly.

Advantages (and Pitfalls) of Single-Particle Imaging in the Analysis of HIV-1 Assembly

Spectroscopic and biochemical methods have long been used to examine both the structure of the components of macromolecular assemblies and their interactions. Indeed, the dynamics of assembly of bacteriophage and viruses have been used as model systems for understanding macromolecular complex assembly.[3] The strength of spectroscopic and biochemical studies is their ability to obtain large signal-to-noise ratios by pooling many events into a single measurement. However, this strength also leads to a number of limitations in the kinds of information that can be gathered. A major constraint is that dynamic information is usually lost. For example, in the case of viruses such as HIV-1, the assembly of particles is generally not synchronized; thus, information about the temporal ordering of events is lost by averaging. In contrast, imaging of single virions avoids temporal averaging and the loss of information about the dynamics of individual events.

Further weaknesses of macroscopic measurements include the fact that the signal is derived from all molecules in a sample, whether or not they participate in assembly. Indeed, pulse-chase labeling experiments suggest that only a small fraction of the HIV-1 Gag that is synthesized is actually incorporated into particles.[4] This problem is potentially exacerbated when the host machinery that is involved in viral assembly is analyzed. Moreover, assembly events may be too transient to be reflected in the macroscopic measurements. This would be particularly severe if the assembly time of individual particles is short compared to the overall life span of virion components. This is likely to be a problem with imaging HIV-1 assembly, since pulse-chase labeling suggests that several hours can pass between the synthesis of an HIV-1 Gag protein and its appearance in extracellular virions,[4–6] while assembly times for individual particles have been estimated to be on the order of minutes (see the text below).

Conversely, in microscopy, if a set of criteria can be established for determining if a particular signal comes from an assembling virion, analysis of individual particles offers the potential of surmounting many of the problems of macroscopic measurements. Since individual virions do not contain sufficient material for conventional biochemical or spectroscopic analysis, microscopic imaging is employed. Tagging viral and host components with fluorophores can offer the sensitivity to study the formation of these individual virions. By taking this approach, we can assay the dynamics of the recruitment of components to an individual virion, in principle, by measuring the fluorescence associated with each component at a putative site of virion assembly.

Despite the potential advantages, there are a number of important issues to address in the general design and interpretation of imaging experiments: Do the fluorescent tags that are attached to viral or cellular components affect the process to be studied? What kind of imaging modality should be used? How is the level of fluorescence related to the number of molecules present at a particular location in the cell? How can one be confident that the fluorescent signal is actually reporting the event one wishes to study?

Almost all approaches to live-cell imaging of specific molecules involve attaching a tag, usually a fluorophore. For instance, it is common to express proteins as fusions to a fluorescent protein, such as green fluorescent protein (GFP). While such approaches have provided many biological powerful insights,[7] it is important to recognize that tagged proteins can be nonfunctional, can be dominant-negative or can fail to reflect the localization of the native protein, particularly if the fluorescently tagged protein is present at significantly-greater-than-endogenous levels, as is often the case when it is expressed by transient transfection.[8] Moreover, while the presence of a fluorescent tag on a protein has sometimes been used to quantify the number of molecules of a protein, there are a number of associated caveats. For example, untagged endogenous protein is often present in addition to the ectopically expressed fluorescent fusion protein, and the ratio of the two at the particular subcellular site being studied is generally not known. Another problem is relating the fluorescence intensity to the number of fluorophores (reviewed in Ref. 9). This can be affected by various factors, including how effectively the dipole of the fluorophore is excited and how efficiently the photons are captured by the detector, as well as by the effects of the local environment around a fluorophore, leading to differences in fluorescence, even in the absence of differences in the numbers of fluorophores.[9]

For these reasons, it is important to perform a number of control experiments to demonstrate that molecules (in this case, viral and cellular proteins that participate in particle assembly) behave authentically before embarking on a set of imaging experiments. Moreover, quantitative analyses of particle assembly should be interpreted cautiously—while it is clearly possible to track the dynamics of individual components relative to each other, quantifying the numbers of molecules present at a particular location in a cell is challenging.

Biological Properties of HIV-1 Virion Components and Imaging HIV-1 Assembly

Fluorescent images of putatively assembling HIV-1 particles have been obtained using a number of approaches, including standard fixed-cell immunofluorescence microscopy, as well as fixed-cell and live-cell imaging combined with fluorescent protein tagging,[10–13] or biarsenical–tetracysteine tagging of virion components.[14,15] In principle, a number of virion components can be labeled with fluorophores, but using the HIV-1 Gag protein has some key advantages. First, it is the most abundant protein in virion particles by a considerable margin—it is estimated that several thousand molecules of Gag are present in each virion,[16] a number that should be readily detectable by light microscopy if a reasonable fraction of the molecules is labeled with conventional fluorophores. Second, it is the only viral protein that is required to be expressed in cells to cause the formation of virus-like particles.[1] Indeed, expression of Gag alone in cells causes the formation of particles that are morphologically indistinguishable from bona fide immature virions. Third, Gag can tolerate the insertion of monomeric fluorescent proteins either at its C-terminus or at the flexible C-terminus of the matrix (MA) domain of Gag, while retaining the ability to assemble into virions.[11,13] In both cases, the morphological accuracy of particle assembly is improved by coexpressing unlabeled Gag molecules; if this precaution is taken, fluorescent virus-like particles that are morphologically indistinguishable from authentic virions can be assembled and visualized on the surface of cells using fluorescent protein fusions of Gag.[13,17] In the case of the MA insertion site, it is even possible to generate virions that are fluorescently labeled and nearly fully infectious. These sites can also tolerate the insertion of a tetracysteine tag for biarsenical fluorophore labeling, and some studies have been carried out using this approach,[14,15] although this strategy is generally reckoned to be less effective in obtaining high-quality images of particle assembly than fluorescent protein tagging.

In principle, virion components other than Gag (e.g., the Env and Vpr proteins) can also be tagged with fluorophores. However, the fraction of the total protein expressed in virion-producing cells that is associated with particles is significantly less for these virion components than it is for the Gag protein. One other key component of HIV-1 virions that can be labeled is the genomic RNA; this has been achieved by expressing fluorescent protein–RNA binding protein fusions, along with viral RNAs engineered to include multiple copies of the cognate RNA binding site.[18–20] This approach has recently been of great utility in imaging the packaging of viral genomes and in assessing when and where genomic RNA dimerization occurs in cells.

