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Current Opinion in Virology

Live cell imaging of viral entry

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Viral entry encompasses the initial steps of infection starting from virion host cell attachment to viral genome release. Given the dynamic interactions between the virus and the host, many questions related to viral entry can be directly addressed by live cell imaging. Recent advances in fluorescent labeling of viral and cellular components, fluorescence microscopy with high sensitivity and spatiotemporal resolution, and image analysis enabled studies of a broad spectrum across many viral entry steps, including virus-receptor interactions, internalization, intracellular transport, genomic release, nuclear transport, and cell-to-cell transmission. Collectively, these live cell imaging studies have not only enriched our understandings of the viral entry mechanisms, but also provided novel insights into basic cellular biology processes.

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Introduction

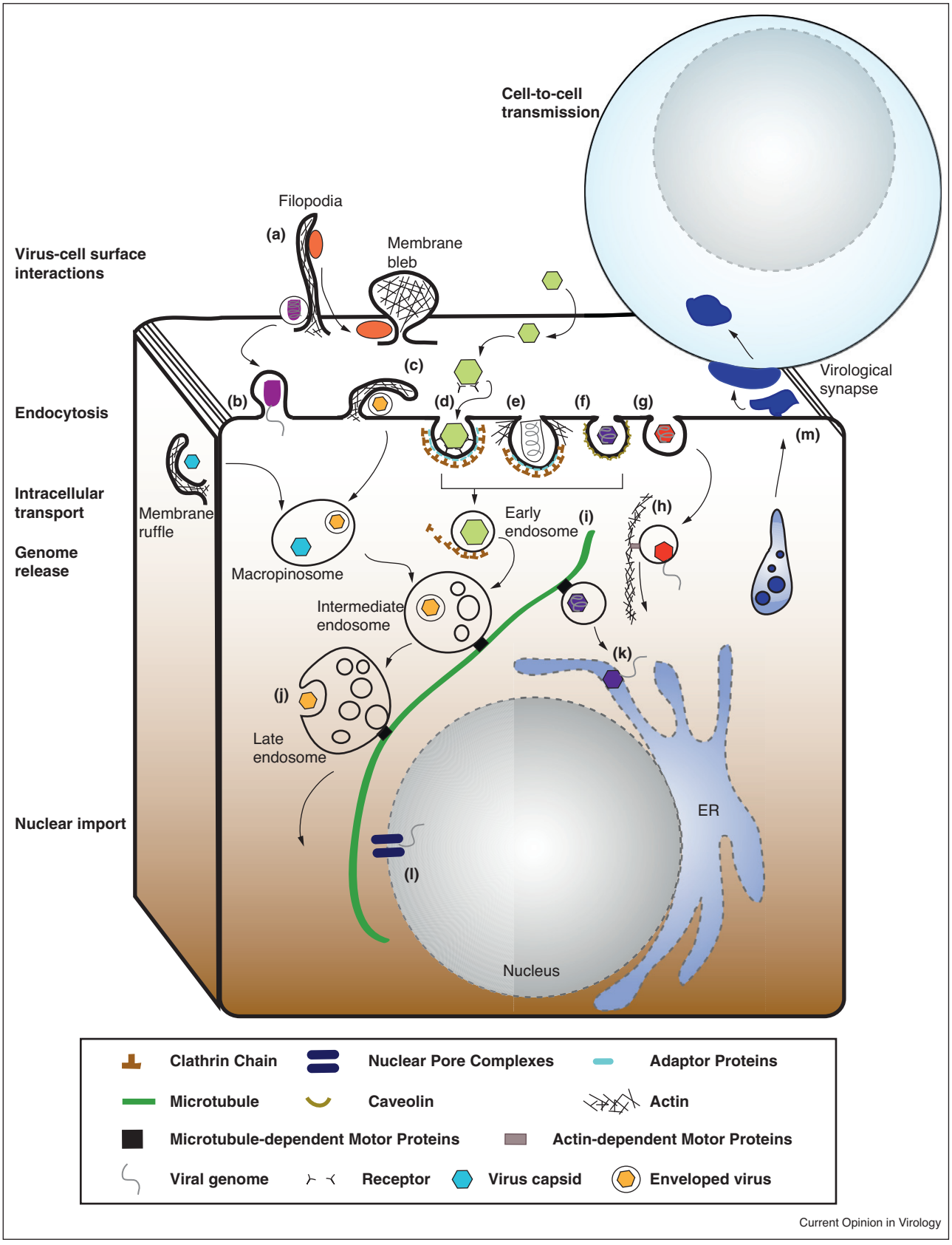
As microscopic Trojan horses, viruses usurp host cell machinery to deliver their genome into the cell for initiating productive infection. Viral entry is extremely dynamic and consists of multiple steps. The process begins with virion attachment to the cell surface, typically via low affinity electrostatic interactions with the glycocalyx, followed by specific binding to receptors. Receptor engagement allows viruses to either directly release their genome into the cell at the plasma membrane, or to enter cells through endocytosis. Endocytosed virus particles typically traffick through endosomal vesicles by actin-dependent and/or microtubule-dependent transport to rapidly navigate through the dense cytoplasm. Specific environmental cues trigger either fusion of enveloped virus with the endosome, or membrane penetration by capsid proteins for non-enveloped viruses, allowing viral genetic material to be released into the cytoplasm. For DNA viruses and a few RNA viruses, nuclear import of the viral genome precedes viral replication, protein expression, and assembly. For most RNA viruses however, subsequent steps of the infection cycle follow immediately after viral genome release (Figure 1) [1,2].

