Chapter 5
Single-Particle Tracking of Virus Entry in Live Cells

Xiaowei Zhang, Wei Li, and Zongqiang Cui

Abstract Novel imaging technologies such as single-particle tracking provide tools to study the intricate process of virus infection in host cells. In this chapter, we provide an overview of studies in which single-particle tracking technologies were applied for the analysis of the viral entry pathways in the context of the live host cell. Single-particle tracking techniques have been dependent on advances in the fluorescent labeling microscopy method and image analysis. The mechanistic and kinetic insights offered by this technique will provide a better understanding of virus entry and may lead to a rational design of antiviral interventions.

Keywords Single-particle tracking · Virus dynamic entry · Fluorescent labeling · Microscopy configurations · Image processing

Introduction

Viruses are obligate intracellular parasites, which contain either an RNA or DNA genome surrounded by the protective virus-encoded protein coat or a membrane of cellular origin. Virus entry typically occurs in several tightly controlled, consecutive steps. These steps start with binding at the cell surface and end with the release of the genome at the site of replication. As the virus progresses in its entry program, it undergoes changes that result in events such as internalization, membrane fusion, and uncoating of the genome (Fig. 5.1). Viruses exploit cellular signals not only to induce changes in the particle but also to coordinate intracellular transport via virus–cell interactions, ensuring that each step in the entry program occurs at the right point in the sequence, at the right time, and in the right place (Marsh and Helenius 2006).

X. Zhang · W. Li · Z. Cui (✉)
State Key Laboratory of Virology, Wuhan Institute of Virology, Center for Biosafety Mega-Science, Chinese Academy of Sciences, Wuhan, China

University of Chinese Academy of Sciences, Beijing, China
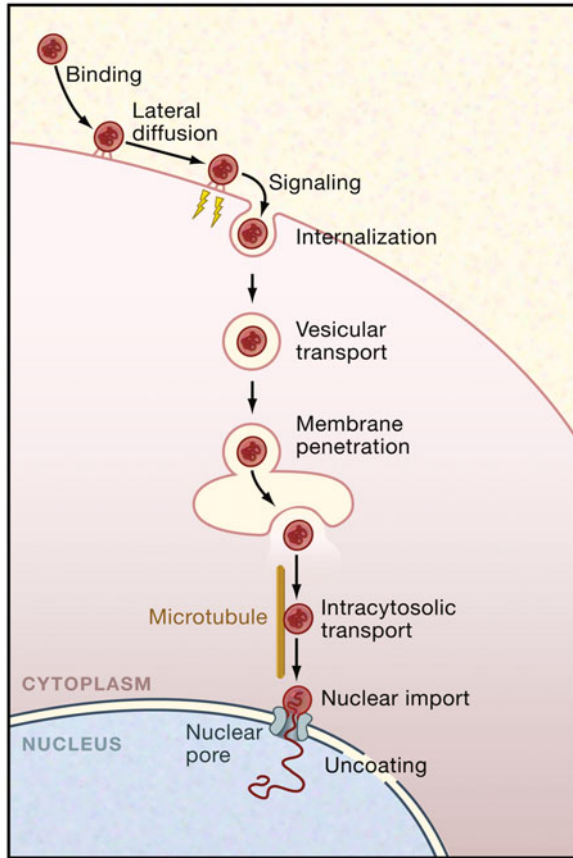e-mail: zhangxw@wh.iov.cn; liwei@wh.iov.cn; czq@wh.iov.cn

Fig. 5.1 Steps in the entry program of viruses. Whether enveloped or non-enveloped, many viruses depend on the host cell's endocytic pathways for entry. They follow a multistep entry and uncoating program that allows them to move from the cell periphery to the perinuclear space. In this example, the virus proceeds to deliver its uncoated genome into the nucleoplasm. The interaction between the virus and the host cell starts with virus binding to receptors on the cell surface, followed by lateral movement of the virus–receptor complexes and the induction of signals that result in the endocytic internalization of the virus particle. After trafficking and delivery into the lumen of endosomes, caveosomes, or the ER, a change in the virus conformation is induced by cellular cues. This alteration results in the penetration of the virus or its capsids through the vacuole membrane into the cytosolic compartment. Enveloped viruses use membrane fusion for penetration, whereas non-enveloped viruses induce lysis or pore formation. After targeting and transport along microtubules, the virus or the capsid binds, as in this example, to the nuclear pore complex, undergoes a final conversion, and releases the viral genome into the nucleus. The details in the entry program vary for different viruses and cell types, but many of the key steps shown here are general. Adapted with permission from reference Marsh and Helenius (2006). Copyright 2006 Elsevier (order number 5393950747869)

Single-particle tracking of virus entry in live cells captures the dynamic, transient, and multistep processes unresolved through traditional ensemble and fixed cell assays. It allows researchers to follow the fate of individual virus particles, probe dynamic interactions between viruses and cellular components, and dissect the steps of the entry program (Brandenburg and Zhuang 2007). In this chapter, we will highlight the dynamic entry of viruses and describe how to use single-particle tracking methods for elucidating individual steps of entry programs. Furthermore, in the following section, we will give an insight on how to use fluorophores for virus labeling, imaging instruments, and data analysis methods.

Dynamic Entry of Viruses into Host Cells

Viruses are obligate intracellular parasites that must gain entry into the host cells and utilize cellular machinery to survive. Virus entry is a highly dynamic motion involving multiple transient steps including binding, plasma membrane surfing, internalization/endocytosis, intracellular trafficking, and uncoating/endosomal fusion. Viruses are relatively simple in composition and lack any locomotive capacity; thus viruses evolved to exploit host cell proteins during entry. The interactions of the virus with different cellular structures during the various entry steps are dynamic, and so are the cellular structures themselves.

Virus–Receptor Interactions

Viruses first exploit cell surface receptors to initiate infection of target cells. Specific virus–receptor interactions will activate the downstream signaling cascades, and trigger the internalization of viruses or conformational alterations in viral structural proteins for genome release. The detailed processes of how viruses navigate along the cell surface to bind specific receptors are difficult to decipher in traditional ensemble measurements. Single-particle tracking provides intricate information on the transient interaction between the virus and receptor molecules, as exemplified by the different patterns of surface movement, spatio-temporal clustering of receptors, and sequential exploitation of multiple receptors.

