Application of super-resolution and advanced quantitative microscopy to the spatio-temporal analysis of influenza virus replication

Emma Touizer[1,2], Christian Sieben[3], Ricardo Henriques[2,4,5], Mark Marsh[2] and Romain F. Laine[2,4]*

[1] Division of Infection and Immunity, University College London, London, UK
[2] MRC Laboratory for Molecular Cell Biology, University College London, London, UK:
[3] Department of Cell Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany
[4] The Francis Crick Institute, London, UK
[5] Instituto Gulbenkian de Ciência, Oeiras, Portugal

* Correspondence: r.laine@ucl.ac.uk

Abstract: With an estimated 3 to 5 million human cases annually and the potential to infect domestic and wild animal populations, influenza viruses are one of the greatest health and economic burdens to our society [1] and pose an ongoing threat of large-scale pandemics. Despite our knowledge of many important aspects of influenza virus biology, there is still much to learn about how influenza viruses replicate in infected cells, for instance how they use entry receptors or exploit host cell trafficking pathways. These gaps in our knowledge are due, in part, to the difficulty of directly observing viruses in living cells. In recent years, advances in light microscopy, including super-resolution microscopy and single-molecule imaging, have enabled many viral replication steps to be visualised dynamically in living cells. In particular, the ability to track single virions and their components, in real time, now allows specific pathways to be interrogated providing new insights to various aspects of the virus-host cell interaction. In this review, we discuss how state-of-the-art imaging technologies, notably quantitative live-cell and super-resolution microscopy, are shedding new nanoscale and molecular insights into influenza virus replication and revealing new opportunities for developing antiviral strategies.

Keywords: super-resolution microscopy, advanced light microscopy, quantitative microscopy, live-cell microscopy, SMLM, STORM, SIM, STED, expansion microscopy, influenza virus, viral replication.

1. Introduction

In humans, influenza viruses are pathogens that can cause acute infections in the respiratory system. Globally, every year 3-5 million people develop severe seasonal influenza with up to 650,000 deaths [1]. Although vaccines and some antivirals (e.g. Tamiflu) against seasonal influenza are available, the rapid antigenic drift and shift [2] have, to date, made it difficult to obtain broadly neutralizing vaccines effective against multiple viral strains. Due to the alarming pandemic potential of emerging zoonotic influenza viruses [3], the WHO recently listed a 'Global Influenza Pandemic' as one of the top 10 threats to global health [4]. The emergence of SARS-CoV-2 and its associated pandemic [5] has greatly emphasised the need to better understand highly transmissible respiratory viruses, including influenza virus.

Influenza viruses consist of an RNA genome encased in a lipoprotein membrane (the viral envelope). The viral genome is made up of eight RNA segments, wrapped by the viral nucleoproteins and the RNA polymerase (RNApol made of PB1, PB2 and PA), termed viral ribonucleoprotein complexes (vRNP). The envelope is derived from the host cell plasma membrane and contains two major glycoproteins, hemagglutinin (HA) and neuraminidase (NA), together with the proton channel M2. The M1 protein forms a proteinaceous matrix underneath the envelope, as seen in Figure 1a. HA and NA are essential for infection as they mediate the first steps of entry. HA binds directly to receptors decorated with sialic acid to promote viral entry, whereas NA is capable of cleaving sialic acid from glycoproteins and is involved in viral release from the plasma membrane upon budding as well as penetration of mucus on the respiratory epithelium. As a consequence, the balance between the amount of HA and NA in the viral envelope has been shown to be critical for virus fitness [6]. From a therapeutic point of view, both HA and NA are exposed on the surface of virions making them key anti-viral and vaccine targets. However, antigenic shift and drift are the main drivers for influenza virus diversity and lead to the limited efficacy of annual Influenza vaccines [7].

To understand the mechanisms underlying influenza virus replication and those of cell-to-cell viral transmission, with a view of developing new anti-viral strategies, one emerging requirement is to be able to directly observe virions and virus-host cell interactions. The small size of influenza virions (~120 nm diameter) has made electron microscopy (EM) the method of choice for these observations. For example, EM has revealed that influenza virus particles can be pleiomorphic, taking shapes from ~120 nm diameter spherical particles to long filamentous forms up to several µm long and ~90 nm in diameter (reviewed in [8,9]), but the relevance of this pleiomorphism to viral infection and pathogenesis *in vivo* is currently unclear. Cryo-EM has been instrumental in structural studies, showing, for example, that M1 forms a helical array that supports the shape of filamentous particles [10], revealing details of the structure of HA molecules [11] and that the organisation of HA is influenced by the curvature of the viral membrane highlighting that the lateral distribution and density of HA on the virus are key for entry [12]. Although EM can resolve viral and cellular structures at near-atomic level, its use is usually limited to fixed samples, only providing a snapshot in time of specific events and no, or only low levels of, molecular identification. In contrast, though typically limited to ~250 nm resolution due to the diffraction of light, optical imaging combined with fluorescence has high molecular specificity and is compatible with live-cell imaging.

In recent years, the development of advanced and quantitative fluorescence microscopy and, importantly, that of super-resolution microscopy (SRM) [13] has opened new possibilities for direct imaging of cells and for understanding host-pathogen interactions by providing a powerful combination of enhanced spatial resolution, very high molecular specificity and practical compatibility with live-cell imaging [14]. As an example of the potential of SRM to reveal new insights to viral biology, its application to studies of human immunodeficiency virus (HIV) (reviewed in [15,16]), has revealed new insights into the nanoscale distribution of the viral envelope protein (Env) on the surface virions [17] and the role of ESCRT complexes in HIV egress [18].

In this review, we present representative examples of the exciting new advances in the study of influenza virus permitted by novel optical imaging tools. We limit our scope to SRM, single-molecule imaging and quantitative live-cell analysis, with a particular emphasis on SRM, since observing virus assemblies at the nanoscale is central to understanding virus replication. Therefore, we first describe these technologies and then present recent studies in the context of the different aspects of influenza viral replication, from entry to viral budding. We finally provide an outlook of what these techniques may unravel in the future.

We identified three key opportunities for advanced fluorescence microscopy techniques to provide novel insights into influenza virus biology, as highlighted in Figure 1b-d: i) understanding virus structures and associated host cell components at the nanoscale [17–20], ii) resolving individual viruses among large populations of viruses [21,22] and iii) observing dynamic processes in real-time [23,24].

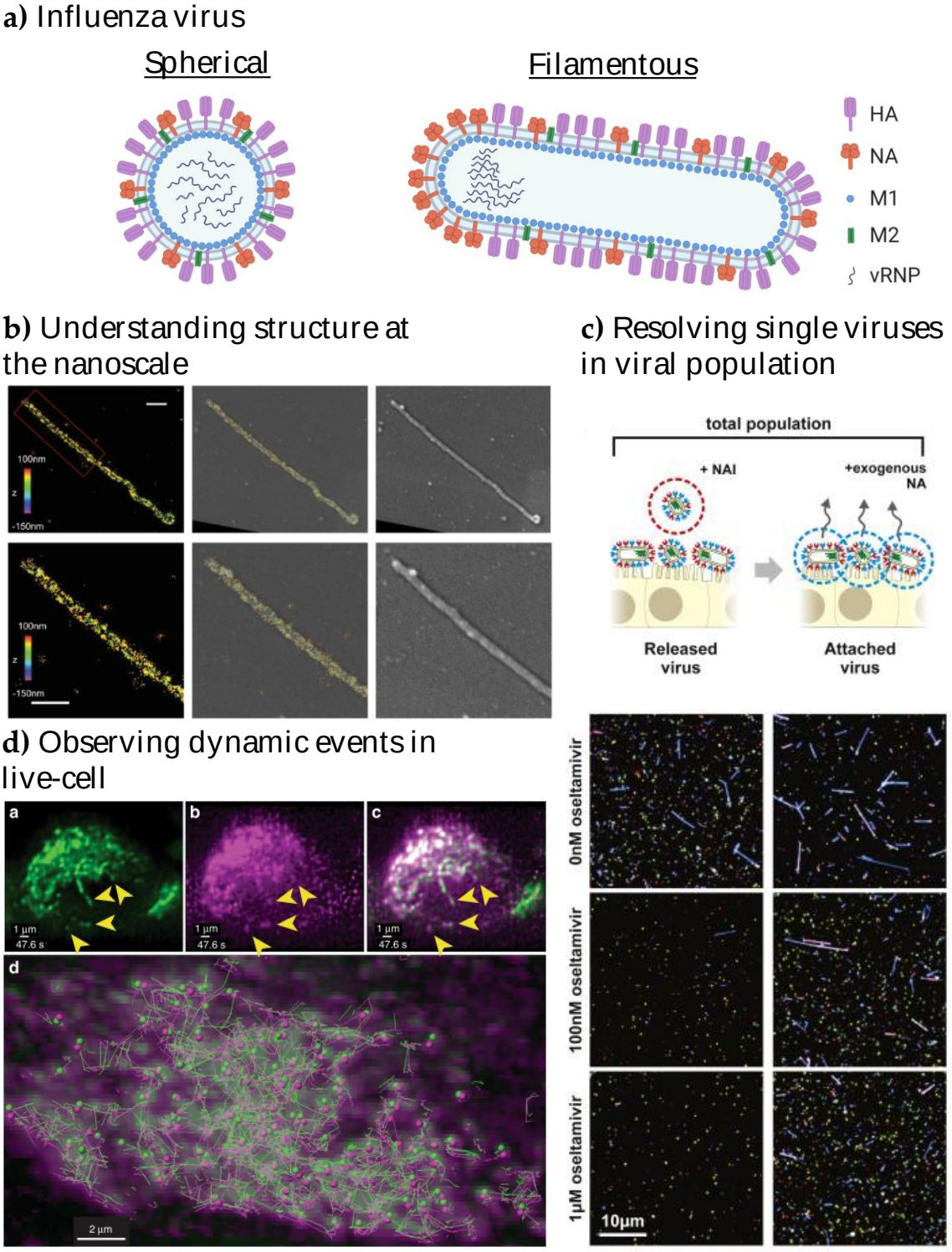

Figure 1: Advanced microscopy gives structural and functional insights into influenza replication. ***(a) Structure of influenza virions.*** *Spherical particles diameter ~120nm; filamentous particles length up to 20µm* [8,9]. ***(b) Understanding viral structure at the nanoscale***. *Correlative 3D-STORM and scanning electron microscopy (SEM) images of filamentous Udorn influenza virus immuno-labelled for HA. Left: STORM image. Right: SEM image. Middle: Overlaid image. Bottom: Magnified views of the region of interest shown on top. Scale bars: 500nm. Adapted from* [19]. ***(c) Resolving single viruses in viral populations***. *Influenza-infected cells were treated with oseltamivir, a neuraminidase inhibitor at different concentrations and the "Released" viruses and the viruses that remained "Attached" were imaged. Adapted from* [22]. ***(d) Observing dynamic events in live cells***. *Live-cell light-sheet three-dimensional (3D) imaging of cells expressing GFP-Rab11A were infected with recombinant influenza expressing mRuby-PA as a marker for the vRNP. Top, left to right: GFP-Rab11A, mRuby-PA, overlay (arrows: colocalizing puncta). Bottom: colocalizing puncta of GFP-Rab11A and mRuby-PA tracks over time. Adapted from* [24].

