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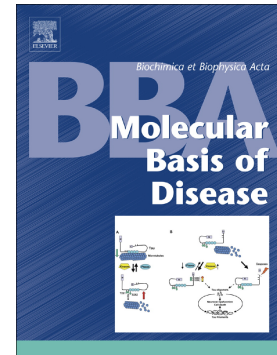
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Virus morphology: insights from super-resolution fluorescence microscopy

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Abstract

As epitomised by the COVID-19 pandemic, diseases caused by viruses are one of the greatest health and economic burdens to human society. Viruses are 'nanostructures', and their small size (typically less than 200 nm in diameter) can make it challenging to obtain images of their morphology and structure. Recent advances in fluorescence microscopy have given rise to super-resolution techniques, which have enabled the structure of viruses to be visualised directly at a resolution in the order of 20 nm. This mini-review discusses how recent state-of-the-art super-resolution imaging technologies are providing new nanoscale insights into virus structure.

Keywords:

Virus, Influenza, HIV, SARS-CoV-2, Super-resolution, Fluorescence Microscopy, STORM, SIM, STED

1. Introduction

Viral diseases result in widespread human and animal morbidity and mortality every year, with significant health, economic and social impact. Viruses are ultramicroscopic (typically 20-300 nm in diameter) infectious agents that only replicate within the cells of living hosts. They are composed of an RNA or DNA core, a protein coat, and, in more complex types, a surrounding lipid envelope derived from the plasma membrane of the host cell. The direct visualization of virus particle structure, and the identification and quantification of virus proteins within virions, is critical to furthering our understanding of the virus lifecycle, to enhance our knowledge about viral replication strategies, and to provide opportunities for detection, diagnosis and the development of antiviral strategies.

Since the first images of virus particles obtained in the 1930s [1], electron microscopy (EM) has been the primary microscopy technique of choice to study and characterize viruses. Although EM can resolve viral structures at near-atomic level, it has several drawbacks; it can lead to sample preparation artifacts, is usually limited to fixed samples, offers no dynamic information, and little, or no, molecular identification of specific proteins. Two alternative single-particle microscopy methods that can be employed to study viral properties at the nanoscale are atomic force microscopy [2], which can be used to topographically scan the virus particle surface with a mechanical probe, or optical tweezers [3], a single-molecule technique that allows the measurement of forces between two samples or between the sample and the environment. Both of these methods have been used successfully to characterise viral systems [reviewed in 4], however possess several limitations such as difficulty in identifying specific structures, slow scanning time, or problems in trapping nanostructures as large as viruses, which restricts their wide spread use.

The application of fluorescence optical microscopy to the study of virus structure and morphology offers a number of powerful advantages, including high specificity through the use of specialized fluorescent probes, multi-colour characterization, 3D imaging, and live-cell compatibility; however, has been constrained by physical limitations in optics. In 1873, Ernst Abbé postulated the diffraction limit for optical microscopes [5], which restricts the resolution of traditional optical microscopy to approximately 250 nm in the lateral and 700 nm in the axial direction. The consequence of this is that in a typical biological sample where many thousands of crowded proteins are labelled; the signals from each of these point sources spread out and merge, obscuring the molecular details of the sample. This phenomenon becomes particularly problematic when studying nanoscale macromolecular complexes, such as viruses [6]. Over the last two decades, light microscopy techniques have experienced phenomenal improvements in sensitivity, functionality and resolution [reviewed in 7], in particular, the implementation of super-resolution microscopy (SRM). SRM techniques use a range of physical or chemical methods to break the diffraction limit of optical microscopy, opening up a multitude of new possibilities for direct imaging of virus particles.

The ground-breaking field of viral optical imaging, which includes studies of virus-host interactions, viral transmission, virus replication, assembly and budding, and the characterization of viral vaccines and anti-viral strategies, is continuously being updated with excellent reviews (for the latest contributions see [4, 7-12]). This mini-review will focus on the application of SRM techniques to the elucidation of virus morphology and architecture. In light of the continuous threat of new viruses, these studies constitute a crucial part of the expansion of our knowledge of these dangerous pathogens and offer exciting new ways of studying and ultimately preventing viral disease.

2. Super-resolution techniques used in virus research

SRM is a family of microscopic techniques, based on different principles, optics and instrumentation, that embrace the same goal: to overcome the resolution constraints imposed by the diffraction limit of light. Stimulated emission depletion (STED) is a laser confocal scanning microscopy technique that achieves a (dye-dependent) maximum resolution of 20-80 nm; reducing the size of the point spread function (PSF) by illuminating the sample with two aligned laser beams: a conventional excitation beam (to excite the fluorophores) and a depletive doughnut-shaped laser to quench the fluorescence signal around the excitation scanning point [13]. STED can be costly to implement and utilizes relatively high laser intensities, but allows fast data acquisition and doesn't require additional data processing to generate a super-resolution image.

