Virus trafficking – learning from single-virus tracking

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Abstract | What could be a better way to study virus trafficking than 'miniaturizing oneself' and 'taking a ride with the virus particle' on its journey into the cell? Single-virus tracking in living cells potentially provides us with the means to visualize the virus journey. This approach allows us to follow the fate of individual virus particles and monitor dynamic interactions between viruses and cellular structures, revealing previously unobservable infection steps. The entry, trafficking and egress mechanisms of various animal viruses have been elucidated using this method. The combination of single-virus trafficking with systems approaches and state-of-the-art imaging technologies should prove exciting in the future.

Viruses live brief but eventful lives. During its short life cycle, a virus interacts with various cellular structures, taking advantage of different cellular environments to optimize the chances of replication. The infection starts with the virus binding to specific receptors or attachment factors on the cell surface[1]. Although some viruses release their genomes directly into the cell by breaking the plasma membrane barrier, most viruses enter cells by endocytosis[2,3]. After internalization, virus particles are sequestered in endocytic organelles until the proper conditions are met for viral-genome release. Two main strategies are used for genome release: enveloped viruses fuse with a cell membrane, thereby exposing the genome or capsid to the cytosol, whereas non-enveloped viruses partially disrupt cellular membranes to release viral RNA or DNA. After entry, the viral genome is transported to specific sites in the cytoplasm or nucleus for replication and expression. The newly synthesized viral proteins and genetic material are then transported to specific sites for assembly into progeny viruses. Although virus assembly can occur at the plasma membrane, many viruses begin the assembly process in intracellular organelles, such as the endoplasmic reticulum or multivesicular bodies. Assembled virions exit by exocytosis or after lysis of the host cell. Mature viruses can subsequently infect new target cells.

It is evident that viral infections are complex processes that include many steps and interactions with different cellular structures. Not only are these interactions dynamic, but often the cellular structures are themselves dynamic. Furthermore, the same virus might infect cells by several different routes and most viral entry events might be futile. Therefore, studying viral infection mechanisms should benefit from an experimental approach that allows researchers to follow the fate of individual virus particles, to probe dynamic interactions between viruses and cellular machinery, and to dissect the steps of the infection process so that the mechanisms underlying each step can be determined. Single-virus tracking in living cells offers precisely this opportunity.

Single-virus tracking in live cells

Single-virus tracking is a real-time imaging technique that uses fluorescence microscopy to monitor individual virus particles or viral components in live cells. To successfully track a single virus particle, the virus and cellular structures of interest must be fluorescently labelled, the microscope must be sufficiently powerful for the detection of single viruses or viral components in real time, and the acquired image series must be analysed to extract useful information.

Technical aspects of single-virus imaging. The first step towards single-virus tracking in live cells is labelling the viral components and relevant cellular structures. A crucial requirement is that both viruses and cellular structures need to be labelled with a sufficient number of fluorophors for detection at the single particle level without inhibiting viral infectivity and cell functions. The external components of a virus, such as the capsid of a non-enveloped virus or the membrane of an enveloped virus, can be readily labelled with fluorescent dyes (BOX 1). Tracking virions that are labelled in this manner can provide important insights into the transport or uncoating mechanisms of viruses (FIG. 1a; Supplementary information S1 (Movie)). Internal components, such as the capsid or genome of an enveloped virus, are typically inaccessible for labelling after the

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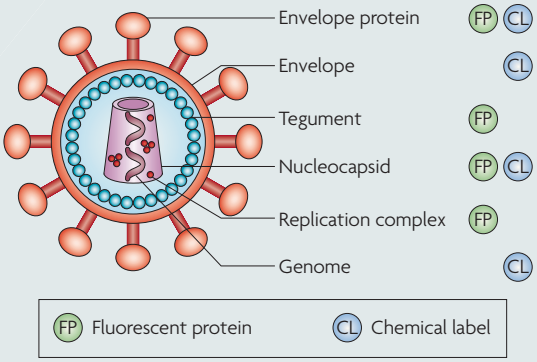
Box 1 | Strategies for labelling various viral components

Two general strategies exist for labelling viruses: the fusion of a target viral protein with a fluorescent protein (FP), or direct chemical labelling with small dye molecules. The palette of FPs has recently expanded to include brighter and more photostable FPs for a broad range of colours[7,80]. In addition, pH sensitive and photo-switchable FPs have also been developed[4]. To fuse an FP with a viral structure, the DNA sequence of the FP has to be inserted into the open reading frame of the target viral protein. Labelling occurs during virus replication in host cells. In order to generate a virus that is visible at the single particle level with a high signal-to-noise ratio, attachment of multiple FPs per virus is necessary. Therefore viral components that exist in multiple copies per virion, such as capsid or tegument proteins, are useful labelling targets (see figure).

In contrast to FPs, chemical labels (CLs) can be attached to viral structures at different time-points during the viral life cycle. An increasing number of CLs with different characteristics can be either covalently attached or non-covalently associated with the target protein[8]. For example, capsids of purified non-enveloped viruses can be covalently labelled with amino-reactive dyes (for example, cynine or Alexa dyes)[6,18,42]. The outer membrane of purified enveloped viruses can be labelled by the incorporation of lipophilic dyes for example, DiD, DiI and analogues[5]. Metabolic labelling can be used to label viruses during viral replication. For instance, cell-permeable biarsenical dyes can specifically bind to viral proteins that contain tetracystein peptide sequences[9,57]. Labelling of viral genomes can be done by infecting cells in the presence of fluorescently labelled nucleotides or nucleic acid-binding dyes (B.B. *et al.*, unpublished data). Antibodies are typically not useful for virus labelling in tracking experiments in live cells because they can block the function of viral proteins after binding.



completion of viral assembly and purification. The development of fluorescent proteins (FPs)[4] solved this technical problem. The key feature of FPs is that they are genetically encoded and therefore allow specific viral components to be labelled during viral replication and prior to assembly. Moreover, FPs can also be used to label endogenous cellular proteins, which facilitates the study of virus–cell interactions (FIG. 1b; Supplementary information S2 (movie)).