For example, different pathways are used by Avian sarcoma and leukosis virus (ASLV) depend upon usage of two natural isoforms of its receptor. The entry of labeled ASLV into cells has been imaged using fluorescence microscopy in live cells in real time. Important differences in ASLV entry have been found, in that virus was attached to a transmembrane receptor or a lipid-anchored receptor. It revealed functional implications of ASLV entry at different rates via alternative receptors (Jha et al. 2011).

Virus Internalization

Virus binding to receptors and ensuing cellular signaling promote endocytosis or induce fusion at the plasma membrane. Most viruses hijack different cellular endocytotic pathways to enter the host cells. Endocytosis is a sequential multistep process mainly involving activation, cargo capture and sorting, endosomal organelle targeting, and specific fusion of cargoes with a target membrane (Doherty and McMahon 2009). Clathrin-mediated endocytosis is the most commonly used pathway for receptor-mediated endocytosis of viruses. In addition, the internalization of viruses may also occur via several other endocytotic pathways, such as caveolae-dependent, clathrin- and caveolae-independent endocytosis, and macropinocytosis (Mercer et al. 2010; Schelhaas 2010). On the other hand, several enveloped viruses such as human immunodeficiency virus (HIV), herpes simplex virus, and Sendai virus and a number of non-enveloped viruses including picornaviruses and polyomaviruses have been reported to release the capsids or genome directly into the cytoplasm. However, many of these viruses are also capable of utilizing endocytosis for penetration.

Single-virus tracking offers an ideal approach to discriminate the internalization pathways and pinpoint the accurate release site of viral nucleocapsids or genome. Single-virus tracking can also be combined with the co-tracking of cellular components. For example, the entry mechanism of the influenza virus has been revealed by tracking the interaction of single viruses with cellular endocytic components using fluorescence microscopy. Thus, it has been found that the influenza virus can simultaneously exploit clathrin-mediated and clathrin- and caveolin-independent endocytic pathways with similar efficiency (Rust et al. 2004).

Virus Transport

Once in the cytoplasm, viruses need to be transported to a particular subcellular location to release the genome and initiate replication. Due to the size of viruses ranging from about 20 to 400 nm and the high viscosity of the cytoplasm, polarized or directional movements of viruses toward concrete regions of cells by free diffusion are difficult (Greber and Way 2006). Therefore, viruses have evolved efficient strategies to hijack the host intracellular trafficking machinery components such as cytoskeletal tracks to transport to sites of replication within the cytosol (most RNA viruses) or the nucleus (most DNA viruses) (Hernandez-Gonzalez et al. 2021). The dynamic and constantly changing cytoskeleton may provide the force necessary to move viruses within cells. On the other hand, cytoskeleton tracks exemplified by microtubules and microfilaments may serve as a "highway" along which viruses can efficiently traffic. Transport of viruses along the cytoskeleton is mediated by microtubule motor proteins (dynein/dynactin and kinesin) or microfilament motor proteins (myosins) (Radtke et al. 2006). As a result, the transport of viral cargo molecules is a

complex and multistep process that requires the orchestration of multiple host and viral components. As shown by single-virus tracking and live cell imaging, HIV-1 was endocytosed and transported to endosomes in a clathrin- and actin-dependent manner in primary macrophages. Furthermore, the authors showed that a dynamic actin cytoskeleton plays a pivotal role in HIV-1 entry and intracellular migration in macrophages (Li et al. 2017). By using single-particle tracking, trajectories of individual, fluorescence-labeled murine polyoma virus on the surface of tissue culture cells were recorded. It has been shown that virus particles underwent free, cholesterol-dependent, lateral diffusion, rapidly followed by a period of confinement through an actin cytoskeleton-dependent mechanism (Ewers et al. 2005).

Virus Uncoating

Following internalization, viruses undergo an uncoating process to release the genome into a particular cellular compartment capable of supporting virus replication. For enveloped viruses, uncoating follows the fusion of their lipid membranes with a host membrane, in most cases of endosomal origin (Mercer et al. 2010). For non-enveloped viruses, it involves direct pore formation or disassembly to deliver the viral genome (Suomalainen and Greber 2013). Membrane fusion plays a pivotal role in the entry of enveloped viruses into cells. The merging of viral and cellular membranes is mediated by viral fusion proteins, which involves multiple sequential steps in the fusion process (Joo et al. 2010). Non-enveloped viruses undergo conformational changes upon entry into cells leading to the release of the genome, which is a stepwise process induced by cellular cues. The dynamics of these fusion and uncoating steps remain unresolved, and the trigger mechanism of some viruses is not fully understood.

Measurement of fusion/uncoating by tracking single-virus particles will allow more accurate characterization of the pathways and mechanisms by which viral entry occurs (Haldar et al. 2020). For example, many non-enveloped viruses such as poliovirus (PV) have been reported to release their genome at the plasma membrane. However, endocytosis of these viruses has also been observed, making it difficult to identify the productive entry pathway. By using the approach of single-virus tracking, it was found that PV uncoating occurs only after the internalization of virus particles (Brandenburg et al. 2007).

Fluorescent Labeling of Viruses and Cellular Components

Virus entry encompasses the initial steps of infection, from virion attachment to genome release. Fluorescent labeling of viral and cellular components and imaging in live cells enable intricate studies on this process. To perform single-virus tracking, both virions and relevant cellular components need to be labeled to visualize them in

live cells. Virions are commonly labeled by fusing fluorescent proteins to viral proteins, by incorporating organic dyes, or by incorporating fluorescent nanoparticles into the virion. Meanwhile, relevant cellular components are also often labeled with fluorescent probes, without impairing their physiological localization and cellular functions.

