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Single-Virus-Force-Spectroscopy unravels molecular details of virus infection

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Abstract:
Virus infection is a multistep process that has significant effects on structure and function of both the virus and the host cell. The first steps of virus replication include cell binding, entry and release of the viral genome. Single-Virus-Force-Spectrosocpy (SVFS) has become a promising tool to understand the molecular details of those steps. SVFS data complemented by biochemical and biophysical including theoretical modeling approaches provide valuable insights into molecular events that accompany virus infection. Properties of virus-cell interaction as well as structural alterations of the virus essential for infection can be investigated on a quantitative level. Here we will review applications of SVFS to virus binding, structure and mechanics. We demonstrate that SFVS offers unexpected new insights not accessible by other methods.

Insight Box:

With this review, we aim to introduce single-virus force spectroscopy (SVFS) as a recent branch in biophysical virology. Innovative technical improvements integrated into virology and cell biology provide new insights into virus infection, while challenging the classical view of related processes. We will briefly introduce technical requirements and continue with providing examples and discussing how SVFS can help to study virus-cell interaction at unforeseen detail to better correlate virus (and cell) structure with function.

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Viruses are a major class of pathogens and have been identified to infect a variety of organisms from animals and plants to bacteria and even other viruses[1]. Viruses were first identified as filtratable, transmissible and infectious agents in the late 19th century although related diseases were documented much earlier[2]. Since then, boosted by the invention of the electron microscope in the 1920s, a great variety of virus families were identified. Early virological experiments measured infection after virus transmission as the main readout. These approaches form the fundamental basis of experimental virology and provide important information about virus infection behavior.

The methods to study viruses underwent a rapid development. Nowadays, the potential to isolate, propagate and purify many viruses makes them accessible for a variety of modern biological and biophysical techniques. Among them, single molecule manipulation techniques allow the study of individual virus particles in an environment, that can closely mimic the natural situation.

Indeed, in contrast to the study of viruses in a bulk approach it has important advantages to study them at the single-virus level. The ensemble approach is very robust, but the result is only an average of a population of viruses. Taking into account that viruses behave differently, the average does not account for the biological variability. Already electron microscopy revealed that depending on the species, viruses may feature a high degree of pleomorphism[3, 4]. Many viruses have an intrinsically high mutation rate, allowing them to quickly adapt to environmental conditions, i.e. to evade an antiviral response or adapt to a new host tissue or organism. Even more important, several properties of viruses may not even be accessible by bulk approaches. As an example and as we will outline, single virus approaches allow to quantitatively describe the mechanical properties of the multilayer shells of enveloped viruses impossible by bulk methods. Not least, taking into account the variability of the host cell especially in an infection context[5], it is important to study virus infection at the level of individual cells.

Here we review recent advances in single-virus manipulation, imaging and force spectroscopy. Without being too technical, the aim of this review is to summarize and discuss essentially the *status quo* in single virus biophysics with a focus on force spectroscopy. We will discuss methods that allow to quantitatively explore biophysical

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properties of single virus particles as well as their host cell interaction and how obtained results enabled very unexpected insights into virus replication. Further, we intend to highlight the advantages of interdisciplinary approaches and how they can open new horizons in the field of virus biophysics.

1. Introduction

Viruses are a large group of pathogens that appear in many different shapes, protein and nucleic acid configurations as well as host tropism[1]. As many viruses remain a constant threat for the human population, it is of paramount importance to understand the many aspects of virus biology. As we will discuss, virus biophysical methods enable to study viral characteristics and processes along the infection cycle at molecular resolution that in turn provide insights into their replication and cell biology.

Most viruses are of rather simple organization and only contain in the range of tens of different protein species, while some complex viruses can encode more than 200 genes[6]. There are multiple ways of classifying viruses in different groups. A more recent and widely used categorization was introduced by Baltimore in 1971 and refers to the nucleic acid genome that can be made of RNA or DNA of either (+) or (-) polarity[7]. However, the classical system developed by Lwoff and colleagues in 1962 also refers to structural properties such as the virus dimensions, symmetry or the

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presence of a lipid envelope[8]. We will introduce how such structural properties can be studied applying biophysical approaches to study single viruses and discuss their role in virus replication. For a virus being either enveloped or non-enveloped includes several mechanical properties and their modulation, that are important for the infection cycle and can be measured at the level of single viruses.

A fundamental feature of viruses is that they cannot reproduce without a susceptible host cell. Hence, virus infection starts with host cell binding. This process involves all kinds of biomolecules such as proteins, carbohydrates and lipids. Recognition of the host cell surface should preferably be specific to ensure that the virus enters a cell that is actually capable of producing progeny viruses. Such a high level of specificity can be accomplished for instance by using a co-receptor as in the case of Rotavirus, HIV or Adenovirus[9]. In this case, the presence of the first receptor mediates virus attachment to the cell surface (also attachment factor), while association with the second receptor produces a specific response either inside the virion (e.g. HIV, Rotavirus) or the cell (e.g. endocytosis). An example for the latter case is the binding of Coxsackievirus B, which involves the sequential interaction with decay-accelerating factor (DAF) and the Coxsackievirus and adenovirus receptor (CAR)[10], which induces a conformational change in the virus capsid and promotes endocytosis. Using multivalency represents another strategy to accomplish and support specific cell binding[11, 12]. Protein-carbohydrate interactions are typically of low affinity at the single recognition level, but can, involving multiple interactions of the same kind, sum up to constitute a very stable and specific interaction. Bacterial toxins such as Cholera or Shiga circumvent low affinity binding by using multiple (4 and 5, respectively) simultaneous interactions[13], while viruses such as influenza virus can engage with up to hundreds of receptors[12]. Thinking about the complexity of a mammalian plasma membrane[14], specialized techniques are required to investigate virus binding as well as subsequent downstream events. Indeed, as we will illustrate, only single virus approaches allow a quantitative study of both the degree of multivalency and the forces involved in cell binding.

