Viruses as probes for systems analysis of cellular signalling, cytoskeleton reorganization and endocytosis
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It is well known that mammalian viruses hijack the cellular signalling and internalization machineries to enter and to infect their host cells; however, only in the past six years have researchers started to follow individual virus particles and to investigate the events that they induce in living cells. The relative ease of imaging individual virus particles with time-lapse microscopy, despite being limited by light-diffraction, allows for specific and local kinetic analysis of individual events in signalling, cytoskeleton reorganization and endocytosis. Furthermore, virus infection is an easy-to-use endpoint readout, which is ideally suited for functional genomics approaches. The combined information from these studies will be crucial for the development of models that describe the underlying systems of cellular signalling, cytoskeleton reorganization and membrane trafficking during virus entry.

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Introduction
Viruses are dependent on the machinery of the host cell for replication of their genetic material and for production of their capsid and envelope proteins. To reach the site of replication, a virus particle must bind to the host cell, penetrate the cellular membrane to enter the cytosol and often also enter the nuclear envelope to allow replication of viral DNA. Therefore, viruses have evolved remarkable mechanisms to exploit every possible cellular system that might aid them in this path. Furthermore, many viruses do not kill the cell upon infection, but use their cellular machinery for efficient spread between cells and tissues.

Viruses are excellent tools to study these cellular processes. Most can be easily purified and genetically or chemically modified. The three-dimensional structure of an increasing number of viruses is now known, some with atomic resolution. Another advantage is that several viruses down-regulate processes in the host cell that might otherwise produce background signals in assays. In recent years, these advantages, combined with the availability of sophisticated light microscopes for time-lapse imaging, have culminated in experiments in which the ways that individual virus particles enter and spread within cells have been monitored. In this review, I will describe the general approach that is used and give examples in which such studies have revealed exciting new insights into the life cycle of viruses and the cellular processes they hijack (see Figure 1).

Methodology to follow individual fluorescent virus particles
The general approach to study the pathway of individual virus particles is as follows. Either virus particles are purified and chemically labelled with a fluorophore or a recombinant virus is generated that has a fluorescent protein tag in one of its structural proteins. These modifications are not always easy. The virus particles should remain mono-dispersed and should maintain infectivity so that their interactions with the host cell are not altered. Incorporation of at least 50 fluorophores in an individual virus particle is required to generate a signal bright enough to be detected with standard confocal or wide-field microscopes. An appropriately diluted amount of such fluorescent virus particles will generate images with spots of uniform size and intensity (Figure 2). One spot represents an individual virus particle, which can be followed during cell attachment and internalization and can be used as a probe to study virus-induced signalling events, cytoskeleton reorganization, endocytosis and transport through the cell.

Although fluorescent labelling to monitor virus internalization is an old technique [1], six years ago time-lapse confocal fluorescence imaging was first used to specifically follow individual particles of the non-enveloped virus adenovirus 2 (Ad2) [2]. Purified Ad2 capsids were labelled in vitro with ∼1000 Alexa Fluor molecules. These displayed directional movement along microtubules after internalization into cells. Tracking the particles allowed determination of the mode (processive or iterative), speed and directionality of movement. Similarly, particles of the non-enveloped virus simian virus 40 (SV40) that were labelled in vitro with ∼500 Alexa Fluor molecules were used to monitor their internalization by
individual caveolae and to determine the movement of the vesicles containing these virus particles along microtubules [3]. Enveloped viruses can also be labelled in vitro, either by the addition of protein-reactive fluorophores that label the envelope proteins or by fluorescent lipid dyes that intercalate with the lipids in the viral envelope. The latter method can also be used to monitor the local fusion of the envelope of the viral particle with the cellular membrane [4], capitalizing on the dequenching properties of certain lipid dyes upon dilution. This is similar to classical biochemical assays that monitor fusion of membranes in a test tube.

In another example, a recombinant herpes simplex virus (HSV-1) was generated that expresses protein (VP26) fused to green fluorescent protein (GFP), which is incorporated in the viral capsid [5]. If each capsid contains about ~400 GFP molecules, the signal of individual virus particles is bright and can easily be followed during cell entry. Similarly, researchers constructed a human immunodeficiency virus (HIV) that encodes Vpr, a structural protein of the reverse transcription complexes, which is fused to GFP [6]. This allowed them to monitor the dynamics of individual reverse transcription complexes as they move along microtubules towards the nucleus.