Given the dynamic and multi-step nature of viral entry, many questions can benefit from studying viral entry in live cells. For example, how does the virus initiate internalization? How are host proteins spatially and temporally recruited during viral entry? Furthermore, since viruses may enter through multiple uptake pathways, yet not all routes lead to productive infection, what are the specific entry pathways that result in successful viral genome release and where does this process happen? Answering these questions not only elucidates mechanisms of viral entry, but also — oftentimes — provides new insights into the cellular uptake pathways [2].

Live cell imaging with fluorescent microscopy offers a powerful tool for studying the dynamic viral entry events. To allow successful detection, viruses and relevant cellular components are often labeled with fluorescent probes, but cautionary steps should be taken to ensure that viral infectivity and cellular functions are not impaired by fluorescent labeling (Table 1). Depending on the purpose of study, viral membrane, capsid proteins or viral genome contents are often labeled separately or simultaneously (Table 2). A number of different imaging modalities such as confocal, total internal reflection (TIR) and Epi-illumination fluorescence microscopy have been used. Furthermore, development of image analysis algorithms has enabled researchers to track a large number of viral entry events in three-dimensions with high speed and precision [3,4,5•,6,7]. We refer the reader to several reviews on instrumentation and analysis for live cell virus tracking [3,8–10].

Live cell imaging has substantially improved our understanding of viral entry. Because of the limited space, we can highlight only a small subset of recent studies, though additional studies of interest are summarized in Table 3. The following section reviews the virological insights garnered from live cell imaging in combination with other cell biological and virological assays. We will focus on how dynamic imaging of living cells can be used

Figure 1


Cell-to-cell transmission
Virological synapse
Virus-cell surface interactions
Endocytosis
Intracellular transport
Genome release
Nuclear import
Filopodia
(a)
Membrane bleb
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
Membrane ruffle
Macropinosome
Early endosome
Intermediate endosome
Late endosome
ER
Nucleus
Clathrin Chain
Nuclear Pore Complexes
Adaptor Proteins
Microtubule
Caveolin
Actin
Microtubule-dependent Motor Proteins
Actin-dependent Motor Proteins
Viral genome
Receptor
Virus capsid
Enveloped virus
Current Opinion in Virology

to enhance our understanding of viral entry, including virus-receptor engagement, internalization, intracellular trafficking, viral genome release, and cell-to-cell transmission (Figure 1).

