

Super-Resolution Microscopy

Subjects: Virology

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1. Introduction

In humans, influenza viruses cause acute infections in the respiratory tract. Every year, three to five million people develop severe seasonal influenza with up to 650,000 deaths, globally ^[1]. Although vaccines and some antivirals (e.g. Tamiflu) against seasonal influenza are available, the rapid antigenic drift and shift have ^[2], 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 virions 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 thus mainly 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 of cell-to-cell viral transmission, with a view to 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 pleomorphic, 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 pleomorphism 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. This highlights 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, while 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 of virions [17] and the role of ESCRT complexes in HIV egress [18].

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][18][19][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 virus replication. **(a)** Structure of influenza virions. Spherical particles diameter ~120 nm; filamentous particles length up to 20 μm [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: 500 nm. 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 three-dimensional (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. 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 (~250 nm). 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 the excitation scanning point. This effectively reduces the volume of the fluorescence measurement and typically improves resolution down to 50–70 nm. 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]). 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 150 nm. In Figure 2b, 3D SIM imaging is used to show that ubiquitin is packaged inside purified influenza virions [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 virions [21].

A third approach to SRM is to exploit the capability of certain fluorescent dyes to blink (photoswitch) 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 possible to precisely localize them in space despite each molecule producing a diffraction-limited spot on the camera, and to then build a nanoscale map of fluorophore distribution. SMLM techniques routinely achieve a localization precision of 10–20 nm (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) [37].

An exciting variant of these latter techniques 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 [38].

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: 5 μ m. 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 *d*STORM reconstructions at \sim 30 nm resolution of influenza viruses labelled for NA (red) and HA (blue). Bottom: *d*STORM reconstructions of a filamentous virion labelled for HA and NA at \sim 30 nm resolution with corresponding intensity profile. HA and NA are shown to exclude one another. Scale bar: 200 nm. Adapted from [39]. **(d)** ExM. BS-C-1 cell labelled for tyrosinated tubulin (green) and de-tyrosinated tubulin (magenta). (i): Comparison of ExM image (bottom) with corresponding pre-expansion image (top). Scale bars: 2 μ m. Insert, pre-(ii) and post-expansion (iii) with corresponding intensity profiles (iv). Scale bars: 500 nm. Adapted from [40].

During influenza virus assembly, it was shown using a biochemistry approach that HA preferentially accumulates in so-called lipid rafts [41][42], mediated through its transmembrane domain [43]. Some of the earliest fPALM experiments, which looked at this association at the plasma membrane of HA-expressing fibroblasts [44], could visualize that HA forms irregular, lipid raft-associated clusters with a similar size range to that of budding virions. The high-resolution of live-cell fPALM enabled different models of membrane organisation to be discerned and revealed the molecular dynamics within the clusters. Further, an early form of SMLM called 'Blink' [45] showed that HA at the plasma membrane of infected cells forms dynamic nanodomains of around 80 nm [46]. The small size of these microdomains would be challenging to observe with conventional fluorescence imaging techniques.

Figure 2c shows a filamentous influenza virus imaged with *d*STORM. The nanoscale resolution achieved 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) [47]. 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 \sim 4 \times [47] and up to \sim 20 \times with iterative ExM (iExM) [48]. The approach can even preserve and resolve the integrity of bio-macromolecular assemblies, as successfully demonstrated by visualizing the molecular organisation of the centrosome [49]. In Figure 2d, we show an example of ExM used 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 [50][51][52], including for bacteriophage T5 [53], 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, provides a novel way to enhance resolution in tissue in 3D.

Beyond SRM, quantitative long-term live-cell imaging has great promise for the study of host-pathogen interactions at 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 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 [54], 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 FIAsh [55], Sfp [56], transglutaminase 2 [57], sortase A [58] with bright fluorescent markers, offer new opportunities for improved live-cell imaging. These new technologies enable the behaviours of viral and cellular components 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 [59][60][61]. In the context of SPT, a powerful approach has been to use quantum dots (QDs) [62][63][64], for long-term imaging, but with limitations due to the relative complexity of labelling steps.

Single-molecule Förster Resonance Energy Transfer (smFRET) [65] constitutes a powerful optical method to observe nanoscale changes in conformations within biomolecules, especially when using 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 [66]. The direct spatial localization of individual RNA transcripts can be performed by single-molecule fluorescence using in situ hybridization (FISH) [67]. FISH is also well-suited to studies of the assembly of influenza virions and has been used to observe vRNPs in the cytoplasm en route to budding sites [68][69].

We note that these imaging approaches can also benefit from quantitative approaches such as single-particle averaging [24][25][31], machine learning [70][71] and modelling [24][25][72], in order to tease out the most informative features from the imaging data.

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