**Signalling**

To prepare a cell for the invasion, virus particles trigger events as soon as they bind to the plasma membrane. This generally involves the binding to and the cross-linking of cell-surface molecules such as glycosphingolipids, receptor tyrosine kinases and integrins. By following an individual virus particle, one has the possibility to study the actual cross-linking process, initiation of signalling, and propagation of the downstream cascade from a single site on the plasma membrane. To date, it is remarkable what little use has been made of individual virus particles to study cellular signalling, because their fixed size and defined number of receptor-binding sites are likely to allow more controlled analysis of local signalling events than soluble growth factors or cytokines, or even than coated latex beads [7].
A good example is the analysis of the signalling events induced by human cytomegalovirus (HCMV). HCMV is a herpesvirus that is known to activate several signalling pathways including phosphatidylinositol-3-OH kinase (PI3K), G-protein and mitogen-activated protein kinase cascades [8]. Recently, the epidermal growth factor receptor (EGFR) was identified to be a cellular receptor of HCMV [9]. Time-lapse imaging of individual fluorescent HCMV particles combined with fluorescent sensors of the EGF cascade will be a powerful means to study local activation of EGFR signalling as well as signal propagation. Furthermore, because EGFR signalling results in actin cytoskeleton remodelling and stimulates endocytosis [10], such studies will undoubtedly shed light on the entry process of HCMV, which is currently not well understood.

Upon binding to its cellular receptors, the coxsackievirus B adenovirus receptor (CAR) and αV integrins, Ad2 induces signalling cascades through PI3K and Rac1, a small GTPase of the Rho family [11]. Local signal activation beneath a membrane-bound Ad2 particle has not yet been analysed, and thus details are still lacking. However, recent work has shown that Ad2 induces signalling for two reasons [12]: first, the signalling leads to sequestration of the integrin-bound virus particle into clathrin-coated pits, which are subsequently internalized; second, the actin cytoskeleton is reorganized to form membrane ruffles and macropinocytosis is increased. Interestingly, echovirus 1, a virus that utilizes a different combination of integrins as a receptor (α2β1), appears to activate other downstream events. Upon cross-linking, the integrin complexes re-localize to caveolae and are subsequently internalized in a protein kinase c-dependent manner [13,14]. This is an important observation for studies of cell adhesion, as it suggests that the dynamic association with caveolae and the caveolae-mediated endocytosis might, under certain conditions, modulate integrin functions.

SV40 binds to glycosphingolipids [15] and induces a signalling cascade that results in the tyrosine phosphorylation of proteins that localize to caveolae [16]. It is unclear how the multivalent binding to and the clustering of glycosphingolipids in the extracellular leaflet induces phosphorylation of tyrosine residues on the cytosolic leaflet, and if transmembrane proteins, such as major histocompatibility complex class I molecules, are involved [17]. One theory is that the multivalent binding of glycosphingolipids on the extracellular leaflet leads to coalescence of lipid rafts, which are small microdomains composed of tightly packed glycosphingolipid and cholesterol molecules [18]. If the two leaflets of lipid rafts are coupled the clustering would lead to coalescence of raft molecules on the inside of the membrane. This coupling could be achieved through interactions between the long saturated fatty acids of the glycosphingolipids in the outer...
leaflet and the lipid-modified or membrane-inserted (but not spanning) proteins (such as c-Src or caveolin-1, respectively) in the inner leaflet. The resulting increase in the local concentration of signalling molecules, such as c-Src, might activate a downstream signalling cascade [18]. This theory is largely hypothetical, and SV40 particles could serve as ideal tools to test it. Indeed, recent observations suggest that caveolin-1 contributes to the coupling of raft membrane leaflets and assembles a stable membrane domain, which sequesters SV40 particles [19].

The actin cytoskeleton
In many cases, virus-induced signalling leads to dynamic changes in the actin cytoskeleton. This might have several purposes. One is to increase or to activate endocytic activity. For instance, after binding to the cell surface SV40 stimulates breakdown of both actin stress fibres and the cortical actin cytoskeleton to activate endocytosis of virus-loaded caveolae. Interestingly, by studying the dynamics of individual particles and GFP-tagged actin, it was observed that local signalling events stimulate short bursts of actin polymerization beneath individual surface-bound virus particles, which is necessary for productive infection [16].