An important pitfall in imaging the HIV-1 Gag protein is that it can exist in several different states and different locations in the cell before, during and after its assembly into particles.[21–25] Light microscopic techniques lack the resolving power to determine whether a fluorescent signal is emitted by unassembled labeled Gag protein, individual virions or clustered virions. More important, if the signal that is examined is truly that of a virion in the act of assembly, then that signal is expected to be rare. A rough calculation (based on the assumption that 10^6 infected or transfected cells can produce $\sim$1 μg of p24 capsid protein per day) means that individual cells generate approximately several thousand particles per day. Current estimates of the time to assemble an HIV-1 particle are on the order of a few minutes (see the text below). Thus, at any particular time, there may be only a few dozen virions in the act of assembly (and at different stages of the process) in a background of hundreds or thousands of particles that have already assembled, as well as in a background of virion components that are not yet assembled or, perhaps, will never be assembled. When an assembly event occurs, it is but one source of fluorescent signal in a background that is much larger. Thus, the challenge is how to resolve a genuine assembly event from a fluke anecdotal observation or from events that precede or follow assembly.

It is instructive to consider the overall behavior of the Gag protein in designing a strategy to maximize the chances of observing assembly events. Infected cells, or cells expressing HIV-1 Gag protein alone, contain a pool of Gag that appears to be diffusely distributed throughout the cytoplasm,[25] along with apparent concentrations of the protein at the plasma membrane and/or at intracellular sites.[21,22,24,26,27] Experiments that have employed epifluorescence microscopy combined with fluorescence recovery after photobleaching (FRAP) and photoactivation indicate that the diffusely distributed pool of Gag is highly mobile and circulates throughout the cytoplasm within $<$60 s.[25] It is likely that this mobile pool of Gag is essentially equivalent to the cytoplasmic Gag protein that is unprocessed and behaves as a

monomer or as a low-order multimer when Gag-expressing or HIV-1-infected cells are subjected to biochemical fractionation and membrane flotation analysis.[28,29] This mobile, diffusely distributed, cytoplasmic pool of HIV-1 Gag can be reasonably assumed to represent Gag protein that has yet to be incorporated into particles. Conversely, the concentrations of Gag that are visualized at the plasma membrane or coincident with endosomal compartments could potentially represent Gag proteins that are in the act of assembling into virions. Deciding whether accumulations or concentrations of Gag represent populations of the protein before, during or after its assembly into particles is a critical factor in interpreting an imaging experiment. Indeed, this key decision has been a source of controversy over where in the cell HIV-1 assembly occurs.[2] However, several studies have revealed that endosomal accumulations arise later than concentrations at the plasma membrane,[6,21–24] and their appearance can be inhibited by blocking endocytosis in the absence of effects on extracellular particle yield.[21,22] Moreover, the accumulation of Gag protein in endosomes is exacerbated by tetherin, a restriction factor that is expressed in cells that are commonly used in imaging experiments and traps virions at the plasma membrane after they have assembled, and thereafter causes their internalization.[30] Thus, intracellular concentrations of Gag occur largely through internalization of virions that have already assembled at the cell surface. Alternatively, in macrophages, portions of the plasma membrane are deeply involuted; therefore, assembly at these areas of the plasma membrane can give the illusion that assembly occurs at intracellular compartments.[31,32] Overall, it has become quite clear that HIV-1 Gag protein accumulates first at the plasma membrane, and this finding is key for interpreting imaging experiments and for selecting optimal techniques for imaging the assembly of individual particles.

Selection of Microscopy Techniques for Imaging HIV-1 Assembly

Selecting an optimal imaging modality for the analysis of any given biological process is not always straightforward. Techniques that improve spatial resolution often do so at the expense of temporal resolution. For example, electron microscopy can resolve finer spatial detail than light microscopy, but at the cost of a complete loss of temporal resolution: the samples are fixed and, thus, not changing. Even the newest superresolution optical techniques trade spatial resolution for temporal resolution. Confocal microscopy has the advantage of providing sharper images by discarding light that is not derived from the plane of focus. However, there are two fees that must be paid for this advantage. First, since photons are being discarded, the signal is weaker; this often necessitates stronger illumination, resulting in faster photobleaching. Second, if one is observing a moving object, the narrower is the plane of focus, then the sooner the object is lost from view. Multiphoton microscopy has the advantage of allowing one to image deeper into tissue. However, it cannot achieve as fine a spatial resolution as other forms of microscopy.

A technique that has proved especially useful in the analysis of HIV-1 assembly is total internal reflection fluorescence microscopy (TIR-FM), a variation of epifluorescence microscopy.[9] TIR-FM creates an excitatory field that exponentially decays away from the coverslip with a space constant of ~70 nm. Thus, if one is studying events occurring at the surface of the cell, TIR-FM has the advantage of exciting only those fluorophores that are in close proximity to the coverslip (the ventral surface of the cell). This results in excellent spatial and temporal resolutions in imaging experiments, and the application of this approach has allowed the visualization of the genesis of individual virions.

Insights into HIV-1 Assembly from Imaging Studies

Dynamics of the assembly of Gag into particles

TIR-FM studies have yielded results that are entirely consistent with the notion that HIV-1 particle genesis is initiated and completed at the plasma membrane, both in model cell lines[33,34] and in primary macrophages, which are physiological targets of HIV-1 infection (N.J., S.M.S. and P.D.B., unpublished data). Specifically, if particles assemble on the cell surface, then they should appear one at a time, and the time course with which each individual particle appears should reflect the time course of assembly. Two key differences would be seen if virions assembled on internal membranes and were then delivered to the surface. First, a number of particles would be expected to appear together simultaneously at the plasma membrane as a vesicle containing preassembled virions fuses with it, delivering a bolus of virions to the cell surface. Second, the rate at which virions appeared would reflect the rate of delivery of vesicles to the cell surface (which would appear almost instantaneously relative to the rates that images are actually acquired during the observation of HIV-1 assembly, which occurs over several minutes).

