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Live cell imaging of the HIV-1 life cycle

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Abstract

Technology developed in the past 10 years has dramatically increased the ability of researchers to directly visualize and measure various stages of the HIV type 1 (HIV-1) life cycle. In many cases, imaging-based approaches have filled critical gaps in our understanding of how certain aspects of viral replication occur in cells. Specifically, live cell imaging has allowed a better understanding of dynamic, transient events that occur during HIV-1 replication, including the steps involved in viral fusion, trafficking of the viral nucleoprotein complex in the cytoplasm and even the nucleus during infection and the formation of new virions from an infected cell. In this review, we discuss how researchers have exploited fluorescent microscopy methodologies to observe and quantify these events occurring during the replication of HIV-1 in living cells.

Visualizing the HIV life cycle

HIV-1 is a primate lentivirus that infects cells of the human immune system, an infection that ultimately culminates in the disease state known as AIDS. As an enveloped retrovirus, HIV-1 infection begins with the interaction of the viral envelope proteins and cell surface proteins on the surface of T cells and macrophages, which culminates in the fusion of host and viral membranes (for a review of the HIV-1 life cycle, see [1]). Following the deposition of the viral core in the host cell cytoplasm, the viral nucleoprotein complex traffics to the nucleus and the viral RNA genome is reverse transcribed into DNA, which is ultimately integrated into the host cell chromosome. This establishment of proviral infection leads to the expression of viral accessory and structural proteins, which must assemble within the target cell to form functional progeny virions capable of initiating a new round of infection. In the 25 years that have passed since the discovery of HIV-1, we have come to understand a great deal about how the virus executes its life cycle. During this time, our appreciation of various stages of the viral life cycle has coincided with the development of new techniques and technologies that have facilitated novel lines of investigation. The advances afforded by fluorescent microscopy are one such example. The ability to visualize the trafficking of viral proteins in their natural context has provided the capacity to observe and understand the progression of the viral life cycle where it occurs, in living cells. Moreover, advances in imaging technology have recently begun shifting the field of fluorescent microscopy from a technique that can provide qualitative reinforcement to more classical methods to an independent assay system capable of quantifying biological phenomena. This transition has been particularly apparent in the field of live cell microscopy, with the development of novel techniques that have allowed the scientist to observe and measure dynamic cellular processes as they occur. This review focuses on how this increasing ability to both observe and measure cellular events in the context of live and fixed cell microscopy has advanced our understanding of the HIV-1 life cycle.

Viral fusion

In recent years, it has become increasingly obvious that specific regions of the membrane, known as lipid rafts, play a critical role in mediating the biological activity of membrane associated proteins. These membrane microdomains, which are rich in cholesterol, facilitate the lateral mobility of membrane proteins and are also required for efficient transmembrane signaling events in many cell types [2]. Our understanding of these regions has grown with the development of microscopy-based assays that measure the dynamic activity inherent to these membrane microdomains [3], and these methodologies have been used to examine the events and interactions that culminate in the fusion of viral and target cell membranes.

Examination of the membrane biology of the CD4 receptor and coreceptors has underscored the importance of membrane dynamics in the events leading to viral fusion. Preliminary examination revealed that CD4 and the CCR5 coreceptor localized with markers indicative of dynamic membrane activity such as actin and ezrin [4,5]. Measurement of protein trafficking to and from discrete regions of the membrane (or other areas of the cell) is ideally analyzed using fluorescence recovery after photobleaching (FRAP). FRAP involves the photobleaching of fluorescent fusion proteins in a discrete region of the cell region and measuring the rate at which fluorescence returns (or does not return) to that region [6]. It has been demonstrated, using FRAP of CD4 and CCR5 fluorescent fusion proteins, that these receptors demonstrate significant lateral mobility within the plasma membrane [7,8]. Cholesterol is required for the accumulation of CD4 and the CXCR4 coreceptor in these dynamic regions of the membrane, because cholesterol depletion inhibits receptor localization to these regions [5]. This localization was also shown to be functionally relevant, as fusion between cells expressing the HIV envelope and cells expressing CD4 and coreceptor was reduced after cholesterol depletion [5], providing evidence that the ability of these receptors to move laterally within the membrane and accumulate in specific membrane microdomains is relevant to their ability to mediate fusion with HIV-1 envelope. A more recent study confirmed the correlation between the lateral mobility of CD4 and its ability to mediate fusion. In this case, the ability of CD4 to mediate cell-cell fusion with cells expressing HIV-1 envelope was directly correlated with its mobility within the plasma membrane using FRAP analysis [9].