Another purpose is to bring cell surface-bound virus particles to sites of high endocytic activity. This is nicely illustrated in the recently discovered mechanism of virus-particle surfing. By studying the local binding to and the dynamics of fluorescent virus particles on the surface of cells, it was found that particles from several unrelated extracellular viruses bind to a filopodium and are then transported to endocytic hotspots located in the cell body in an actin- and myosin 2-dependent manner (W Mothes, unpublished). The activity is provided by retrograde F-actin flow, and might represent a cellular process to change the location of signalling receptors on the cell surface to modulate signalling cascades.

Enveloped viruses that fuse directly with the plasma membrane need to overcome the cortical actin barrier to efficiently infect the cell. HSV-1 probably tackles this problem by activation of Ca^{2+} signalling pathways [20], which are well known to induce cortical actin depolymerization and destabilization of focal adhesions [21].

Actin rearrangements might also aid in the efficient spread of progeny virus particles, as observed for vaccinia virus (VV) [22]. Individual VV particles can be observed by time-lapse microscopy to stimulate the formation of actin tails. Although this was initially believed to mediate both intracellular propulsion and intercellular spread, similar to that seen in the bacterium *Listeria* [23], more careful analysis revealed that VV only uses actin tails for spread between cells after fusion of its outer envelope with the plasma membrane [24].

Endocytosis
Actin rearrangements contribute to virus particle internalization, in most cases, by endocytosis. Ironically, although virus endocytosis might have been intended as a cellular defence mechanism aimed at destruction of the particle within lysosomes [25,26], many enveloped viruses hijack the process as the low pH in early and late endosomes provides a convenient cue for the virus to initiate membrane penetration [1]. As a result, the virus takes a convenient ride into the cell and escapes before it is exposed to degradative lysosomes [27]. For influenza (flu) virus, the process of fusion of the envelope with the endosomal membrane has now been followed live and *in vivo* by observation of the dequenching of a fluorescent lipid dye that is incorporated in the virus’ envelope [28*].

Recent studies that use individual fluorescent virus particles and time-lapse imaging are revealing new insights into the endocytic process that have been previously overlooked. For flu and reovirus particles, it was observed that individual particles do not directly bind to or laterally move into pre-assembled clathrin-coated pits on the surface, but initiate the formation of the coat directly under them after binding [28*,29*]. This appears to be a general principle of clathrin-coated pit formation, in which a limited amount of clathrin monomers undergo transient polymerization anywhere on the cell surface (the exploratory mode), and only commit to the formation of a full coat when a nucleation factor, such as a receptor-bound virus particle, is present [29*].

Our knowledge about alternative clathrin-independent endocytic routes has greatly increased in recent years owing to the use of viruses [3,16,19,30,31]. Studies of polyomavirus and SV40 have revealed a new endocytic itinerary that is activated by the virus particle and involves internalization by caveolae or lipid rafts, intracellular traffic through caveosomes (a novel type of endocytic organelles that have a neutral pH and are enriched in caveolin-1), sorting from caveosomes to tubular membranes, and accumulation in the smooth endoplasmic reticulum. This itinerary is, at least in part, shared by echovirus 1 [32*]. Cholera toxin is also partly internalized by caveolae and might be transported to the endoplasmic reticulum by way of a similar route or might diverge at one of the intersections between the caveolar and the classical endocytic pathway [19]. Although we are far from having a complete understanding of these endocytic pathways, it is clear that they would not have been revealed without the use of viruses.

Finally, several recent studies that employ detailed analysis of virus entry events come to unprecedented conclusions. For instance, many flu particles are not internalized by clathrin-coated pits, but by way of an alternative endocytic pathway [33]. Another surprise is the recent finding that in HeLa cells, HIV is, to a large
extent, dependent on clathrin-mediated endocytosis for infectious entry [34**]. Also, HSV-1 enters HeLa cells by endocytosis instead of by direct membrane fusion [35**]. The continuation of comprehensive analysis of the exact itineraries that viruses take will probably reveal many more unknown features of the endocytic pathway, and will force us to change our, sometimes dogmatic, views.