We will describe experimental approaches that can be used to accomplish this task with a highlight on two single molecule manipulation techniques, optical tweezers (OT) and atomic force microcopy (AFM). We will discuss advantages and show how these methods as well as the combination with theoretical advances can unravel

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biophysical properties of viruses not accessible with other techniques.

2. Introduction to single molecule manipulation techniques. Optical tweezers and atomic force microcopy.

Optical tweezers. Optical tweezers originate from an observation made by Arthur Ashkin in 1970[15]. Ashkin observed, that the momentum transported by light can exert forces on particles with different refractive indices compared to the surrounding medium. The forces that determine an optical trap are sketched in Fig. 1. Importantly, optical traps use high-power lasers with a Gaussian beam profile, i.e. the center of the beam has the highest intensity. To minimize interaction with biological specimen by the high-energy lasers (3W), they operate at near-infrared light wavelength of 1064 nm[16]. In comparison, conventional confocal microscopes use lasers at 10-20 mW. However, only a small fraction of the energy will eventually interact with the biological specimen. If the laser light hits a spherical particle (e.g. a bead, Fig.1B) the light is refracted at the edges of the bead, changing the direction of the deflected beam[15]. Since light carries a momentum, this will cause a momentum change of the laser beam and induce a momentum transfer onto the bead. The amount of the momentum transferred onto the bead depends on the intensity of the deflected light and thus on the Gaussian beam profile. Hence, the total force exerted on the particle is the sum of forces produced by the different rays within the beam Fig.1D. This is called the gradient force and represents the most important force component of optical traps. The second component is also based on the particle characteristic of the light, which can induce radiation pressure. The so-called scattering force pushes the particle in the direction of the propagating beam. The gradient force must overcome the scattering force, which is realized experimentally by using a high-numerical aperture (NA) objective and creating a steep light intensity gradient[17]. As a result of both components, the particle is stabilized in the center of the beam slightly downstream of the focus. The x-y position of the trapped particles can be measured by detecting the laser beam with nm-precision on a quadrant photodiode (QPD), a light-sensitive diode that is divided into four equal segments. The total light intensity reaching the detector provides information about the particles axial position. After calibrating the optical trap, displacement of a particle from the center of the beam can be directly translated into the applied force. This allows to investigate processes at the level of single molecules.

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Atomic force microscopy. In contrast to optical tweezers, the atomic force microscope represents a contact tool, whereby a sample is probed by a mechanical sensor (scanning probe microscopy (SPM)). The sensor is a very sharp tip at the end of a cantilever arm. Typically, the tip is of pyramidal shape, with a tip radius of only a few nm. The AFM tip scans surfaces and creates a height contour map of the sample. The position of the tip is measured by pointing a laser beam on the cantilever and detecting its reflection using a photodiode (Fig. 1A). If the tip scans an uneven surface the cantilever will bend and alter the signal position at the photodiode. In combination with precise sample scanning, the topography of a biological specimen can be determined at high resolution, i.e. corresponding to the radius of the tip. AFM can reach spatial resolutions of down to 1 nm on biological samples, which allows imaging of single proteins such as rhodopsin[18] or connexin 26 of gap junctions[19]. The precise detection of the cantilever position enables force measurements in axial direction. AFM cantilevers can be functionalized using highly specific and resistant attachment methods[20, 21] with proteins, viruses, bacteria or cells[21, 22, 23, 24, 25, 26, 27]. Since the cantilever acts as a Hookean spring, it can in addition to topographic imaging, also be used to sense forces in axial direction. In force spectroscopy mode, a functionalized cantilever is lowered on an interacting surface (e.g. a cell) until binding occurs or the cantilever touches the surface. The cantilever is retracted and in case of a binding event will bend until the underlying bond fails and the cantilever returns into the zero-force position (see force-distance-cycle in Fig. 2A).

Force spectroscopy in microbiology

Although the focus of this article is on single-virus force spectroscopy, we will briefly outline related studies conducted using other microbes such as bacteria fungi or protozoic parasites[28, 29, 30, 31].

The attachment of single bacteria to modified AFM cantilevers allowed to study bacterial cell adhesion in a variety of different systems[24, 29, 32, 33, 34, 35, 36, 37]. Using AFM, the effect of antibiotics on bacterial cell membranes was tested for *Escherichia coli* and *Staphylococcus aureus* indicating that bacterial cell wall stiffness is not entirely uniform and responds to antibiotic treatment [38, 39]. Substrate adhesion of *Lactobacillus plantarum* was probed and revealed strong binding to both, biotic and abiotic surfaces[24]. *Vice versa*, also the binding of ligands to bacterial surfaces can be

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studied using force spectroscopy by combining specific ligands attached to AFM cantilevers with surface-immobilized bacteria[34]. Probing the surface of fungal spores revealed dynamic changes during the course of germination, which correlate with modified cell adhesion[40]. More recently, it was shown for *Aspergilus formigatus* that regularly arranged rodlets shield the hydrophilic inner cell wall during dormant phases presumably to promote spore dispersion[41]. Larger microbes such as *Trypanosoma* or *Plasmodium* allow the use of optical trapping in order to study motility or cell binding[42, 43, 44]. The motility pattern of African trypanosomes as well as their flagellar force generation was studied using microfluidics and a dual-optical trap set up[43]. Similarly, two optical traps were used to study the adhesion between Plasmodium and human erythrocytes thereby testing the effect of known cell invasion inhibitors[42].