In its basic form, monitoring the assembly of individual HIV-1 particles in TIR-FM essentially involved measuring the signal associated with a punctum of fluorescently tagged Gag protein at the

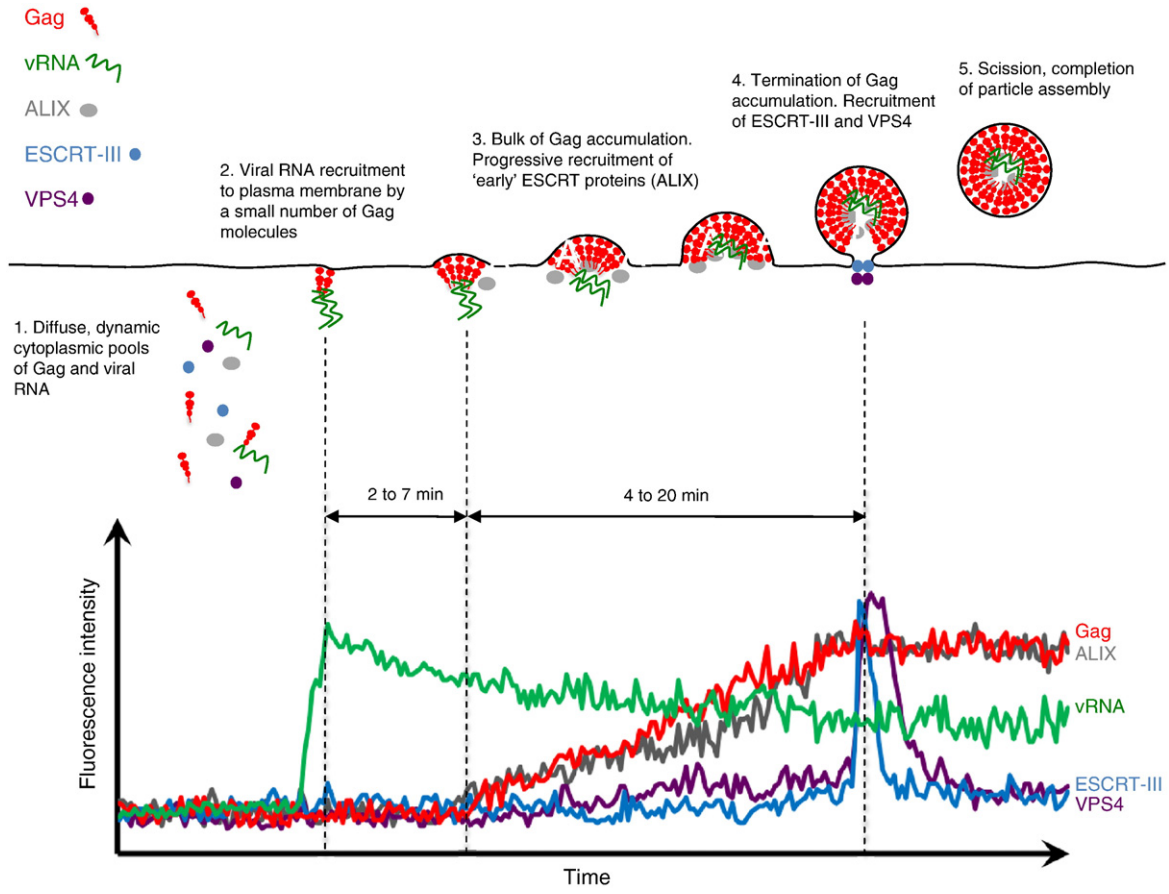

Fig. 1. Stereotypical behavior of viral and cellular components (compiled from several different actual experiments) during sequential steps in the assembly of HIV-1 and EIAV particles, imaged by TIR-FM. The fluorescence intensity over time at a site of virion particle assembly is plotted on the chart, and an interpretation of the fluorescence data is represented by the diagram. The various virion components and host factors are color-coded in the same way on the chart and the diagram [Gag, red; viral RNA (vRNA), green; ALIX, gray; ESCRT-III, blue; VPS4, purple].

plasma membrane over time.[33,34] These experiments show that following the initial detection of a punctum whose intensity is marginally greater than the surrounding diffuse Gag background, its fluorescence increases steadily until it reaches a plateau (Fig. 1). The "assembly time" is defined as the time elapsed between the initial detection of a punctum and the point at which its intensity reaches a plateau. In the context of Gag–GFP transiently expressed in HeLa cells, the mean assembly time of individual particles is approximately 8–9 min.[34] However, assembly times are quite variable and range from as little as 4 min to as much as 20 min, even within the same cell. More important, particles generated with HIV-1 Gag–GFP or with a proviral construct, in which GFP is inserted into the flexible C-terminus of MA, assemble with broadly similar kinetics.[33,34] The variation in assembly times can be explained, in part, by variation in Gag expression level, which is known or predicted to influence self-interaction between Gag molecules and its propensity to accumulate at the plasma membrane.[35] Indeed, assembly times are longer for Gag–GFP particles that appear at early times after transfection, but decrease thereafter as Gag concentration in the cell increases, stabilizing at ~5 min,[34] suggesting that the rate of assembly of individual virions is accelerated as Gag protein accumulates. Another key determinant of assembly time that likely contributes to the observed variability is the number of Gag molecules packaged into virions, which is known to vary among individual particles.[16]

The aforementioned assembly times are likely to represent underestimates of the true time required to complete assembly. This is because a certain number of Gag molecules must be already assembled in order to be detected as a nascent punctum. The number of Gag molecules required to assemble to cross this detection threshold is unknown and is dependent on the sensitivity and resolution of the imaging equipment, as well as on the "background" level of fluorescent Gag that is yet to be incorporated

into virions. Based on the detection limit of our own microscope, we estimate that this first signal of a particle, as defined by a detectable punctum of fluorescent Gag protein, is likely to occur when a few tens of molecules of Gag have assembled. The time required for these molecules to gather at the site of assembly is not defined, but studies in which the viral RNA is also imaged (see the text below) suggest that it is likely to be on the order of a few minutes.