Virus-receptor interactions

To initiate infection, viruses typically first nonspecifically bind to attachment factors on the plasma membrane. After attachment, several viruses, such as murine polyoma virus-like particle (MPy VLP), murine leukemia virus (MLV), avian leukosis virus (ALV), human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV), vaccinia virus (VV), human papillomavirus-16 pseudovirus (HPV-16 PsV), hepatitis c virus (HCV), and herpes simplex virus type 1 (HSV-1) navigate along the cell body in an actin-dependent manner [11•,12,13••,14•,15,16]. For HSV-1, MLV, VSV, ALV, VV, HPV-16 PsV, and HCV, attachment is followed by transport along microvilli or filopodia toward the cell at a speed consistent with the actin retrograde flow [11•,13••,14•,15,16]. In addition, many viruses require actin-polymerization for plasma membrane surfing, and only exhibit firm attachment after engaging in multivalent interactions with specific receptors. Virus-induced clustering of receptors can either trigger conformational changes in the receptor-bound viral protein to prime viral genome release directly at the plasma membrane, or more commonly, transduce intracellular signaling to initiate endocytosis [2]. Because of the transient interaction between the virus and receptor molecules, the detailed mechanisms of how virus binding induces receptor clustering or signal transduction is difficult to investigate by biochemical assays. Live cell imaging of canine parvovirus (CPV) and its receptor, transferrin receptor (TfR), revealed that CPV capsids cluster limited TfRs on the cell surface and a small fraction of receptor-bound capsids rapidly diffuse into clathrin-coated pits after viral attachment. The low affinity binding between CPV capsids and TfR causes 76% of the initially bound virus particles to detach before engaging the endocytic machinery [17]. Another live-cell receptor-virus study determined how the presence or deletion of TVA receptor transmembrane domain affects avian sarcoma and leukosis virus (ASLV) internalization and fusion kinetics. The authors found that ASLV particles infecting cells expressing the full length TVA receptor exhibited an increased rate of internalization, but resulted in a longer hemifusion state than ASLV particles infecting cells expressing only the GPI-linked TVA protein. How ASLV differentially engages with the full-length or GPI-linked TVA receptor remains unclear [18•]. Additional live cell studies have been conducted to visualize receptor-virus interactions between CD81 and HCV, DC-SIGN and Uukuniemi virus (UUKV), and GM1 and simian virus 40 (SV40) [4,15,19,20]. An interesting future direction regarding virus-receptor interactions is to probe the spatiotemporal sequence of events for multiple receptor-engaging viruses such as HCV and coxsackievirus B (CVB) [2,21–23]. For CVB, viral entry begins on the apical domain with attachment to Decay Accelerating Factor (DAF), which initiates Rac-dependent actin rearrangements required for lateral transport of the virus to the tight junctions. Transport to tight junctions is required for CVB engagement with its receptor, coxsackievirus and adenovirus receptor (CAR), and for receptor-mediated endocytosis [24•]. Similar to CVB, HCV viral entry into polarized cells also begins with binding to co-receptors on the apical domain followed by lateral transport to tight junctions, the viral entry site. However, the process is even more complicated, requiring engagement with up to five different protein receptors [23]. One can expect that visualization of these complex viral entry processes will provide interesting new insights about entry dynamics.

Endocytosis/internalization of viruses

In order to enter cells, viruses hijack different cellular endocytotic pathways for internalization, among which clathrin-mediated endocytosis is commonly used, though viruses may also internalize through other pathways such as macropinocytosis, caveolar/raft-dependent endocytosis, and clathrin–caveolin/raft-independent pathways. We refer readers to several reviews on this topic [25,26,27••,28–33], and describe only some examples here. Multicolor live cell imaging offers a powerful tool to probe the orchestrated recruitment of endocytic machinery during the virus internalization process. Using this technique, researchers found that influenza A virus (IAV), reovirus, and VSV induce *de novo* assembly of clathrin machinery [34••,35–37], rather than diffusing into nascent assembling clathrin-coated pits, as exhibited by