3. Virus-cell binding.

Virus-cell binding marks the first interaction between virus and host cell. Important decisions are made during this process, most importantly for the virus is attachment to a cell that is suitable for generating progeny viruses. Surprisingly, some virus receptors are highly abundant molecules. Examples are glycan receptors such as sialic acid or gangliosides but also proteinaceous receptors like integrin or CD4. This would in principle allow systemic infection, however a variety of other host cell factors, such as endosomal proteases, necessary to cleave and activate membrane fusion proteins of enveloped viruses, the genome replication machinery, but also the transmission and infection route mostly prevent this. However, virus receptor specificity has long been an important characterization parameter for the description of viruses and the evaluation of their zoonotic potential[45, 46].
Different assays have been developed to measure or screen virus receptor binding. Among others, solid-phase binding assays allow the screening of a variety of receptor molecules such as glycan libraries[47, 48]. Here, the desired ligand is coupled to a flat surface and can either be probed with intact viruses[49] or purified protein[50], which is then detected using antibody binding. To measure thermodynamic properties of virus-receptor binding, surface plasmon resonance (SPR) has become widely used[51, 52]. Receptor molecules are flushed into a sample chamber, where they

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are allowed to interact with the gold-coated sensor chip. Flushing the sample into the chamber then allows to measure association as well as dissociation kinetics. However, both, the solid phase assay as well as SPR have some limitations, which render them only applicable for specific needs. The ligand density and orientation, which likely affects binding[53], is not easily controllable. In addition, SPR is known to often underestimate dissociation due to high re-association rates, a problem that becomes apparent when using multivalent ligands such as viruses. More recently, microscale thermophoresis (MST) was used to study the interaction of purified glycoprotein-receptor complexes[54]. MST has the advantage that binding and dissociation kinetics can be measured in solution under defined conditions. However, the complex environment of a three-dimensional host cell plasma membrane, where receptor molecules might be embedded in a glycocalyx or mucus layer further make it difficult to isolate and test the effect of specific molecules.

In order to measure virus-cell specificity of individual viruses at the level of single cells, more sensitive techniques are required. AFM and optical tweezers can be used to measure forces with single molecule resolution. Well-developed specific attachment protocols allow immobilization of viruses and other biomolecules on AFM cantilevers or micrometer-sized beads[22, 23]. This type of measurement, summarized as single-virus force spectroscopy (SVFS), allows to characterize virus binding using intact viruses and living cells under conditions that mimic the natural situation very closely.

Using AFM-based SVFS, it was shown that human rhinovirus forms multiple parallel interactions with living host cells, where binding forces could be confirmed by measurements on artificial receptor surfaces[22]. The correlation between individual receptor molecules involved in virus binding and cell adhesion might not be linear and can be of parallel and serial organization, a property that can only be investigated using single-molecule measurements. Measurements usually include force distance cycles (Fig. 2) while varying the loading rate, i.e. the pulling speed. Loading rate versus rupture force distributions can then be described using theoretical models to reveal thermodynamic parameters of the investigated interaction, such as the dissociation rate k_{off} [23]. The association rate k_{on} for virus-cell binding can be studied by varying the contact time and probing the binding probability[22, 55]. Dividing on- by off rate provides direct access to the overall affinity of the interaction, a parameter that can be compared with *in vitro* measurements of

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purified ligand-receptor pairs to study the modulation of binding properties due to the environment of the plasma membrane.

Multivalency is an intrinsic design principle for many virus-host cell interactions. Some viruses, such as influenza, VSV or Ebola are coated in a layer of spike proteins that simultaneously engage with receptors on the host cell. The affinity for the individual pair might be very low (mM-range for protein-glycan interactions[12, 56]), but can sum up to remarkable avidity values in the nM range[12]. On living cells, these interactions, although originating from the same type of molecular interaction pair might not be strictly self-similar. It was shown for influenza virus that single binding events underlie some intrinsic scattering that might be classified as measurement noise[23]. However, not only potential binding to other cell surface molecules, but also the structural variability of a living cell, i.e. the specific environment and orientation of each individual receptor can have an effect on the observed unbinding characteristics. The variability of single molecule interactions for influenza virus was studied using OT and AFM measurements on different living host cell types[23]. Using various cell types, that differ with respect to their sialic acid surface composition revealed that HAs receptor specificity might not be a direct indicator for binding to a living cell showing that specific glycan. This notion of cell and receptor specificity of being partly independent was also hypothesized for HRV[22]. The combination with *in silico* force spectroscopy provided an explanation for the intrinsic variability and reduced self-similarity of the measured force values. For influenza HA-sialic acid interaction, it was found that each individual unbinding event follows a unique unbinding trajectory resembling different kinetics, length and energy[23]. These insights emphasize the potential of combining experimental and modeling approaches for getting quantitative details of molecular interactions, while also showing that only specialized techniques such as SVFS are suitable to investigate virus-cell specificity on living cells. In addition, adapted data analysis protocols allow to account for the elasto-mechanical properties of the plasma membrane during force spectroscopy on living cells[57]. Here, a maximum likelihood approach can be used to consider each individual force trace thereby avoiding binning of loading rates and potential misinterpretation[57, 58].

Also for HIV, the interaction of the spike protein with co-receptors was studied using SVFS. It could be shown that engagement with the primary receptor CD4 is very stable but only for a short lifetime until the viral glycoprotein gp120, organized with

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gp41 in a homotrimeric complex, finds its preferred co-receptor molecule[59]. This secondary interaction is virus strain specific for the respective co-receptor, either CCR5 or CXCR4.