Analogous assembly times have been measured for other retroviruses and using other techniques. Specifically, the assembly of individual equine infectious anemia virus (EIAV) Gag–GFP particles, measured with TIR-FM, occurs in a mean of 11.5 min.[36] Additionally, the assembly of individual murine leukemia virus (MLV) particles has been imaged with spinning-disk confocal microscopy.[37] Mean assembly times for MLV were measured to be 15 or 20 min depending on the specific cell line used, with variation in the range of 8–30 min.[37] Individual MLV assembly events have also been characterized by atomic force microscopy, where the progress of assembly was determined by measuring the viral protrusion height of the infected cell surface over time.[38] In these studies, the nascent virion bud grew to maximal height, presumably indicating the completion of assembly, in a mean of 20 min. Again, these assembly times are likely to represent underestimates because a detection threshold must be crossed in order to define the commencement of assembly. Nonetheless, it is noteworthy that broadly similar assembly dynamics are evident across a range of retroviruses and with a variety of imaging techniques.

Use of FRET, FRAP and photoconvertible fluorophores to monitor Gag behavior during viral assembly

A number of refinements of the aforementioned optical techniques have been applied to reveal further insight into the process of Gag assembly and to provide additional evidence that the appearance of fluorescent puncta represents genuine assembly events. For example, inserting the photoconvertible fluorescent protein mEosFP into the stalk of MA has revealed the origin of Gag proteins that are incorporated into nascent virions.[33] When excited with 405-nm light, mEosFP irreversibly photoconverts such that its emission changes from green to red. Thus, if cells are subjected to 405-nm illumination in a TIR microscope, Gag–mEosFP molecules present at the plasma membrane will have red emission thereafter. Analysis of particle assembly following a pulse of 405-nm excitation revealed that Gag–mEosFP molecules that are newly recruited to particles emit green light.[33] This strongly suggests that Gag molecules that are recruited into assembling particles do so from the rapidly diffusing cytosolic pool or from Gag molecules that have only been recently (after the 405-nm excitation) delivered to the plasma membrane.

Analyses of fluorescence resonance energy transfer (FRET) have been used to demonstrate retroviral Gag oligomerization in the cytosol and at the plasma membrane, at the scale of the whole cell,[24,39,40] suggesting that initiating events in particle assembly, specifically Gag multimerization, can occur in the cytoplasm. FRET has also been used to characterize Gag proximity during assembly of individual particles.[34] At the early stages of assembly, when Gag puncta are initially detected, the FRET coefficient between Gag–GFP and Gag–mCherry is similar to the FRET coefficient measured in areas containing only diffuse Gag–GFP and Gag–mCherry. During assembly, the FRET coefficient increases, reaching a maximum at the same time that recruitment terminates. This maximum FRET coefficient in Gag puncta on the plasma membrane is similar to FRET coefficients measured within individual cell-free particles, again providing evidence in support of the notion that Gag puncta that appear at the plasma membrane represent bona fide particles.[34]

FRAP experiments performed on assembling virus-like particles have demonstrated that the plateau that defines the end of the aforementioned assembly time genuinely represents the completion of the assembly of a particle.[34] Specifically, fluorescence recovery is observed in particles that have increasing fluorescence during the prebleach period. Conversely, Gag–GFP particles whose intensity is high and stable during the prebleach period do not recover after bleaching. Thus, particles that were recruiting Gag molecules before the bleach continued to do so postbleach; those with steady fluorescence represent particles in which Gag recruitment was completed and irreversible, the expected signature of virion assembly events.

Overall, such fluorescence-based techniques have strongly suggested that assembly of HIV-1 particles is nucleated at the plasma membrane and proceeds via a constant recruitment of Gag molecules from a rapidly diffusing cytosolic pool. These Gag molecules become closely—and eventually irreversibly—associated with each other, until some threshold is reached and Gag recruitment terminates. As such, the puncta of fluorescently tagged Gag proteins that appear at the plasma membrane exhibit several properties expected of assembling virion particles.

Behavior of viral genomic RNA during assembly of individual particles

Techniques developed for imaging the localization and movement of cellular mRNAs in living cells[41] have been applied in the analysis of HIV-1

genome packaging during particle assembly.[18–20] Specifically, an RNA binding protein from the bacteriophage MS2 is fused to GFP and a nuclear localization signal. The MS2 coat protein ordinarily recognizes a stem loop within the phage RNA with high affinity and high specificity. Thus, for visualization of viral or cellular RNA, multiple ($n=24$) copies of this stem loop are inserted into the target RNA, and the RNA is expressed in cells that also express the nuclear MS2–GFP fusion protein. Following transcription, the MS2 stem–loop-containing RNAs bind the MS2–GFP fusion protein in the nucleus, and the complexes move to the cytoplasm where they can be visualized as fluorescent puncta. For the imaging of HIV-1 genomes, the MS2 stem loops were inserted into a minimal viral genome within the *gag* intron such that only unspliced viral mRNA, which constitutes the viral genome, is labeled. More important, these modified viral genomes were found to be incorporated into virions in a packing-signal-dependent manner and to be compatible with the generation of infectious virions, suggesting that they should behave similarly to bona fide viral RNA.[18,19]

HIV-1 RNA can be readily visualized in the cytoplasm with a variety of microscopic techniques. Interestingly, there is also some evidence from this technique that HIV-1 RNA, as well as feline immunodeficiency virus RNA, accumulates at the nuclear envelope, suggesting that transport through the nuclear pore might be rate-limiting.[19] In the cytoplasm, HIV-1 RNA appears highly dynamic. Observation by TIR-FM reveals that individual HIV-1 RNA molecules move rapidly in and out of the proximity of the plasma membrane, remaining visible in the TIR field no more than a few seconds, with high and variable lateral mobility (0.05–0.5 μm/s). However, when Gag is coexpressed, a fraction of the RNA molecules dock at the membrane and remain there for several minutes, where they exhibit slower lateral drift (~0.01 μm/s).[18] Although Gag is not initially detected in association with these membrane-anchored RNAs, the fact that this behavior is not observed in the absence of Gag, or when either the Gag myristoylation signal or the viral RNA packaging signal is mutated, strongly suggests that a subdetectable number of Gag molecules are responsible for anchoring viral RNA to the plasma membrane[18] (Fig. 1). Notably, when Gag assembly is imaged in the presence of labeled viral RNA, ~75% of assembly events are observed to occur coincident with a membrane-anchored viral RNA. On average, ~4.5 min elapses between the appearance of the viral RNA at the plasma membrane and the first detection of a coincident Gag protein signal (Fig. 1). The lateral drift of the viral RNA slows further and then ceases as Gag assembly proceeds. Particle assembly appears to be necessary to irreversibly anchor the viral RNA because a Gag mutant that retains membrane-binding and RNA-binding activities but does not assemble into particles causes viral RNAs to dock at the plasma membrane, but these RNAs continue to drift laterally and then dissociate from the membrane after a mean of ~8 min. Overall, these observations indicate that an early intermediate in particle assembly is a membrane-bound complex containing viral RNA and a small number of Gag molecules, which nucleates the further recruitment of Gag protein that forms the nascent virion.