In Figure 1b, the use of correlative EM and 3D-SRM allowed the distribution of HA in filamentous influenza viruses to be observed at the nanoscale [19], corroborating the structural information from EM to the chemical identity information at the single-molecule level from the SRM technique used here. The nanoscale resolution achieved by SRM is capable of giving information at the molecular organisation level, highlighting specific nanoscale assemblies both in the viral structure [17,25] and in the infected cell [26]. Observing large virus populations at the single-virus level can also be used to directly investigate the diversity of structures within pleiomorphic virus population [21] or the functional effects of drugs, as seen in Figure 1c where adding the NA inhibitor oseltamivir reduces the release of filamentous viral particles, thus highlighting a virus shape-specific effect of the drug [22]. Live-cell imaging can offer a wide-range of possibilities to observe the replication cycle of influenza virus. In Figure 1d, fluorescence light-sheet microscopy in living cells combined with quantitative particle tracking in 3D showed the co-transport of Rab11A-containing recycling endosomes with influenza viral RNA (vRNA) and the exploitation of this trafficking pathway by the virus [24].

2. Overview of super-resolution microscopy and novel imaging methods to study influenza virus replication

SRM comprises optical imaging techniques that offer resolution beyond the diffraction limit of light (~250nm). Figure 2 shows graphical explanations and examples of the main SRM techniques applied to the study of influenza virus replication.

SRM was developed in the mid-1990s, first with stimulated emission depletion (STED) microscopy [27]. STED is a scanning technique based on laser confocal scanning microscopy, which exploits the phenomenon of stimulated emission to quench the fluorescence signal around an excitation scanning spot. This effectively reduces the volume of the fluorescence measurement and typically improves resolution down to 50-70nm. For influenza virus, the resolution gain of STED has been used to follow, for example, vRNPs during viral trafficking along the endosomal pathway in dendritic cells (Figure 2a; [28]).[28]. By allowing nanoscale localization of structures, STED provides a powerful way to determine the identity and the structure of the organelles involved in vRNP trafficking with high spatial precision.

A second technique called structured illumination microscopy (SIM) [29] relies on the acquisition of a sequence of fluorescence images obtained under a set of structured illuminations (typically made of stripes), each creating Moiré patterns with the underlying structure of interest. The measured Moiré patterns contain information about high spatial frequencies (high-resolution information) in the sample that can be recovered by image reconstruction of the raw data, typically achieving resolution down to 150nm. In Figure 2b, 3D SIM imaging is used to show that ubiquitin is packaged inside purified influenza viruses [30]. This study showed that ubiquitin is then used during virus entry to recruit the cellular aggresome pathway to facilitate capsid disassembly and release of vRNPs. Despite the limited resolution increase of SIM, it has been successfully used to describe the substructure of viruses such as vaccinia [31] and the heterogeneous morphology of influenza [21].

A third approach to SRM is to exploit the capability of certain fluorescent markers to blink under specific experimental conditions. This set of techniques, collectively called single-molecule localization microscopy (SMLM), relies on the use of fluorophores that can be effectively switched on and off in a stochastic manner. By isolating single fluorophores spatio-temporally, it is therefore possible to precisely localize them in space despite each molecule producing a diffraction-limited spot on the camera, and then build a nanoscale map of fluorophore distribution. SMLM techniques commonly achieve a resolution of 10-20nm (Figure 2c). Some of earliest described SMLM techniques, PALM (photo-activated localization microscopy) [32] and fPALM (fluorescence PALM)[33], use genetically engineered photo-activatable fluorescent proteins, whereas STORM (stochastic optical reconstruction microscopy) [34] uses conventional photo-switchable organic dyes such as Cy5. Variations on these original SMLM approaches have been developed including *d*STORM (*direct* STORM) [13,35], PAINT (points accumulation for imaging in nanoscale topography) [36] and GSD (ground state depletion) [39].

An exciting variant is single-particle tracking PALM (sptPALM), a technology capable of deciphering the spatial organisation and dynamics of individual molecules by randomly photo-activating single-molecules and tracking them in living cells. This approach was originally demonstrated by tracking individual HIV Gag proteins at the plasma membrane of Gag expressing cells [40].

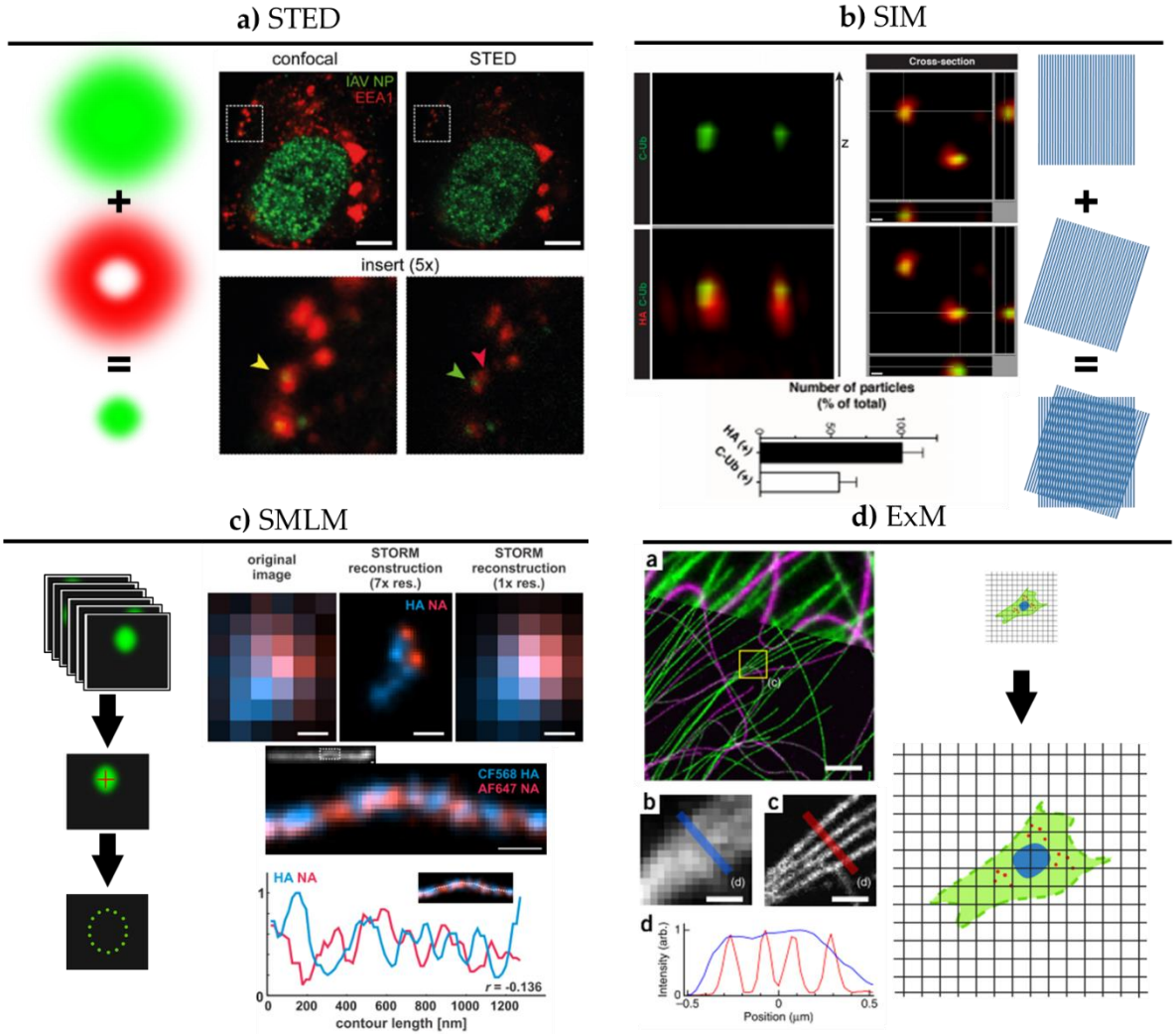

Figure 2: Super-resolution microscopy for the study of influenza virus replication. ***(a) STED.*** *Confocal (left) and STED (right) images of a dendritic cell 4 hours post-infection with influenza virus vRNP (green) and the early endosomal marker EEA1 (red). Scale bar: 5µm. Adapted from* [28]. ***(b) SIM.*** *3D SIM z-stack and cross-section images of purified influenza viruses labelled for hemagglutinin (HA) (red) alongside immuno-labelling of the C-terminus of ubiquitin (C-Ub) (green). Bottom: quantification of HA and C-Ub colocalization. Scale bar: 100 nm. Adapted from* [30]. ***(c) SMLM.*** *Top: diffraction-limited image and dSTORM reconstructions at ~30 nm resolution of influenza viruses labelled for NA (red) and HA (blue). Bottom: dSTORM reconstructions of a filamentous virion labelled for HA and NA at ~30 nm resolution with corresponding intensity profile. HA and NA are shown to exclude one another. Scale bar: 200 nm. Adapted from* [37]. ***(d) ExM.*** *BS-C-1 cell labelled for tyrosinated tubulin (green) and de-tyrosinated tubulin (magenta). Top: Comparison of ExM image (bottom) with corresponding pre-expansion image (top). Scale bars: 2 µm. Bottom: insert, pre- and post-expansion with corresponding intensity profiles. Scale bars: 500nm. Adapted from* [38].

Interestingly, some of the earliest fPALM experiments looked at the association of influenza virus HA with lipid rafts [41]. This study showed that HA forms dynamic irregular clusters of varying size in lipid rafts. Due to the small size of these microdomains at the plasma membrane, it would be very challenging to observe them with conventional fluorescence imaging techniques. Further, an early form of SMLM called 'Blink' [42] was used to observe that HA at the plasma membrane of infected cells forms dynamic nanodomains of around 80 nm [43].

Figure 2c shows a filamentous influenza virus imaged with *d*STORM. The nanoscale resolution allowed by *d*STORM reveals the spatially alternating distribution of HA and NA-rich regions along a filamentous influenza virion.

A newcomer in the SRM field is expansion microscopy (ExM) [44]. This method turns the diffraction limit problem on its head by expanding the sample isotropically in a hydrogel, practically improving the resolution of images by the expansion factor, typically of ~4x [44] and up to ~20x with iterative ExM (iExM) [45]. The approach can even preserve and resolve the integrity of bio-macromolecular assemblies, as successfully demonstrated by visualizing the molecular organisation of the centrosome [46]. In Figure 2d, we show an example of ExM use to study the spatial organisation of cytoskeletal structures in cells. Although some early studies show that ExM can be used to study viral infection and viral assembly [47–49], including for bacteriophage T5 [50], ExM remains under-used in the context of virology despite its potential versatility and ease of use.