In future studies, also other infection events downstream of receptor binding might be accessible in SVFS. Indeed, endocytosis events of coated quantum dots can be detected within the cantilever approach force traces[60, 61]. Such force dips during cell contact could also be observed for influenza virus SVFS, but only during slow retraction speed, potentially giving the cell enough time to initiate plasma membrane invagination (Fig.3). This also illustrates the complexity of measuring interactions between viral glycoproteins and cellular receptors in systems mimicking closely the biological situation.

Multiparametric imaging

Multiparametric imaging provides a powerful combination of different AFM-based methods and can dramatically increase the information content of an AFM measurement. Force-distance (FD) curve-based AFM offer a unique combination of topographic imaging and simultaneous probing of biophysical properties by recording an FD curve at each xy position across the sample[62, 63, 64]. The resulting curves can be analyzed to extract properties such as adhesive forces as well as deformation and elasticity. Recognition imaging provides another method to correlate topography with adhesion by detecting changes in the cantilever oscillation amplitude due to molecular interactions[65, 66]. All obtained parameters can be combined to assemble a map of a biological surface showing its physical and chemical properties along with its 3D topography (Fig.2B).

Multiparametric imaging has been applied to study cellular and microbial surfaces, with the later including bacteria as well as viruses. FD curve-based imaging of Herpes simplex virus 1 (HSV1), minute virus of mice (MVM) and φ 29 bacteriophage revealed structural properties of individual viruses while mapping mechanical stability directly onto individual capsid proteins [67, 68,Carrasco, 2006 #96](see also below). Recent progress further allows studying processes at high temporal resolution to follow dynamic processes such as virus budding from infected cells. Using functionalized AFM tips, the budding of single bacteriophages could be visualized and mapped on

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the topographic and mechanical properties of the infected cell[26].

The wide applicability and immense information content of multiparametric imaging make it a powerful technique to study complex biological systems and processes.

4. Virus structure and mechanics.

Mechanics of non-enveloped viruses.

Although Arthur Ashkin reported the manipulation of cells, bacteria and viruses using optical tweezers already in 1987[69, 70], a direct observation of single viruses using AFM was not possible until 1992[71, 72, 73]. In these early works on virus imaging, the particles were not only used as a calibration tool to characterize and highlight the performance of the used instrument[72], but also to unravel structural details such as steaming DNA bundles form individual T4 bacteriophage virions[71]. Satellite Tobacco mosaic virus (STMV), a non-enveloped virus with an icosahedral shell, was imaged using AFM in 1995[74]. The viruses formed crystal lattices, of which the kinetics could be followed revealing a 2D and 3D growth upon nucleation[74]. AFM imaging could reveal structural features that were not accessible before, while maintaining the sample under physiological conditions. Human rhinoviruses (HRV) bound to planar bilayers via a Ni^{2+}-NTA coupling of HIS-tagged virus receptors were also shown to form crystalline hexagonal arrangements of virions[75]. High-resolution imaging of single HRV could reveal structural features, such as regular surface protrusions that could be correlated with bound receptor, flushed into the sample chamber during image acquisition.

First AFM nano-indentation experiments demonstrated the accessibility of internal mechanical properties of viruses in a quantitative manner, also reviewed in [76, 77]. Some non-enveloped viruses such as Simian Virus 40[78], Polio Virus[79] or bacterial phages Qbeta[80] are small enough to be subjected to x-ray crystallography in order to elucidate their structure. Their high symmetry, controlled assembly and low number of proteins also make them an appealing target for the development of nano-containers[81]. Due to the small size of most non-enveloped viruses, DNA packaging represents an enormous thermodynamic investment, raising the question about accompanying mechanical properties. However, AFM nano-indentation experiments

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on HSV1 could show that empty and DNA containing capsids are mechanically indistinguishable[67]. It was further shown that the mechanical stability is caused by the DNA packaging but then independent of its presence[67]. Nano-indentation experiments on MVM revealed that virus stiffness depends on the orientation of the virus, i.e. on which symmetry axis the force was exerted[82]. A combination with finite element modeling revealed that this anisotropic mechanical reinforcement can be explained by local capsid-bound DNA patches[82].

In addition to structural characterization of virus particles, the combination with specific biochemical treatments, AFM nano-indentation experiment could further broaden our understanding of virus infection processes. Adenovirus (ADV) capsid stiffness was followed throughout the maturation process showing that immature particles are more stable and elastic than fully matured particles, which may be important to prevent their uncoating and render them uninfectious[83]. Further, Snijder *et al.* could show that the stiffness of ADV changes upon binding to the host cell proteins defensin and integrin. While binding to integrin, the natural receptor of ADV, led to capsid softening possibly enabling uncoating, binding to defensin, a host restriction factor, had the opposite effect of stabilizing the virion[84]. Such a detailed insight cannot be provided by bulk/ensemble approaches.

Mechanics of enveloped viruses.

The development of softer cantilevers and highly-sensitive force detectors allowed imaging of enveloped viruses such as influenza A virus. Giocondi and colleagues could resolve the envelope organization of the hemagglutinin spike protein under physiological conditions[85]. Influenza viruses were deposited on mica and imaged under neutral and low pH conditions. The influenza HA undergoes a well-characterized conformational change at low pH, leading to the merger of endosomal and viral membrane inside the host cell[86]. AFM imaging revealed a hexagonal arrangement of spike proteins that was maintained after acid treatment, indicating the existence of functional assemblies of envelope glycoproteins on the virus surface. This was previously observed by freeze-thaw electron microscopy of virus-liposome fusion events and suggested that multiple HAs in a defined arrangement cooperatively engage in fusion pore formation[87].