Schematic of the different viral entry pathways. Viral entry encompasses the following steps: virus-cell surface interactions, endocytosis, intracellular transport, genome release, and in some cases, nuclear import. To begin an infection, virus particles absorb directly onto the cell surface or onto filopodia **(a)**. Cell surface interactions are typically characterized by low electrostatic interactions with the glycocalyx before specific binding with cellular receptors. Virus binding to receptors may directly lead to genome release at the plasma membrane **(b)**. More commonly, however, virus-receptor engagement induces downstream signaling events, resulting in internalization through one or more of the following pathways: macropinocytosis **(c)**, clathrin-mediated endocytosis **(d and e)**, caveolin-dependent endocytosis **(f)**, or alternative, less characterized uptake pathways **(g)**. Upon endocytosis, viruses hijack either the actin cytoskeleton **(h)** or microtubule network **(i)** to navigate through the dense cytoplasm. In addition to relying on the cytoskeleton for transport, viruses also utilize compartment-specific environmental cues, such as low pH or enzymatic cleavage, to trigger genome release through viral fusion for enveloped viruses or membrane penetration for non-enveloped viruses **(j and k)**. For DNA viruses and a few RNA viruses, genome translocation into the nucleus **(l)** precedes subsequent replication steps, while all other viruses replicate within the cytoplasm immediately after uncoating. After viral replication, some viruses such as HIV have the capability of mediating direct transfer of virions from the infected cell to another neighboring, uninfected cell **(m)**.

Table 1

Example cellular and organelle markers used previously for studying viral entry in live cell

Markers for probing virus–host interactions	Genetically encoded [reference]	Chemical label or cargo [reference]
Plasma membrane	Actin [13••], LifeAct [84]	Octadecyl rhodamine [7], cell mask, DiI
Filopodia surfing		Microinjection of fluorescently labeled phalloidin, or plasma membrane markers
Virus-receptor engagement	Virus dependent (e.g. TfnR for CPV [17], TVA for ASLV [18•])	
Endocytosis		
Clathrin-mediated endocytosis	Clathrin light chain, clathrin heavy chain [33], AP2 (e.g. σ2 subunit) [36], dynamin [34••], auxillin [34••], epsin [55•], SNX9, synaptojanin, amphiphysin [33]	Transferrin, EGF, LDL [68]
Macropinocytosis	Sorting nexin 5 [46], actin, PAK1, PI3K, Ras, Src, HDAC6 [33]	Fluid phase marker (e.g. dextran) [33]
Caveolar or lipid raft dependent	Caveolin 1 [41], src, PKC, actin [33]	Cholera toxin [33]
Phagocytosis	Dynamin [34••], actin, RhoA, RohG, Ras, PKC [27••]	Fibronectin-coated beads
Flotillin	Flotillin-1, Flotillin-2 [33]	
Arf6	Arf6, Arf1 [27••]	
Intracellular transport		
Microtubule-dependent	Tubulin [41]	Microinjected chemically labeled Tubulin [57]
Actin-dependent	Actin, cortactin, Arp3 [34••], LifeAct [84]	Microinjection of fluorescently labeled phalloidin
Endosomal trafficking		
Macropinosome	Sorting nexin 5 [46], Rab8 [33]	Fluid phase marker (e.g. dextran) [33]
Recycling endosome	Rab4, Rab11 [85]	Transferrin
Early endosomes	Rab5 [68], EEA1, Rabadaptin-5, Rabenosyn-5 [85]	
Intermediate compartment	ESCRT (e.g. Hrs) [71], Rab5 + Rab7 [68]	
Late endosome and lysosomes	Rab7 [68], Lamp, Rab9 [71], ESCRT, HOPS [64]	LysoSensor or Lysotracker
Endoplasmic reticulum	CellLight ER-GFP	ER tracker
Nuclear import	H2B [61•]	Dapi/Hoechst, DRAQ5, Syto dyes

CPV and dengue virus (DenV) [17,38]. HCV, DenV, and VSV internalize cells exclusively through clathrin-mediated endocytosis [15,34••,35,38,39], while SV40 and PyV may enter cells through a clathrin-independent and caveolin-independent pathways [40–42]. A number of viruses, including adenovirus type 35 (AdV), VV, Ebola, CVB, and IAV internalize through macropinocytosis [13••,22,28,43–46,47•]. Particularly for WB VV strain, virus binding to the cell body triggers dramatic transient membrane blebbing — a process referred to as