Although each of these techniques provides nanoscale imaging, they also have their own advantages and drawbacks which need to be considered when choosing a method. If maximum resolution is required and the imaging is performed on fixed cells, then SMLM can provide an optimal solution. On the other hand, when imaging tissue or thick samples, STED can be an effective method due to its inherent optical sectioning capabilities. SIM, on the other hand, provides an easy and fast solution for live-cell imaging at medium resolution. ExM is not compatible with live sample imaging but constitutes a sample-based approach to SRM that can easily be combined with the other SRM techniques and thus provide a unique way to enhance resolution and image in tissue in 3D.

Beyond the potentials of SRM, quantitative long-term live-cell imaging has great promises for the study of host-pathogen interactions at the relevant temporal and spatial scales. For instance, by being able to visualize viruses and their components interacting with host cells, quantitative live-cell fluorescence microscopy can be used to study the range of cellular pathways exploited by viruses at the single-cell and single-virion level [8,9,22]. SRM and quantitative live-cell fluorescence microscopy was used to study the uncoating of HIV in living cells with a high temporal resolution providing evidence that the HIV capsid can remain intact while entering the cell nucleus [23]. The study of live-cell dynamics has been further enhanced through light-sheet microscopy, which allows high speed, long-term imaging in 3D with low phototoxicity [51], but this method remains largely underexploited in the context of viral replication, potentially due to the lack of availability of such tools in appropriate containment conditions for live virus imaging.

Further, recent advances in microscopy sensitivity for the detection of single molecules, and the introduction of new and non-invasive labelling strategies such as FlAsH [52], Sfp [53], transglutaminase 2 [54], sortase A [55] with bright fluorescent markers, offer new opportunities for improved live-cell imaging. These new technologies will enable the behaviours of viral and cellular component to be mapped dynamically in super-resolution [14], multi-label structural studies using intact viruses [22] and single-particle tracking (SPT) to follow individual virus particles during infection and individual viral components during replication [56–58]. In the context of SPT, a powerful approach has been to use quantum dots (QDs) [59–61], for long term imaging, but with limitations due to the relative complexity to perform the labelling steps. Single-molecule Förster Resonance Energy Transfer (smFRET) [62] constitutes a powerful optical method to observe nanoscale changes in conformations within biomolecules, especially when leveraging small peptide labels and quantitative analysis. In the context of influenza, this approach has been used to study the dynamics of fusion-associated low pH-induced HA conformational changes [63]. The direct spatial localisation of individual RNA transcripts can be performed by single-molecule fluorescence using *in situ* hybridization (FISH) [64]. FISH is also well suited to studies of the assembly of influenza virions has been used to observe assembled vRNPs in the cytoplasm *en route* to budding sites [65].

We note that these imaging approaches can also benefit from quantitative approaches such as single-particle averaging [21,25,31], machine learning [66,67] and modelling [21,25,68], to tease out the most informative features from the imaging data.

3. Understanding virus structure

To study the structure and composition of influenza viruses, it can be useful to combine nanoscale resolution and chemical specificity, therefore, SRM is perfectly suited to this task. Since such studies are typically carried out using purified viruses, SMLM and STED have exceptional potential. For instance, correlative STORM and EM have made it possible to observe the nanoscale distribution of HA, M1 and vRNP in a filamentous strain of influenza virus [19]. This correlative technique provided sufficient resolution to distinguish the layers of HA inserted into the viral envelope on each side of the ~100 nm diameter tubular filament structure and confirmed previous observations that the vRNPs are located at the distal end of budding filaments (Figure 1a). Here, the multi-colour capabilities of SMLM were essential to identify the different components within the viral particle. These original studies demonstrated that SMLM can be powerful for virus structural studies while highlighting its complementarity with EM.

Multi-colour fluorescence microscopy makes it possible to spatially resolve many different components of a virion. But in order to effectively image these structures, there is a need for efficient labelling of the biomolecules of interest. Although immunofluorescence provides a robust way to label fixed samples, live virus imaging can prove challenging. For instance, labelling viral components with genetic tags often compromises the infectivity of the engineered viruses. Therefore, using novel small-peptide labelling strategies can provide a great solution. A recent study used innovative site-specific labelling strategies, combining enzymatic approaches and small size tags, that were minimally functionally disruptive, to label 8 different influenza virion proteins (HA, NA, NP, N, M, PA, PB1 and PB2) to study both the morphology and the replicative fitness of live viruses (Figure 3a) [22]. Interestingly, the authors were able to observe that a large heterogeneity in shape and composition of the virions can be generated during virus assembly: one virus infecting a cell can result in the production of pleiomorphic progenies. This study also showed that elongated viruses are polarized with one NA-rich pole and one HA-rich pole. This polarity was found to be crucial for influenza virus motility on pathogenically relevant mucosal surfaces. Further, *d*STORM imaging allowed the observation of the nanoscale distribution of HA and NA on filamentous viruses [37], revealing an alternating pattern of the proteins on virions (see Figure 1c). The biological relevance of this precise pattern is currently unclear, but the authors provided evidence through modelling that specific spatial HA and NA organisation can support the clearing of mucosal surfaces by the virus through the unique combination of binding and cleaving of sialic acid groups.

SRM microscopy techniques can also be efficiently combined with advanced quantitative analysis to quantify the structural diversity of the virions within a virus population. In particular, TIRF-SIM (total internal reflection fluorescence SIM) was used in combination with machine learning to develop a high-throughput methodology capable of classifying influenza viruses based on their shape [21]. This automated framework was applied to different strains of live-attenuated influenza viruses (LAIV) to report on their morphology and the potential association of form to functionality.

Although STED has not been used to study the structure of individual influenza viruses, it has allowed the demonstration that HIV envelope protein (Env) distribution on virions is regulated by the structural polyprotein Gag proteolysis/maturation, a rearrangement that is key for efficient receptor engagement and viral entry [17]. Additionally, STED has been used in combination with fluorescence correlation spectroscopy (FCS) [69] to study the diffusion properties of Env proteins during this maturation process within individual HIV particles [70]. This study of dynamic processes within a virus using SRM highlights its potential for the study of structural re-organisation during infection, especially prominent during virus uncoating. Together, these studies illustrate the potential for STED microscopy as a powerful tool for the structural analysis of viruses.

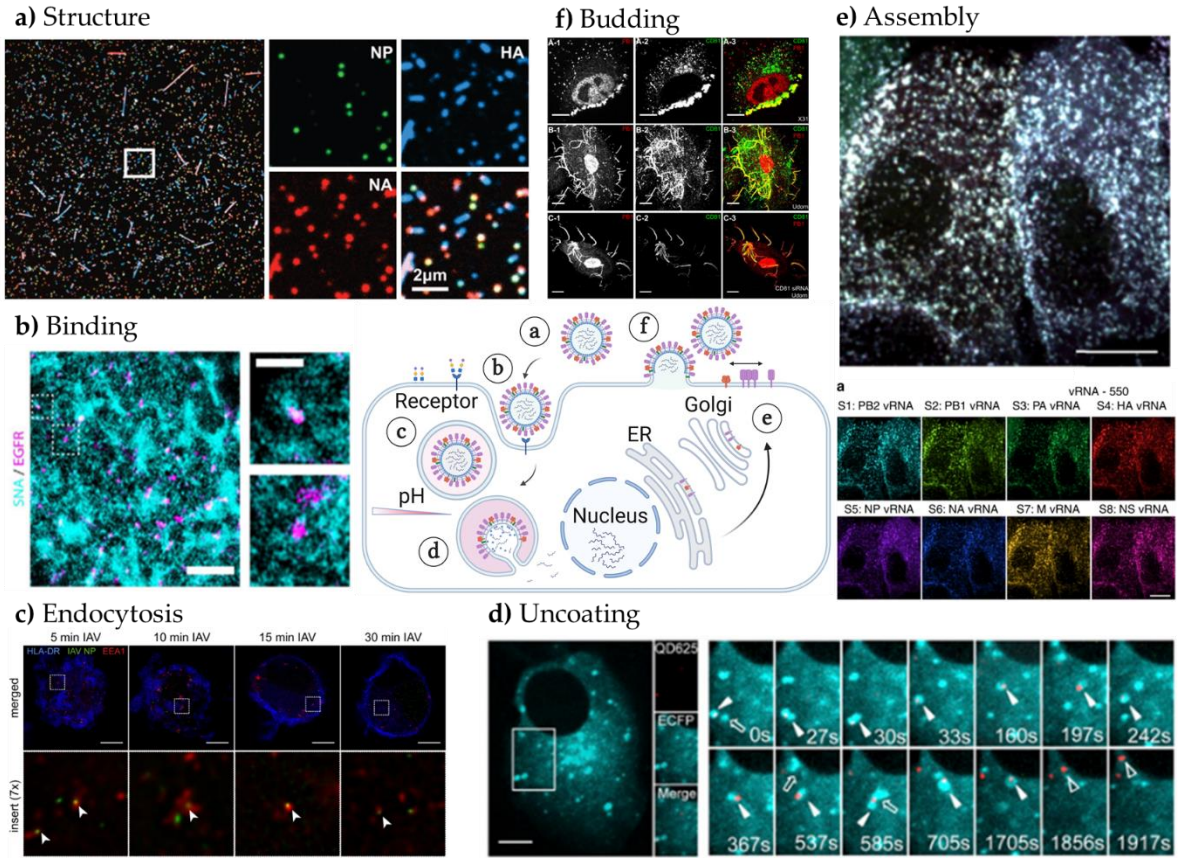

Figure 3: Analysis of influenza virus replication using advanced microscopy. ***Scheme of the influenza life cycle and corresponding microscopy images showing, anti-clockwise: (a) Virus particle structure and composition:*** *Spherical and filamentous influenza virions labelled for NP (green), HA (blue) and NA (red) and immobilized on a coverslip exhibit diverse morphology and composition. Adapted from* [22]. ***(b) Binding:*** *Left: Two-colour STORM image of A549 cells labelled for sialic acid (SNA) (blue) and anti-EGFR (magenta). Scale bar: 500nm. Right: insert of left. Scale bars: 200nm. Adapted from* [26]. ***(c) Endocytosis:*** *STED images of dendritic cells labelled for HLA-DR (blue), influenza virus NP (green) and EEA1 (red). Arrows point to nanoscale colocalization of EEA1 and NP signals. Scale bar: 5µm. Adapted from* [28]. ***(d) Uncoating:*** *MDCK cells expressing Rab7-ECFP (blue) infected with influenza vRNPs labelled with quantum dot QD625 (red). Scale bar: 5µm. Snapshots of tracked influenza and Rab7 over time. Filled triangles: colocalization of QD and Rab7 signals, hollow triangles: released vRNPs, arrows: virus-negative endosomes. Adapted from* [61]. ***(e) Assembly:*** *each of the 8 segments of viral genomic RNA is stained by fluorescence in-situ hybridisation (FISH) using Atto550 as label. Scale bar: 10µm. Adapted from* [71]. ***(f) Budding:*** *Top row: A549 cells were infected with influenza virus X-31 for 16h and labelled with anti-CD81 (green) and anti-PB1 (red). CD81 is recruited to the virus budding zone in X-31 infected cells. Middle row: A549 cells infected with Udorn virus. Same staining as top. CD81 is incorporated into budding filamentous virions of Udorn-infected cells. Bottom row: same to middle row, but in CD81-KO cells. Remaining CD81 in CD81-KO cells is incorporated into budding filamentous viruses of Udorn infected cells. Scale bar: 10mm. Adapted from* [72].