Another study by Yi *et al.* also found that cholesterol depletion prevents the interaction between the CD4 receptor and CCR5 coreceptor that is induced by the binding of HIV-1 envelope to CD4 [11]. These authors used fluorescence resonance energy transfer (FRET) to measure the association between a CD4 tagged with yellow fluorescent protein (CD4-YFP) and a CCR5 tagged with cyan fluorescent protein (CCR5-CFP). FRET exploits the fact that excitation of some fluorophores can transfer nonradiative energy to an acceptor fluorophore in very close proximity, such that the wavelength-specific excitation of one fluorescent protein can result in the excitation of a separate, neighboring fluorescent protein. FRET signals can therefore be used to monitor the close association (~5 nm) between proteins in cells by exciting one protein (called the donor) and measuring emission occurring from a separate protein (called the acceptor) [10]. Yi and colleagues observed that the FRET signal was induced between CD4-YFP and CCR5-CFP fusion proteins by the addition of HIV-1 envelopes to cells, but that cholesterol depletion prevented the association between these two proteins induced by HIV-1 envelope [11]. A similar observation was also made by Furuta *et al.*, who used similar donor and acceptor pairs to measure an interaction between CD4 and CCR5 occurring only in the presence of HIV-1 envelope. They also observed that this association between CD4 and CCR5, as measured by FRET, was inhibited by the addition of drugs that inhibit CCR5-mediated fusion [12]. Gaibelet and colleagues also found similar results examining FRET between CD4 tagged with blue fluorescent protein (CD4-BFP) and CCR5 tagged with a green fluorescent protein (GFP), observing that FRET

was measurably increased between these donor and acceptor proteins after the addition of CCR5 tropic envelope [13]. However, these authors also observed measurable FRET activity between CCR5 and CD4 in the absence of HIV-1 envelope protein [13], an observation seemingly at odds with the previously mentioned studies [11,12]. This discrepancy could be explained by differences in the donor and acceptor proteins, cell lines or means of analysis used by these laboratories. However, these studies collectively provide insight into the importance of receptor localization in the process of fusion. They also demonstrate the ability of live cell imaging techniques to quantitatively examine the biology of the HIV-1 life cycle.

The ability to fluorescently label the membranes of individual virions has also afforded the opportunity to monitor the process of fusion occurring between individual virions and target cells in real time. This method, first used to monitor the fusion of avian sarcoma and leukosis virus (ASLV) with target cells [14], relies on the ability to observe the mixing of target cell and fluorescently labeled viral membranes. This technique has demonstrated the existence of numerous discrete steps occurring during fusion, including an early step termed hemifusion (the mixing of membrane contents), subsequent fusion pore formation, and ultimately fusion pore enlargement, which allows the viral nucleoprotein complex to enter the target cell [14]. This ability to detect the mixing of the viral and target cell membranes during fusion was used by Markosyan and colleagues to dissect the events occurring during fusion mediated by HIV-1 envelope. These authors used both a fluorescent lipid dye (DiD) to label the viral membrane and a fluorescent nucleocapsid-GFP protein that freely diffuses away from virions after the permeabilization of virions or effective mixing of viral and cytoplasmic compartments [15]. Using this assay system, these authors could quantitatively dissect lipid mixing of membrane components from the formation of a fusion pore large enough to allow mixing of cytoplasm and viral contents. They observed that, similar to ASLV, hemifusion occurred more rapidly and before content mixing [15], demonstrating that fusion pore formation is a multistep process occurring during HIV-1 envelope fusion. This system was elegantly used to identify a temperature-sensitive intermediate of the fusion process occurring between virions possessing HIV-1 envelope and target cells expressing CD4 and coreceptor [16]. A kinetic delay in the ability of HIV-1 virions to fuse with target cells at 37°C was observed, but this delay could be overcome by first exposing cells to virions at a nonpermissive temperature that did not allow fusion to occur (23°C) and then subsequently elevating the temperature to 37°C. This preincubation at the nonpermissive temperature appears to allow the formation of ternary Env (envelope glycoprotein), CD4 and coreceptor complexes, even though fusion itself is inhibited. This idea is supported by the finding that preincubation at a non-permissive temperature reduced the efficacy of fusion inhibitors that prevent fusion by binding chemokine coreceptors [16].