Labelled viruses are visualized in live cells using a fluorescence microscopy set-up. Three imaging geometries are used most often. Among these, epi-fluorescence microscopy is the simplest to set-up (BOX 2). This method also has the largest imaging depth and is often the method of choice when long-range viral trafficking or transport is being studied. However, owing to the background autofluorescence (noise) of cells, epi-detection is inadequate for imaging virus particles that contain only a few fluorescent molecules. To reduce the background noise, confocal detection, or excitation by total internal reflection is used (BOX 2). Confocal microscopy requires a rapid scanning scheme (laser scanning confocal) or multiplexed detection (spinning disc confocal) to acquire images of living cells (BOX 2). By using confocal imaging it is possible to acquire 3-dimensional images, but this advantage is mitigated by a relatively severe signal loss. Total internal reflection fluorescence (TIRF) microscopy is a wide-field imaging technique that has minimum signal loss. However, the excitation depth of an evanescent wave generated by total internal reflection is only 100–200 nm, limiting the application to events that occur close to the cell surface. Therefore TIRF imaging is typically used to monitor the early events of viral entry. In addition to these background-reduction techniques, the development of charge-coupled device (CCD) cameras with high quantum yields (60–80%) and rapid frame rates (1–500 ms) was also an important technological advance that benefited single-virus-imaging technology.

Finally, live-cell imaging is a technique with a high-information content. Each frame of an image contains megabytes of data and each viral infection movie typically comprises hundreds to thousands of frames. Recently, developments in automated image analysis have enabled us to convert these movies into large statistical ensembles of viral trajectories that show viral transport mechanisms and virus–cell interactions. We briefly describe the image-analysis principles that are crucial for single-virus tracking in BOX 3.

Challenges in single-virus tracking

Virus and cell labelling. Viruses are tiny — the smallest viruses have a diameter of only 20–30 nm, limiting the number of fluorescent probes that can be attached to a virus particle without causing self-quenching effects between dye molecules or affecting viral infectivity. For example, whereas hundreds to thousands of dye molecules can be attached to a relatively large influenza virion without affecting its infectivity[5], only 30–50 dye molecules can be attached to a polio capsid owing to its small size (B.B. *et al.*, unpublished data). In extreme examples, such as the adeno-associated virus, attaching more than a few dye molecules to the capsid renders the virus non-infectious[6]. This limit can be even more stringent for the labelling of internal viral components, such as the genome. Because cells also fluoresce, it can be difficult for microscopists to distinguish between the signal from a single-virus particle and the background fluorescence of a cell. As the cell auto-fluorescence background levels tend to be weaker at longer excitation wavelengths, it is helpful to use fluorescent probes with longer wavelengths for virus labelling. The red fluorescent dyes, such as Cy5, Alexa 647 and DiD (excitation maximum ~ 650 nm) and red fluorescent proteins, such as mCherry (excitation maximum ~ 590 nm[7]) are often good choices for virus labelling. The situation becomes more troublesome when studying viral assembly, because fluorescent viral proteins are usually overexpressed in an infected cell, which produces a considerable amount of background signal and makes it difficult to identify assembling virions. In addition, the relatively bulky size of fluorescent proteins might interfere with viral structures and functions, especially when attached to the internal components of tightly packed viruses. One advance that might help to solve this problem is the development of small molecules that specifically bind to genetically encoded peptide sequences[8,9].

Evanescent wave
An evanescent wave is an electromagnetic wave exhibiting exponential decay with distance. Optical evanescent waves are commonly found during total internal reflection.

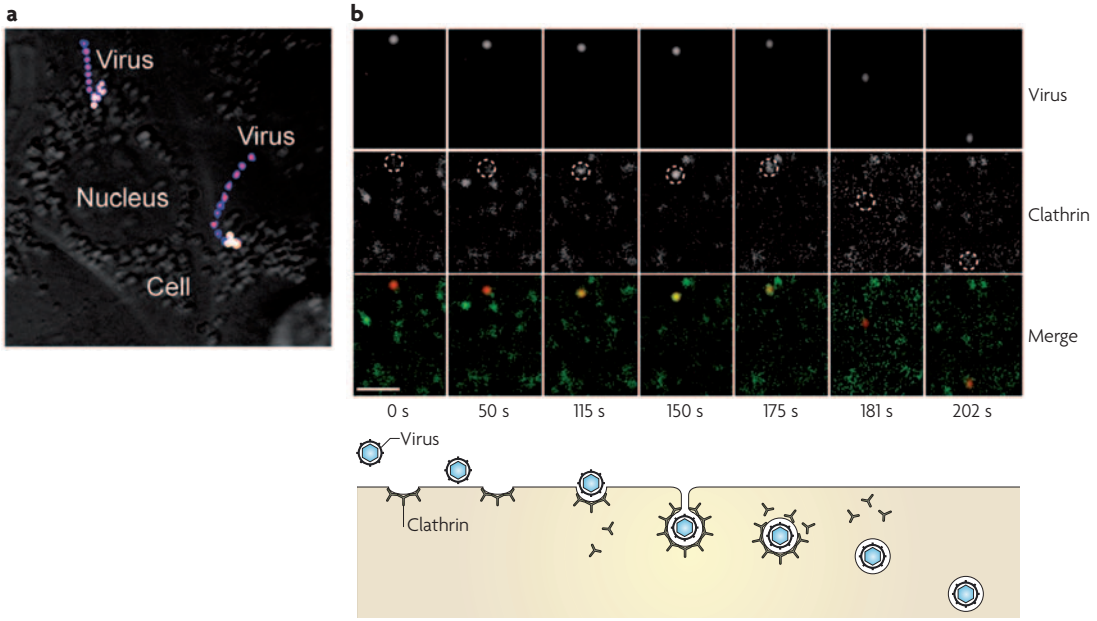


Figure 1 | **Time-lapse images of influenza viruses in live cells. a** | Stacked, time-lapse images of influenza viruses in living cells, revealing actin and microtubule-dependent transport. The virus is labelled with the lipophilic dye, DiD. The sudden colour change from blue/pink to yellow/white indicates a dramatic increase in the fluorescence signal of DiD, indicating the fusion of the virus with an endosome. A movie showing the time course of one of these virus particles can be found in the Supplementary Information S1 (movie). **b** | Simultaneous images of a DiD-labelled virus (upper panels and red in lower panels) and fluorescent protein-labelled clathrin (middle panels and green in lower panels) in a cell show the internalization of the virus by a clathrin-coated vesicle. The centres of dotted circles in the middle panels indicate the virus positions. Overlay of green and red signals appears yellow. The time (in seconds) after viral attachment and different stages of viral entry are shown below the images. A movie showing the time course of this virus particle is shown in Supplementary Information S2 (movie). Part (**a**) reproduced with permission from REF.5 © (2003) National Academy of Sciences, USA. Part (**b**) reproduced with permission from *Nature Structural and Molecular Biology* REF.13 © (2004) Macmillan Publishers Ltd.