Table 2

Virus labeling strategies for live cell imaging

Labeling strategy	Location	Specific examples [references]
Lipophilic dye (DiI, R18, DiD)	Envelope	IAV [37,46,55•,62,68,86•,87], DenV [38], HCV [15], Ebola [46], HIV or HIV pseudovirus (HIV PsV) [57,74••,88,89], hepatitis b surface antigen particles (HBsAg particles) [63], UUKV [19], ASLV [18•], VSV [90]
Non-specific covalent linkage (NHS, hydrazine, maleimide)	Membrane protein, capsid, tegument	MPy VLP [12], HPV-16 PsV [14•], IAV [62,68,91], CPV [17], VSV [34••,35,39], reovirus [36], SV40 [40,41,53,71], PyV [42], AdV [45], AAV [54,59], SFV [58], FMDV [60], PV [7,73••], Seneca valley virus (SVV) [7], HBsAg particles [63], UUKV [19,67], echovirus-1 (EV-1) [92]
Genetically encoded FPs (GFP, mcherry, ecliptic pHluorin)	Incorporated viral or host protein	VV [13••], MLV, VSV, ALV, HIV or HIV PsV [11•,57,72,74••,77••,88,89,93], HSV-1 [16], ASLV [18•]
Genetically encoded small peptides (FlAsH)	Membrane or encapsulated protein	HIV [61•]
Enzymatic labeling (SNAP, sortase, biotin ligase)	Membrane protein	VSV-G pseudotyped lentivirus or retrovirus [94], IAV [95]
Quantum dots	Membrane protein	AAV [54], SV40 virus-like particle (SV40 VLP) [4], VSV-G pseudotyped lentivirus or retrovirus [94], IAV [95]
pH sensitive dye	Membrane protein	IAV [62], PV [73••]
Nucleic acid (intercalating dyes)	Genome	PV [73••]
Diffusible fluorescent probe	Internal viral compartment	IAV [86•]

Table 3

List of studies using live cell imaging to probe the different viral entry steps

Live cell imaging and viral entry	Virus examples [references]
Viral entry step	
Virus-cell surface interactions	
Actin-mediated mobility	MPy VLP [12], VV [13••], ALV, HIV, MLV, VSV [11•], HPV-16 PsV [14•], HCV [15], HSV-1 [16]
Virus-receptor engagement	CPV [17], HCV [15], UUKV [19], ASLV [18•]
Endocytosis	
Clathrin-mediated endocytosis	IAV [37,55•], CPV [17], reovirus [36], VSV [34••,35,39], DenV [38], AAV [54]
Macropinocytosis	VV [13••], Ebola [46], AdV [45],
Caveolar or lipid raft dependent	SV40 [41], PyV [42], AAV [54]
Other	IAV [37,55•], SV40 [40], PyV [42]
Intracellular transport	
Microtubule-dependent	IAV [62], SV40 [41], SFV [58], AAV [54,59], HIV [57,61•], FMDV [60]
Actin-dependent	PV [7], HBsAg particles [63], HIV [61•]
Endosomal trafficking/low pH trigger (yes/no)/site of membrane penetration (yes/no)	
Early endosomes	IAV/(yes/no) [68], DenV/(yes/no) [38], SV40/(yes/no) [96], pseudotyped lentivirus or retrovirus [94]
Intermediate compartment or late endosome	DenV/(yes/yes) [38], SFV/(yes/yes) [58], Ebola/(yes/yes) [46], IAV/(yes/yes) [68], UUKV/(yes/yes) [67], VSV(yes/debated) [90], SV40(yes/no) [71]
Other	PV/(no) [73••], HIV PsV/(yes) [74••,93]
Genome release	PV [73••], HIV [74••]
Nuclear import	IVA [91], HIV [57,61•]
Cell-to-cell transmission	HIV [77••]
Hemifusion and Fusion kinetics	ASLV [89], HIV PsV [72,88], IAV [87]
In vitro systems	SV40 (virus particle orientation) [4] SV40 (label free method) [20] IAV (hemifusion and fusion) [86•]

apoptotic mimicry — induced by the high phosphotidyl serine content on the virion. After triggering host cell signaling indicative of macropinocytosis, VV internalizes the cell during the bleb retraction process [13••]. However, different VV strains use distinct forms of macropinocytosis for entry, with IHD-J VV inducing filopodia on host cells rather than apoptotic mimicry [44].