Visualizing the cytoplasmic trafficking of individual virions

The ability to directly observe individual virions in target cells has provided critical insights into the biology of HIV-1 infection that had been difficult to explore using classical molecular and cellular biology techniques. This is because many of the virions that enter a target cell are nonspecifically endocytosed and never enter the cytoplasm of target cells by envelope-mediated fusion, making the activity of the small population of relevant viral cores difficult to assay. Moreover, biologically important events might only occur in the context of intact viral cores, which are unstable and difficult to purify biochemically. This obstacle has been overcome by developing the ability to fluorescently label HIV-1 viral particles with a GFP-Vpr (viral protein R) fusion protein [17]. Because Vpr remains associated with the viral genome until the viral complex enters the nucleus [18], labeling individual virions with this protein allows the cytoplasmic behavior of individual viral complexes to be visualized in both live and fixed cell microscopy experiments. Such studies have benefited from the

ability to pseudotype HIV-1 virions with the pH-dependent envelope glycoprotein of vesicular stomatitis virus (VSV-g), which allows a majority of virions to productively enter the host cell, compared with the small percentage of virions that achieve productive entry using the HIV-1 envelope protein. Live cell observation of viral trafficking demonstrated that virions can traffic in the cell at speeds consistent with microtubule-mediated movement in cells, resulting in the perinuclear accumulation of viral complexes [17]. This was confirmed by the microinjection of target cells with fluorescently labeled tubulin, which allowed the trafficking of individual viral cores along microtubules to be visualized in living cells (Figure 1) [17]. This trafficking required the minus-end microtubule motor dynein, as microinjection of target cells with antidynein antibodies prevented the perinuclear accumulation of viral complexes.

A method for reliably discriminating between virions that have productively entered the target cell via fusion from those virions that have been nonspecifically endocytosed and remain within the endosomal compartment has recently been developed [19]. The ability to separate these two viral populations has allowed a more careful analysis and quantification of those virions that are actively undergoing postentry steps during infection [19–21].

The Charneau laboratory has developed an alternative method to visualize the trafficking of individual virions. These researchers inserted a tetracysteine motif into the HIV-1 integrase protein [22]. This tetracysteine motif can be bound and labeled by fluorescent biarsenical derivatives [23]. Labeling is achieved with membrane-permeable biarsenical compounds that fluoresce in either green (FlAsH) or red (ReAsH) wavelengths. The primary disadvantage of this labeling technique is a relatively high degree of background signal detectable even in the absence of proteins with introduced tetracysteine labeling motifs [24]. Arhel *et al.* overcame this obstacle by labeling virions *in vitro* and subsequently purifying them by ultracentrifugation. Using these integrase-labeled virions, they measured the trajectories assumed by these virions using live cell microscopy. They observed that virions exhibited rapid, curvilinear, saltatory trafficking, indicative of microtubule-based movement, which resulted in the perinuclear accumulation of viral complexes [22]. These authors also could visualize a decrease in the motility of viral complexes after their perinuclear deposition, which the authors suggest represents a ‘docking’ interaction between the nuclear membrane and reverse transcription complex [22]. Lastly, they could detect the accumulation of these complexes within the nuclear compartment. They observed that, inside the nucleus, movement of the viral nucleoprotein complex becomes severely restricted, and they could occasionally observe the disappearance of their integrase-labeled signal, which could represent the visualization of individual integration events during infection [22].

Another recent study used an alternative technique to label preintegration complexes (PICS). Virion incorporation of a GFP-integrase fusion protein into virions was induced by fusing the protein to Vpr [25]. A protease cleavage site between Vpr and the integrase fusion protein allows the subsequent release of the GFP-integrase construct after protease activation during viral maturation. PICs labeled with this GFP-integrase fusion protein preferentially accumulate in regions of the nucleus occupied by decondensed chromatin rather than regions containing heterochromatin [25], with the number of nuclear PICS reaching a maximum 6 h after infection. These two systems represent novel tools to better understand the factors that mediate nuclear trafficking and integration of the PIC.