**The microtubule cytoskeleton**

After internalization and/or penetration of the plasma or endosomal membrane, virus particles or the vesicles that carry them interact with the microtubule (MT) cytoskeleton. This was first demonstrated for HSV-1 [36] and with time-lapse microscopy for Ad2 [2]. From the latter study, it immediately became clear that MT-mediated transport does not simply mean unidirectional movement, but rather a sum of motile events in both directions with a net transport towards one end. Similar observations have been made for intracellular HSV-1 particles (A Vonderheft and B Sodeik, personal communication) and HIV particles [6] on their way to the nucleus, as well as for VV particles during exit [24]. Also, endocytic vesicles that carry internalized viruses, such as caveolar vesicles and caveosomes that contain SV40 particles [3] and early endosomes that contain Semliki Forest virus (L. Pelkmans, unpublished) or flu particles [4], display complex movements in both directions, which indicates track-switching from one microtubule to the other. This implies that both intracellular virus particles and vesicles that carry virus particles are able to bind to multiple motors with different directional specifications and are able to regulate their activities. We are far from understanding how this system works, but we know that the motor complex dynein–dynactin is commonly used for transport to the cell centre, and conventional kinesin is used for motility towards the cell periphery. However, it is probable that multiple motors are used in a coordinated fashion. Interestingly, during transport of VV from the cell centre to the periphery and its subsequent spread, VV switches from conventional kinesin- and MT-mediated transport to actin-mediated transport. It does this by a mechanism that is controlled by c-Src [37]. This mechanism could be the reverse of what endocytic vesicles use to switch from actin- to MT-mediated transport during movement from the periphery to the cell centre [38]. Also, *in vitro* assays that use fluorescent microtubules and virus capsids together with cytosol components will aid in deciphering these complex events.

**Viruses as tools in systems biology**

Viruses will be tremendously useful in the emerging field of molecular systems biology. The aim of a systems biologist is to formulate models that comprehensively describe a biological process in quantitative terms to reveal the system’s design principles and to predict the behaviour of that system in different environments. Two directions that should finally meet will be essential to reach this goal. First, the components and the regulatory circuits involved in this process have to be determined. Second, the dynamics and flow of information and material have to be recorded quantitatively, both in steady-state situations and after a series of perturbations, and the role of the identified components to establish these dynamics needs to be determined. In this respect, the careful analysis of the actin-based motility of *Listeria monocytogenes* (although this intracellular parasitic bacterium is a very simple system composed of only nine essential components) [39], including the recent development of a sophisticated computational model that is able to reproduce many characteristics of this system [40], serves as a good example.

Indeed, recent advances indicate that viruses are ideal for analysis of this type. First, considerable progress has been made in the development of automated image-analysis software that is able to automatically detect and to track small fluorescent spots in a time-lapse series acquired with confocal, wide-field or total internal reflection microscopy [29*,38,41,42] (I. Sbalzarini and Y. Kalaidzidis, personal communication). These software tools, facilitated by the appearance of viruses as spots of uniform size and intensity, will allow us to obtain kinetic and quantitative measurements of thousands of individual virus particles. By including fluorescent cellular proteins, we will be able to accurately measure the subcellular positions of virus particles and their interactions with cellular components, and to reconstruct particle movement from series of images taken with high time-resolution. This will provide information on the directionality and the speed of movement.

Second, because the cellular processes hijacked by viruses are rate-limiting for successful infection and spread, their functionality can be easily and systematically analysed by measurement of viral gene expression. The complete sequencing of several mammalian genomes, the advent of RNA interference (RNAi), and the production of genome-wide arrayed cDNA and short interfering RNA (siRNA) libraries allows systematic analysis of most, if not all, genes involved in these processes. Recombinant viruses that express a fluorescent or a luminescent protein provide an easy readout that can be automated for use in high-throughput screening. Furthermore, by analysis of the infectious entry pathway of many viruses in parallel in this way, a functional profile can be assembled for each cellular gene, which allows identification of entry pathway-specific functional groups.