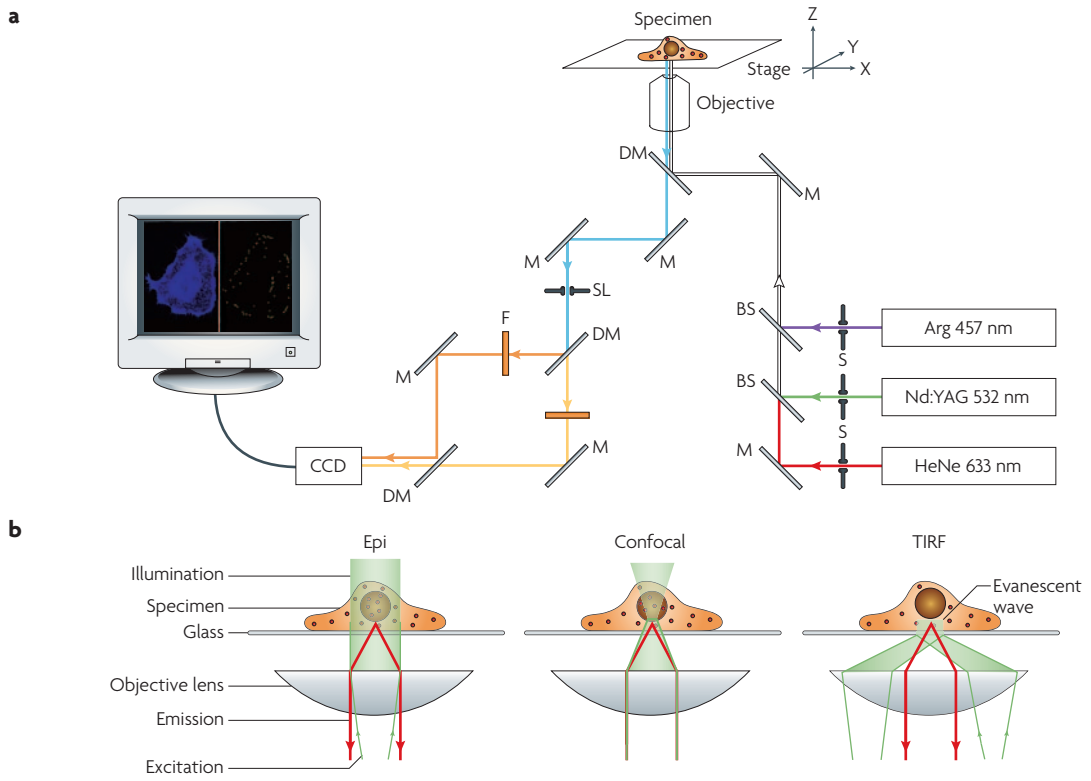
Inorganic nanoparticles, such as semiconductor quantum dots, are also promising for use as probes[10,11]. Quantum dots are much brighter and more robust than fluorescent dyes and proteins, but their bulky size places severe limits on their application. Research is underway to reduce the size of quantum dots (M. Bawendi, A. Ting and S. Weiss, personal communication) and to synthesize other small inorganic probes[12].

In order to study the interaction of viral particles with cellular structures, it is often helpful to label cellular proteins with FPs. Although this can be accomplished by fusing the FP gene with the gene of interest and expressing the fusion protein in host cells, this approach can be fraught with problems. It is sometimes challenging to obtain an optimum level of fusion-protein expression that allows the detection of individual cellular structures yet does not perturb cellular processes. Production of fluorescently tagged proteins needs to be accompanied by a proof-of-function study. For example, when clathrin-coated pits are marked with FP-tagged clathrin, the uptake of well-characterized cargos that are known to enter cells by clathrin-dependent endocytosis, such as transferrin and low-density lipoprotein (LDL), should be similar to the uptake behaviour that is observed in wild-type cells. Even when such conditions are met, endogenous unlabelled proteins might still complicate quantitative analysis and leave open the question of whether all relevant cellular structures are indeed fluorescently labelled. Functional and morphological control experiments are necessary to remove such doubt. Again, using clathrin-coated pits as an example, we and others have shown that when FP-tagged clathrin-light chain A (CLa) was expressed in cells in the presence of endogenous CLa, more than 96% of clathrin-coated pits visualized by immunofluorescence or with fluorescently labelled transferrin co-localize with FP-marked structures[13,14], showing that nearly all clathrin-coated pits are detected by the FP signal.

Large particle-to-pfu ratio. Many viruses have high particle-to-pfu (plaque forming unit) ratios; that is, hundreds to thousands of virus particles are necessary to infect a single cell. How can one be sure of tracking an infectious (as opposed to a non-infectious) virus particle? It is advisable to simultaneously track the maximum possible number of virus particles that are entering one cell and separate tracked particles into groups that have similar behaviours. Among these viruses, it should be possible to focus on the group of virus particles that are most likely to cause a productive infection. For example, fusion between viral and

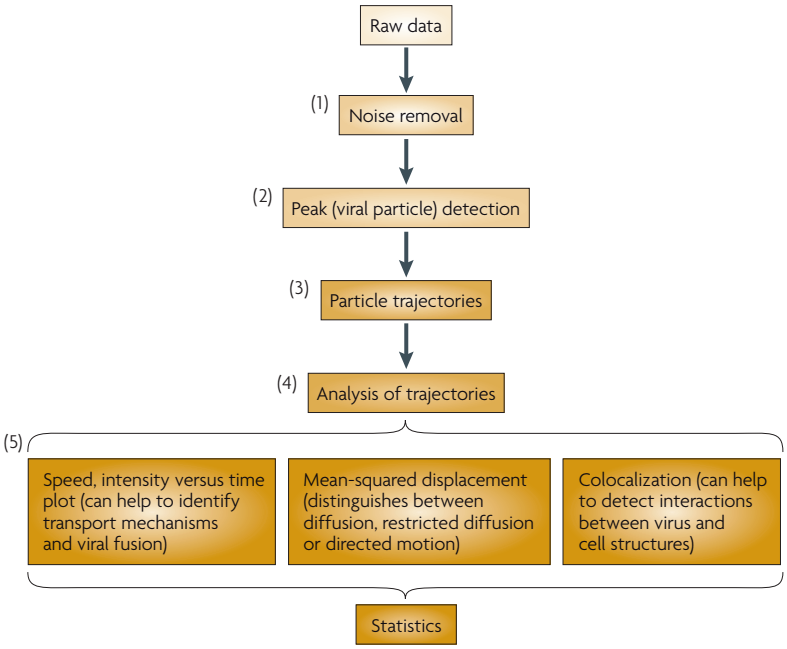
cellular membranes allows the virus to release its contents into the cell and is therefore a crucial infection step for enveloped viruses. So monitoring the fusion event of enveloped viruses, by fluorescence dequenching[5], enables the researcher to selectively focus on a subgroup of viruses that will have a productive infection. For non-enveloped viruses, viral genome release can be monitored as a marker of a productive infection. This can be done by separately labelling the viral capsid and genome with different fluorescent probes (B.B. *et al.*, unpublished results) as a marker for successful viral entry. In most cases, single-virus-tracking experiments cannot cover a full cycle of infection. In order to confirm that the tracked and analysed viral behaviours