Fluorescent Proteins

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its homologs from diverse marine animals and its mutant variants are widely used as genetically encoded fluorescent probes (Chudakov et al. 2005). Identification and development of fluorescent proteins offer a powerful toolkit for the visualization of structural organization and dynamic processes in live cells. Up to now, fluorescent proteins of different colors and properties have been used in a variety of applications to visualize morphology, location, and movement of proteins of interest, and the emission wavelength of fluorescent proteins spans from blue to near-infrared spectrum (Chudakov et al. 2010).

Fluorescent proteins can be used as a convenient fluorescent probe to label viruses and relevant cellular components for virus tracking. Recombinant viruses encoding fluorescent fusion proteins facilitate real-time analysis of the entry motility of viruses. For example, EGFP fusions to tegument proteins, which are located between the lipid envelope and the capsid shell, have been used to follow the complex anterograde and retrograde directional movements of herpesviruses in synaptically connected neurons (Greber 2005; Luxton et al. 2005). The pseudorabies virus (PRV) protein Us9 is a small membrane protein that is highly conserved among alpha herpesviruses and is essential for the anterograde axonal spread in neurons. PRV strain that expresses functional green fluorescent protein (GFP)-Us9 fusion proteins has been constructed to visualize its anterograde direction transportation in living neurons during infection (Taylor et al. 2012; Lyman et al. 2008).

Some viruses can enter cells via the endocytosis pathway, along which the pH drops from moderately acidic early and recycling endosomes maturing into more acidic late endosomes to most acidic in the lysosomes (Sorkin and Von Zastrow 2002). Viral structural proteins will pass through acidic compartments at some point in the virus entry phase. Therefore, when using fluorescent fusion proteins to monitor viral entry in live cells, it is important to consider how fluorescent proteins will behave in these acidic compartments. The current generations of blue, cyan, and red fluorescent proteins (e.g., ECFP and mKate2) are relatively pH insensitive and will maintain relatively high fluorescence in even the most acidic intracellular compartments, like lysosomes (pH 4.5–5.0). These kinds of fluorescent proteins are suitable for imaging viral particles in acidic organelles (Shaner et al. 2005). Other fluorescent proteins such as EYFP, pHluorin, and pHuji are highly pH sensitive. These proteins present high fluorescence under neutral pH conditions, while the fluorescence signal decreases markedly at acid pH. The pH sensitivity of such

fluorescent proteins can be exploited as a pH sensor to study virus fusion and entry (Melikyan et al. 2005; Miyauchi et al. 2011). By co-labeling an avian retrovirus with pH-sensitive pHluorin and pH-resistant mKate2 simultaneously, single-virus imaging showed that the virus co-trafficked and fused with intracellular compartments tagged with fluorescent Rab5 and Rab7 proteins in live cells (Padilla-Parra et al. 2014).

Organic Dyes

Organic dyes are important fluorophores used in fluorescence imaging in live cells (Gonçalves 2009). Due to their relatively small size of 1–2 nm, organic dyes are suitable for fluorescence labeling of viruses, which have a size range of 20–400 nm in diameter. Besides, organic dyes have several outstanding advantages, such as excellent optical properties, good biocompatibility, commercial availability, and a wide range of color palettes, which confer their broad usage for single-virus tracking (Miyawaki et al. 2003; Resch-Genger et al. 2008).

   Viral glycoproteins (envelope proteins) or capsid proteins for non-enveloped viruses can be readily labeled with amine-reactive dyes, as exemplified by cyanine dyes and Alexa Fluor dyes. Cy3 and Cy5 are the most commonly used cyanine dyes for single virus tracking. Cy3 has been used to label the envelope protein of the influenza virus (Lakadamyali et al. 2003, 2006). As a long-wavelength fluorophore, Cy5 has the advantage of reduced interference from autofluorescence in live cells. Therefore, Cy5 has been much more popularly applied to label viruses, including the envelope protein of Semliki forest virus (SFV) (Vonderheit and Helenius 2005) and rabies virus (Xu et al. 2015), and the capsid protein of PV (Brandenburg et al. 2007) and adeno-associated virus (Seisenberger et al. 2001). It is worthy of note that researchers have viewed live scenes of viral infection by tagging individual viruses with one or two Cy5 molecules (Wang et al. 2014). Besides, virions and cellular components could be labeled by cyanine dyes or fluorescent proteins, respectively, for imaging in live cells. For instance, the intracellular pathway taken by individual Cy5-labeled SFV particles was followed using triple-color fluorescence microscopy in live cells transfected with GFP- and RFP-tagged Rab5, Rab7, Rab4, and Arf1, the cellular components that mediate cargo transfer (Vonderheit and Helenius 2005).

   Alexa Fluor dyes are another group of amine-reactive dyes that are frequently used for single-virus tracking alone or in combination with other fluorophores. Alexa Fluor dyes cover an extremely broad range of excitation/emission wavelengths spanning from ultraviolet (UV) to visible and near-infrared regions. They are derivatives of certain well-known organic dyes such as rhodamine, fluorescein, and cyanine dyes. Alexa Fluor dyes possess highly demanded photophysical properties including high quantum yields, large molar extinction coefficients, and high photostabilities. Moreover, Alexa Fluor dyes tend to be more resistant to fluorescence quenching and retain their intense fluorescence even at high degrees of labeling compared to the cyanine dyes (Berlier et al. 2003; Gebhardt et al. 2021).

Thus, Alexa Fluor dyes have been widely used for investigating the cellular uptake and trafficking of viruses. For example, tracking of Alexa Fluor 594-labeled simian virus 40 revealed that individual viruses moved laterally in a random fashion until trapped in stationary spots positive for caveolin-1 tagged with a green fluorescent protein (Pelkmans et al. 2002). Alexa Fluor derivatives have also been applied to label other non-enveloped viruses, including foot-and-mouth disease virus (Martín-Acebes et al. 2011) and human papillomavirus (Schelhaas et al. 2008), or enveloped viruses such as vesicular stomatitis virus (VSV) (Cureton et al. 2010) and dengue virus (Dejarnac et al. 2018).