The feasibility of this approach was tested in a high-throughput screening of the function of the human complement of lipid, protein and carbohydrate kinases (the kinome) in the infectious entry pathways of SV40 and vesicular stomatitis virus (VSV) [43**]. This analysis generated numerous new insights. It revealed that the
kinome plays a particularly prominent role in virus infection and that these two infectious entry pathways are differentially regulated. It showed that most kinases involved in VSV infection are required (their silencing reduces infection), whereas half of the kinases involved in SV40 infection act as suppressors (their silencing enhances infection). It revealed that the pathways are under reciprocal and extensive control by various cellular processes and by most of the known signalling pathways. For example, nutrient-sensing by the target of rapamycin (TOR) cascade specifically controls the VSV pathway, whereas integrin signalling controls the SV40 pathway. Using secondary assays, it was estimated that around 70% of the kinases have functions in endocytosis besides their known roles in signalling and in the regulation of the cytoskeleton. Among the other 30% of kinases, some genes were found to be essential for viral infection but not for the general fitness of the cell. Taken together, these first results have already revealed general principles in the regulation of these two infections. They show how much we still have to learn to gain an understanding of well-characterized virus entry pathways and demonstrate the future potential of this approach to reach that goal.

Conclusions

I see two main future directions in this area of research. One will continue with the characterization of virus infection pathways, including several viruses that have evolved yet unknown mechanisms of cell entry or spread. Detailed and careful analysis of these viruses will reveal new cellular processes and will add to our fundamental understanding of the cell.

A second area for future research will utilize well-characterized virus model systems. The viruses of which the molecular biology is known should be engineered in every possible way to allow a systematic analysis of the complete infectious cycle, including cell attachment, entry, replication and translation, as well as the spread of newly synthesized virus particles. We will be able to follow the dynamics of thousands of virus particles under many different conditions (using systematic screening of small compounds, cDNAs, siRNAs, etc) for many different virus systems in parallel. This ‘systems virology’ approach will not only contribute to the systematic analysis of cell biological processes but will also allow us to define pathway-specific sets of genes and to formulate a genetic fingerprint of virus entry pathways, providing new means to classify mammalian viruses. This might be crucial for the development of future anti-viral drugs as it will allow the identification of cellular components and their small molecule inhibitors, crucial for virus infection but that are dispensable for cellular physiology. Targeting of cellular components instead of viral proteins will be less hindered by the development of viral resistance and could thus serve as a much better approach for anti-viral therapies.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

14. Upla P, Marjomaki V, Kankaanpaa P, Ivaska J, Hyytiä T, Van Der Goot FG, Heino J: Clustering induces a lateral redistribution of α2β1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. Mol Biol Cell 2004, 15:625-636. This study shows that the multivalent clustering of certain integrin combinations induces their redistribution into caveolae. Not only might this be the mechanism for how echovirus 1 gets sequestered into caveolae but it also suggests how caveolae might be able to regulate integrin function, a link that was long known to exist.
Old literature predicted that murine polyoma virus and SV40 virus use protein and lipid components as cellular receptors; this study identified the lipid component — ganglosides. These lipids are enriched in caveolae and in lipid rafts, which explains, in part, their modes of viral entry. Nevertheless, differences to other gangloside-binding ligands as well as the strong ability to activate signalling cascades suggest that additional receptors, which have not yet been identified, are involved.


This study richly documents the dynamics and itinerary of echovirus 1 entry, showing similarities, but also differences, to the entry pathways of SV40 and polyomavirus. It will be interesting to compare the host machineries involved in these three related entry pathways [43**].


This study and [35**], foremost the one from Nicola et al., have successfully and convincingly challenged dogmatic views in the virus entry field. They show that viruses that were believed to fuse directly with the plasma membrane are actually more efficient at entering the cell via endocytosis. Although this might be specific for HeLa cells, which were used in both studies, it shows that HIV and HSV-1 are able to hijack the endocytic machinery with important implications for strategies to fight these viral infections.


See annotation to [34**].


This study is the first to systematically compare the host (human) cellular genes that are involved in two different infectious virus entry pathways using RNAi methodology. By doing this, major differences between the regulatory principles of the endocytic pathways they hijack are revealed. In general, when expanded to several viral systems and complete host genomes, such studies can lead to a re-classification of virus families according to the host machineries they hijack, and could change our view on anti-viral treatments, focussing on the host instead of on the pathogen.