Box 2 | **Fluorescence microscopes for single-virus imaging**



A typical single-virus tracking setup includes an inverted microscope with a temperature controlled stage, several lasers, optics and a sensitive detector. In the figure, part (**a**) shows the microscope setup. Various laser lines, such as Argon ion (Arg; 457 nm, 488 nm and 514 nm), Krypton ion (647 nm; not shown), helium-neon (HeNe; 543 nm, 594 nm and 633 nm) and Nd:YAG (532 nm) lasers, can function as light sources to excite different fluorophors. Multicolour imaging is enabled by a combination of several different laser lines with specific excitation and detection optics that allow different coloured excitation to be combined at the sample, and different coloured fluorescence emissions to be separately detected. In the figure, part (**b**) shows imaging geometries. In the epi-fluorescence geometry (left panel), a collimated light beam illuminates a large sample area (~ 100 µm in linear dimension)[81]. Fluorescence emission from the sample is collected by a high numerical aperture objective and detected by a CCD camera. The advantages of this scheme are the low signal loss, rapid wide-field detection and large excitation depth, which allow single particles to be tracked in a large sample volume. The disadvantage is its poor rejection of the fluorescence background signal from the cell. Confocal microscopy (middle panel) uses a focused light beam and spatial filtering techniques (pinholes in excitation and detection paths) to eliminate out-of-focus background fluorescence, but at the cost of signal reduction[82]. For the observation of fast dynamics in live cells, a spinning disk confocal setup is often preferred over the relatively slow scanning confocal microscope, which relies on rotating mirrors to scan a focused laser beam in the imaging plane and a point detector, such as avalanche photodiode or photomultiplier tubes, for signal acquisition[81]. The spinning disk confocal microscope relies on a pair of rapidly rotating discs, one with an array of pinholes and the other with correspondingly aligned micro-lenses. Thereby the excitation light is subdivided into thousands of beams, which simultaneously scan the entire field at a rate of >1000 frames per second. This scheme effectively creates a wide-field image detected by a CCD camera. When combined with Z-direction scanning of the sample stage, both confocal schemes allow the construction of 3-dimensional images. In a TIRF microscope (right panel), the incident light strikes the interface between two optical media of different refractive indices (for example, a glass substrate and a cell) at a sufficiently large angle to induce total reflection[83]. As a result, an evanescent excitation field is generated, extending only a few hundred nanometers into the second medium. This wide-field imaging geometry offers the best rejection of background signal, but can only be used to detect events occurring close to the adhering surface of the cell. In order to simultaneously track a large number viruses, a camera chip with a high number of pixels is needed, which allows a large field of view. BS, beam splitter; CCD, charge-coupled device; DM, dichroic mirrors; F, filter; M, mirrors; SL, optical slits to control image size; S, shutters; TIRF, total internal reflection fluorescence.

Box 3 | **Image analysis**



Single particle-tracking algorithms have made it possible to capture the detailed dynamics of viral entry and trafficking mechanisms. The algorithms convert the raw stack of fluorescence images into a set of trajectories that specify the coordinates and fluorescence intensity of each particle as a function of time. The analysis typically begins with processing each image frame with a spatial filter to remove noise (step 1 in the figure) and is followed by automated identification of isolated fluorescent peaks that correspond to virus particles (step 2)[13]. To identify peaks, filtered images are scanned for local maxima above a threshold, giving an estimate of the coordinates of the particles[84]. Trajectories can be established by connecting particles from one frame to the next using nearest-neighbour association and the motion history of individual particles (step 3)[13,57,85]. Furthermore, a split-and-merge mechanism allows the continued tracking of particles even when they merge or separate into smaller entities[86]. The overlap in fluorescence emission between two or more coloured channels can be used to probe the assembly or disassembly of different viral components or the interactions between viral and cellular structures. Analysis of the spatial and temporal trajectories of viruses allows us to identify the crucial events in infection pathways (such as viral fusion), to analyse the transport mechanisms of viruses and to detect interactions between virus particles and cellular structures (step 4). Other analyses are also possible at this stage. The automated analysis of particle trajectories is typically followed by a manual check for ambiguities and errors. A large number of trajectories need to be analysed to generate a statistically sound description of viral movement and virus–cell interaction (step 5). The variety of viral behaviours should be observed in multiple independent experiments and ideally in different cell types.