4. Understanding viral entry and trafficking

Entry of influenza virus into cells is a multi-step process. Following the engagement of HA with sialic acid-decorated cell surface receptors, influenza virions undergo endocytosis through clathrin-mediated mechanisms [73] or macropinocytosis [74], with some possible cell dependence [75]. Although HA will bind various sialylated glycoproteins and glycolipids, the engagement of specific cell surface glycoproteins appears to be necessary for successful infection. The identity of these receptors is still a source of debate [76,77], but candidate proteins include the epidermal growth factor receptors (EGFR) which trigger clathrin-mediated endocytosis upon activation by influenza virus [78]. Following uptake from the plasma membrane, influenza viruses traffic through early endosomes to late endosomes where the acidic pH activates the viral membrane proton channel M2. This triggers

conformational changes in the viral core required for efficient uncoating [79,80] and HA-mediated fusion with the endosomal membrane [81]. Importantly, late endosomes are located close to the nuclear periphery, thus uncoating is spatially coupled to the import of vRNPs into the nucleus for replication.

Advanced microscopy and especially SPT has provided essential insights into the dynamics and pathways exploited by influenza virus for entry [82]. Here, the viruses were labelled with a common labelling approach for enveloped viruses using the lipophilic dye DiD, that inserts directly into the viral membrane. Individual virions were tracked and quantified in living cells to demonstrate that similar percentages of influenza particles colocalized with Epsin-1, a cargo-specific adaptor for clathrin-mediated endocytosis, and clathrin, hinting that Epsin-1 may promote clathrin-mediated endocytosis. Recently, a similar live-cell SPT study suggested that influenza virus dynamics at the plasma membrane of a target cell are determined by the spatial distribution of sialylated glycans, with single influenza particles dwelling for longer periods of time on glycan-rich regions [26]. This highlighted the existence of regions of the plasma membrane where the virus may have more time to engage with receptors and trigger endocytosis. In the same study, dual-colour STORM was used to measure the size and density of the sialylated glycan islands as well as the nanoscale organisation of EGFR at the plasma membrane. This showed that EGFR forms nanoclusters that partially colocalize with the sialylated glycans islands (Figure 3b), an association that may provide enough time for EGFR clusters to initiate endocytosis at the virus binding site. This study shows that a combination of advanced microscopy techniques can lead to a complementary set of dynamic and structural information that builds a more complete picture of the biological processes.

Within early endosomes, influenza viruses exploit retrograde trafficking along the microtubule network of the host cell to reach perinuclear late endosomes where fusion and uncoating occurs. Vesicular identity (e.g. early or late endosomes) can be done by simple colocalization microscopy studies, using well-established markers, and lead to an understanding of the dynamics of internalization. However, the higher resolution provided by SRM and colocalization at the nanoscale can discriminate between virions exploiting specific endosomal carriers as opposed to being in close proximity. In addition, SRM can be used to observe possible nanoscale structural re-organisations of endosomes and other organelles as a consequence of viral infection. Focusing on early trafficking events through the endosomal pathway, STED was exploited to study the entry dynamics of spherical influenza particles in human dendritic cells [28] (see Figure 2a and Figure 3c). However, this study did not explore structural changes of the endosomal system and was performed in fixed cells at specific time points post-infection, it is technically possible to do this in living cells with SRM.

Recently, the use of QDs was shown to provide a powerful way to label viral components for long term tracking within living cells, allowing the dynamics of viral infection to be studied in near real-time over the extended periods. This approach was used to label the viral envelope and track viruses inside living infected cells, as they traffic along the microtubule network towards the perinuclear region, revealing the complex range of motion exhibited by the virus during trafficking towards the nucleus and highlighting the impact of the virus on molecular transport processes within the infected cell [59,60].

Studying virus entry and trafficking using SRM can therefore interrogate the identity and organisation of viral receptors, the organelle identity and structure dynamics of each step. However, the modulation of these phenomena with respect to dynamics and viral structure remain poorly understood despite viral entry being a critical step to target for anti-viral therapy.

5. Understanding fusion and uncoating

On reaching the perinuclear region of a cell, influenza viruses need to release their genetic material into the cytoplasm. This release requires the fusion of the viral membrane with the limiting endosomal membrane and the disassembly of the M1 proteinaceous layer lining the inside of the viral envelope (uncoating). The viral envelope fusion with the endosome membrane is HA-mediated and occurs through a multi-step process involving a hemifusion intermediate, where only the apposed leaflets of the membranes merge, followed by the formation of a pore and finally complete fusion.

To study the dynamics of this process under the microscope, it is necessary to have access to a readout of the state of the viral envelope in real-time. For this, a conventional microscopy method involves observing the dequenching of the fluorescence signal from a lipophilic dye incorporated into the viral envelope. For instance, an *in vitro* study on supported lipid bilayers used two-colour TIRF to show the kinetics of influenza virus membrane fusion at the single-particle level [83]. The interior of the virus particles were loaded with a fluorescent marker and the envelope labelled with a lipophilic dye, which remained quenched in intact particles. Dequenching of the lipophilic dye indicated the formation of hemifusion intermediates while the release of the dye from the interior of the virus indicated full fusion. By tracking changes in the intensity of both dyes over time, the authors were able to quantify the precise timing of different phases of the fusion reaction from hemifusion to pore-formation. A similar approach was also demonstrated in cells [84] to study fusion dynamics, though without the possibility to pinpoint hemifusion. Although this approach does not constitute a recent imaging methodological development, it remains a powerful and commonly used microscopy tool that can benefit from analytical methods such as SPT and probe development for longer-term imaging.

By contrast, recent single-molecule imaging revealed important molecular dynamics in the context of viral envelope fusion. This was demonstrated via smFRET to observe the dynamics of the conformational changes of HA in intact virions during the fusion process [63]. The study leveraged single-molecule sensitivity, small peptide labels and quantitative analysis to show that HA is capable of sampling a range of conformations in its pre-fusion state, but its equilibrium is shifted towards a more fusion-favourable state upon acidification. Such an approach can reveal excellent temporal resolution of conformational dynamics *in vitro* using TIRF microscopy but has not yet been demonstrated in a cellular context. Potential optical configurations such as light-sheet microscopy may offer the single-molecule sensitivity for such assays in infected cells [85].

Following fusion, influenza virus uncoats progressively: first the matrix layer of M1 proteins dissociates followed by the release of the 8 vRNPs into the cytosol. A recent study has shown that influenza viruses can exploit the cellular aggresome to promote uncoating following M2-mediated acidification of the viral core [30]. For this, 3D-SIM was used to highlight the presence of unanchored ubiquitin chains packaged into virions (see Figure 2b) that recruit histone deacetylase 6 (HDAC6) to fusion sites on late endosomes to help influenza uncoating. In a follow-up study, the authors demonstrated that the karyopherin TNPO1 is also involved in uncoating by promoting the dissociation of M1 and disassembly of the vRNPs [86]. Specifically, SIM was used to observe TNPO1-associated de-bundling of vRNPs in the cytoplasm before nuclear import. Here, the resolution improvement of SIM was sufficient to show the molecular associations involved in these steps of uncoating. Also, the compatibility of SIM with a wide range of labelling approaches makes SIM an excellent complementary tool to biochemical studies.

After uncoating in late endosomes, vRNPs need to travel through the cytoplasm and into the nucleus where the viral genome replication and expression occurs. This step was shown to be a critical bottleneck for efficient infection due to the potential for immediate RNA degradation in the cytoplasm [68]. This was indicated by mathematical modelling of the key steps of viral trafficking up to viral genome nuclear import. The model was calibrated using a combination of biochemical assays, cytometry and microscopy. Quantitative live-cell microscopy may support the refining of such modelling approaches, providing a fully quantitative biophysical framework for viral infection.

The diffusion of the vRNPs from the release site to the nucleus has been studied using SPT and QD labelling strategy [61] (Figure 3d). By using multi-colour quantum dot labelling, this study was able to elegantly show that vRNP segments are released sequentially from late endosomes during uncoating and enter the nucleus through a multi-stage movement process.

Thus, the capacity of advanced quantitative live-cell imaging to perform fast-tracking of multiple viral components simultaneously can provide dynamic information that enhances our understanding of the underlying biophysical processes. This was also demonstrated elegantly in a study of HIV uncoating [23] where fast, sensitive live-cell imaging of early infection events from

trafficking, nuclear import to uncoating. Specifically, this study provided the first direct demonstration that the HIV capsid crosses the nuclear envelope intact, supporting the notion that capsid integrity is essential to protect HIV from innate sensing.

Although fusion and uncoating involve fundamental structural reorganisations of viruses at the nanoscale, SRM has yet to be used to observe these phenomena in the context of influenza. This may be a consequence of the difficulty of observing these events as they occur, since the highest-resolution SRM techniques like SMLM mainly work in fixed samples rather than live-cell.

6. Understanding assembly, budding and release

To produce new infectious virions, the essential viral proteins and the viral genome must be transported from their sites of replication: the cytoplasm or ER and the nucleus respectively, to the plasma membrane where virions assemble. To study the trafficking events leading to virus assembly, it is necessary to be able to follow the spatio-temporal dynamics of viral components. It is particularly important to understand how the segmented genome is capable of bundling into infectious virions, since this genomic reassortment is linked to the appearance of emerging viral strains, with pandemic potentials.

Fluorescence *in situ* hybridisation (FISH) [64] methods have been used to understand the spatio-temporal dynamics of segment bundling [65,87]. Recent advances in microscopy have allowed multiplexing FISH observations of up to 4 segments to show that segments are capable of forming subcomplexes throughout the cytoplasm during infection [88]. A recently developed method, called Multiple Sequential FISH (MuSeq-FISH) based on sequential single-molecule imaging of vRNA segments enabled a large-scale colocalization study between the 8 vRNA segments of influenza in assembly [71] (see Figure 3e). By observing the relative occurrences of specific bundling of vRNPs in infected cells combined with mathematical modelling, a set of possible bundling order of the vRNPs was suggested. Here, the authors highlighted that this selective packaging may be central to RNA re-assortment and its associated pandemic potential. Again, the combination of single-molecule imaging and advanced quantitative analysis can provide clear biophysical insights into the mechanisms of viral assembly.