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More recently, not only the surface but also inner structural features of influenza A virus were investigated unraveling a remarkable mechanical stability. AFM nano-indentation was used to characterize influenza virus stiffness under different infection-relevant conditions[88, 89]. Thereby, the structural components determining the mechanical properties of influenza viruses could be precisely identified. The virus has three essential structural components namely, the spike proteins, the lipid envelope and the underlying matrix protein layer. For the virus, being enveloped has some crucial advantages. As many enveloped animal viruses enter cells trough endocytosis[90], this enables them to travel inside the host cell without being (1) in contact with the cytoplasm and (2) risking recognition by the host cells innate immunity. The complex maturating program of endosomal vesicles[91] then provides a way for the virus to sense its localization and respond to external cues such as decreasing pH[92], increasing calcium levels[93] or the presence of specific proteases[94] in the endosomal lumen. However, being encapsulated in a lipid bilayer might be a trade-off since enveloped viruses loose structural stiffness compared to a proteinaceous virus capsid. AFM nano-indentation could show that influenza A viruses are about 10 times softer that non-enveloped viruses such as cowpea chlorotic mottle virus or bacteriophages[95, 96]. Surprisingly, the lipid envelope provides two thirds of the overall virus stiffness, as shown by measurements on liposomes made from viral lipids and compared to intact viruses[89]. Further, the unique lipid composition does not show a major temperature-driven phase transition and allows even wall-to-wall deformation[97]. These studies provide important information about the biology of enveloped viruses, suggesting that a lipid envelope might, compared with a more rigid protein capsid, be more adaptable to environmental changes like climatic conditions, host tissue or adaptation to a new host. It provides a soft but flexible shell that is reinforced by the underlying matrix protein M1, the membrane-spanning glycoproteins and the genome segments.

This modular composition together with the unique properties of its components perfectly adapt the virus to the host cells environment and its own life cycle. To illustrate, several studies suggested that the virus envelope, i.e. its structural-mechanical properties have to be primed for fusion between the envelope and the target membrane to release the viral genome. Obviously, it is a technical challenge to explore the mechanical properties and their structural basis of enveloped viruses, in

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particular along the entry pathway. SFVS methods turned out to be very helpful to address these properties (see below). Precise knowledge of the specific (physico-chemical) conditions of the local environment of viruses along the different stages of virus entry, in particular along the endocytic pathway of virus entry, enables to mimic the native conditions during measurements.

Human immunodeficiency virus (HIV) was shown to regulate its mechanical stiffness during the replication cycle. HIV virions show a dramatic drop in stiffness from budding virions to mature particles entering a cell[98]. This stiffness switch could be linked to the cytoplasmic tail of the viral membrane protein Env, which probably involves its bridging function between the viral envelope and the HIV matrix protein Gag[99] and illustrates how viruses prime their structure to enable host cell entry.
Indeed, to successfully infect a cell, viruses must uncoat to release and deliver their genome into the cell. Influenza HA responds to low pH levels by inducing the merger of viral and endosomal membrane during the endosomal passage. Another viral membrane protein, the proton channel M2, mediates an influx of protons eventually leading to disassembly of the M1 capsid layer and release of the viral genome. However, accompanying structural changes were not known until recently. AFM nano-indentation experiments on influenza viruses treated with different pH conditions mimicking those of the lumen of maturating endosomes could show that the virus stiffness decreases, responding to the pH in two major steps[88, 100]. Starting with a reversible softening at pH conditions resembling early endosomes, the stiffness decreases down to the level of that of liposomes at late endosomal pH conditions. Using viruses devoid of HA and liposomes as comparison, it could be shown that the M1 protein layer is the major structural components responsible for the pH response[88]. Importantly, using infection assays that simulate endosomal passage, it could be shown that both steps are necessary for efficient infection, a results that was later also shown using biochemical assays[101].

However, of critical note, due to the high variability of mechanical properties, in particular of enveloped viruses with non-regular/symmetric structure as influenza viruses, a rather high number of measurements are necessary to identify changes/differences in the mechanical properties. At a first glance, this might argue for ensemble measurements. However, as already outlined such detailed and

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specific information on the structural basis of mechanical properties of viruses as obtained by SFVS are not accessible by any bulk approach.

5. Single virus tracking and lateral force spectroscopy.

Single virus tracking (SVT) is an important tool to study virus-cell invasion with high spatial precision and it has been used to follow a variety of viruses during cell infection[102]. Although light microscopy techniques are not the main focus of this review, we want to discuss a few reports that indicate its use for studying virus receptor binding.

Viruses can be fluorescently labeled using expression of viral fusion proteins[103, 104], chemical protein or genome modification[105, 106] or incorporation of fluorescent lipids[107]. These virus-labeling strategies were shown not to interfere with viral replication and hence provide easy access to study virus infection by SVT. Regarding the cell, emerging evidence from different areas suggested nanoclustering and protein organization in microdomains as a major feature of cellular plasma membranes[108]. Recently, using super-resolution microscopy techniques, it was shown that cellular immunological as well as neurophysiological proteins appear in nanoclusters of different size. DC-SIGN and lymphocyte function-associated antigen 1 (LFA-1) form nanoclusters with diameter between 50-100 nm[109], while *Bruchpilot*[110] and synaptic SNARE proteins[111] appear in structures between 20 – 100 nm. These examples suggest that the studied proteins might form functional aggregates, a notion that was also suggested for epidermal growth factor receptor (EGFR)[112]. Not least, viral proteins form dense membrane-associated clusters during virus assembly and budding (HA, HIV-Gag)[109, 113], processes that also include the co-clustering of cellular proteins[114].