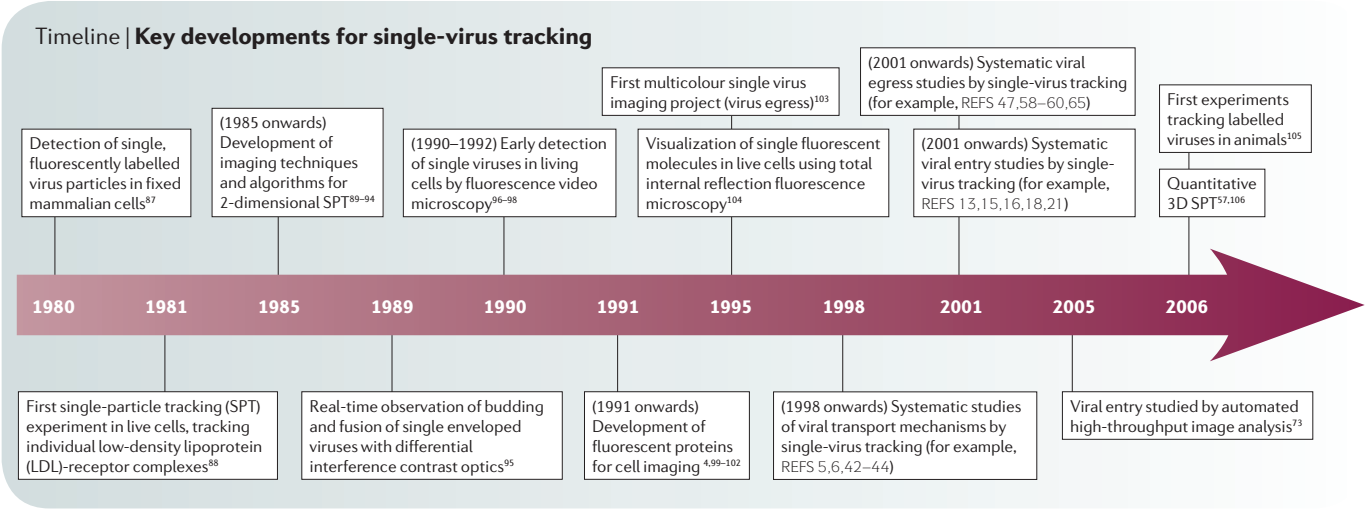
are indicative of those of the infectious virus particles, a combination of single-virus tracking with virological assays, such as a plaque assay, is necessary. For example, when inhibitors (drugs or siRNA) of viral entry are monitored using both single-particle tracking and viral titre assays, complete correlation between the two assays should give confidence that the single-particle tracking experiments are monitoring entry pathways that are relevant to infection.

Challenges associated with image instrumentation and analysis. In common with other imaging techniques, other challenges for single-virus tracking arise from the limited spatial and time resolution of fluorescence imaging and the need to analyse a rapidly increasing amount of image data, both discussed later.

Despite the challenges described above, recent advances in optics, device physics, chemistry and molecular biology have enabled us to track individual virus particles in living cells and to probe dynamic interactions between viruses and cellular machinery in real time (TIMELINE). In the following sections, we will review advances made in viral entry, transport and egress due to single-virus tracking in live cells.

Viral entry

Viruses exploit specific receptors on the cell surface to identify and infect target cells. However, these receptors are often rare or distributed in regions that are not readily accessible to incoming virions. Therefore, many viruses first bind to relatively non-specific attachment factors, such as carbohydrates, and migrate along the cell surface to locate specific receptor(s)[1] (FIG. 2). Specific virus–receptor interactions activate signalling cascades, guide the virus into endocytic pathways and/or trigger conformational changes in the virus envelope or capsid proteins for genome release[1] (FIG. 2). Single-virus tracking offers an ideal method for the visualization of virus movement. Multicolour live-cell imaging that allows tracking of single-virus particles together with fluorescent-protein labelled cellular structures provides a powerful tool for studying viral entry mechanisms. Using this approach, even transient interactions between viruses and cellular proteins can be detected. Multicolour labelling of distinct virus components also allows viral disassembly during entry to be monitored. Such dynamic information is crucial for a more complete understanding of viral entry mechanisms.



The trajectories of single influenza viruses in live cells revealed actin-dependent movement of the virus before the particles were taken up by endocytosis[5,13]. Several viruses, such as murine leukaemia virus, Avian leukosis virus, HIV and vesicular stomatitis virus (VSV) use the cortical actin cytoskeleton, together with myosin II, for directed movement along microvilli or filopodia towards entry 'hot spots' at the cell body[15]. Murine polyoma virus-like particles have distinct modes of movement on the cell surface: an initial phase of free diffusion is followed by actin-dependent, confined movement in small domains[16]. A virus-imaging experiment shows that, in order to reach specific receptors located at tight junctions, coxsackieviruses first binds to the attachment factor DAF (decay-accelerating factor), which through Abl kinase activates a Rac-dependent actin rearrangement that allows the virus particle to move towards the tight junction[17]. Together, these imaging experiments have substantially improved our understanding of viral entry and revealed an early stage of entry that had been largely overlooked previously: viral attachment is followed by organized transport on the cell surface, often mediated by actin, probably to locate specific receptors or special active zones that are efficient in virus uptake.

Multicolour live-cell imaging provides additional information on the interactions between virus and cellular structures, further elucidating viral-entry mechanisms. Using this approach, single Simian virus 40 (SV40) particles were simultaneously tracked with GFP-tagged caveolae. These experiments revealed the dynamic SV40 entry process and a new endocytic organelle, the caveosome[18,19,20] (FIG. 2). Most SV40 co-localizes with caveolae, activating tyrosin kinases and inducing rearrangement of actin stress fibres to form actin tails on virus-loaded caveolae[21] (FIG. 2). After a dynamin-dependent pinching process, the SV40-loaded caveolae leaves the plasma membrane and enters the caveosome before finally reaching the ER through microtubule-dependent transport. Polyoma virus and echovirus 1 also enter cells through a similar pathway to SV40 (REFS 22–24).

Influenza virus uses a distinct entry mechanism[13,25,26]. As directly visualized in a virus tracking experiment, influenza viruses can simultaneously use two pathways to enter cells[13] (FIG. 2): most virus particles are internalized through clathrin-mediated endocytosis by promoting the *de novo* formation of clathrin-coated pits (CCPs) at the viral binding site; the remaining virus particles enter cells through a clathrin- and caveolin-independent pathway. After entry by these pathways the virus particles have similar post-endocytic trafficking behaviour, which leads to viral fusion with similar efficiencies. As obligatory parasites that exploit constitutive cellular functions for their own replication, viruses are also natural probes for understanding cell biology. Experiments tracking fluorescent Rab proteins together with single influenza particles (as well as transferrin, LDL and EGF (epidermal growth factor)) in live cells reveal two distinct populations of early endosomes[27]: a dynamic population that matures rapidly to form late endosomes, and a relatively static population that matures much more slowly. Different cargos are differentially targeted to the two endosomal populations. Influenza viruses are preferentially delivered, by microtubule-dependent transport, to the dynamic early endosomes[27]. Viral fusion typically occurs in the midst of the endosomal maturation process with both early and late endosomal markers present on the endosome — an intermediate state of endosomal maturation that has only recently been discovered[27–29].