Enveloped viruses are surrounded by a continuous lipid bilayer derived from the host cell plasma membrane or intracellular membrane (Lenard 2008). Fluorescent lipophilic dyes are popular for membrane labeling and particle tracing applications and thus can be utilized to label viruses by targeting the lipid envelope. It has been known that hydrophobic–lipophilic interactions facilitate the incorporation of lipophilic dyes into the outer membrane of enveloped viruses (McClelland et al. 2021). Up to now, the most commonly used fluorescent lipophilic dyes are rhodamine derivatives and long-chain dialkylcarbocyanines such as DiO, DiI, and DiD. Lipophilic dyes at high concentrations tend to fluorescence self-quench due to close proximity constraints (Klymchenko et al. 2012). Upon fusion where viral membranes merge with the membranes of host cells, the lipophilic dyes will diffuse resulting in dequenching and an increase in fluorescence indicating the occurrence of membrane fusion. For instance, the self-quenching rhodamine derivatives R18 and R110 have been applied to study influenza virus envelope fusion with cellular membranes (Lowy et al. 1990; Floyd et al. 2008). By labeling respiratory syncytial virus (RSV) particles with DiD, the fluorescent signal was used for real-time monitoring of the virus fusion and entry upon clustering with surface trafficked NCL, the coreceptor for RSV fusion (Griffiths et al. 2020). It is noteworthy that this approach using fluorescent lipophilic dyes detects fusion based on the lipid mixing activity, but not the actual release of the interior core/content of virus, which is a prerequisite for infection. More accurate approaches involve co-labeling the viral core and the viral membrane with different fluorescent probes are needed.

Quantum Dots

Quantum dots (QDs), a kind of semiconductor nanocrystals, are among the most investigated fluorescent nanoparticles in bioimaging applications (Michalet et al. 2005; Wegner and Hildebrandt 2015). QDs offer a wide range of useful optical properties for bioimaging assays with single-molecule readouts, including high photostability, high quantum yield, large absorption coefficients, and narrow emission spectra (Zhou et al. 2015). The emission wavelengths of QDs range from UV to infrared and can be tuned by controlling their size and composition (Wegner and Hildebrandt 2015). Their excellent brightness (10−100 times higher than organic dyes or fluorescent proteins) and resistance to photobleaching (100−1000 times
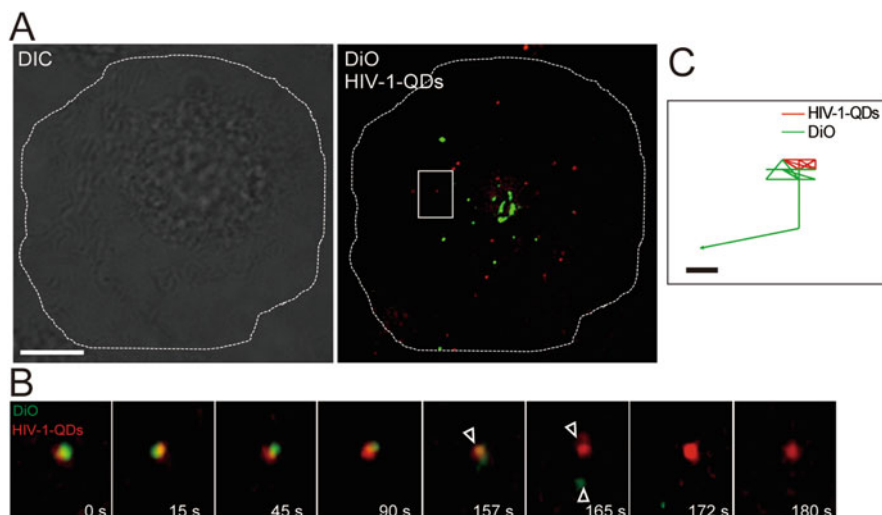
Fig. 5.2 Real-time imaging of the HIV-1-QD viral core release in primary macrophages. The cellular boundary of the macrophage is highlighted by a dashed line. (**a**) Dual-labeled HIV-1-QD-DiO particle in a macrophage (shown in the rectangular region) was tracked. Scale bar: 10 μm. (**b**) Sequential snapshots are shown for the separation of QD-Vpr and the DiO-labeled membrane of the viral particle that is shown in (**a**) in the macrophage. Arrowhead indicates the separation of QD and DiO signals. (**c**) Trajectories of HIV-1-QD (red) and DiO-labeled membrane (green) for the viral particle that is shown in rectangular region of (**a**). Scale bar: 0.2 μm. Adapted with permission from reference Li et al. (2017). Copyright 2017 American Chemical Society

higher than organic dyes or fluorescent proteins) make them especially useful for live-cell imaging and single-particle tracking (Alivisatos et al. 2005; Liu et al. 2020). The wide emission wavelength and distinct emission spectra of QDs also facilitate multicolor imaging where various components of a virion can be labeled and tracked simultaneously.

In a noteworthy study, QDs were successfully exploited to site specifically label enveloped viruses, leading to the real-time monitoring of the intracellular movement of single viruses in live cells (Joo et al. 2008). Specific internal viral components can also be labeled with QDs during viral replication and prior to assembly. The use of this approach could obviate incorporation of QDs on the viral surface which may affect the virus–host interaction and thus the efficiency of viral entry. For example, QDs conjugated with modified genomic RNAs, which contain a packaging signal sequence, can be successfully incorporated into intact virions in live cells without modification of the viral surface. This QD-containing VSV-G pseudotyped lentivirus demonstrated a Rab5+ endosome- and microtubule-dependent entry route (Zhang et al. 2013). In the context of QDs-based labeling of viral interior proteins, our group demonstrated that QDs could be encapsulated in HIV-1 virions by incorporating viral accessory protein Vpr-conjugated QDs during virus assembly. With the HIV-1 particles encapsulating QDs, we not only monitor the entry route of the virus at a single-particle level in live human primary macrophages (Li et al. 2017)

(Fig. 5.2), but also explored detailed scenarios of HIV dynamic entry and crossing the cortical actin barrier in resting CD4+ T lymphocytes (Yin et al. 2020). In addition, we have constructed a type of infectious HIV-1 virus encapsulating QDs through the site-specific decoration of the viral matrix protein and used it to visualize virus entry in human primary macrophages by single-particle imaging (Li et al. 2018).