On the other hand, advanced live-cell imaging can provide direct information about the dynamics of viral component transport to the assembly sites and the mechanisms involved. Fast 3D tracking using light-sheet microscopy was exploited to follow the co-transport of multiple influenza vRNA segments (using GFP-tagged PA) [88] and with the recycling endosomal marker Rab11A (here using mRuby-tagged PA) [24] in living cells (see Figure 1c). Following vRNA transport in 3D at high speed enabled parallelisation of tracking observations captured in the cell volume. These studies suggested an assembly of vRNPs *en route* to the budding site, facilitated by the association with and modulation of Rab11A-associated vesicular transport, demonstrating the profound effect of infection on molecular and vesicular transport dynamics [24].

In some cases, following the assembly process in living cells through the visualisation of newly expressed viral proteins in living cells can constitute a challenge for fluorescence labelling since genetic modifications of viral proteins can lead to loss of infectivity and/or assembly defects. Therefore strategies for non-invasive labelling of newly expressed viral proteins were designed, notably through the use of a split green fluorescent protein (GFP) approach [89], to fluorescently tag the influenza polymerase PB2 protein [90] in order to observe vRNPs trafficking in live infected cells. This labelling strategy was shown to be minimally disruptive and allowed the accumulation of nascent vRNPs at the plasma membrane, alongside PB2, to be observed in live cells.

Influenza virus assembly, budding and release are also closely tied to HA, NA and M2 distribution on the host cell plasma membrane [91]. Previous studies have shown that whilst HA and NA are found in lipid rafts [92], M2 is excluded [93], despite the fact that M2's location at the budding site is key to facilitate ESCRT-independent membrane scission [94]. Therefore, the organisation and regulation of these microdomains containing HA/NA/M2 are critical for all steps of viral assembly and release. Here, SRM can provide a powerful solution to studying the nanoscale organisation of

these viral components at and around the site of assembly. Live-cell fPALM combined with quantitative spatial correlation analysis showed that the cluster distribution of HA in the plasma membrane of infected cells correlates positively with dense regions of actin and correlates negatively with cofilin at the nanoscale [95]. Single-molecule tracking approaches also showed that the mobility of HA diffusion was reduced in actin-rich regions. These findings point to a potential two-way feedback between HA clustering and actin dynamics. A further study using the same techniques recently revealed that the nanoscale clusters of HA confine the diffusion of PIP2 [96], suggesting that HA acts on intracellular pathways involved in PIP2 regulation of actin re-modelling.

Interfering with these dynamic microdomains can potentially be used as a therapeutic intervention. STORM imaging of budding filamentous influenza viruses revealed that antibodies against the extracellular part of M2 (M2e) were able to disrupt virus production [97]. Interestingly, the study also observed the presence of HA-filled protrusions connecting infected cells with neighbouring uninfected cells, highlighting a potential role of filamentous viruses in direct cell-to-cell infection.

Advanced microscopy can be leveraged to investigate the role of specific host proteins during infection. A study unveiled CD81, a tetraspanin previously shown to be a co-receptor for hepatitis C virus entry [98], as a key host factor for influenza uncoating and virus release [72]. This study showed that CD81 is recruited to influenza virus budding sites (Figure 3f) and is even found clustered in nascent virions during budding. The authors found using 3D STORM that CD81 clusters in an alternating fashion with PB1 (every 150-200nm) on the surface of budding filamentous viruses. Although the importance of this pattern, if any, is currently not understood, SRM helped reveal the nanoscale co-organisation of a host protein, CD81, with a viral component, PB1.

It is worth highlighting that SRM has proven very powerful in the study of budding mechanisms for HIV. In particular, PALM and *d*STORM have been used to study how Env is recruited to Gag protein clusters during HIV assembly at the plasma membrane [99], and 3D SMLM to delineate the distribution and potential role of the ESCRT machinery in and around HIV budding sites [18,20]. More recently, a similar 3D SMLM strategy allowed the nanoscale observation that HIV Env does not distribute randomly within the budding virion but rather accumulates around the neck of the nascent virions, suggesting that Gag lattices form prior to Env assembly [20]. This observation was also later supplemented by single-molecule tracking of Env at the budding site, showing that Env cytoplasmic tail is necessary for its interaction with Gag [100]. These studies show that advanced SPT and SRM approaches can help to identify mechanisms of infection and virus host-cell interactions at the molecular level. Their application to influenza virus may contribute to understanding the host-cell interactions that determine viral shape at the single virus level.

7. Understanding viral restriction by the host cell and supporting the development of antiviral strategies

Host cell restriction and innate immune responses are important intrinsic suppressors of viral replication. The interferon-induced antiviral protein IFITM3 has previously been identified as an influenza restriction factor that inhibits viral entry prior to uncoating [101] and increases viral degradation through the lysosomal pathway [102]. To further understand the mechanism of IFITM3 restriction, two-colour STED was used to visualize and quantify the clustering of IFITM3 in influenza-positive endosomal vesicles [103]. The study showed that IFITM3 clustering increased following uptake of influenza inside the cell, with IFITM3 coating early endosomal vesicles and recycling endosomes that contained viral NP (i.e. pre-fusion), potentially preventing influenza from achieving a productive infection at an early timepoint. A recent study demonstrated the use of novel photo-switchable probes for SMLM called Super Beacons and provided the first observation of IFITM1 nanoscale organisation at the plasma membrane [104]. The nanoscale organisation and localization of viral restriction factors during infection may then be revealed by SRM and help understand the underlying mechanism of restriction.

Uncoating is a critical step in viral replication essential for release of the viral genome into the cytoplasm of a new host cell. However, this release exposes the vRNA to cellular innate immune sensors that may initiate interferon responses and viral restriction. A study showed that influenza is capable of suppressing the RIG-I-mediated interferon response and that this effect is dependent on a specific amino-acid motif in the viral polymerases PB1/PA [105]. Multi-colour 3D STORM revealed a close interaction of the vRNPs with the innate immune sensors RIG-I and MAVS at the mitochondrial membrane early in infection, which may act as a direct suppressor of the interferon response. Here, the capability to perform nanoscale imaging supports a model of direct molecular interactions between vRNPs and intracellular innate sensors.

Advanced microscopy, and especially quantitative microscopy, may also offer robust understanding of the effect of an antiviral drug at the single-virus level. By studying the structures of live viruses shed from cells treated with the NA inhibitor oseltamivir, quantitative microscopy and the use of non-invasive labelling enabled the observation that oseltamivir preferentially decreases the release of filamentous viruses as opposed to smaller spherical ones [22]. This suggests that the pleiomorphic diversity of influenza may help improve viral escape when challenged with oseltamivir, highlighting the potential importance of this diversity *in vivo* (see Figure 1b). This highlights the capabilities of advanced microscopy to relate structure to function, in the design and characterisation of anti-viral drugs.

In the context of drug development for influenza antiviral therapy, high-throughput microscopy approaches offer great promise for quantitative and large-scale analysis of compound in the search of specific cellular and viral phenotypes. For instance, a high-throughput quantitative imaging methodology was developed to provide assays capable of probing different stages of the early influenza infection: initial binding, endocytosis, viral membrane fusion, uncoating, nuclear import and translation [106]. The imaging approach in these assays is conventional: for example, looking at the position and intensity of anti-M1 signal to monitor uncoating. The novelty comes with the authors' use of machine learning to automate and quantify the distinction between the different visual endpoint phenotype of the assays and the optimization of the timing of the read-outs. One key application of such technology is the identification of host factor as a drug target for the prevention and/or treatment of influenza virus infection.

8. Conclusions

Our review summarizes recent applications of SRM and quantitative advanced microscopy techniques to study influenza replication. It highlights important techniques such as SRM for structural studies of molecular assemblies either in the virus particles [22] or in interactions with host-cell components [30], SPT via QD labelling for tracking and trafficking dynamics [61], single-molecule imaging via smFRET to study conformational dynamics of HA [63] or smFISH to study the bundling of viral genome segments during assembly [71], all of which provided important new mechanistic insights into influenza virus replication. In addition, the application of live-cell approaches, in particular SPT of different viral components (such as vRNPs) [61] and single-virus studies [19,56], enabled by novel labelling strategies such as QD and small enzymatic tagging, have uncovered important dynamic behaviours of influenza virus infection in cells, highlighting the complexity of trafficking and assembly [28,71], and the shape-biased effect of oseltamivir on pleiomorphic viruses [22]. This diversity can only be uncovered by advanced microscopy techniques capable of high sensitivity and molecular specificity, combined with high spatial and temporal resolution.

An important underlying principle to all these microscopy methods is the combination of imaging with quantitative data analysis to determine e.g. the relative levels and spatial distributions of envelope proteins in filamentous viral particles [37] or the spatio-temporal behaviour of endosomal vesicles and vRNA by SPT [24]. Quantitative approaches [22,24,71,106] and the study of the virus at the population level, especially those using novel machine-learning-based methods, have the potential to make a transformative change in how we analyse imaging data [66,67] in an objective and unbiased approach, especially as they become more widely available [107]. Several computational approaches have already been developed to build and map nanoscale structural

features of virus morphology [21,25,108]. The strength of these approaches is that they can be combined with the above-mentioned microscopy techniques in order to largely benefit from their respective advantages. For instance, single-molecule tracking can be performed in combination with SRM imaging of the host cell cytoskeleton or endosomal network, to precisely assign observed features to underlying cellular structures. As we have highlighted throughout this review, a number of approaches remain under-exploited such as the use ExM both for the study of viral assembly and the modulation of host organelle organisation in infection.

For the first time, these new imaging technologies allow us to follow the fate of individual virions in live cells, to understand how the choice of entry pathway or assembly may be modulated depending on the cellular (e.g. activation or suppression of relevant host factors including restriction factors) and viral context (e.g. virus composition, multiple concomitant infections), and to quantify the effect of potential anti-viral drugs at the single-virus level. We expect the combination of these advanced imaging technologies with new labelling methods, and new computational approaches including machine learning, will reveal new knowledge of influenza virus replication enabling the development of effective therapeutics. These technical advances will be important tools in efforts to understand virus-host interactions that determine infection outcomes and pandemic potential.

Author Contributions: Writing—original draft preparation, E.T. and R.F.L.; writing—review and editing, E.T., C.S., R.H., M.M. and R.F.L.; supervision, R.F.L. All authors have read and agreed to the published version of the manuscript.

Funding: E.T. is supported by an MRC DTP studentship (MR/N013867/1). C.S. is funded by the Helmholtz Association. R.H. is supported by UK Medical Research Council (MR/K015826/1), the Wellcome Trust (203276/Z/16/Z) and the Gulbenkian Foundation. M.M. was supported by the UK Medical Research Council funding to the MRC-UCL LMCB University Unit (MC_UU00012/1 and MC_U12266B). R.F.L. would like to acknowledge the support of the MRC Skills development fellowship (MR/T027924/1).