Two studies on VSV entry represent intriguing examples of how large virus particles (~70 nm × 200 nm) can hijack canonical endocytic pathways, despite having dimensions larger than conventional clathrin-coated vesicles (diameter ~120 nm). Live cell imaging, in combination with electron microscopy, showed that wildtype VSV enters cells through partially coated clathrin vesicles. Complete enclosure of the VSV-containing clathrin-coated pits requires actin polymerization. Transient recruitment of actin machinery including actin, Arp3 and cortactin follow after clathrin and adaptor proteins. Drug treatments that inhibit actin polymerization reduce the efficiency of viral uptake [34••]. Interestingly, a mutant VSV with a shorter length enters cells through completely coated clathrin vesicles, and does not require actin polymerization [39]. These two studies exemplify how live cell imaging can be utilized for studying non-spherical virus entry, and future applications to highly pleomorphic, filamentous influenza and paramyxoviruses particles will likely further elucidate the entry mechanisms of these viruses [2,48–50,51•].

Many viruses such as IAV, SV40, and adeno-associated virus (AAV) enter cells through multiple endocytic routes [37,40,47•,52–54]. In some cases, blocking one pathway — for example, inhibiting clathrin-mediated endocytosis for IAV — only shunts the virus to the alternative pathway, but does not affect overall infectivity, making the study of host protein involvement in viral entry very challenging. This problem can be overcome by tracking individual virus trajectories that lead to successful infection. Using this approach, it was found that epsin 1 is recruited in synchrony with clathrin at IAV entry sites, while viruses that enter through the alternative pathway do not require epsin 1. Depleting epsin 1 or overexpressing a dominant-negative mutant only affects the clathrin-dependent entry pathway, with most of the virus particles routed through a clathrin-independent pathway. Therefore, the total amount of internalized virus particles and the viral infectivity are unaffected upon epsin 1 knockdown [55•].

Intracellular transport

Upon internalization, viruses are usually sorted into endosomal vesicles and transported toward the perinuclear region along microtubules. As shown by live cell imaging, IAV, SV40, HIV, semliki forest virus (SFV), AAV, and foot and mouth disease virus (FMDV) traffick along microtubules at a speed of several microns per second [30,41,56–60,61•,62]. Surprisingly, by tracking poliovirus (PV) after internalization, Vaughan *et al.* discovered that a

substantial number of internalized virus particles display actin-dependent fast, undirected movement with speeds of up to 5 μm/s. The unusually high actin-dependent transport speed observed in this study is nearly a magnitude greater than previously reported actin-dependent motility. Therefore, the underlying mechanism for the rapid actin-dependent transport remains unclear [7]. Recently, HbsAg particles have also been found to hijack actin for rapid intracellular transport [63]. Future work is required to determine whether this novel trafficking pattern contributes to productive infection, and what endosomal populations and molecular motors mediate the rapid actin-dependent transport.

Trafficking in endosomes and viral genome release

Cargos that are internalized through different endocytic pathways are delivered to different endosomal compartments. For example, cargos internalized through clathrin-mediated endocytosis, caveolin-mediated endocytosis and some cargos internalized through clathrin-independent and caveolin-independent pathways, are first delivered to early endosomes, before being trafficked either back to the plasma membrane directly by recycling endosomes, or to late endosomes and lysosomes for eventual degradation [64]. Cargos internalized *via* macropinocytosis are first delivered to macropinosomes before sorting to degradative or recycling endosomes [28].