To track the intricate sequential entry process of a single-virus particle in live cells, labeling of both the interior and exterior components of viruses is needed without an impact on the infectivity of viruses. Our group constructed dual-color IAV particles by combining internal encapsulation and surface decoration using QDs with different colors or by incorporating differently colored QD-vRNP segments into single virions. By applying single-particle imaging, the virus entry process including internalization, transport, uncoating, and intranuclear vRNP trafficking of individual IAV virions was visualized in real time in live cells (Qin et al. 2019; Yamauchi 2019).

Microscopy Methods and Image Processing

Microscopy Configurations

To visualize and track the dynamic entry processes of viruses, a number of different microscopy configurations need to be employed.

Wide-field epifluorescence microscopy is the simplest optical implementation for single virus tracking experiments among the available microscopy configurations. The epifluorescence configuration illuminates a relatively large area (approximately 100 μm × 100 μm) of the sample via the excitation light and the fluorescence signal can be captured quickly and easily by a highly sensitive camera (Rust et al. 2011). This method has large excitation depth and low signal loss, allowing the study of the long-range movement of individual viruses. However, owing to a large amount of background autofluorescence of cells, epifluorescence detection often shows weak signals originating from individual virions that contain only a few fluorescent molecules (Liu et al. 2020). This method needs to extract an in-focus signal from a high-noise background.

The confocal microscopy modality has a thinner depth of field and higher resolution compared to wide-field epifluorescence microscopy. It uses point illumination and pinhole optics to eliminate the background noise but at the cost of signal reduction. In order to track the dynamic behavior of viruses in live cells, different sites of volume must be scanned simultaneously. The usage of spinning-disk confocal microscopy (SDCM) meets the demand. Cross sections of a sample with a fast piezo z-scan device can be combined with SDCM to reconstruct all three-dimensional images (X, Y, and Z), which has been used for single-virus tracking in live cells (Han et al. 2012). Besides, SDCM can markedly improve imaging speed and allows the elucidation of dynamic events of viruses in live cells. The main

disadvantage of SDCM is the cross-talk between pinholes, which can be circumvented by increasing the inter-pinhole distance or by decreasing the out-of-focus light. Besides, exposure to strong light can be damaging to live cells (Shimozawa et al. 2013).

Total internal reflection fluorescence microscopy (TIRFM) is useful to observe fluorescence signals occurring at interfaces. The TIRFM makes good use of total internal reflection to produce an evanescent field. Typically, the evanescent wave extends only a few hundred nanometers into the cell. Therefore, TIRFM facilitates the visualization of biological events occurring on the plasma membrane of live cells, such as clathrin-based entry kinetics of VSV (Johannsdottir et al. 2009).

Image Processing

After choosing and utilization of a virus labeling method and microscopy configuration, the dynamic information of virus entry can be extracted from a time series of images. Several challenges have to be overcome with the analysis of viral entry in live cells. It is important to eliminate background noise and thereby enhance the signal-to-noise ratio. To combat the noise of concern, several particle detection and image restoration techniques have been developed specifically for single-virus tracking. For example, in each frame of images containing fluorescent particles, the location of the individual particle with high precision will be determined. To locate the center position, the point spread-function (PSF) is regarded as a suitable parameter to measure the shape of individual particles, as well as the 3D diffraction pattern (Liu et al. 2020). Then the isolated fluorescent peaks that correspond to virus particles will be automatically identified by PSF-fitting methods. The Gaussian nonlinear least-squares fitting method is the most common way for single-virus tracking. It possesses very high localization accuracy and is compatible with localization-based super-resolution techniques such as stochastical optical reconstruction microscopy (STORM) and photoactivated-localization microscopy (PALM). The radial symmetry approach is another type of the geometric-based method which can be utilized for localizing particle centers. In terms of computation speed, it is significantly faster than that of the Gaussian fitting method (Liu et al. 2013).

Trajectories of virus particles can be reconstructed by connecting particles in each frame by using nearest-neighbor association and the motion history of individual virus particles (Brandenburg and Zhuang 2007; Shen et al. 2017; Sbalzarini and Koumoutsakos 2005). Then mean squared displacements are applied to determine whether the virus particles display different types of movement models, such as directed, normal, or anomalous diffusion. Viruses tend to change their motion behavior from 2D diffusion on the cell membrane to directed transport and then to 3D anomalous diffusion within the cytosol (Brandenburg and Zhuang 2007). The diffusion type can reveal the type of interactions between different viral structures or the interactions between viral and cellular structures. By measuring the
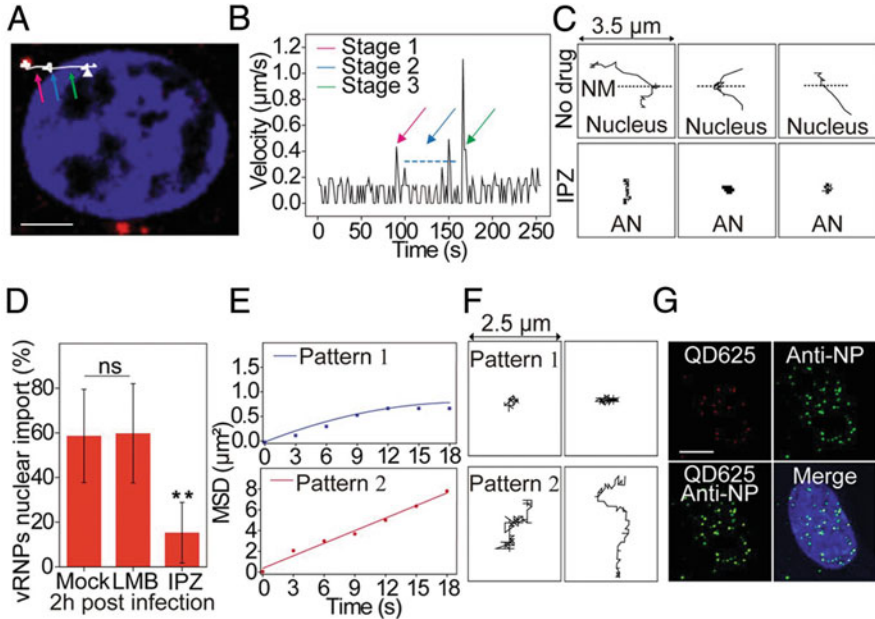
Fig. 5.3 Characterization of vRNP nuclear import and intranuclear movement. (**a**) Trajectory of a representative QD625-vRNP transport. The colored arrows indicate stage 1 (pink), stage 2 (blue), and stage 3 (green) (scale bar: 5 μm). (**b**) Analysis of the mean velocities of QD625-labeled vRNPs shown in **a**. (**c**) Trajectories of the QD signals in untreated and importazole (IPZ)-treated cells. NM, nuclear membrane; AN, adjacent nuclear. (**d**) Statistical analysis of /IAV vRNP nuclear import with drug treatment. ns, no significant difference; $n = 3$; **$P < 0.01$. (**e**) Analysis of the MSD plots of two vRNP diffusive patterns in the nucleus. (**f**) Trajectories of two vRNP diffusive patterns. (**g**) Immunofluorescence assay of QD625-vRNPs in the nucleus at 2 hpi (scale bar: 5 μm). Adapted with permission from reference Qin et al. (2019). Copyright © 2019 the Author(s). Published by PNAS. This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND)