Acknowledgments: Schematics were created with BioRender.com.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. WHO Factsheet on Influenza (Seasonal) Available online: https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal) (accessed on May 13, 2020).
2. Houser, K.; Subbarao, K. Influenza vaccines: Challenges and solutions. *Cell Host Microbe* **2015**, *17*, 295–300, doi:10.1016/j.chom.2015.02.012.
3. Mostafa, A.; Abdelwhab, E.M.; Mettenleiter, T.C.; Pleschka, S. Zoonotic potential of influenza A viruses: A comprehensive overview. *Viruses* **2018**, *10*, 497.
4. WHO Ten threats to global health in 2019 Available online: https://www.who.int/news-room/feature-stories/ten-threats-to-global-health-in-2019 (accessed on Jun 29, 2020).
5. Wu, F.; Zhao, S.; Yu, B.; Chen, Y.M.; Wang, W.; Song, Z.G.; Hu, Y.; Tao, Z.W.; Tian, J.H.; Pei, Y.Y.; et al. A new coronavirus associated with human respiratory disease in China. *Nature* **2020**, *579*, 265–269, doi:10.1038/s41586-020-2008-3.
6. Wagner, R.; Matrosovich, M.; Klenk, H.-D. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. *Rev. Med. Virol.* **2002**, *12*, 159–166, doi:10.1002/rmv.352.
7. Gamblin, S.J.; Skehel, J.J. Influenza hemagglutinin and neuraminidase membrane glycoproteins. *J. Biol. Chem.* **2010**, *285*, 28403–28409, doi:10.1074/jbc.R110.129809.
8. Badham, M.D.; Rossman, J.S. Filamentous Influenza Viruses. *Curr. Clin. Microbiol. Reports* **2016**, *3*, 155–161, doi:10.1007/s40588-016-0041-7.
9. Dadonaite, B.; Vijayakrishnan, S.; Fodor, E.; Bhella, D.; Hutchinson, E.C. Filamentous influenza viruses. *J. Gen. Virol.* **2016**, *97*, 1755–1764, doi:10.1099/jgv.0.000535.
10. Calder, L.J.; Wasilewski, S.; Berriman, J.A.; Rosenthal, P.B. Structural organization of a filamentous influenza A virus. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 10685–10690, doi:10.1073/pnas.1002123107.
11. Benton, D.J.; Gamblin, S.J.; Rosenthal, P.B.; Skehel, J.J. Structural transitions in influenza haemagglutinin at membrane fusion pH. *Nature* **2020**, *583*, 150–153, doi:10.1038/s41586-020-2333-6.
12. Wasilewski, S.; Calder, L.J.; Grant, T.; Rosenthal, P.B. Distribution of surface glycoproteins on influenza A virus determined by electron cryotomography. *Vaccine* **2012**, *30*, 7368–7373, doi:10.1016/j.vaccine.2012.09.082.
13. Huang, B.; Babcock, H.; Zhuang, X. Breaking the diffraction barrier: Super-resolution imaging of cells. *Cell* **2010**, *143*, 1047–1058, doi:10.1016/j.cell.2010.12.002.
14. Lelek, M.; Di Nunzio, F.; Henriques, R.; Charneau, P.; Arhel, N.; Zimmer, C. Superresolution imaging of HIV in infected cells with FlAsH-PALM. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 8564–8569, doi:10.1073/pnas.1013267109.
15. Müller, B.; Heilemann, M. Shedding new light on viruses: Super-resolution microscopy for studying human immunodeficiency virus. *Trends Microbiol.* **2013**, *21*, 522–533, doi:10.1016/j.tim.2013.06.010.
16. Chojnacki, J.; Eggeling, C. Super-resolution fluorescence microscopy studies of human immunodeficiency virus. *Retrovirology* **2018**, *15*, 1–16, doi:10.1186/s12977-018-0424-3.

17. Chojnacki, J.; Staudt, T.; Glass, B.; Bingen, P.; Engelhardt, J.; Anders, M.; Schneider, J.; Müller, B.; Hell, S.W.; Kräusslich, H.G. Maturation-dependent HIV-1 surface protein redistribution revealed by fluorescence nanoscopy. *Science (80-.).* **2012**, *338*, 524–528, doi:10.1126/science.1226359.

18. Van Engelenburg, S.B.; Shtengel, G.; Sengupta, P.; Waki, K.; Jarnik, M.; Ablan, S.D.; Freed, E.O.; Hess, H.F.; Lippincott-Schwartz, J. Distribution of ESCRT machinery at HIV assembly sites reveals virus scaffolding of ESCRT subunits. *Science (80-.).* **2014**, *343*, 653–656, doi:10.1126/science.1247786.

19. Kim, D.; Deerinck, T.J.; Sigal, Y.M.; Babcock, H.P.; Ellisman, M.H.; Zhuang, X. Correlative stochastic optical reconstruction microscopy and electron microscopy. *PLoS One* **2015**, *10*, e0124581, doi:10.1371/journal.pone.0124581.

20. Buttler, C.A.; Pezeshkian, N.; Fernandez, M. V.; Aaron, J.; Norman, S.; Freed, E.O.; Van Engelenburg, S.B. Single molecule fate of HIV-1 envelope reveals late-stage viral lattice incorporation. *Nat. Commun.* **2018**, *9*, 1861, doi:10.1038/s41467-018-04220-w.

21. Laine, R.F.; Goodfellow, G.; Young, L.J.; Travers, J.; Carroll, D.; Dibben, O.; Bright, H.; Kaminski, C.F. Structured illumination microscopy combined with machine learning enables the high throughput analysis and classification of virus structure. *Elife* **2018**, *7*, e40183, doi:10.7554/eLife.40183.

22. Vahey, M.D.; Fletcher, D.A. Low-Fidelity Assembly of Influenza A Virus Promotes Escape from Host Cells. *Cell* **2019**, *176*, 281-294.e19, doi:10.1016/j.cell.2018.10.056.

23. Burdick, R.C.; Li, C.; Munshi, M.H.; Rawson, J.M.O.; Nagashima, K.; Hu, W.S.; Pathak, V.K. HIV-1 uncoats in the nucleus near sites of integration. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 5486–5493, doi:10.1073/pnas.1920631117.

24. Bhagwat, A.R.; Le Sage, V.; Nturibi, E.; Kulej, K.; Jones, J.; Guo, M.; Tae Kim, E.; Garcia, B.A.; Weitzman, M.D.; Shroff, H.; et al. Quantitative live cell imaging reveals influenza virus manipulation of Rab11A transport through reduced dynein association. *Nat. Commun.* **2020**, *11*, 23, doi:10.1038/s41467-019-13838-3.

25. Laine, R.F.; Albecka, A.; Van De Linde, S.; Rees, E.J.; Crump, C.M.; Kaminski, C.F. Structural analysis of herpes simplex virus by optical super-resolution imaging. *Nat. Commun.* **2015**, *6*, 5980, doi:10.1038/ncomms6980.

26. Sieben, C.; Sezgin, E.; Eggeling, C.; Manley, S. Influenza A viruses use multivalent sialic acid clusters for cell binding and receptor activation. *PLOS Pathog.* **2020**, *16*, e1008656, doi:10.1371/journal.ppat.1008656.

27. Hell, S.W.; Wichmann, J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt. Lett.* **1994**, *19*, 780, doi:10.1364/ol.19.000780.

28. Baharom, F.; Thomas, O.S.; Lepzien, R.; Mellman, I.; Chalouni, C.; Smed-Sörensen, A. Visualization of early influenza A virus trafficking in human dendritic cells using STED microscopy. *PLoS One* **2017**, *12*, e0177920, doi:10.1371/journal.pone.0177920.

29. Gustafsson, M.G.L. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J. Microsc.* **2000**, *198*, 82–87, doi:10.1046/j.1365-2818.2000.00710.x.

30. Banerjee, I.; Miyake, Y.; Philip Nobs, S.; Schneider, C.; Horvath, P.; Kopf, M.; Matthias, P.; Helenius, A.;

Yamauchi, Y. Influenza A virus uses the aggresome processing machinery for host cell entry. *Science (80-.).* **2014**, *346*, 473–477, doi:10.1126/science.1257037.

31. Gray, R.D.M.; Albrecht, D.; Beerli, C.; Huttunen, M.; Cohen, G.H.; White, I.J.; Burden, J.J.; Henriques, R.; Mercer, J. Nanoscale polarization of the entry fusion complex of vaccinia virus drives efficient fusion. *Nat. Microbiol.* **2019**, *4*, 1636–1644, doi:10.1038/s41564-019-0488-4.

32. Betzig, E.; Patterson, G.H.; Sougrat, R.; Lindwasser, O.W.; Olenych, S.; Bonifacino, J.S.; Davidson, M.W.; Lippincott-Schwartz, J.; Hess, H.F. Imaging intracellular fluorescent proteins at nanometer resolution. *Science (80-.).* **2006**, *313*, 1642–1645, doi:10.1126/science.1127344.

33. Hess, S.T.; Girirajan, T.P.K.; Mason, M.D. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys. J.* **2006**, *91*, 4258–4272, doi:10.1529/biophysj.106.091116.

34. Rust, M.J.; Bates, M.; Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **2006**, *3*, 793–795, doi:10.1038/nmeth929.

35. Heilemann, M.; van de Linde, S.; Schüttpelz, M.; Kasper, R.; Seefeldt, B.; Mukherjee, A.; Tinnefeld, P.; Sauer, M. Subdiffraction-Resolution Fluorescence Imaging with Conventional Fluorescent Probes. *Angew. Chemie Int. Ed.* **2008**, *47*, 6172–6176, doi:10.1002/anie.200802376.

36. Sharonov, A.; Hochstrasser, R.M. Wide-field subdiffraction imaging by accumulated binding of diffusing probes. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 18911–18916, doi:10.1073/pnas.0609643104.

37. Vahey, M.D.; Fletcher, D.A. Influenza a virus surface proteins are organized to help penetrate host mucus. *Elife* **2019**, *8*, e43764, doi:10.7554/eLife.43764.

38. Chozinski, T.J.; Halpern, A.R.; Okawa, H.; Kim, H.J.; Tremel, G.J.; Wong, R.O.L.; Vaughan, J.C. Expansion microscopy with conventional antibodies and fluorescent proteins. *Nat. Methods* **2016**, *13*, 485–488, doi:10.1038/nmeth.3833.

39. Fölling, J.; Bossi, M.; Bock, H.; Medda, R.; Wurm, C.A.; Hein, B.; Jakobs, S.; Eggeling, C.; Hell, S.W. Fluorescence nanoscopy by ground-state depletion and single-molecule return. *Nat. Methods* **2008**, *5*, 943–945, doi:10.1038/nmeth.1257.

40. Manley, S.; Gillette, J.M.; Patterson, G.H.; Shroff, H.; Hess, H.F.; Betzig, E.; Lippincott-Schwartz, J. High-density mapping of single-molecule trajectories with photoactivated localization microscopy. *Nat. Methods* **2008**, *5*, 155–157, doi:10.1038/nmeth.1176.