Visualizing HIV-1 budding

Imaging HIV-1 particles generated with a Gag fused to pHluorin (a GFP variant whose fluorescence is diminished at acidic pH)[42] has been used as a tool to determine whether particles have undergone scission from the cell surface.[34] This assay is based on the assumption that once virions have detached from the cell membrane, they should not be able to exchange any molecules—even protons—with the cell cytoplasm. Because the exceptionally high turnover enzyme, carbonic anhydrase, which catalyzes the reaction $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$, is highly abundant in the cytoplasm, increasing pCO_2 in cell culture medium should acidify the cytosol more rapidly efficiently than the interior of particles that have completed budding and have separated from the cytosplasm. Indeed, after a brief pulse of pCO_2, two populations of Gag–pHluorin particles on the surface of cells that differ in their sensitivity to pCO_2 are detected. The comparatively pCO_2-resistant population exhibits the same property as virion particles that are harvested from the cell-free culture supernatant and have, by definition, completed budding.[34] More important, the appearance of these pCO_2-resistant, putatively budded particles is abolished when mutations are introduced into the so-called late domain that recruits ESCRT (*e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport)) proteins.[34] While the pHluorin-based approach does not currently allow precise measurements of the time at which budding occurs, this approach does reveal that the mobility of particles does not increase, nor do they move away from the site of assembly, after budding[34] at the ventral surface–coverslip interface. Nonetheless, other investigators have used changes in particle mobility or their complete disappearance in TIR-FM experiments as a surrogate for viral budding.[33] Intuitively, the motility of particles might increase once scission has occurred, since the constraint of a membranous neck is removed. Estimates on the order of 10–15 min between the completion of Gag accumulation and particle release have been obtained using this approach.[33] However, when this criterion is used, only a small minority of particles are observed

to be released.[33] It is likely that in most cases, the movement of completely budded particles is inhibited by cell surface adhesion molecules, as well as by the limited space between the ventral surface of the cell and the coverslip (~40 nm), which is smaller than the typical diameter of a virion (~100 nm). Therefore, increases in particle mobility might be a consequence of their internalization rather than budding. This interpretation is consistent with the observation that, simultaneous with a particle beginning to move, it disappears from the evanescent field (which decays over 100 nm from the coverslip while still being visible under epifluorescent illumination, demonstrating that it is moving away from the coverslip).[33] Thus, the increased particle mobility that can sometimes be observed after assembly has been completed should be interpreted with caution, in our view.

Behavior of ESCRT proteins during the assembly of individual particles

Studies of the ESCRT proteins during retrovirus assembly have recently revealed the kinetics of recruitment of the cellular machinery required for budding.[36,43] The ESCRT machinery, which is composed of approximately 20 proteins organized into a coherent interaction network, functions in cellular membrane fission events, such as multivesicular body formation, and at the terminal stages of cytokinesis.[44,45] Thus, studies of viral budding may serve as a model for the function of the ESCRT pathway in cellular processes. The complete ESCRT machinery contains three defined protein complexes, ESCRT-I, ESCRT-II and ESCRT-III, and other associated proteins such as ALIX and the ATPase VPS4.[44,45] ESCRT proteins and complexes are soluble and are thought to be recruited at the site of membrane fission in an ordered manner, with certain components, such as the ESCRT-I complex and ALIX, acting early in the pathway, and with the ESCRT-III complex, which is composed of numerous CHMP proteins, acting later. In mammalian cells, ALIX bridges ESCRT-I to ESCRT-III proteins via direct interactions.[46–50] Recent *in vitro* data suggest that ESCRT-III proteins are responsible for the scission of the membrane neck and that VPS4 acts during or after the scission step to recycle the complex.[51]

Numerous enveloped viruses, including all retroviruses, exploit the ESCRT machinery directly or indirectly by means of specific sequences, called late domains, that mediate ESCRT protein recruitment. There are various types of late domain among retroviruses; for example, HIV-1 encodes two late domains within the p6 domain of Gag: a PTAP motif, which binds ESCRT-I,[52–54] and an LxxLF motif, which binds ALIX with relatively low affinity.[47,48] Conversely, EIAV encodes a single known late domain motif, YPDL, which binds ALIX with higher affinity.[46–48,55]

By means of stable cell lines expressing moderate levels of a subset of GFP-tagged ESCRT proteins[36] or transiently expressed VPS4,[43] the dynamics of ESCRT protein recruitment during the assembly of HIV-1 and EIAV have recently been defined with TIR-FM-based approaches. Specifically, ALIX progressively accumulates at EIAV assembly sites, with dynamics that are virtually indistinguishable from the accumulation of EIAV Gag (Fig. 1). ALIX is not observed to dissociate from assembly sites and, concordantly, ALIX is found in EIAV and HIV-1 virions.[47,48] In stark contrast, the CHMP proteins that have been examined (CHMP1b, CHMP4a and CHMP4c) are only transiently recruited to HIV-1 and EIAV assembly sites at around the time that Gag protein accumulation is completed,[36] typically remaining detectable at the site of budding for only ~2 min (Fig. 1). The VPS4 protein behaves similarly to the CHMP proteins, presumably because it is recruited by them, and is observed only transiently at the site of assembly around the time that Gag accumulation is completed.[36,43] The two completely different behaviors of the ESCRT pathway components suggest different modes of recruitment. In the case of ALIX, the amount recruited appears to be simply proportional to the number of ALIX binding sites that are present at the site of particle assembly. It is even possible that Gag and ALIX are recruited into a nascent virion as a complex that has preformed in the cytoplasm. Conversely, the CHMP and VPS4 proteins behave as if some triggering event that occurs upon termination of Gag accumulation causes their rapid recruitment. This trigger could perhaps be the accumulation of a threshold number of CHMP protein binding sites as the "early" ESCRT proteins such as ALIX accumulate to a particular level, or perhaps the attainment of a critical-degree membrane curvature. The "activation" of CHMP proteins, whereby a binding site for the next member of a CHMP protein polymer is revealed as each monomer is recruited and activated, may also play a role in this characteristic.[56] ALIX and the CHMP proteins also differ in that ALIX is not efficiently removed through the action of VPS4, while CHMP proteins and VPS4 itself dissociate from assembly sites soon after their deposition.[36] Since the recruitment of the proteins that are responsible for membrane fission occurs at the time that assembly is completed and since the proteins remain at the site of assembly for ~2 min, these lead to the hypothesis that budding occurs quite rapidly after completion of assembly. Notably, catalytically inactive forms of VPS4 that cause budding arrest lead to the accumulation of CHMP proteins at sites of particle budding, demonstrating that VPS4 activity is actually required for the release of virions and not simply for the recycling of the