Insights into restriction factor biology

Live and fixed cell microscopy techniques have also proven particularly valuable in understanding the cell biology of the retroviral restriction factor TRIM5α [26,27]. Members

of the TRIM family localize to various regions of the cytoplasm and nucleus [28], and TRIM5 α is known to multimerize into discrete regions in the cytoplasm known as cytoplasmic bodies [27]. It was originally suggested that these structures represented nonfunctional, static accumulations of protein, but live cell microscopy has revealed that these bodies are dynamic structures that appear relevant to the ability of TRIM5 α to restrict HIV-1 virions during infection. Using TRIM5 α proteins fused to GFP and YFP proteins, TRIM5 α cytoplasmic bodies were shown to utilize the microtubule cytoskeleton to traffic throughout the cell [29]. Although it is unclear if it is cytoplasmic bodies themselves or instead properties inherent to the TRIM5 α protein that allow it to dynamically multimerize into such structures that are critical to mediate restriction, the ability to visualize the interactions occurring between virions and restriction factors *in situ* has provided a better understanding of this process.

Live and fixed cell imaging techniques have also been used to directly observe the interactions that occur between TRIM5 α and HIV-1 virions to better understand the process of restriction. A stable accumulation of individual viral complexes associating with TRIM5 α cytoplasmic bodies after proteasome inhibition was subsequently observed using fixed cell microscopy [20], providing a mechanistic explanation for previous biochemical studies suggesting that proteasome function was required for a step in the restriction process. Live cell microscopy was used to observe the interactions between viral complexes and restriction factors in real time. These experiments revealed that restriction-sensitive HIV-1 viral complexes could both interact with preexisting TRIM5 α cytoplasmic bodies and induce the *de novo* formation of cytoplasmic bodies around individual virions [20].

Virus assembly and budding

The complex interactions that occur between viral and host cell proteins to affect the formation and release of individual virions has also been an area of research that live cell imaging has proved particularly useful. The HIV-1 Gag precursor protein associates with cellular membranes by virtue of a myristoylation signal and a short stretch of basic amino acids that mediate electrostatic interactions with the host cell membrane [30]. Attempts to dissect the mechanism by which Gag proteins multimerize and mediate subsequent assembly steps in living cells have benefited greatly from studies that visualize and quantify these phenomena. These approaches utilize the ability to observe the formation of virus-like particles (VLPs) in cells by tethering fluorescent markers to the viral Gag proteins. Fixed cell studies using correlative light and electron microscopy, which allows individual fluorescent punctum to be examined by electron microscopy, clearly demonstrate that fluorescent puncta observable by light microscopy are indeed budding virions [31]. However, one of the difficulties in the field has been determining if a given punctum is a developing VLP or one that has formed, been released from the cell and has become reassociated with the cell after release. This could partly explain number of conflicting reports in this area of study, and demands that all such studies be interpreted with caution.

Aspects of cell biology that govern VLP behavior in cells have been examined with microscopy. An examination of the intracellular mobility of Gag-GFP signals in cells demonstrated that the Gag-GFP protein is extremely mobile in cells, and that mobility is increased when the determinants that mediate the later stages of assembly are removed, suggesting that as the assembly process proceeds, Gag mobility becomes reduced [32]. In addition, FRAP and photoactivatable-GFP (PA-GFP) analysis revealed that the mobility of membrane-associated forms of Gag-GFP requires cholesterol [32]. PA-GFP is a GFP variant that is only weakly fluorescent before its activation by 413 nm light [33]. Activation of this protein in a discrete region of the cell allows the trafficking of the protein originally present in this region to be followed over time. Depletion of cholesterol reduced Gag-GFP or Gag-

PA-GFP motility in live cell experiments, which could be rapidly restored by cholesterol replenishment. This work, consistent with the observation that cholesterol depletion reduces viral production [34], cumulatively suggests that VLP formation can be mediated by interactions that occur as a result of the lateral mobility of membrane-associated Gag molecules in a cholesterol-dependent manner.