instantaneous speed versus time, the different motions from a trajectory can be distinguished and analyzed separately. For instance, it has been shown that individual QD-labeled vRNPs of influenza viruses display a three-stage movement to the cell nucleus and show two diffusion patterns when inside the nucleus during the entry process (Qin et al. 2019) (Fig. 5.3).

Concluding Remarks

Single-particle tracking techniques are powerful tools for investigating the dynamic entry processes of viruses into cells with high spatio-temporal resolution. They can help elucidate the distinct entry pathway of the same virus or similar entry routes of different viruses. With the development of new fluorescent probes, better labeling

strategies, and novel microscope configurations in the upcoming decade, we will witness an ongoing and broader application of single particle tracking in the field of virology. Particularly, over recent years, functional genetic screens have been carried out using genome-wide siRNA libraries, insertional mutagenesis of haploid cell lines, and CRISPR-Cas9 knockout screens. A subset of host factors required to support virus entry has been identified (Hirsch 2010; Puschnik et al. 2017; Carette et al. 2011). Given that viruses exploit a variety of cellular factors to get entry into host cells, single-particle tracking of viruses in live cells will undoubtedly provide an important tool for dissecting the dynamic roles of the identified host proteins in viral entry. To reduce the influence of fluorescent labeling on viral infectivity, only a few fluorescent molecules can be used to label viral structures. Fluorescent nanoparticles such as QDs are useful labeling schemes and have the potential to track the whole entry process of individual viruses in live cells. Lastly, most live cell imaging studies on virus entry are currently performed with 2D culture cells. Viral tracking in conditions that more accurately mimic in vivo circumstances such as 3D organoids or physiological barriers (Strange et al. 2019; Premeaux et al. 2021) is likely to give a better understanding of the virus entry process.

References

Alivisatos AP, Gu W, Larabell C (2005) Quantum dots as cellular probes. Annu Rev Biomed Eng 7: 55–76

Berlier JE, Rothe A, Buller G, Bradford J, Gray DR, Filanoski BJ, Telford WG, Yue S, Liu J, Cheung CY, Chang W, Hirsch JD, Beechem JM, Haugland RP (2003) Quantitative comparison of long-wavelength Alexa Fluor dyes to Cy dyes: fluorescence of the dyes and their bioconjugates. J Histochem Cytochem 51(12):1699–1712

Brandenburg B, Zhuang X (2007) Virus trafficking—learning from single-virus tracking. Nat Rev Microbiol 5(3):197–208

Brandenburg B, Lee LY, Lakadamyali M, Rust MJ, Zhuang X, Hogle JM (2007) Imaging poliovirus entry in live cells. PLoS Biol 5(7):10

Carette JE, Guimaraes CP, Wuethrich I, Blomen VA, Varadarajan M, Sun C, Bell G, Yuan B, Muellner MK, Nijman SM, Ploegh HL, Brummelkamp TR (2011) Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. Nat Biotechnol 29 (6):542–546

Chudakov DM, Lukyanov S, Lukyanov KA (2005) Fluorescent proteins as a toolkit for in vivo imaging. Trends Biotechnol 23(12):605–613

Chudakov DM, Matz MV, Lukyanov S, Lukyanov KA (2010) Fluorescent proteins and their applications in imaging live cells and tissues. Physiol Rev 90(3):1103–1163

Cureton DK, Massol RH, Whelan SP, Kirchhausen T (2010) The length of vesicular stomatitis virus particles dictates a need for actin assembly during clathrin-dependent endocytosis. PLoS Pathog 6(9):1001127

Dejarnac O, Hafirassou ML, Chazal M, Versapuech M, Gaillard J, Perera-Lecoin M, Umana-Diaz C, Bonnet-Madin L, Carnec X, Tinevez JY, Delaugerre C, Schwartz O, Roingeard P, Jouvenet N, Berlioz-Torrent C, Meertens L, Amara A (2018) TIM-1 ubiquitination mediates dengue virus entry. Cell Rep 23(6):1779–1793

Doherty GJ, McMahon HT (2009) Mechanisms of endocytosis. Annu Rev Biochem 78:857–902

Ewers H, Smith AE, Sbalzarini IF, Lilie H, Koumoutsakos P, Helenius A (2005) Single-particle tracking of murine polyoma virus-like particles on live cells and artificial membranes. Proc Natl Acad Sci U S A 102(42):15110–15115

Floyd DL, Ragains JR, Skehel JJ, Harrison SC, van Oijen AM (2008) Single-particle kinetics of influenza virus membrane fusion. Proc Natl Acad Sci U S A 105(40):15382–15387

Gebhardt C, Lehmann M, Reif MM, Zacharias M, Gemmecker G, Cordes T (2021) Molecular and spectroscopic characterization of green and red cyanine fluorophores from the alexa fluor and AF series*. Chemphyschem 22(15):1566–1583