CHMP proteins and the replenishment of soluble pools of ESCRT-III components.[36]

Concluding Remarks and Opportunities for Further Study

Much has been learned about the assembly of HIV-1 and other retroviruses by visualizing the genesis of individual particles in living cells. However, there are several more questions in the biology of HIV-1 assembly that might be usefully tackled with such imaging-based approaches. For example, a number of viral and cellular molecules, in addition to those already investigated, are incorporated into HIV-1 particles to enhance or inhibit viral replication (e.g., envelope, clathrin, tetherin, APOBCE3G); how and when these molecules are packaged have only been partly understood and could be illuminated by the imaging of individual particles. One key question that remains only partly resolved, and is probably best addressed by single-particle imaging studies in live cells, is whether and how HIV-1 selects specific locations on the plasma membrane at which to initiate assembly. Lipid rafts,[57–59] tetraspanin-enriched microdomains[60] and sites of cell-to-cell contact[61,62] have each been posited to be selected by HIV-1 as locations for particle assembly, but how these domains are selected remains unclear. In principle, HIV-1 Gag may contain at least two different targeting activities. Specifically, Gag binds to $PI(4,5)P_2$ (phosphatidylinositol 4,5-bisphosphate), a plasma-membrane-associated phospholipid,[63,64] and also likely binds to the cytoplasmic tail of the HIV-1 envelope protein.[65] Each of these interactions could strongly influence the location on the plasma membrane where the Gag–viral RNA complexes that initiate assembly are enriched prior to virion formation.

A number of imaging studies have indicated that HIV-1 Gag and Env proteins accumulate at sites of cell-to-cell contact, particularly in cultures of infected T cells.[61,62,66] These accumulations have been termed as "virological synapse" and have been posited to be sites at which virion particles are assembled and directly transferred from an infected cell to a target cell. Virological synapses have been primarily studied by light and electronic microscopic studies of fixed infected cells, where their formation appears to be dependent on the HIV-1 receptors in the target cell and on the HIV-1 envelope in the infected cell.[66] Some live-cell imaging of virological synapses has been performed using spinning-disk confocal microscopy techniques and a replication-competent HIV-1 molecular clone that carries a GFP at the C-terminus of MA. The transfer of fluorescent signal from a donor to a target T cell was tracked, as was infection of neighboring cells.[62] While this approach has allowed the direct visualization of the translocation of large quantities of fluorescent Gag from a donor to a target cell, this technique currently lacks the resolution to allow the tracking of individual particles through the virological synapse or to allow the conclusion that assembly is specifically directed to sites of cell-to-cell contact. However, the notion that assembly might be directed to specific regions of the plasma membrane that are advantageous for virus transfer to new target cells has been quite well developed using MLV.[37] In this instance, single-particle analyses have been used to demonstrate that assembly events occur preferentially at zones of cell-to-cell contact. This apparently directed assembly of individual particles was shown to be dependent on the cytoplasmic tail of the envelope protein and on viral receptors in the neighboring target cell, suggesting that the viral envelope protein may recruit the Gag molecules that initiate assembly.[37]

Overall, the particular advantages of individual particle imaging in live cells have yielded findings that would have been difficult or impossible to obtain using macroscopic or fixed-cell microscopic techniques. Moreover, these techniques should be applicable to many other viruses that assemble at the plasma membrane. With further technological and conceptual development, a deeper understanding of virus particle assembly processes awaits.

Acknowledgements

The authors' work is supported by NIH grants: K99AI87368 (to N.J), R01 AI089844 (to SMS), R01AI50111 and RO1 AI52774 to PDB.

References

1. Gottlinger, H. G. (2001). The HIV-1 assembly machine. *AIDS*, **15**, S13–S20.
2. Bieniasz, P. D. (2009). The cell biology of HIV-1 virion genesis. *Cell Host Microbe*, **5**, 550–558.
3. Opella, S. J., Zeri, A. C. & Park, S. H. (2008). Structure, dynamics, and assembly of filamentous bacteriophages by nuclear magnetic resonance spectroscopy. *Annu. Rev. Phys. Chem.* **59**, 635–657.
4. Tritel, M. & Resh, M. D. (2000). Kinetic analysis of human immunodeficiency virus type 1 assembly reveals the presence of sequential intermediates. *J. Virol.* **74**, 5845–5855.
5. Schubert, U., Ott, D. E., Chertova, E. N., Welker, R., Tessmer, U., Princiotta, M. F. *et al.* (2000). Proteasome inhibition interferes with Gag polyprotein processing, release, and maturation of HIV-1 and HIV-2. *Proc. Natl Acad. Sci. USA*, **97**, 13057–13062.
6. Finzi, A., Orthwein, A., Mercier, J. & Cohen, E. A. (2007). Productive human immunodeficiency virus