Other viruses have also been observed to co-localize with CCPs in live cells, including reovirus and adenovirus[30,31]. In common with influenza, reovirus enters by the *de novo* formation of CCPs[30]. Experiments tracking reoviruses, transferrin, LDL and labelled CCPs revealed a random initiation behaviour of coated pits, which are only stabilized after cargo binding[30]. For HIV, although direct fusion with the plasma membrane is the main pathway to deliver the capsid into the cytoplasm, a smaller fraction of viruses also infect cells by clathrin-mediated endocytosis[32]. Besides clathrin- or caveolin-mediated endocytosis, various viruses, including polyoma virus and herpes simplex virus (HSV)[33–35], exploit other clathrin- and caveolin-independent entry pathways.

As shown by these examples, the ability to monitor time-dependent behaviour of individual virus particles

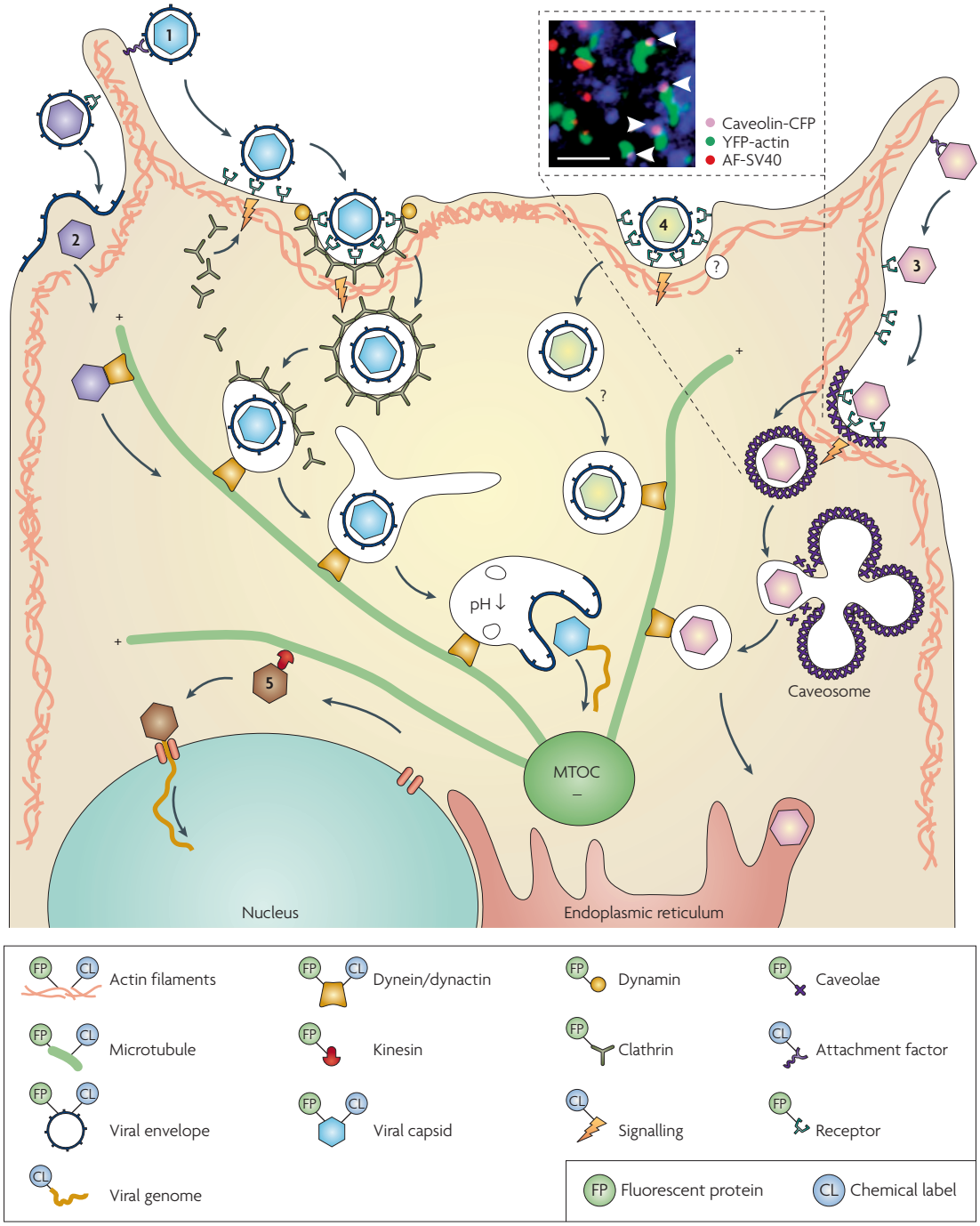


Figure 2 | **Viral entry and transport.** Viruses attach to the plasma membrane, surf on the cell surface or along the filopodia (**1–3**), and bind to specific receptors before entering the cell. Viruses can directly fuse with the plasma membrane (**2**). They also hijack endocytic pathways, including clathrin-dependent (**1**), caveolin-dependent (**3**) or clathrin- and caveolin-independent (**4**) pathways for internalization. After internalization and transport through the actin matrix, vesicles that contain virus are transported by dynein or dynactin along microtubules towards the microtubule organizing centre (MTOC). This might include trafficking of viruses through endosomes, caveosomes or the endoplasmic reticulum, prior to the release of the virus into the cytoplasm. Capsids can also be transported by dynein or dynactin along microtubules. From the MTOC, capsids can be transported by kinesin towards the replication site of the nucleus (**5**). Some viruses release their genetic material into the cytosol whereas others transport their genomes into the nucleus. The key shows how the different components have been labelled previously. The inset panel shows the caveolin-mediated endocytosis of Simian virus 40 (SV40). The arrowheads indicate SV40-containing caveolae co-localized with actin tails. The dye-labelled SV40 particles are shown in red and the fluorescent protein-labelled caveolin and actin are in purple and green, respectively. Scale bar represents 3μm. Inset panel reproduced with permission from REF.21 © (2002) American Association for the Advancement of Science.

and to probe dynamic interactions between viral and cellular structures has substantially enriched our understanding of viral-entry mechanisms at the molecular level. These studies have not only revealed previously unknown virus–cell interactions that are crucial for infection, but have also shed new light on fundamental cellular mechanisms and pathways. Emerging single-virus tracking experiments have also begun to study fusion mechanisms of enveloped viruses in live cells[36–38]. Compared with enveloped viruses, the uncoating mechanisms used by non-enveloped viruses are less well understood, but we expect future single-virus experiments to contribute to this important area of virus research.