However, the composition, size, biogenesis and function of plasma membrane nanodomains remain largely unknown. A lipid-mediated compartmentalization has been suggested since 1987 and led to the formulation of the lipid-raft hypothesis[115]. Another well-studied concept is based on submembraneous actin and spectrin meshworks that restrict the motion of transmembrane but not lipid-anchored proteins leading to the formation of diffusion barriers and hence compartmentalization[116].

As we discussed earlier, virus-receptor binding is highly crucial for cell recognition and binding. Tracking single viruses during this process can help to infer structural

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properties of the plasma membrane as a particles movement is critically controlled by features of the underlying surface. Mathematical models are generally used to characterize single particle trajectories and extract parameters such as mean squared displacement (MSD), diffusion time and velocity[117]. However, these parameters are mostly useful to characterize specific particle transport mechanisms such as microtubule-associated or actin-mediated flow[118] with an emphasis on diffusion-controlled processes. In contrast, other analysis protocols are based on high-density tracking of particles and allow to extract biophysical properties about the cellular organization as well as attraction energies of specific cellular regions[119, 120, 121]. In these studies the movement of single proteins in a biological membrane is described as a combination of a friction-controlled diffusion and an energy potential due to molecular interactions. The method hence describes a powerful new concept to identify lateral forces from minimal invasive two dimensional tracking data[119, 120, 121, 122, 123, 124]. It has been used to characterize the motion of single AMPA and glycine receptor molecules but can also be applied to single virus trajectories[120, 121]. Indeed, using trajectories of influenza A virus obtained on living MDCK cells, it was shown that viruses show preferred localization in small nanodomains (diameter 100-200 nm) (Fig.4), visualized as converging trajectories of viruses returning to a nanodomain several times[120]. Output parameters of this analysis are size and probability of the observed potential well as well as quantitative force field parameters.

The identification of such plasma membrane potential wells from trajectories of confined diffusing single viruses allows to draw maps of attractive forces, which could be used to identify areas of preferred virus binding or cell entry. Hence, the lateral mobility of viruses can provide tremendous information about virus binding and infection routes as well as structural features supporting viral surface movements.

6. Conclusion

Virus-cell interaction can be measured on at least three levels of complexity. (1) Measurements on the cell population level such as using virus mediated agglutination of (un)modified red blood cells or flow cytometry with labeled viruses represent global whole-cell binding parameters. On the other hand, (2) *in vitro* assays such as SPR or solid phase binding assay can be used to study receptor specificity.

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These approaches offer precise control over the studied ligands and can also be upscaled to screen ligand libraries. (3) SVFS fills the gap between these two levels of complexity and allows to investigate virus-cell specificity using intact viruses and living cells. As we have described, results obtained at these different levels are not easily comparable and make it necessary to discriminate between cell and receptor specificity. The cell is much more complex and should not be simplified to a receptor-presenting surface. In contrast, it will be of great importance to study the ultrastructure of the host cell plasma membrane to better understand its importance for virus binding. SVFS in combination with glycomics or proteomics approaches can help to identify essential components of the plasma membrane facilitating virus-cell interaction. Not only cell binding, also other steps of virus replication become accessible for SVFS. Studying virus mechanics helped to understand the uncoating of multilayered enveloped viruses as well as the unique packaging of DNA in capsid viruses. However, the study of highly dynamic processes like virus endocytosis or budding as well as fast conformational or mechanical changes of the virion require increased time resolution, a technical challenge where high-speed AFM will be particularly helpful.

However, as soon as cellular processes are studied, light microscopy becomes indispensable. A field that undergoes a rapid development itself and enabled super resolution microscopy has also provided tremendous insights into virus biology[113, 114, 125]. It will be tempting to combine mechanical or topographical AFM studies with the specificity and spatial resolution of super resolution microscopy.

In this review, we have shown how SFVS can significantly improve our understanding of virus infection. Viruses are highly specialized pathogens and only the development and combination of new techniques allows the study of virus properties in detail that was not accessible before. We believe it will be of great importance to merge the fields of and support the communication between biophysics, virology and cell biology. Processes and whole systems can be modeled to proof hypothesis or challenge and extend the current understanding of virus replication. New insights are expected to foster our understanding and support the development of new antiviral strategies.