type 1 assembly takes place at the plasma membrane. *J. Virol.* **81**, 7476–7490.
7. Tsien, R. Y. (2005). Building and breeding molecules to spy on cells and tumors. *FEBS Lett.* **579**, 927–932.
8. Rappoport, J. Z. & Simon, S. M. (2008). A functional GFP fusion for imaging clathrin-mediated endocytosis. *Traffic*, **9**, 1250–1255.
9. Simon, S. M. (2009). Partial internal reflections on total internal reflection fluorescent microscopy. *Trends Cell Biol.* **19**, 661–668.
10. Perrin-Tricaud, C., Davoust, J. & Jones, I. M. (1999). Tagging the human immunodeficiency virus Gag protein with green fluorescent protein. Minimal evidence for colocalisation with actin. *Virology*, **255**, 20–25.
11. Sandefur, S., Smith, R. M., Varthakavi, V. & Spearman, P. (2000). Mapping and characterization of the N-terminal I domain of human immunodeficiency virus type 1 Pr55(Gag). *J. Virol.* **74**, 7238–7249.
12. Hermida-Matsumoto, L. & Resh, M. D. (2000). Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. *J. Virol.* **74**, 8670–8679.
13. Muller, B., Daecke, J., Fackler, O. T., Dittmar, M. T., Zentgraf, H. & Krausslich, H. G. (2004). Construction and characterization of a fluorescently labeled infectious human immunodeficiency virus type 1 derivative. *J. Virol.* **78**, 10803–10813.
14. Rudner, L., Nydegger, S., Coren, L. V., Nagashima, K., Thali, M. & Ott, D. E. (2005). Dynamic fluorescent imaging of human immunodeficiency virus type 1 gag in live cells by biarsenical labeling. *J. Virol.* **79**, 4055–4065.
15. Gousset, K., Ablan, S. D., Coren, L. V., Ono, A., Soheilian, F., Nagashima, K. *et al.* (2008). Real-time visualization of HIV-1 GAG trafficking in infected macrophages. *PLoS Pathog.* **4**, e1000015.
16. Briggs, J. A., Simon, M. N., Gross, I., Krausslich, H. G., Fuller, S. D., Vogt, V. M. & Johnson, M. C. (2004). The stoichiometry of Gag protein in HIV-1. *Nat. Struct. Mol. Biol.* **11**, 672–675.
17. Larson, D. R., Johnson, M. C., Webb, W. W. & Vogt, V. M. (2005). Visualization of retrovirus budding with correlated light and electron microscopy. *Proc. Natl Acad. Sci. USA*, **102**, 15453–15458.
18. Jouvenet, N., Simon, S. M. & Bieniasz, P. D. (2009). Imaging the interaction of HIV-1 genomes and Gag during assembly of individual viral particles. *Proc. Natl Acad. Sci. USA*, **106**, 19114–19119.
19. Kemler, I., Meehan, A. & Poeschla, E. M. (2010). Live-cell coimaging of the genomic RNAs and Gag proteins of two lentiviruses. *J. Virol.* **84**, 6352–6366.
20. Chen, J., Nikolaitchik, O., Singh, J., Wright, A., Bencsics, C. E., Coffin, J. M. *et al.* (2009). High efficiency of HIV-1 genomic RNA packaging and heterozygote formation revealed by single virion analysis. *Proc. Natl Acad. Sci. USA*, **106**, 13535–13540.
21. Jouvenet, N., Neil, S. J., Bess, C., Johnson, M. C., Virgen, C. A., Simon, S. M. & Bieniasz, P. D. (2006). Plasma membrane is the site of productive HIV-1 particle assembly. *PLoS Biol.* **4**, e435.
22. Neil, S. J., Eastman, S. W., Jouvenet, N. & Bieniasz, P. D. (2006). HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. *PLoS Pathog.* **2**, e39.
23. Harila, K., Prior, I., Sjoberg, M., Salminen, A., Hinkula, J. & Suomalainen, M. (2006). Vpu and Tsg101 regulate intracellular targeting of the human immunodeficiency virus type 1 core protein precursor $Pr55^{gag}$. *J. Virol.* **80**, 3765–3772.
24. Hubner, W., Chen, P., Del Portillo, A., Liu, Y., Gordon, R. E. & Chen, B. K. (2007). Sequence of human immunodeficiency virus type 1 (HIV-1) Gag localization and oligomerization monitored with live confocal imaging of a replication-competent, fluorescently tagged HIV-1. *J. Virol.* **81**, 12596–12607.
25. Gomez, C. Y. & Hope, T. J. (2006). Mobility of human immunodeficiency virus type 1 $Pr55^{Gag}$ in living cells. *J. Virol.* **80**, 8796–8806.
26. Nydegger, S., Foti, M., Derdowski, A., Spearman, P. & Thali, M. (2003). HIV-1 egress is gated through late endosomal membranes. *Traffic*, **4**, 902–910.
27. Pelchen-Matthews, A., Kramer, B. & Marsh, M. (2003). Infectious HIV-1 assembles in late endosomes in primary macrophages. *J. Cell Biol.* **162**, 443–455.
28. Kutluay, S. B. & Bieniasz, P. D. Analysis of the initiating events in HIV-1 particle assembly and genome packaging. *PLoS Pathog.* 6, e1001200.
29. Paillart, J. C. & Gottlinger, H. G. (1999). Opposing effects of human immunodeficiency virus type 1 matrix mutations support a myristyl switch model of Gag membrane targeting. *J. Virol.* **73**, 2604–2612.
30. Neil, S. J., Zang, T. & Bieniasz, P. D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature*, **451**, 425–430.
31. Deneka, M., Pelchen-Matthews, A., Byland, R., Ruiz-Mateos, E. & Marsh, M. (2007). In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. *J. Cell Biol.* **177**, 329–341.
32. Welsch, S., Keppler, O. T., Habermann, A., Allespach, I., Krijnse-Locker, J. & Krausslich, H. G. (2007). HIV-1 buds predominantly at the plasma membrane of primary human macrophages. *PLoS Pathog.* **3**, e36.
33. Ivanchenko, S., Godinez, W. J., Lampe, M., Krausslich, H. G., Eils, R., Rohr, K. *et al.* (2009). Dynamics of HIV-1 assembly and release. *PLoS Pathog.* **5**, e1000652.
34. Jouvenet, N., Bieniasz, P. D. & Simon, S. M. (2008). Imaging the biogenesis of individual HIV-1 virions in live cells. *Nature*, **454**, 236–240.
35. Perez-Caballero, D., Hatziioannou, T., Martin-Serrano, J. & Bieniasz, P. D. (2004). Human immunodeficiency virus type 1 matrix inhibits and confers cooperativity on Gag precursor–membrane interactions. *J. Virol.* **78**, 9560–9563.
36. Jouvenet, N., Zhadina, M., Bieniasz, P. & Simon, S. (2011). Dynamics of ESCRT proteins recruitment during retroviral assembly. *Nat. Cell Biol.* **13**, 394–401.
37. Jin, J., Sherer, N. M., Heidecker, G., Derse, D. & Mothes, W. (2009). Assembly of the murine leukemia virus is directed towards sites of cell–cell contact. *PLoS Biol.* **7**, e1000163.
38. Gladnikoff, M. & Rousso, I. (2008). Directly monitoring individual retrovirus budding events using atomic force microscopy. *Biophys. J.* **94**, 320–326.
39. Derdowski, A., Ding, L. & Spearman, P. (2004). A novel fluorescence resonance energy transfer assay demonstrates that the human immunodeficiency virus type 1 $Pr55^{Gag}$ I domain mediates Gag–Gag interactions. *J. Virol.* **78**, 1230–1242.