Viral transport

After uncoating, the viral contents need to be transported to proper sites for replication. Objects larger than 20 nm (or significantly heavier than 500 kDa) cannot freely diffuse through the crowded cytoplasm[39]. Instead, molecular motors shuttle on cytoskeletal tracks to actively transport cargo. Kinesin and dynein motors move on microtubule tracks, whereas myosin motors interact with actin filaments[40,41]. The polymerization of actin can also propel a cargo particle in and out of the cell[40]. All of these transport systems have been successfully hijacked by viruses.

As is evident in single-virus trajectories, many viruses are transported by minus and plus end-directed motor proteins along microtubules towards the microtubule organizing centre (MTOC), either by directly interacting with molecular motors or through inclusion in a motor-interacting vesicle (FIG. 2). This was observed for reovirus, adenovirus, HSV, influenza virus and HIV[5,42–45]. Viral transport is often highly regulated as with, for example, HSV in neurons. After fusion of incoming virions with the plasma membrane, HSV capsids are transported towards the minus end of microtubules by dynein and its cofactor dynactin[43]. In the axons of sensory neurons, incoming HSV capsids are moved by retrograde transport, whereas newly assembled capsids undergo bidirectional and saltatory motions, indicating a modulation of the plus-end directed motility[46]. This modulation involves HSV tegument proteins[47–51], the removal of which precedes the retrograde transport of the incoming capsid to the cell body, whereas the addition is coupled to anterograde transport of progeny capsids to the distal axon[52,53]. Important signalling events might occur during viral transport. During its dynein-dependent motion along microtubules, adenoviruses transiently activate distinct signalling cascades, such as cAMP-dependent protein kinase A, p38/mitogen activated protein kinase (MAPK) and the downstream MAPK activated protein kinase 2, to balance nuclear targeting and enhance infection[42,54].

The mechanisms by which capsids are transported to the nuclear pore complex for the import of viral genomes are less well known. Capsids might be transported by kinesin from the MTOC towards the nuclear pore complex and nuclear import factors might be involved in the unloading of capsids from microtubules that are proximal to the nucleus[55] (FIG. 2). The direct tracking of an incoming viral genome in live cells has been challenging due to difficulties encountered in generating infectious virions harbouring labelled viral DNA or RNA. Tracking microinjected viral ribonucleoprotein (vRNP) particles from influenza showed diffusion as the predominant mechanism for the transport of vRNPs both towards the nuclear pore complexes and inside the nucleus[56]. In a recent study, the HIV genome-associated integrase was labelled, and automated 3-dimensional particle tracking revealed that HIV-1 complexes underwent both microtubule- and actin-dependent movements toward the nucleus and more diffuse movements once inside the nucleus[57]. Remarkably, reverse transcription of the HIV genome seems to begin while the virus is being transported to MTOC[44].

Viral assembly and egress

A successful viral infection is ultimately marked by the assembly and release of progeny virus particles. After genome replication and protein synthesis, the subviral components are transported to the assembly site and progeny virions leave the cell by directly budding from the plasma membrane, controlled exocytosis or lysis of the cell (FIG. 3). Labelling viral structural components with fluorescent proteins allows the assembly of individual virions to be monitored, and shows the location and kinetics of assembly as well as the exit mechanisms of matured viruses.

A series of imaging experiments by time-lapse microscopy revealed the exit mode of vaccinia virus[40]. Intracellular enveloped vaccinia virus particles move from their perinuclear assembly site to the plasma membrane along microtubules[58–63]. These particles fuse with the plasma membrane, but remain attached as cell-associated enveloped virus (CEV)[64]. The envelope protein B5R activates the Src family kinases, which phosphorylate another viral protein A36R, which in turn recruits the actin polymerization machinery, forming a characteristic actin tail beneath the CEV to enhance cell-to-cell spread of the virus[65] (FIG. 3). Interestingly, the phosphorylation of A36R also triggers the release of kinesin from the virus[65], indicating that the viral envelope protein coordinates the transition from microtubule-based to actin-based transport, which is crucial for the egress of the virus. The actin-tail formation can also be independently initiated by the Abl-family kinase, which induces the release of CEV from the cell[66]. It was recently discovered that African swine fever virus also shows a microtubule-dependent movement towards the plasma membrane, followed by actin polymerization that propels the virus away from the cell[67,68].

Compared with imaging the entry and transport of single virions, which typically involves labelling incoming viruses and therefore does not suffer from strong background cell fluorescence, it is more challenging to probe the assembly and egress of viruses at the single-particle level. The background fluorescence from newly synthesized viral components makes it extremely difficult to monitor early assembly steps. Determining the distribution of viral proteins in living cells is currently