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Figures

Fig. 1

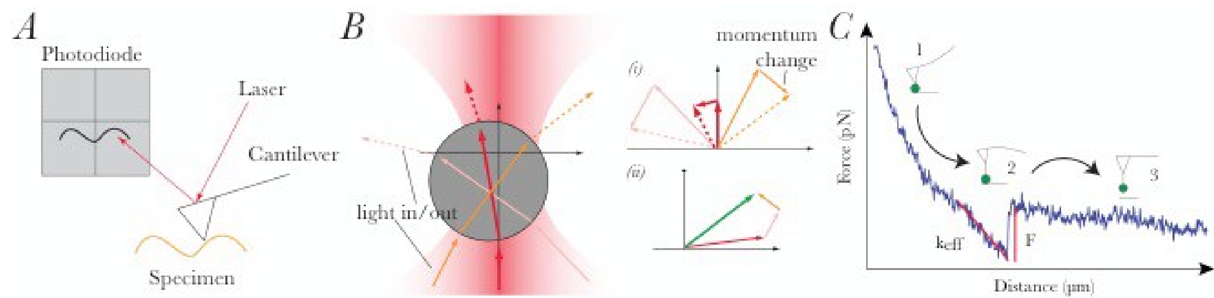

Fig. 1. Mechanism of atomic force microscopy and optical trapping. (**A**) In atomic force microscopy, the sample is scanned with a very sharp tip (tip radius 5-10 nm) at the end of a cantilever arm. The cantilever acts as a Hookean spring and hence bending can be translated into applied force. The deflection of the cantilever is measured by pointing a laser on the back of the cantilever and detecting the reflection on a quadrant photodiode (QPD). (**B**) Optical tweezers operate with high power near-infrared lasers with a Gaussian beam profile (i.e. the center beam has the highest intensity), which are focused by a high NA microscopic objective. In case of a spherical particle getting into proximity of the beam focus, the beam will be deflected at the edges of the particle, which leads to a changed direction of the deflected beam. This causes a momentum change of the light beam (**i**) and a resulting momentum transfer on the bead directed in the opposite direction (**ii**). The total force applied on the particle (green arrow) is the sum of forces generated by the different rays within the beam (**ii**) and is directed towards the center of the optical trap. During a force-distance cycle (**C**) the cantilever is lowered on the cell until touching the surface (1). Subsequently, the cantilever is retracted at a defined velocity. In case of an interaction, the cantilever will bend towards the sample (2) until the underlying bond fails and the cantilever returns into the zero-force position (3). The corresponding regions of the force-distance cycle are labeled on the retraction curve. The curve shows a single unbinding event, where the slope at rupture gives the effective spring constant k_{eff}, which is used to calculate the loading rate r using $r=k_{eff}*v$, where v is the pulling velocity. The obtained force vs. loading rate plot can be fitted to a single energy barrier model to obtain the dissociation rate without force k_{off} as well as the distance to the transition state x_u [23].

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Fig. 2

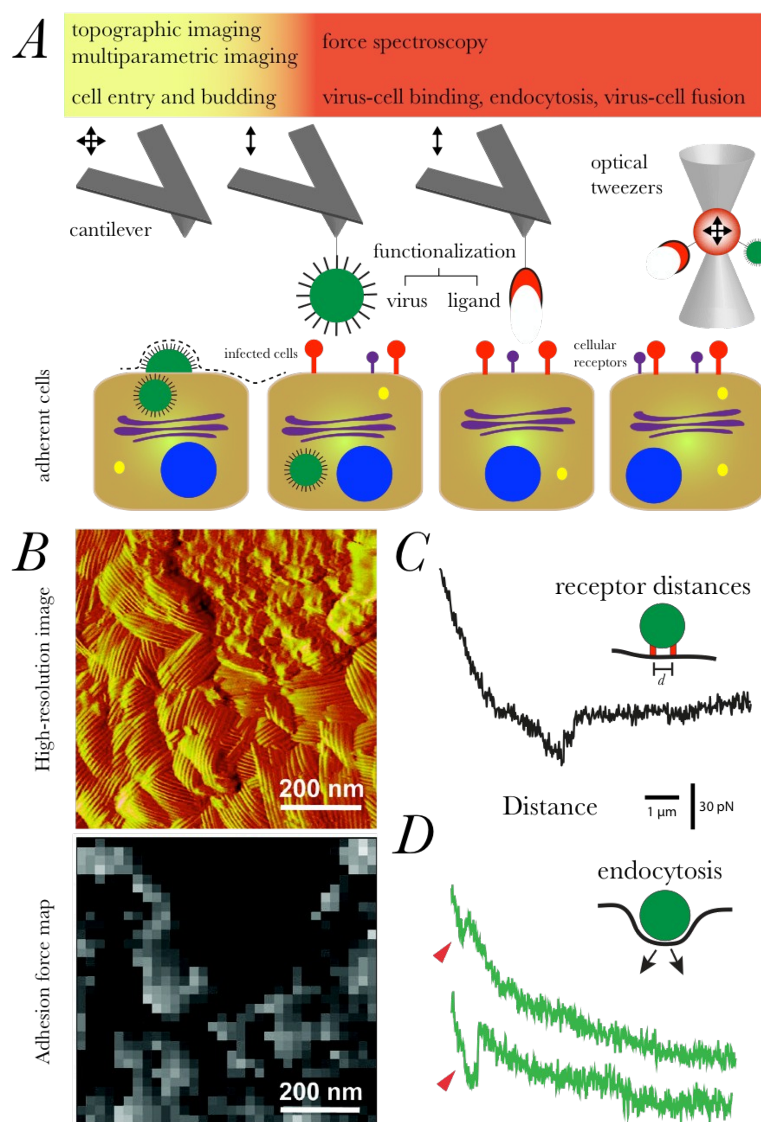

Fig. 2 AFM and OT in microbiology. (A) AFM and OT have various fields of applications in microbiology. While topographic as well as multiparametric imaging is only possible using AFM (yellow part), both techniques allow force spectroscopy measurements (red part). The cantilever can be modified with purified ligands, viruses or bacteria, which allows to measure adhesive forces in axial direction. Optical tweezer experiments typically use small spherical beads as a sample carrier to allow precise optical trap calibration and force measurements. Hereby the bead can be moved in three dimensions. (**B**) Correlation of a high-resolution topographic image with a simultaneously obtained adhesion map during multiparametric imaging. With permission reproduced from [126]. Force-distance curves can further be used to study other properties of virus-cell interaction. (**C)** shows an example of a force-distance retraction curve showing a stepwise unbinding event, indicating rupture of multiple bonds. From the distance between the two sequential rupture events, distances between viral proteins or receptors on the cell surface can be inferred. During a slow cantilever approach or in a force-clamp situation it is possible to observe endocytosis events **C**, characterized by a kink (red arrow) of the force-distance curve, indicating an applied force from the direction of the cell[60]. Shown are two examples with different attractive force, indicating varying cellular processes or endocytosis stages. Force traces in **C** and **D** are from SVFS measurement of influenza A virus interacting with living mammalian cells.