Viruses have evolved mechanisms to co-opt these endosomal compartments not only for efficient navigation through the dense cytoplasm, but also to access specific intracellular environments for triggering viral genome release. Viruses including VSV and respiratory syncytial virus (RSV) undergo viral uncoating within early endosomes [35,65,66]. Many other viruses, such as IAV, Ebola, DenV, SFV, and UUKV, require trafficking from early to maturing or late endosomes before uncoating [38,46,58,62,67,68]. The pH drop during the endosomal maturation process is likely to trigger conformational changes in viral proteins that lead to viral fusion, although viruses like Ebola and severe acute respiratory syndrome coronavirus (SARS-CoV) require additional acid-dependent enzymatic cleavages [69,70]. SV40 is sorted from late endosomes to the ER, where the ER-associated degradation machinery facilitates the disruption of the capsid for viral genome release [41,71]. Live cell imaging with proper labeling schemes not only helps to detect where genome release occurs (Table 1), but also sheds light on the fundamental endosomal trafficking pathways.

Live-cell tracking of Rab5 (early endosome marker) and Rab7 (late endosome marker) shows that early endosomes consist of two populations: a dynamic population that rapidly matures into late endosomes and a static population with much slower kinetics. Interestingly, IAV and other degradative cargoes, primarily sort into the dynamic population of early endosomes, while recycling cargoes such as Tfn non-discriminately sort into both populations. The study revealed that sorting signals can come from the plasma membrane rather than beginning at the early endosomes. Furthermore, tracking IAV within the dynamic population revealed that viral fusion occurs within Rab5+ and Rab7+ endosomes [68], which is different from DenV, a virus that primarily fuses within Rab7+ late endosomes [38]. Recent studies have also defined macropinocytosis as an important entry pathway for IAV [47•]. For this pathway, it remains unclear whether IAV can undergo fusion directly within macropinosomes, or whether IAV in macropinosomes eventually are sorted into degradative, low pH endosomal compartments for uncoating.

In contrast to viruses that require endocytosis for viral fusion, several enveloped viruses including HIV, HSV, sendai virus (SendV), and a number of non-enveloped viruses such as PV, have been reported to release their genome at the plasma membrane [29,72]. These viruses often rely on viral protein-receptor conformational changes that are usually pH-independent. However, endocytosis of these viruses is often observed, making it difficult to identify the productive entry pathway. Single-virus tracking provides an ideal approach to directly probe the exact viral genome release site. Using this approach, PV RNA release was shown to occur rapidly near the plasma membrane by using Syto82 to label the vRNA and Cypher5, a pH sensitive dye, to the label the capsid. By quenching the non-internalized Cypher5 fluorescence with alkaline buffer, the authors found that PV RNA release occurs only after the virus particles internalized. Taken together, these PV studies decisively proved that PV vRNA release occurs within the cell in vesicles close to the plasma membrane [73••]. A single-virus tracking study was also performed to revisit the exact HIV entry site using membrane-labeled HIV containing a diffusible core content marker. Three categories of HIV-cell fusion events were observed: partial fusion at the plasma membrane without releasing its content, bona fide endosomal viral fusion, and a more complicated two-step fusion event with membrane mixing at the cell surface, followed by content release inside the cell. Furthermore, internalized HIV particles require dynamin before fusion within endosomal compartments, which likely leads to productive infection [74••,75]. Future work using similar assays will help to identify the sites of genome release for other viruses that do not require low pH for membrane fusion or penetration.

Nuclear import

After delivery into the cytoplasm, the viral genome contents of DNA viruses and a few RNA viruses must undergo nuclear import to initiate subsequent steps of

the infection. A recent study using FlAsH-labeled integrase incorporated into infectious HIV-1 particles showed that the HIV-1 complexes sequentially exhibit four distinct stages of movement: first, microtubule-dependent transport; second, actin-directed trafficking; third, confined mobility upon docking at the nuclear membrane; and fourth, diffusive intranuclear movement [61•]. Interestingly, data suggest that HIV reverse transcription may already occur sometime during stage 1 movement [57]. In another study, Strunze *et al.* found that kinesin-1 mediates the interaction between adenovirus viral capsid and nuclear pore complex components, and further disrupts the capsid to facilitate nuclear import [76]. Future studies of interest will likely use similar techniques to elucidate how other viruses hijack the host cell machinery to deliver their viral genome into the nucleus.