Gonçalves MS (2009) Fluorescent labeling of biomolecules with organic probes. Chem Rev 109(1):190–212

Greber UF (2005) Viral trafficking violations in axons: the herpesvirus case. Proc Natl Acad Sci U S A 102(16):5639–5640

Greber UF, Way M (2006) A superhighway to virus infection. Cell 124(4):741–754

Griffiths CD, Bilawchuk LM, McDonough JE, Jamieson KC, Elawar F, Cen Y, Duan W, Lin C, Song H, Casanova JL, Ogg S, Jensen LD, Thienpont B, Kumar A, Hobman TC, Proud D, Moraes TJ, Marchant DJ (2020) IGF1R is an entry receptor for respiratory syncytial virus. Nature 583(7817):615–619

Haldar S, Okamoto K, Dunning RA, Kasson PM (2020) Precise triggering and chemical control of single-virus fusion within endosomes. J Virol 95(1):01982-20

Han JJ, Kiss C, Bradbury AR, Werner JH (2012) Time-resolved, confocal single-molecule tracking of individual organic dyes and fluorescent proteins in three dimensions. ACS Nano 6(10):8922–8932

Hernandez-Gonzalez M, Larocque G, Way M (2021) Viral use and subversion of membrane organization and trafficking. J Cell Sci 134(5):252676

Hirsch AJ (2010) The use of RNAi-based screens to identify host proteins involved in viral replication. Future Microbiol 5 (2):303–311

Jha NK, Latinovic O, Martin E, Novitskiy G, Marin M, Miyauchi K, Naughton J, Young JA, Melikyan GB (2011) Imaging single retrovirus entry through alternative receptor isoforms and intermediates of virus-endosome fusion. PLoS Pathog 7(1):1001260

Johannsdottir HK, Mancini R, Kartenbeck J, Amato L, Helenius A (2009) Host cell factors and functions involved in vesicular stomatitis virus entry. J Virol 83(1):440–453

Joo KI, Lei Y, Lee CL, Lo J, Xie J, Hamm-Alvarez SF, Wang P (2008) Site-specific labeling of enveloped viruses with quantum dots for single virus tracking. ACS Nano 2(8):1553–1562

Joo KI, Tai A, Lee CL, Wong C, Wang P (2010) Imaging multiple intermediates of single-virus membrane fusion mediated by distinct fusion proteins. Microsc Res Tech 73(9):886–900

Klymchenko AS, Roger E, Anton N, Anton H, Shulov I, Vermot J, Mely Y, Vandamme TF (2012) Highly lipophilic fluorescent dyes in nano-emulsions: towards bright non-leaking nano-droplets. RSC Adv 2(31):11876–11886

Lakadamyali M, Rust MJ, Babcock HP, Zhuang X (2003) Visualizing infection of individual influenza viruses. Proc Natl Acad Sci U S A 100(16):9280–9285

Lakadamyali M, Rust MJ, Zhuang X (2006) Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell 124(5):997–1009

Lenard J (2008) Viral membranes. Encycl Virol 2008:308–314. https://doi.org/10.1016/B978-012374410-4.00530-6. Epub 2008 Jul 30

Li Q, Li W, Yin W, Guo J, Zhang ZP, Zeng D, Zhang X, Wu Y, Zhang XE, Cui Z (2017) Single-particle tracking of human immunodeficiency virus type 1 productive entry into human primary macrophages. ACS Nano 11(4):3890–3903

Li Q, Yin W, Li W, Zhang Z, Zhang X, Zhang XE, Cui Z (2018) Encapsulating quantum dots within HIV-1 virions through site-specific decoration of the matrix protein enables single virus tracking in live primary macrophages. Nano Lett 18(12):7457–7468

Liu SL, Li J, Zhang ZL, Wang ZG, Tian ZQ, Wang GP, Pang DW (2013) Fast and high-accuracy localization for three-dimensional single-particle tracking. Sci Rep 3(2462)

Liu SL, Wang ZG, Xie HY, Liu AA, Lamb DC, Pang DW (2020) Single-virus tracking: from imaging methodologies to virological applications. Chem Rev 120(3):1936–1979

Lowy RJ, Sarkar DP, Chen Y, Blumenthal R (1990) Observation of single influenza virus-cell fusion and measurement by fluorescence video microscopy. Proc Natl Acad Sci U S A 87(5): 1850–1854

Luxton GW, Haverlock S, Coller KE, Antinone SE, Pincetic A, Smith GA (2005) Targeting of herpesvirus capsid transport in axons is coupled to association with specific sets of tegument proteins. Proc Natl Acad Sci U S A 102(16):5832–5837

Lyman MG, Curanovic D, Brideau AD, Enquist LW (2008) Fusion of enhanced green fluorescent protein to the pseudorabies virus axonal sorting protein Us9 blocks anterograde spread of infection in mammalian neurons. J Virol 82(20):10308–10311

Marsh M, Helenius A (2006) Virus entry: open sesame. Cell 124(4):729–740

Martín-Acebes MA, Vázquez-Calvo A, González-Magaldi M, Sobrino F (2011) Foot-and-mouth disease virus particles inactivated with binary ethylenimine are efficiently internalized into cultured cells. Vaccine 29(52):9655–9662

McClelland RD, Culp TN, Marchant DJ (2021) Imaging flow cytometry and confocal immunofluorescence microscopy of virus-host cell interactions. Front Cell Infect Microbiol 11:749039

Melikyan GB, Barnard RJ, Abrahamyan LG, Mothes W, Young JA (2005) Imaging individual retroviral fusion events: from hemifusion to pore formation and growth. Proc Natl Acad Sci U S A 102(24):8728–8733

Mercer J, Schelhaas M, Helenius A (2010) Virus entry by endocytosis. Annu Rev Biochem 79:803–833

Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S (2005) Quantum dots for live cells, in vivo imaging, and diagnostics. Science 307(5709):538–544