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Fig. 3

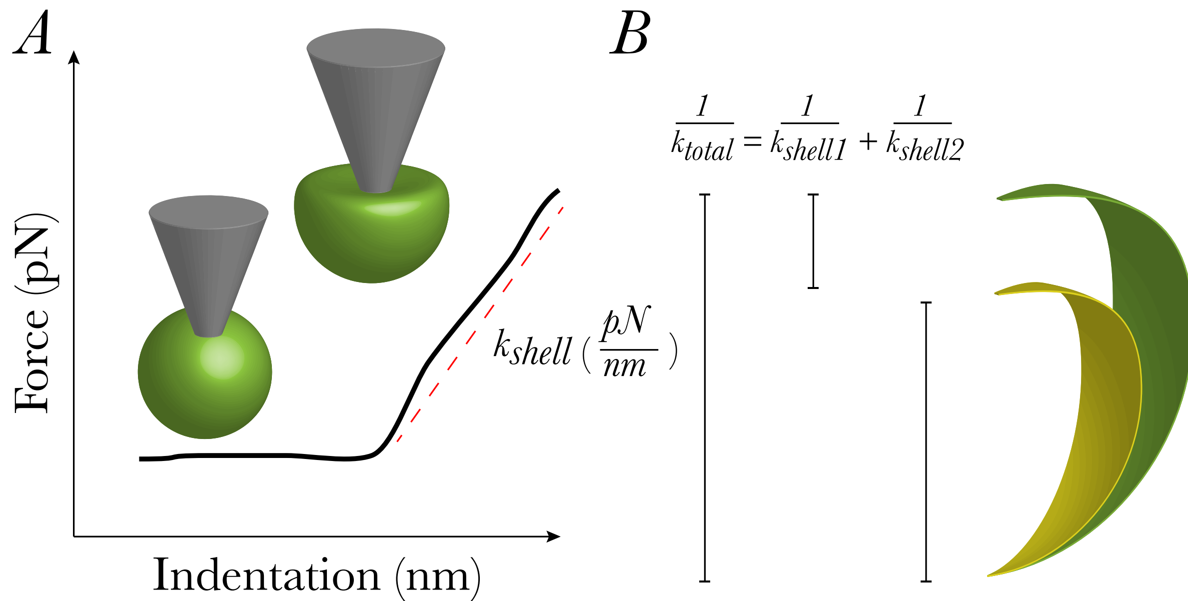

Fig. 3 Studying virus mechanics using AFM nano-indentation. Viral mechanics can be probed in AFM-based nano-indentation experiments (**A**). Viruses are adsorbed or specifically attached to a planar surface, which allows their identification and imaging at high resolution[75]. Targeting a specific region of an individual virus particle and moving the cantilever in axial direction produces a characteristic force-distance curve, where the slope of the linear part upon touching the viral surface can be used to infer the viral stiffness k_{shell}. (**B**) Comparing stiffness measurements after different treatments in combination with a serial spring model can further help to understand and isolate the properties of multilayered particles such as enveloped viruses comprising a layer of spike proteins, a membranous envelope and a protein capsid[88, 89].

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Fig. 4

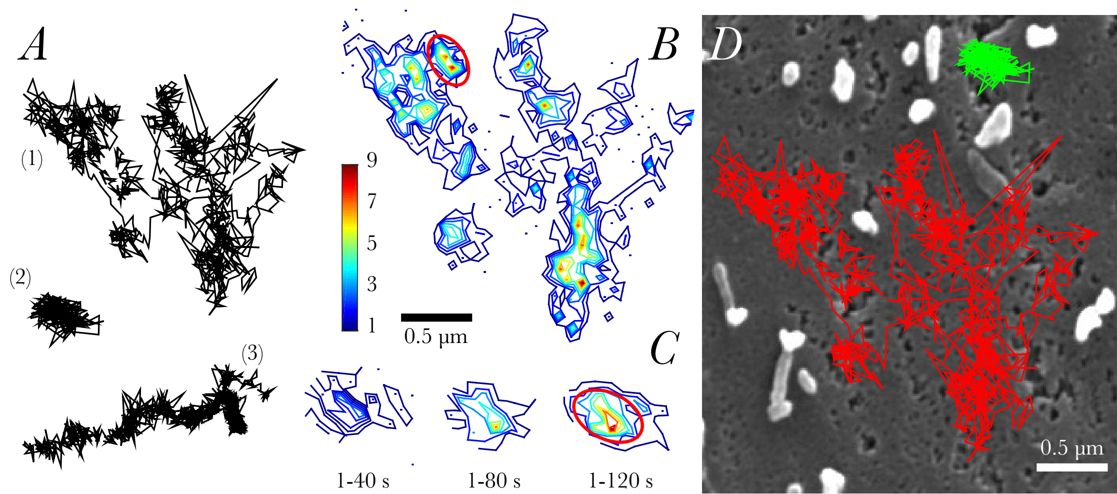

Fig. 4 Virus tracking can reveal structural features of cellular plasma membranes. Following individual influenza virus particles on the plasma membrane of living MDCK cells unraveled different types of movement (**A**). A common feature of those trajectories are regions of preferred residence, characterized by a recurrent motion[120]. A localization histogram helps to reveal high-density regions, shown as a maximal projection of trajectory 1 (**B**) and as time evolution for trajectory 2 (**C**). The color indicates localization probability (a.u.). A stochastic algorithm can be used to identify potential wells (red circles), representing plasma membrane microdomains of higher attracting force[120]. Hence, virus tracking combined with the identification of areas with high lateral affinity might help to construct a map of the cellular plasma membrane with respect to virus infection. Such a map could be correlated with other cellular features as for example the topological 3D structure of the cell surface. **D** shows the two recurrent-step trajectories (1, 2) drawn on-scale onto a scanning electron micrograph of an MDCK cell surface. The origin and nature of those potential wells should be the objective of future studies.

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