A second technique known as structured illumination microscopy (SIM) [14] allows super-resolution by using structured patterns of illumination of light across the sample. When the sample contains features smaller than the diffraction limit, the patterned illumination applied creates an interference pattern known as Moiré fringes. By changing the position and orientation of the structured shape, several Moiré fringes are created that can be mathematically deconvolved, producing an image with a resolution of 100-150 nm. SIM uses a relatively low illumination power compared with other methods of SRM and is very versatile, working well with conventional microscopy fluorophores and fluorescent proteins; however, this technique offers a relatively modest increase in resolution, particularly when it comes to virus studies.

Single molecule localization microscopy (SMLM) is a set of techniques that exploit the ability of fluorescent dyes to stochastically blink (photoswitch) using specific physical or chemical experimental conditions. The number of fluorophores that are in the 'on' state is carefully controlled, such that only a sparse subset is imaged at any one time, hence images from individual fluorophores can be discriminated spatially on wide-field detectors (Figure 1). SMLM achieves super-resolution via the precise localization of the signals emitted from individual molecules by fitting a Gaussian profile, which allows the reconstruction of an image with a localization precision of 10-20 nm. Notable examples of SMLM include photoactivated localization microscopy (PALM) [15] and stochastic optical reconstruction microscopy (STORM) [16], which are based on photo-activable proteins and photo-switchable probes respectively, and points accumulation for imaging in nanoscale Topography (PAINT) [17], which employs labelled probes that transiently bind to targeted molecules. SMLM methods offer the highest spatial resolution to date and are typically based on relatively low-cost total internal reflection fluorescence (TIRF) microscopy systems; however, can require lengthy acquisition and extensive post-acquisition image processing to reconstruct image.

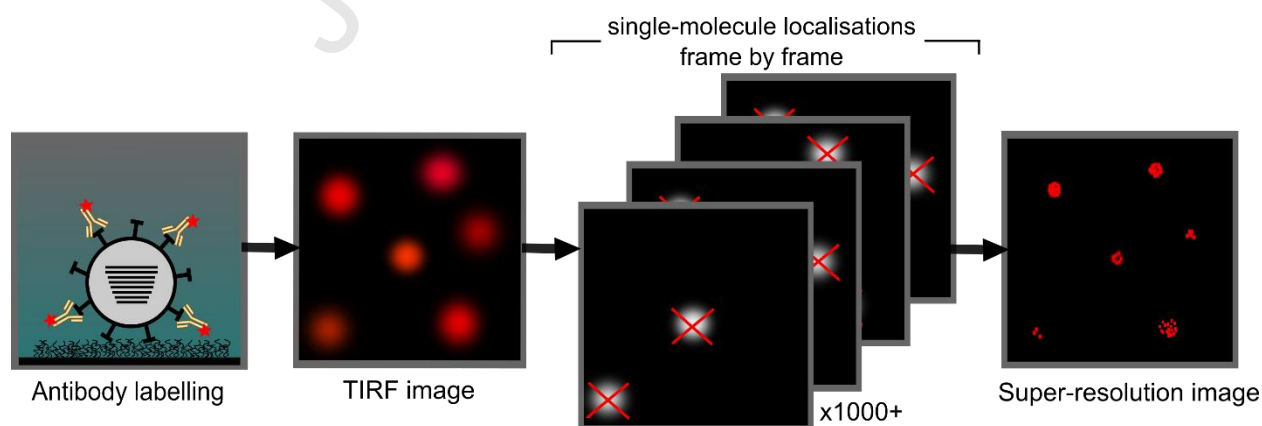


Figure 1. Schematic of STORM imaging of an antibody-labelled virus. Virus particles are immobilised on a glass slide and stained with fluorescent antibodies against a specific protein. Total internal reflection fluorescence

(TIRF) imaging provides a diffraction-limited image of the virus particles. Enhanced resolution is achieved by recording the 'blinking' of the fluorophores and obtaining their precise localisation in each frame of the acquisition. The individual localisations from all frames are used to reconstruct a "super-resolution" image.

A newcomer in the SRM field is expansion microscopy (ExM) [18]. This method enables high resolution imaging of preserved cells and tissues on conventional diffraction-limited microscopes via isotropic physical expansion of the specimens in a hydrogel. The potential of ExM for imaging of viruses has only just started to be explored.