Cell-to-cell transmission

Most live cell viral entry studies are performed with purified virus particles infecting cultured cells. However, many viruses such as HIV, human T cell leukemia virus type (HTLV-1), and VV can propagate themselves through cell-to-cell transmission [29]. Virus spread through cell-to-cell transmission may mimic pathogenesis *in vivo*. Live cell imaging provides a powerful tool to study the transmission process in real time. A recent study shed light on how HIV virus transfers through virological synapses by visualizing an infectious fluorescent HIV clone with GFP inserted within the Gag interdomain (HIV Gag-GFP). During the transmission process, time-lapse imaging revealed that Gag accumulates at the synaptic buttons after forming stable adhesion with adjacent primary T cells. Proximal to the existing buttons, Gag puncta are highly dynamic and individual puncta can penetrate, release, and infect the neighboring target cells [77••].

Conclusions

Viruses have evolved sophisticated mechanisms to hijack host cell machinery for the purpose of delivering its genome into the cell for initiating productive infection. Before the broad application of live cell imaging, many viral entry pathways had been extensively characterized. However, as discussed in this article, live cell imaging of viral entry captures the dynamic, transient, and multi-step processes unascertainable through traditional ensemble and fixed cell assays. Despite the extensive body of knowledge obtained through live cell virus imaging, the technique has its own limitations and future improvement is definitely needed. Firstly, due to the small size of virus particles, only a limited amount of fluorescent probes can be attached to the virus without impairing viral infectivity. This problem poses a challenge for studies tracking slow entry processes that require long imaging acquisition times. Furthermore, contents within the virus are typically labeled during the growth of the virus and incorporated during viral packaging [8,73••]. However, few probes exhibit sufficient brightness and stability without significantly perturbing viral assembly and subsequent infectivity. Future technical advances in fluorescent probe development and application of novel labeling schemes will prove extremely useful for real-time tracking of viral genome release in living cells, and for labeling viruses that can tolerate only a few dye molecules before affecting infectivity. Secondly, the low throughput nature of single particle live cell imaging proves a daunting barrier for scaling-up to high throughput studies. Particularly, over the recent years, a number of genome-wide studies have identified host dependency and susceptibility proteins involved in the virus infection cycle, many of which play critical roles in viral entry [78]. Given that viruses exploit a variety of cellular machineries to enter cells, live cell imaging will undoubtedly provide an indispensible tool for dissecting the dynamic roles of the identified host proteins in viral entry. Development of high-throughput and high-content live cell imaging methods for analyzing the large body of data will tremendously abet future work [79,80•]. Thirdly, given that each virus trajectory contains a wealth of information, improvements in analysis algorithms are needed for maximal extraction of information on the different types of viral transport behavior and interactions between viruses and cellular machinery in an unbiased manner. Integrating such tracking algorithms with high-throughput imaging may not only provide further insights into individual particle behavior, but also define dynamic patterns of virus-host interactions at a population level [81]. Fourthly, interactions between virus and cellular factors occur at the nanometer scale. For instance, initiation of clathrin-mediated endocytosis by viruses and other cargoes lead to a highly specific, well-orchestrated and ordered cellular protein distribution within the clathrin-coated pit [82]. Under conventional light microscopy, the precise localization of the proteins is blurred by the diffraction limit of light. The recent development of superresolution techniques that overcome the diffraction limit and provide nanometer-scale image resolution will open up a window for more detailed investigation of virus-cell interactions [83•]. Lastly, as highlighted by the visualization of HIV cell-to-cell transmission, most live cell imaging studies on virus entry are performed with tissue culture systems. Viral tracking in conditions that more accurately mimic *in vivo* conditions are likely to give additional insights in the entry process. The exciting discoveries made possible by live cell virus tracking within the recent years represent just the tip of the iceberg of knowledge that may be acquired through live cell imaging in the future. In combination with the development of new technologies and discovery of novel biological processes and unknown

viruses, future live cell viral entry studies will undoubtedly further enrich our understanding of virology and basic cellular biology.

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- • of special interest
- •• of outstanding interest

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