41. Hess, S.T.; Gould, T.J.; Gudheti, M. V.; Maas, S.A.; Mills, K.D.; Zimmerberg, J. Dynamic clustered distribution of hemagglutinin resolved at 40 nm in living cell membranes discriminates between raft theories. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 17370–17375, doi:10.1073/pnas.0708066104.

42. Steinhauer, C.; Forthmann, C.; Vogelsang, J.; Tinnefeld, P. Superresolution microscopy on the basis of engineered dark states. *J. Am. Chem. Soc.* **2008**, *130*, 16840–16841, doi:10.1021/ja806590m.

43. Itano, M.S.; Steinhauer, C.; Schmied, J.J.; Forthmann, C.; Liu, P.; Neumann, A.K.; Thompson, N.L.; Tinnefeld, P.; Jacobson, K. Super-resolution imaging of C-type lectin and influenza hemagglutinin nanodomains on plasma membranes using blink microscopy. *Biophys. J.* **2012**, *102*, 1534–1542, doi:10.1016/j.bpj.2012.02.022.

44. Chen, F.; Tillberg, P.W.; Boyden, E.S. Expansion microscopy. *Science (80-.).* **2015**, *347*, 543–548, doi:10.1126/science.1260088.

45. Chang, J.B.; Chen, F.; Yoon, Y.G.; Jung, E.E.; Babcock, H.; Kang, J.S.; Asano, S.; Suk, H.J.; Pak, N.; Tillberg, P.W.; et al. Iterative expansion microscopy. *Nat. Methods* **2017**, *14*, 593–599, doi:10.1038/nmeth.4261.

46. Gambarotto, D.; Zwettler, F.U.; Le Guennec, M.; Schmidt-Cernohorska, M.; Fortun, D.; Borgers, S.; Heine, J.; Schloetel, J.G.; Reuss, M.; Unser, M.; et al. Imaging cellular ultrastructures using expansion microscopy (U-ExM). *Nat. Methods* **2019**, *16*, 71–74, doi:10.1038/s41592-018-0238-1.

47. Mascheroni, L.; Scherer, K.M.; Manton, J.D.; Ward, E.; Dibben, O.; Kaminski, C.F. Combining sample expansion and light sheet microscopy for the volumetric imaging of virus-infected cells with optical super-resolution. *bioRxiv* **2020**, 2020.04.10.035378, doi:10.1101/2020.04.10.035378.

48. Aho, V.; Mäntylä, E.; Ekman, A.; Hakanen, S.; Mattola, S.; Chen, J.H.; Weinhardt, V.; Ruokolainen, V.; Sodeik, B.; Larabell, C.; et al. Quantitative microscopy reveals stepwise alteration of chromatin structure during herpesvirus infection. *Viruses* **2019**, *11*, 935, doi:10.3390/v11100935.

49. Gao, R.; Yu, C.C. (Jay); Gao, L.; Piatkevich, K.D.; Neve, R.L.; Upadhyayula, S.; Boyden, E.S. A highly homogeneous expansion microscopy polymer composed of tetrahedron-like monomers. *bioRxiv* 2019, *1*, 814111.

50. Gambarotto, D.; Hamel, V.; Guichard, P. Ultrastructure expansion microscopy (U-ExM). In *Methods in Cell Biology*; Elsevier Inc., 2020; pp. 1–25.

51. Power, R.M.; Huisken, J. A guide to light-sheet fluorescence microscopy for multiscale imaging. *Nat. Methods* **2017**, *14*, 360–373, doi:10.1038/nmeth.4224.

52. Griffin, B.A.; Adams, S.R.; Tsien, R.Y. Specific covalent labeling of recombinant protein molecules inside live cells. *Science (80-.).* **1998**, *281*, 269–272, doi:10.1126/science.281.5374.269.

53. Yin, J.; Lin, A.J.; Golan, D.E.; Walsh, C.T. Site-specific protein labeling by Sfp phosphopantetheinyl transferase. *Nat. Protoc.* **2006**, *1*, 280–285, doi:10.1038/nprot.2006.43.

54. Lin, C.W.; Ting, A.Y. Transglutaminase-catalyzed site-specific conjugation of small-molecule probes to proteins in vitro and on the surface of living cells. *J. Am. Chem. Soc.* **2006**, *128*, 4542–4543, doi:10.1021/ja0604111.

55. Theile, C.S.; Witte, M.D.; Blom, A.E.M.; Kundrat, L.; Ploegh, H.L.; Guimaraes, C.P. Site-specific N-terminal labeling of proteins using sortase-mediated reactions. *Nat. Protoc.* **2013**, *8*, 1800–1807, doi:10.1038/nprot.2013.102.

56. Lakadamyali, M.; Rust, M.J.; Babcock, H.P.; Zhuang, X. Visualizing infection of individual influenza viruses. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 9280–9285, doi:10.1073/pnas.0832269100.

57. Brandenburg, B.; Zhuang, X. Virus trafficking – learning from single-virus tracking. *Nat. Rev. Microbiol.* **2007**, *5*, 1197–208, doi:10.1038/nrmicro1615.Virus.

58. Liu, S.L.; Wang, Z.G.; Xie, H.Y.; Liu, A.A.; Lamb, D.C.; Pang, D.W. Single-Virus Tracking: From Imaging Methodologies to Virological Applications. *Chem. Rev.* **2020**, *120*, 1936–1979, doi:10.1021/acs.chemrev.9b00692.

59. Liu, S.-L.; Zhang, Z.-L.; Tian, Z.-Q.; Zhao, H.-S.; Liu, H.; Sun, E.-Z.; Xiao, G.F.; Zhang, W.; Wang, H.-Z.; Pang, D.-W. Effectively and Efficiently Dissecting the Infection of Influenza Virus by Quantum-Dot-Based Single-Particle Tracking. *ACS Nano* **2012**, *6*, 141–150, doi:10.1021/nn2031353.

60. Liu, S.L.; Zhang, L.J.; Wang, Z.G.; Zhang, Z.L.; Wu, Q.M.; Sun, E.Z.; Shi, Y.B.; Pang, D.W. Globally visualizing the microtubule-dependent transport behaviors of influenza virus in live cells. *Anal. Chem.* **2014**, *86*, 3902–3908, doi:10.1021/ac500640u.

61. Qin, C.; Li, W.; Li, Q.; Yin, W.; Zhang, X.; Zhang, Z.; Zhang, X.E.; Cui, Z. Real-time dissection of dynamic uncoating of individual influenza viruses. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 2577–2582, doi:10.1073/pnas.1812632116.

62. Weiss, S. Fluorescence spectroscopy of single biomolecules. *Science (80-.).* **1999**, *283*, 1676–1683, doi:10.1126/science.283.5408.1676.

63. Das, D.K.; Govindan, R.; Nikić-Spiegel, I.; Krammer, F.; Lemke, E.A.; Munro, J.B. Direct Visualization of the Conformational Dynamics of Single Influenza Hemagglutinin Trimers. *Cell* **2018**, *174*, 926-937.e12, doi:10.1016/j.cell.2018.05.050.

64. Femino, A.M.; Fay, F.S.; Fogarty, K.; Singer, R.H. Visualization of single RNA transcripts in situ. *Science (80-.).* **1998**, *280*, 585–590, doi:10.1126/science.280.5363.585.

65. Chou, Y. ying; Heaton, N.S.; Gao, Q.; Palese, P.; Singer, R.; Lionnet, T. Colocalization of Different Influenza Viral RNA Segments in the Cytoplasm before Viral Budding as Shown by Single-molecule Sensitivity FISH Analysis. *PLoS Pathog.* **2013**, *9*, e1003358, doi:10.1371/journal.ppat.1003358.

66. von Chamier, L.; Laine, R.F.; Henriques, R. Artificial intelligence for microscopy: What you should know. *Biochem. Soc. Trans.* **2019**, *47*, 1029–1040, doi:10.1042/BST20180391.

67. Belthangady, C.; Royer, L.A. Applications, promises, and pitfalls of deep learning for fluorescence image reconstruction. *Nat. Methods* **2019**, *16*, 1215–1225, doi:10.1038/s41592-019-0458-z.

68. Schelker, M.; Mair, C.M.; Jolmes, F.; Welke, R.W.; Klipp, E.; Herrmann, A.; Flöttmann, M.; Sieben, C. Viral RNA Degradation and Diffusion Act as a Bottleneck for the Influenza A Virus Infection Efficiency. *PLoS Comput. Biol.* **2016**, *12*, 1–23, doi:10.1371/journal.pcbi.1005075.

69. Schwille, P.; Korlach, J.; Webb, W.W. Fluorescence correlation spectroscopy with single-molecule sensitivity on cell and model membranes. *Cytometry* **1999**, *36*, 176–182, doi:10.1002/(SICI)1097-0320(19990701)36:3<176::AID-CYTO5>3.0.CO;2-F.

70. Chojnacki, J.; Waithe, D.; Carravilla, P.; Huarte, N.; Galiani, S.; Enderlein, J.; Eggeling, C. Envelope glycoprotein mobility on HIV-1 particles depends on the virus maturation state. *Nat. Commun.* **2017**, *8*, 545, doi:10.1038/s41467-017-00515-6.

71. Haralampiev, I.; Prisner, S.; Nitzan, M.; Schade, M.; Jolmes, F.; Schreiber, M.; Loidolt-Krüger, M.; Jongen, K.; Chamiolo, J.; Nilson, N.; et al. Selective flexible packaging pathways of the segmented genome of influenza A virus. *Nat. Commun.* **2020**, *11*, 1–13, doi:10.1038/s41467-020-18108-1.

72. He, J.; Sun, E.; Bujny, M. V.; Kim, D.; Davidson, M.W.; Zhuang, X. Dual Function of CD81 in Influenza Virus Uncoating and Budding. *PLoS Pathog.* **2013**, *9*, e1003701, doi:10.1371/journal.ppat.1003701.

73. Sieczkarski, S.B.; Whittaker, G.R. Characterization of the host cell entry of filamentous influenza virus. *Arch. Virol.* **2005**, *150*, 1783–1796, doi:10.1007/s00705-005-0558-1.

74. de Vries, E.; Tscherne, D.M.; Wienholts, M.J.; Cobos-Jiménez, V.; Scholte, F.; García-Sastre, A.; Rottier, P.J.M.; de Haan, C.A.M. Dissection of the influenza a virus endocytic routes reveals macropinocytosis as an alternative entry pathway. *PLoS Pathog.* **2011**, *7*, e1001329, doi:10.1371/journal.ppat.1001329.

75. De Conto, F.; Covan, S.; Arcangeletti, M.C.; Orlandini, G.; Gatti, R.; Dettori, G.; Chezzi, C. Differential infectious entry of human influenza A/NWS/33 virus (H1N1) in mammalian kidney cells. *Virus Res.* **2011**, *155*, 221–230, doi:10.1016/j.virusres.2010.10.008.

76. Edinger, T.O.; Pohl, M.O.; Stertz, S. Entry of influenza A virus: Host factors and antiviral targets. *J. Gen. Virol.* **2014**, *95*, 263–277, doi:10.1099/vir.0.059477-0.