40. Larson, D. R., Ma, Y. M., Vogt, V. M. & Webb, W. W. (2003). Direct measurement of Gag–Gag interaction during retrovirus assembly with FRET and fluorescence correlation spectroscopy. *J. Cell Biol.* **162**, 1233–1244.
41. Fusco, D., Accornero, N., Lavoie, B., Shenoy, S. M., Blanchard, J. M., Singer, R. H. & Bertrand, E. (2003). Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. *Curr. Biol.* **13**, 161–167.
42. Miesenbock, G., De Angelis, D. A. & Rothman, J. E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*, **394**, 192–195.
43. Baumgartel, V., Ivanchenko, S., Dupont, A., Sergeev, M., Wiseman, P. W., Krausslich, H. G. et al. Live-cell visualization of dynamics of HIV budding site interactions with an ESCRT component. *Nat. Cell Biol.* 13, 469–474.
44. Saksena, S., Sun, J., Chu, T. & Emr, S. D. (2007). ESCRTing proteins in the endocytic pathway. *Trends Biochem. Sci.* **32**, 561–573.
45. Carlton, J. G. & Martin-Serrano, J. (2009). The ESCRT machinery: new functions in viral and cellular biology. *Biochem. Soc. Trans.* **37**, 195–199.
46. Martin-Serrano, J., Yarovoy, A., Perez-Caballero, D. & Bieniasz, P. D. (2003). Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. *Proc. Natl Acad. Sci. USA*, **100**, 12414–12419.
47. von Schwedler, U. K., Stuchell, M., Muller, B., Ward, D. M., Chung, H. Y., Morita, E. *et al.* (2003). The protein network of HIV budding. *Cell*, **114**, 701–713.
48. Strack, B., Calistri, A., Craig, S., Popova, E. & Gottlinger, H. G. (2003). AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. *Cell*, **114**, 689–699.
49. Williams, R. L. & Urbe, S. (2007). The emerging shape of the ESCRT machinery. *Nat. Rev. Mol. Cell Biol.* **8**, 355–368.
50. Langelier, C., von Schwedler, U. K., Fisher, R. D., De Domenico, I., White, P. L., Hill, C. P. *et al.* (2006). Human ESCRT-II complex and its role in human immunodeficiency virus type 1 release. *J. Virol.* **80**, 9465–9480.
51. Wollert, T., Wunder, C., Lippincott-Schwartz, J. & Hurley, J. H. (2009). Membrane scission by the ESCRT-III complex. *Nature*, **458**, 172–177.
52. VerPlank, L., Bouamr, F., LaGrassa, T. J., Agresta, B., Kikonyogo, A., Leis, J. & Carter, C. A. (2001). Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). *Proc. Natl Acad. Sci. USA*, **98**, 7724–7729.
53. Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E. *et al.* (2001). Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell*, **107**, 55–65.
54. Martin-Serrano, J., Zang, T. & Bieniasz, P. D. (2001). HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. *Nat. Med.* **7**, 1313–1319.
55. Fisher, R. D., Chung, H. Y., Zhai, Q., Robinson, H., Sundquist, W. I. & Hill, C. P. (2007). Structural and biochemical studies of ALIX/AIP1 and its role in retrovirus budding. *Cell*, **128**, 841–852.
56. Zamborlini, A., Usami, Y., Radoshitzky, S. R., Popova, E., Palu, G. & Gottlinger, H. (2006). Release of autoinhibition converts ESCRT-III components into potent inhibitors of HIV-1 budding. *Proc. Natl Acad. Sci. USA*, **103**, 19140–19145.
57. Nguyen, D. H. & Hildreth, J. E. (2000). Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J. Virol.* **74**, 3264–3272.
58. Ono, A. & Freed, E. O. (2001). Plasma membrane rafts play a critical role in HIV-1 assembly and release. *Proc. Natl Acad. Sci. USA*, **98**, 13925–13930.
59. Lindwasser, O. W. & Resh, M. D. (2001). Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains. *J. Virol.* **75**, 7913–7924.
60. Nydegger, S., Khurana, S., Krementsov, D. N., Foti, M. & Thali, M. (2006). Mapping of tetraspanin-enriched microdomains that can function as gateways for HIV-1. *J. Cell Biol.* **173**, 795–807.
61. Jolly, C. & Sattentau, Q. J. (2007). Human immunodeficiency virus type 1 assembly, budding, and cell–cell spread in T cells take place in tetraspanin-enriched plasma membrane domains. *J. Virol.* **81**, 7873–7884.
62. Hubner, W., McNerney, G. P., Chen, P., Dale, B. M., Gordon, R. E., Chuang, F. Y. *et al.* (2009). Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. *Science*, **323**, 1743–1747.
63. Ono, A., Ablan, S. D., Lockett, S. J., Nagashima, K. & Freed, E. O. (2004). Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. *Proc. Natl Acad. Sci. USA*, **101**, 14889–14894.
64. Saad, J. S., Miller, J., Tai, J., Kim, A., Ghanam, R. H. & Summers, M. F. (2006). From the cover: structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. *Proc. Natl Acad. Sci. USA*, **103**, 11364–11369.
65. Wyma, D. J., Kotov, A. & Aiken, C. (2000). Evidence for a stable interaction of gp41 with Pr55(Gag) in immature human immunodeficiency virus type 1 particles. *J. Virol.* **74**, 9381–9387.
66. Jolly, C. & Sattentau, Q. J. (2004). Retroviral spread by induction of virological synapses. *Traffic*, **5**, 643–650.