The Resh laboratory has used FLaSH labeling to observe Gag protein trafficking after its ribosomal translation. One of the advantages of FLaSH labeling is that labeling can be achieved concurrently with translation, without the requirement for protein maturation that can be a factor in the observation of fluorescent fusion proteins. Moreover, a combinatorial use of FLaSH and ReAsH reagents can allow the trafficking of newly synthesized protein populations to be followed. This is accomplished by labeling existing proteins in a cell with one reagent, then exposing cells to the second reagent. Under these circumstances, the second reagent used will occupy the binding sites present on newly synthesized protein and previously existing protein, which is labeled by both reagents. The Resh group has used this technique to follow newly synthesized Gag proteins from an initial, perinuclear localization to localization in a multivesicular body-like compartment before its accumulation at the plasma membrane in COS-1 cells [35]. However, Rudner *et al.* used a similar technique to observe a similar phenomenon in HeLa cells, but observed that the perinuclear loci in which newly synthesized protein appeared to be located did not contain appreciable levels of the p6^{Gag} epitope by immunostaining, leading the authors to conclude that these intracellular FLaSH-positive loci represent background staining rather than authentic Gag protein [24]. Given that Gag trafficking is known to differ significantly in different cell types, these discrepant results could either reflect that fact or result from other, more technical differences between these studies.

One of the more elegant and effective approaches used to study the interaction of Gag proteins during VLP formation has been the use of FRET to measure the interaction between Gag molecules fused to fluorescent FRET partners. Larson and colleagues first used this approach to measure the interactions between Gag proteins from Rous sarcoma virus [36]. It is worth mentioning that they observed a significant amount of Gag-Gag interaction in the cytoplasm, which increased when the membrane-targeting sequence of Gag was disrupted [36], though the case appears to be significantly different for HIV-1 Gag, based on the works described below.

Derdowski *et al.* used FRET to measure the multimerization occurring between YFP-labeled and CFP-labeled HIV-1 Gag constructs in cells during VLP formation [37]. Protein-protein interactions between the individual Gag proteins required for FRET occurred predominantly at the plasma membrane and required Gag myristoylation. Gag mutants with the G2A mutation, which prevents myristoylation, did not exhibit similar FRET activity. Furthermore, plasma membrane localization was required for Gag-Gag multimerization and measurement of FRET signal, but by itself plasma membrane localization was not sufficient to induce FRET between individual Gag proteins. Regions within the I domain of Gag, which is known to mediate the ability of Gag to multimerize [38], were required to allow plasma membrane localized Gag proteins to generate FRET signal [37]. A similar finding was made by Hubner and colleagues, who performed FRET analysis of Gag protein assembly [39]. These authors inserted a fluorescent protein between the matrix and capsid regions of the Gag precursor protein, flanked by protease cleavage sites. Unlike traditional Gag-GFP constructs, this elegant achievement allows events to be observed during the formation of infectious virions rather than VLP formation. They could also observe FRET between Gag donor and acceptor proteins, in this case iVenus and cerulean fluorescent proteins, which are recently developed variants of YFP and CFP exhibiting improved brightness and FRET characteristics [40]. FRET measurements were greatly impaired when

I domain mutations were present in Gag, consistent with the findings of Derdowski *et al.* [37].

FRET analysis has also been used to quantify interactions occurring in the final stages of viral assembly. During virus release, components of the host cell endocytic sorting machinery are required to facilitate the release of infectious virions from the target cell [41]. Tsg101 is a component of this machinery and was identified as a Gag binding protein that is required for the release of virions from cells [42]. A CFP-Gag and YFP-Tsg101 construct interacts in living cells during virus assembly. Gag expression induced Tsg101 to localize to the plasma membrane from a cytoplasmic and vesicular localization that was assumed in the absence of Gag, and this interaction was sufficient to induce FRET between these two fluorescent proteins [37].