77. Karakus, U.; Pohl, M.O.; Stertz, S. Breaking the Convention: Sialoglycan Variants, Coreceptors, and Alternative Receptors for Influenza A Virus Entry. *J. Virol.* **2019**, *94*, e01357-19, doi:10.1128/jvi.01357-19.

78. Eierhoff, T.; Hrincius, E.R.; Rescher, U.; Ludwig, S.; Ehrhardt, C. The Epidermal Growth Factor Receptor (EGFR) Promotes Uptake of Influenza A Viruses (IAV) into Host Cells. *PLoS Pathog.* **2010**, *6*, e1001099, doi:10.1371/journal.ppat.1001099.

79. Stauffer, S.; Feng, Y.; Nebioglu, F.; Heilig, R.; Picotti, P.; Helenius, A. Stepwise Priming by Acidic pH and a High K+ Concentration Is Required for Efficient Uncoating of Influenza A Virus Cores after Penetration. *J. Virol.* **2014**, *88*, 13029–13046, doi:10.1128/jvi.01430-14.

80. Li, S.; Sieben, C.; Ludwig, K.; Höfer, C.T.; Chiantia, S.; Herrmann, A.; Eghiaian, F.; Schaap, I.A.T. PH-ontrolled two-step uncoating of influenza virus. *Biophys. J.* **2014**, *106*, 1447–1456, doi:10.1016/j.bpj.2014.02.018.

81. Stauffer, S.; Feng, Y.; Nebioglu, F.; Heilig, R.; Picotti, P.; Helenius, A. Stepwise Priming by Acidic pH and a High K+ Concentration Is Required for Efficient Uncoating of Influenza A Virus Cores after Penetration. *J. Virol.* **2014**, *88*, 13029–13046, doi:10.1128/jvi.01430-14.

82. Chen, C.; Zhuang, X. Epsin 1 is a cargo-specific adaptor for the clathrin-mediated endocytosis of the influenza virus. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 11790–11795, doi:10.1073/pnas.0803711105.

83. Floyd, D.L.; Ragains, J.R.; Skehel, J.J.; Harrison, S.C.; Van Oijen, A.M. Single-particle kinetics of influenza virus membrane fusion. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 15382–15387, doi:10.1073/pnas.0807771105.

84. Sakai, T.; Ohuchi, M.; Imai, M.; Mizuno, T.; Kawasaki, K.; Kuroda, K.; Yamashina, S. Dual Wavelength Imaging Allows Analysis of Membrane Fusion of Influenza Virus inside Cells. *J. Virol.* **2006**, *80*, 2013–2018, doi:10.1128/jvi.80.4.2013-2018.2006.

85. Liu, S.; Huh, H.; Lee, S.H.; Huang, F. Three-Dimensional Single-Molecule Localization Microscopy in Whole-Cell and Tissue Specimens. *Annu. Rev. Biomed. Eng.* **2020**, *22*, 155–184, doi:10.1146/annurev-bioeng-060418-052203.

86. Miyake, Y.; Keusch, J.J.; Decamps, L.; Ho-Xuan, H.; Iketani, S.; Gut, H.; Kutay, U.; Helenius, A.; Yamauchi, Y. Influenza virus uses transportin 1 for vRNP debundling during cell entry. *Nat. Microbiol.*

2019, *4*, 578–586, doi:10.1038/s41564-018-0332-2.

87. Chou, Y.Y.; Vafabakhsh, R.; Doğanay, S.; Gao, Q.; Ha, T.; Palese, P. One influenza virus particle packages eight unique viral RNAs as shown by FISH analysis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 9101–9106, doi:10.1073/pnas.1206069109.

88. Lakdawala, S.S.; Wu, Y.; Wawrzusin, P.; Kabat, J.; Broadbent, A.J.; Lamirande, E.W.; Fodor, E.; Altan-Bonnet, N.; Shroff, H.; Subbarao, K. Influenza A Virus Assembly Intermediates Fuse in the Cytoplasm. *PLoS Pathog.* **2014**, *10*, e1003971, doi:10.1371/journal.ppat.1003971.

89. Cabantous, S.; Terwilliger, T.C.; Waldo, G.S. Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nat. Biotechnol.* **2005**, *23*, 102–107, doi:10.1038/nbt1044.

90. Avilov, S.; Magnus, J.; Cusack, S.; Naffakh, N. Time-Resolved Visualisation of Nearly-Native Influenza A Virus Progeny Ribonucleoproteins and Their Individual Components in Live Infected Cells. *PLoS One* **2016**, *11*, e0149986, doi:10.1371/journal.pone.0149986.

91. Chen, B.J.; Leser, G.P.; Morita, E.; Lamb, R.A. Influenza Virus Hemagglutinin and Neuraminidase, but Not the Matrix Protein, Are Required for Assembly and Budding of Plasmid-Derived Virus-Like Particles. *J. Virol.* **2007**, *81*, 7111–7123, doi:10.1128/jvi.00361-07.

92. Takeda, M.; Leser, G.P.; Russell, C.J.; Lamb, R.A. Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 14610–14617, doi:10.1073/pnas.2235620100.

93. Rossman, J.S.; Jing, X.; Leser, G.P.; Balannik, V.; Pinto, L.H.; Lamb, R.A. Influenza Virus M2 Ion Channel Protein Is Necessary for Filamentous Virion Formation. *J. Virol.* **2010**, *84*, 5078–5088, doi:10.1128/jvi.00119-10.

94. Rossman, J.S.; Jing, X.; Leser, G.P.; Lamb, R.A. Influenza Virus M2 Protein Mediates ESCRT-Independent Membrane Scission. *Cell* **2010**, *142*, 902–913, doi:10.1016/j.cell.2010.08.029.

95. Gudheti, M. V.; Curthoys, N.M.; Gould, T.J.; Kim, D.; Gunewardene, M.S.; Gabor, K.A.; Gosse, J.A.; Kim, C.H.; Zimmerberg, J.; Hess, S.T. Actin mediates the nanoscale membrane organization of the clustered membrane protein influenza hemagglutinin. *Biophys. J.* **2013**, *104*, 2182–2192, doi:10.1016/j.bpj.2013.03.054.

96. Curthoys, N.M.; Mlodzianoski, M.J.; Parent, M.; Butler, M.B.; Raut, P.; Wallace, J.; Lilieholm, J.; Mehmood, K.; Maginnis, M.S.; Waters, H.; et al. Influenza Hemagglutinin Modulates Phosphatidylinositol 4,5-Bisphosphate Membrane Clustering. *Biophys. J.* **2019**, *116*, 893–909, doi:10.1016/j.bpj.2019.01.017.

97. Kolpe, A.; Arista-Romero, M.; Schepens, B.; Pujals, S.; Saelens, X.; Albertazzi, L. Super-resolution microscopy reveals significant impact of M2e-specific monoclonal antibodies on influenza A virus filament formation at the host cell surface. *Sci. Rep.* **2019**, *9*, 4450, doi:10.1038/s41598-019-41023-5.

98. Pileri, P.; Uematsu, Y.; Campagnoli, S.; Galli, G.; Falugi, F.; Petracca, R.; Weiner, A.J.; Houghton, M.; Rosa, D.; Grandi, G.; et al. Binding of hepatitis C virus to CD81. *Science (80-.).* **1998**, *282*, 938–941, doi:10.1126/science.282.5390.938.

99. Muranyi, W.; Malkusch, S.; Müller, B.; Heilemann, M.; Kräusslich, H.-G. Super-Resolution Microscopy Reveals Specific Recruitment of HIV-1 Envelope Proteins to Viral Assembly Sites Dependent on the Envelope C-Terminal Tail. *PLoS Pathog.* **2013**, *9*, e1003198, doi:10.1371/journal.ppat.1003198.

100. Pezeshkian, N.; Groves, N.S.; van Engelenburg, S.B. Single-molecule imaging of HIV-1 envelope glycoprotein dynamics and Gag lattice association exposes determinants responsible for virus incorporation. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 25269–25277, doi:10.1073/pnas.1910008116.

101. Feeley, E.M.; Sims, J.S.; John, S.P.; Chin, C.R.; Pertel, T.; Chen, L.-M.; Gaiha, G.D.; Ryan, B.J.; Donis, R.O.; Elledge, S.J.; et al. IFITM3 Inhibits Influenza A Virus Infection by Preventing Cytosolic Entry. *PLoS Pathog.* **2011**, *7*, e1002337, doi:10.1371/journal.ppat.1002337.

102. Spence, J.S.; He, R.; Hoffmann, H.H.; Das, T.; Thinon, E.; Rice, C.M.; Peng, T.; Chandran, K.; Hang, H.C. IFITM3 directly engages and shuttles incoming virus particles to lysosomes. *Nat. Chem. Biol.* **2019**, *15*, 259–268, doi:10.1038/s41589-018-0213-2.

103. Kummer, S.; Avinoam, O.; Kräusslich, H.G. IFITM3 clusters on virus containing endosomes and lysosomes early in the influenza a infection of human airway epithelial cells. *Viruses* **2019**, *11*, 548, doi:10.3390/v11060548.

104. Pereira, P.M.; Gustafsson, N.; Marsh, M.; Mhlanga, M.M.; Henriques, R. Super-beacons: Open-source probes with spontaneous tuneable blinking compatible with live-cell super-resolution microscopy. *Traffic* **2020**, *21*, 375–385, doi:10.1111/tra.12728.

105. Liedmann, S.; Hrincius, E.R.; Guy, C.; Anhlan, D.; Dierkes, R.; Carter, R.; Wu, G.; Staeheli, P.; Green, D.R.; Wolff, T.; et al. Viral suppressors of the RIG-I-mediated interferon response are pre-packaged in influenza virions. *Nat. Commun.* **2014**, *5*, 5645, doi:10.1038/ncomms6645.

106. Banerjee, I.; Yamauchi, Y.; Helenius, A.; Horvath, P. High-Content Analysis of Sequential Events during the Early Phase of Influenza A Virus Infection. *PLoS One* **2013**, *8*, e68450, doi:10.1371/journal.pone.0068450.

107. Von Chamier, L.; Laine, R.F.; Jukkala, J.; Spahn, C.; Krentzel, D.; Nehme, E.; Lerche, M.; Hernández-Pérez, S.; Mattila, P.K.; Karinou, E.; et al. ZeroCostDL4Mic: an open platform to use Deep-Learning in Microscopy. *bioRxiv* **2020**, 2020.03.20.000133, doi:10.1101/2020.03.20.000133.

108. Gray, R.D.M.; Beerli, C.; Pereira, P.M.; Scherer, K.M.; Samolej, J.; Bleck, C.K.E.; Mercer, J.; Henriques, R. VirusMapper: Open-source nanoscale mapping of viral architecture through super-resolution microscopy. *Sci. Rep.* **2016**, *6*, 1–8, doi:10.1038/srep29132.