Miyauchi K, Marin M, Melikyan GB (2011) Visualization of retrovirus uptake and delivery into acidic endosomes. Biochem J 434(3):559–569

Miyawaki A, Sawano A, Kogure T (2003) Lighting up cells: labelling proteins with fluorophores. Nat Cell Biol 7:S1–S7

Padilla-Parra S, Marin M, Kondo N, Melikyan GB (2014) Pinpointing retrovirus entry sites in cells expressing alternatively spliced receptor isoforms by single virus imaging. Retrovirology 11(47):1742–4690

Pelkmans L, Püntener D, Helenius A (2002) Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. Science 296(5567):535–539

Premeaux TA, Mediouni S, Leda A, Furler RL, Valente ST, Fine HA, Nixon DF, Ndhlovu LC (2021) Next-generation human cerebral organoids as powerful tools to advance neuroHIV research. mBio 12(4):00680-00621

Puschnik AS, Majzoub K, Ooi YS, Carette JE (2017) A CRISPR toolbox to study virus-host interactions. Nat Rev Microbiol 15 (6):351–364

Qin C, Li W, Li Q, Yin W, Zhang X, Zhang Z, Zhang XE, Cui Z (2019) Real-time dissection of dynamic uncoating of individual influenza viruses. Proc Natl Acad Sci U S A 116(7):2577–2582

Radtke K, Döhner K, Sodeik B (2006) Viral interactions with the cytoskeleton: a Hitchhiker's guide to the cell. Cell Microbiol 8(3):387–400

Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, Nitschke R, Nann T (2008) Quantum dots versus organic dyes as fluorescent labels. Nat Methods 5(9):763–775

Rust MJ, Lakadamyali M, Zhang F, Zhuang X (2004) Assembly of endocytic machinery around individual influenza viruses during viral entry. Nat Struct Mol Biol 11(6):567–573

Rust MJ, Lakadamyali M, Brandenburg B, Zhuang X (2011) Single-virus tracking in live cells. Cold Spring Harb Protoc 1(9)

Sbalzarini IF, Koumoutsakos P (2005) Feature point tracking and trajectory analysis for video imaging in cell biology. J Struct Biol 151 (2):182–195

Schelhaas M (2010) Come in and take your coat off - how host cells provide endocytosis for virus entry. Cell Microbiol 12 (10):1378–1388

Schelhaas M, Ewers H, Rajamäki ML, Day PM, Schiller JT, Helenius A (2008) Human papillomavirus type 16 entry: retrograde cell surface transport along actin-rich protrusions. PLoS Pathog 4(9):1000148

Seisenberger G, Ried MU, Endress T, Büning H, Hallek M, Bräuchle C (2001) Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. Science 294(5548): 1929–1932

Shaner NC, Steinbach PA, Tsien RY (2005) A guide to choosing fluorescent proteins. Nat Methods 2(12):905–909

Shen H, Tauzin LJ, Baiyasi R, Wang W, Moringo N, Shuang B, Landes CF (2017) Single Particle Tracking: From Theory to Biophysical Applications. Chem Rev 117 (11):7331–7376

Shimozawa T, Yamagata K, Kondo T, Hayashi S, Shitamukai A, Konno D, Matsuzaki F, Takayama J, Onami S, Nakayama H, Kosugi Y, Watanabe TM, Fujita K, Mimori-Kiyosue Y (2013) Improving spinning disk confocal microscopy by preventing pinhole cross-talk for intravital imaging. Proc Natl Acad Sci U S A 110(9):3399–3404

Sorkin A, Von Zastrow M (2002) Signal transduction and endocytosis: close encounters of many kinds. Nat Rev Mol Cell Biol 3(8):600–614

Strange DP, Jiyarom B, Pourhabibi Zarandi N, Xie X, Baker C, Sadri-Ardekani H, Shi PY, Verma S (2019) Axl promotes zika virus entry and modulates the antiviral state of human sertoli cells. mBio 10(4):01372-19

Suomalainen M, Greber UF (2013) Uncoating of non-enveloped viruses. Curr Opin Virol 3(1): 27–33

Taylor MP, Kramer T, Lyman MG, Kratchmarov R, Enquist LW (2012) Visualization of an alphaherpesvirus membrane protein that is essential for anterograde axonal spread of infection in neurons. mBio 3(2):00063-12

Vonderheit A, Helenius A (2005) Rab7 associates with early endosomes to mediate sorting and transport of Semliki forest virus to late endosomes. PLoS Biol 3(7):21

Wang S, Huang X, Huang Y, Hao X, Xu H, Cai M, Wang H, Qin Q (2014) Entry of a novel marine DNA virus, Singapore grouper iridovirus, into host cells occurs via clathrin-mediated endocytosis and macropinocytosis in a pH-dependent manner. J Virol 88(22):13047–13063

Wegner KD, Hildebrandt N (2015) Quantum dots: bright and versatile in vitro and in vivo fluorescence imaging biosensors. Chem Soc Rev 44(14):4792–4834

Xu H, Hao X, Wang S, Wang Z, Cai M, Jiang J, Qin Q, Zhang M, Wang H (2015) Real-time imaging of rabies virus entry into live vero cells. Sci Rep 5:11753

Yamauchi Y (2019) Quantum dots crack the influenza uncoating puzzle. Proc Natl Acad Sci U S A 116(7):2404–2406

Yin W, Li W, Li Q, Liu Y, Liu J, Ren M, Ma Y, Zhang Z, Zhang X, Wu Y, Jiang S, Zhang XE, Cui Z (2020) Real-time imaging of individual virion-triggered cortical actin dynamics for human immunodeficiency virus entry into resting CD4 T cells. Nanoscale 12(1):115–129

Zhang Y, Ke X, Zheng Z, Zhang C, Zhang Z, Zhang F, Hu Q, He Z, Wang H (2013) Encapsulating quantum dots into enveloped virus in live cells for tracking virus infection. ACS Nano 7(5): 3896–3904

Zhou J, Yang Y, Zhang CY (2015) Toward biocompatible semiconductor quantum dots: from biosynthesis and bioconjugation to biomedical application. Chem Rev 115(21):11669–11717