A recent study has also used live cell imaging to examine virus production. This study used total internal reflection fluorescent microscopy (TIR-FM) to monitor the formation of VLPs at the plasma membrane [43]. By using TIR-FM, these authors were able to concentrate their analysis on assembling virions or puncta in specific regions of the plasma membrane. TIR-FM signals decay exponentially with increasing distance from the region of the plasma membrane being analyzed, allowing for exquisite sensitivity in the analyzed region [44]. The formation of individual Gag-GFP puncta could be measured, and events were divided into slow-forming and fast-forming populations. The slowly forming puncta were largely immobile compared with those that rapidly appeared. Moreover, the colocalization of puncta with fluorescently labeled endocytic proteins, such as clathrin and CD63, predominated in the rapidly appearing population, whereas the slowly developing puncta did not associate with these markers. This allowed subsequent analysis to concentrate on these slowly appearing puncta, which were concluded to represent genuine VLP assembly events. Moreover, these slowly appearing puncta shared a similar fluorescent threshold at which no additional fluorescence appeared in these puncta, a point that indicates that a complete VLP has assembled. Careful analysis of these slow appearing puncta allowed the kinetics of VLP assembly to be quantified, with an average of 8.5 min passing between the time a punctum is first visible and when its maximal fluorescence is achieved. Lastly, the final event in viral particle formation, the fission of viral and host cell membranes, could be monitored using an elegant application of a pH-dependent GFP variant called pHlourin [45]. These authors exploited the property of a pHlourin variant that loses fluorescence at low pH to observe fission events by imaging cells under conditions that lowered cytosolic pH. Using Gag-pHlourin fusions, a more dramatic quenching of assembling VLPs that remained physically connected with the cytosol could be observed. Those puncta that had completed fission exhibited a reduced response to environmental acidification. Moreover, these cytosolically exposed VLPs, as well as free cytosolic Gag, exhibited a gradual recovery during the observation period as the cell responded to the increased acidity and returned its cytosolic pH to normal. VLPs that had undergone fission, and therefore do not have the buffering capacity of the cell, did not undergo this restoration in fluorescence intensity [43]. Moreover, mutations in the late domain of Gag, which are required for the final fission events, resulted in the formation of only pH-sensitive puncta [43], demonstrating that this pH-dependent population of puncta are those assembling VLPs that had not yet budded from the producer cell. This elegant dissection of the events occurring during viral assembly demonstrates the power of quantitative, live cell imaging techniques to contribute to our understanding of the viral life cycle.

Visualization of cellular synapses

Contact between cells of the immune system results in the accumulation of numerous cell surface proteins, cytoplasmic proteins and other components at the point of cell-to-cell

contact. The ability to visualize the dynamic redistribution of cellular proteins and compartments that is induced by contact between cells of the immune system has been critical in facilitating our understanding of these events. First coined the immunological synapse [46], it is now clear that these effects of cell-to-cell contact that mediate the spatial reorganization of cell surface proteins and cytoplasmic compartments and proteins also mediate the efficient release of infectious virions onto target cells. As was the case with the immunological synapse, the ability to observe the redistribution of viral components and receptor proteins occurring as a result of cell-to-cell contact has been greatly facilitated by live and fixed cell imaging of these events.

Jolly and coworkers have demonstrated that cell-to-cell transmission of HIV-1 virions is facilitated by exactly the same types of interactions between cells that mediate the immunological synapse [47]. Using immunofluorescence microscopy, they observed that the interactions between infected T cells and uninfected T cell targets induced the accumulation of HIV-1 Env and Gag at the point of cell-to-cell contact, which they defined as the virological synapse (VS) (Figure 2a). This interaction also induced the accumulation of target cell CD4 and CXCR4 coreceptor at the virological synapse. Using imaging-based assay systems, they demonstrated that the accumulation of these molecules at the virological synapse required the actin cytoskeleton and microtubule network, and the transfer of virus to target cells and their subsequent infection was reduced when this accumulation was prevented using pharmacological inhibitors [47,48]. Chen and colleagues have subsequently demonstrated that this means of transmission could be the predominant mechanism by which viral infection occurs, estimating that VS-mediated infection is ~18 000 times more efficient than infection by cell-free virus [49].

A similar phenomenon was also observed between T cells and dendritic cells that had been exposed to HIV-1 virions. Fixed and live cell imaging was used to demonstrate that HIV-1 virions enter the endosomal compartment of dendritic cells and accumulate in regions of the cell that come in contact with neighboring T cells [50]. Imaging-based assays were also used to detect the spatial redistribution of internalized virions in dendritic cells (Figure 2b). Yu *et al.* have recently demonstrated that these internalized virions reside in a compartmentalized invagination of the plasma membrane of the dendritic cell, where they remain susceptible to membrane impermeable gp120 inhibitors [51]. They have also demonstrated the cell-to-cell transfer of these compartmentalized virions from dendritic cell to target T cells. This accumulation of virions at the point of cell-to-cell contact, coined the infectious synapse, provided a mechanistic understanding of how dendritic cells can enhance HIV-1 infection of T cells without becoming infected themselves, a process known as *trans*-infection.

Cell-to-cell transfer of virus from antigen-presenting cells has also been observed in macrophages [52]. In this case, a tetracysteine motif containing Gag construct generated by the Ott laboratory [24] was used by Goussett *et al.* to follow viral Gag protein produced after infection of macrophages [52]. Gag accumulated in multivesicular bodies during virus production (Figure 2c), and these particles contained structures recruited to the point of cell-to-cell contact in a fashion precisely resembling the synapse structures described above [47,50].