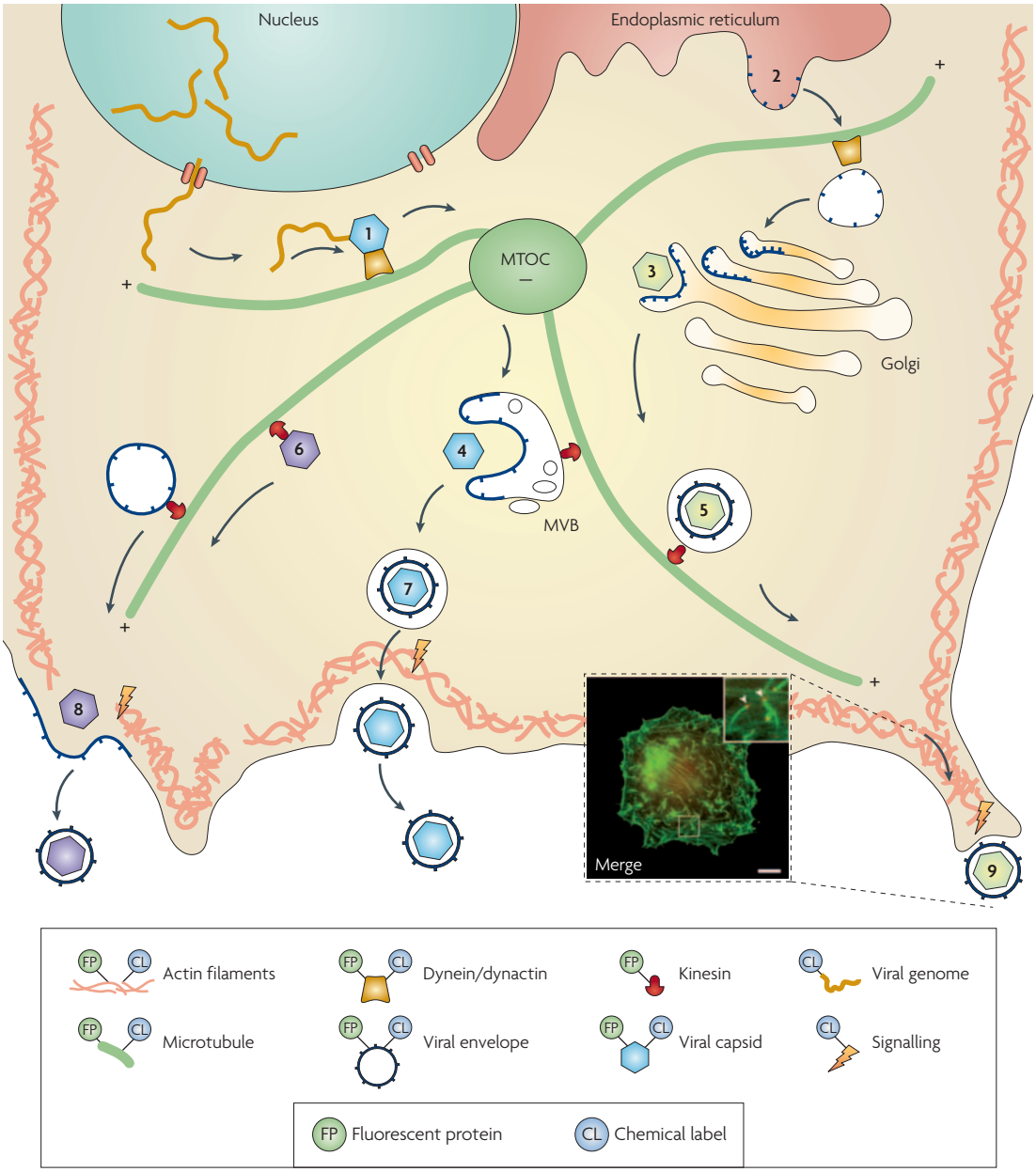


Figure 3 | **Viral assembly and egress.** Viral genomes are packaged into capsids and are transported along microtubules (**1**). Viral membrane proteins are translated at the endoplasmic reticulum membrane (**2**) and transported along microtubules to the Golgi apparatus, where capsids can bud into an envelope (**3**). Viruses also bud into the multivesicular bodies (MVB) (**4**). Complete virions inside transport vesicles (**5**), or subviral particles (**6**), are transported by kinesin on microtubules towards the plasma membrane, and exit the cell by exocytosis (**7**) or budding (**8**) at the plasma membrane. During egress, the actin cortex might propel viruses towards neighbouring cells through a dynamic actin tail (**9**). The key shows how the different components have been labelled previously. The inset image shows actin-tail formation during the egress of vaccinia virus. Actin (green)and the phosphorylated viral membrane protein A36R (red) were detected by immunofluorescence. MTOC, microtubule organization centre. Scale bar represents 10 μm. Inset image reproduced with permission from REF.65 © (2004) American Association for the Advancement of Science.

more tractable. For example, HIV-1 Gag is initially diffuse in the cytosol and then accumulates in perinuclear clusters, after which it transiently passes through multivesicular bodies before moving to the plasma membrane[69]. The bluetongue virus core protein VP3 has highly distinct cellular distributions in the presence and absence of other viral proteins (VP7 and NS2) that are related to assembly[70]. After initial stages of assembly which accumulate sufficient proteins to form a virion, the signal might become sufficiently large to allow viral egress to be tracked at the single-virion level. Experiments with labelled pseudorabies virus tegument and capsid proteins demonstrate viral assembly in the cell body before entering the axon of cultured neurons[71]. The association of human

cytomegalovirus tegument and capsid proteins occurs inside nuclear inclusions[72]. As brighter probes become available and new imaging methods continue to improve background suppression, we expect viral assembly and egress to become as fertile a ground for single-particle tracking experiments as viral entry and transport.

Future perspectives

In this article we have reviewed technical developments that have enabled single-virus imaging in live cells and new insights into viral infection derived from these experiments. Viral infection is complex: viruses can exploit a number of different pathways to infect cells, each pathway consists of many steps and each step features specific interactions between viral and cellular constituents. This level of complexity demands investigation of viral infection at a systems biology level and such research is now taking place. A tour-de-force, high-throughput siRNA screen that compared the involvement of the human kinome (the collection of genes in the human genome that encode kinases) in clathrin-mediated and caveolin-mediated endocytosis revealed distinct but overlapping sets of kinases that regulate VSV and SV40 entry[73]: 92 kinases regulate VSV entry, 80 regulate SV40 entry and 36 kinases are involved in both. Remarkably, most of the overlapping kinases have opposite effects on VSV and SV40. Extending this approach to other viruses and to a much larger set of genes potentially related to viral infection will provide a systematic analysis of viral infection at the molecular level.

It will be extremely fruitful to combine this systems-biology approach with the ability of single-virus tracking to dissect multiple infection pathways and multiple infection steps, such that the mechanism of action can be clearly identified for a large set of host genes that are involved in viral infection. This requires the development of automated acquisition of high-resolution live-cell images that are compatible with high-throughput screening, and algorithms that would allow the analysis of the resulting fast-growing data sets.

Many of the viral infection steps involve the action of only a few viral and cellular molecules. To track these steps in live cells, the development of brighter and smaller probes that allow single-molecule detection in live cells is needed. Furthermore, molecular interactions typically occur at the nanometer scale, beyond the resolving power of conventional optical microscopy. Ultra-structural information is typically obtained by electron microscopy, which is unfortunately not compatible with live-cell imaging. Recently, a number of nanometer-resolution optical imaging methods have been reported, including stimulated emission depletion (STED) microscopy[74], stochastic optical reconstruction microscopy (STORM)[75] photoactivation light microscopy (PALM)[76] and structured illumination microscopy (SIM)[77]. Although substantial improvement in imaging speed is needed before cellular processes can be imaged using these methods in real time, the accomplishment of this goal will definitely open up the investigation of virus–cell interactions.

Finally, it is important to note that cultured cells are simplified model systems. A complete understanding of viral infection would greatly benefit from *in vivo* experiments. It is therefore particularly exciting to track virus particles in live tissues and animals to see how viruses break host defence barriers to reach target cells for infection and how viral infection is transmitted from cell to cell. With the rapid development of *in vivo* imaging methods, such as non-linear optical microscopy[78,79] and endoscopy, this dream might be realized in the not-too-distant future.

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Competing interests statement
The authors declare no competing financial interests.

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