In addition to accumulating at points of cell-to-cell contact, recent reports have also demonstrated that extensions termed membrane nanotubes might also be exploited by viruses during cell-to-cell transmission of virus. These structures were first identified with live cell imaging as structures capable of facilitating the spread of murine leukemia virus [53]. More recently, these structures were shown to mediate the transport of HIV-1 Gag and Env proteins during cell-to-cell transmission (Figure 2d) [54].

These works cumulatively demonstrate the power of imaging techniques to observe and quantify critical aspects of cell biology that are simply not apparent when using more classical approaches. The ability to directly observe the spatial reorganization that defines these structures has been critical in increasing our understanding of how viral infection is facilitated by the events occurring as a consequence of cell-to-cell contact.

Concluding remarks

The ability to visualize events occurring in localized regions of cells has afforded an increased understanding of the way viral proteins interact with host cell factors to facilitate viral replication. The ability to monitor very dynamic, rapidly occurring processes in cells, and quantitatively assay them under various experimental conditions, promises to continue to provide critical insight into processes or activities not readily measurable using more classical methods. As the resolution capabilities of live cell microscopy are improved (Box 1) and as more technology is developed in various fields examining the biology of numerous cellular activities, more opportunities will become available to utilize these new technologies to gain a better understanding of how viruses interact with and manipulate our cell biology to achieve their own replication.

Box 1

Viral life cycle at a resolution of nanometers

One major limitation of using fluorescent microscopy is the inability to resolve objects as small as viruses. Although it is possible for researchers to determine that a virus is present at a particular location, the resolution afforded by fluorescent microscopes is limited by the fact that individual pixels (or, in three dimensions, voxels) represent a spatial area many times the size of a virus. Thus, even with deconvolution microscopy, between 1 and 55 virions could occupy a pixel in which a viral signal is detected. However, the generation of ultra-high resolution systems, such as the OMX system (Optical Microscope eXperimental) created by John Sedat, which can resolve images at 100 nm resolution, lowers the possible number of virions that occupy a pixel to ~1–5 (Figure I). The mechanism behind the OMX technology splits a laser light source into numerous beams that simultaneously image the specimen. This technology appears particularly amenable to live cell studies.

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*TRENDS in Microbiology***Figure 1.**

Trafficking of HIV-1 viral complexes on microtubules. In this time lapse series, human osteosarcoma cells were microinjected with fluorescently labeled tubulin protein and infected with VSV-g pseudotyped HIV-1 virus. The image sequence shows an individual viral complex (green) trafficking on the microtubule network (red). Images were taken every 10 seconds in this movie series.

Figure 2.

Visualizing the cell-to-cell contacts that mediate viral transfer. **(a)** The virological synapse visualized by Jolly and coworkers [47]. Cell-to-cell contact mediates the recruitment of CD4 receptor (red) on the target cell and viral envelope in the infected cell (green) to the VS. Scale bar, 1 μ m. **(b)** The recruitment of individual HIV-1 virions (green) in the dendritic cell (bottom) to regions of the cell in contact with a T cell (top cell), as described by McDonald *et al.* [50]. Cells are visible by nuclear (blue) and actin (red) staining. **(c)** The recruitment of FLAsH labeled Gag protein (green) expressed in primary macrophages (M) to the point of cell-to-cell contact. Scale bar, 15 μ m [51]. The left panel shows the Gag fluorescence, the middle panel shows the macrophages, and the right panel combines these images and the point of cell-to-cell contact is highlighted the boxed region. Panel **(a)** is reprinted with permission from Ref. [46], panel **(b)** is reprinted with permission from Ref. [48], and panel **(c)** is reprinted with permission from Ref. [49].

Figure I.

The power of the OMX imaging system. **(a)** Polystyrene fluorescent beads approximately the size of virions ($0.100\ \mu\text{m}$) were allowed to puddle on a #1.5 coverslip before imaging on a deconvolution fluorescence microscope. **(b)** Images were processed using constrained iterative deconvolution. **(c)** The same field of view imaged by the OMX superresolution microscope. Scale bar, $2.5\ \mu\text{m}$. Image courtesy of Applied Precision.