Many variations on these original SRM approaches have been developed, however all SRM methods, and their derivatives, rely on the efficient and specific labelling of virions with bright and photostable probes that are small enough not to perturb functionality. A number of different strategies to fluorescently label virus particles for imaging exist ([7, 8 and references within], including intercalating and lipophilic dyes, fluorescent nanoparticles such as quantum dots (QDs), metal nanoparticles, fluorescent proteins and covalent labeling strategies. Antibodies, genetically-encoded fluorescent proteins, or self-labelling tags like the Halo tag [19] are commonly used to ensure molecular specificity but can introduce errors in the localization precision of the tagged molecules due to their large size. Smaller tags such as nanobodies or click chemistry with organic fluorophores may help to resolve this issue. More recent alternatives include single-molecule fluorescence in situ hybridization (smFISH) which uses fluorescently labelled DNA or RNA probes to hybridise the viral genome [E.g., 20], molecularly imprinted polymers (MIPs) [21] or a near-instantaneous cation-mediated labelling method that binds small, non-specific fluorescent DNAs to the surface of enveloped viruses [22, 23].

3. The application of SRM to HIV-1 virion architecture

To date, one of the principal applications of SRM imaging to virus research has been on the Human Immunodeficiency Virus type 1 (HIV-1); helped by the detailed understanding of the virus replication cycle and wealth of reagents made available through 30 years of intense research into this important human pathogen, as well as the ability to engineer a subset of HIV-1 proteins with fluorescent transgenes. SRM has contributed to furthering our understanding of all stage of the HIV life cycle, including viral assembly, release, architecture and maturation, cell-to-cell transport and cell entry [11, 12, 24, 25].

During HIV-1 assembly, newly translated viral proteins are trafficked to the host cell membrane and start to assemble into immature virions, within each a dimerised copy of the progeny viral genome is packaged. Approximately 5,000 Gag polyproteins assemble to form a single immature capsid shell [26]; Gag is also responsible for the recruitment of other virus and host cell components which are needed for virus release to the budding site. During particle release, the Gag protein lattice is cleaved into several smaller proteins (MA (matrix), CA (capsid), NC (nucleocapsid) and p6), which reorganizes the virus architecture into a mature and fully infectious form, characterised by a conical capsid [12]. Virions that bud out of the cell are roughly spherical, ~ 100 nm in diameter, and are decorated on the surface by a small number of envelope (Env) glycoproteins, a trimer formed by heterodimers of gp120 and gp41.

SRM has provided novel insights into the architecture of both mature and immature virus particles. Labelling of the viral integrase protein with fluorescein arsenical hairpin binder-ethanedithiol (FIAsH) and PALM imaging allowed longer mature conical cores to be distinguished from immature Gag shells [27], while labelled Gag and STED microscopy allowed the first time-resolved observations of the disassembly of the Gag lattice during maturation [28]. Furthermore, high throughput STED imaging of large numbers of individual particles revealed that while immature viruses generally displayed multiple Env clusters, the majority of mature virions carried a single cluster, indicating a rearrangement of

surface molecules upon core maturation [29], and suggesting a new model where proteolytic maturation of the inner structure of the virus is linked with the cell entry capacity of the virus. This model was supported by measurements of Env mobility on the surface of individual virus particles via the combination of STED with fluorescence correlation spectroscopy (FCS), which confirmed that Env mobility is dependent on virus maturation status [30]. Finally, 2D and 3D STORM on single HIV-1 particles confirmed that Env glycoproteins formed clusters in the membrane of mature virions, and that the host antiviral factor Serine Incorporator Protein 5 (SERINC5) disrupts Env clusters, likely interrupting viral fusion [31].

STORM imaging has previously been used to compare the size of the HIV-1 matrix shell and capsid core in free virions with particles that had just entered a host cell [32], as well as to obtain high-resolution information on HIV-1 virion size by labelling either the integrase or the accessory Vpr protein using genetically-encoded tags, or using antibodies directed against the CA, MA or gp120 proteins [33]. These studies demonstrate the ability to efficiently label and image a wide variety of HIV-1 proteins. The potential of SRM techniques to explore the initiation and dynamics of the inner structural rearrangements of the particle during maturation is an exciting topic requiring further research, that may benefit from the improved spatial resolution that SRM offers.

4. New insights into influenza virion morphology

Influenza virions are surrounded by a lipid bilayer, in which two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA), are embedded. Beneath the lipid bilayer lies the structural matrix protein (M1), which forms a core within which lies the viral RNA segments, each of which is bound by multiple nucleoproteins (NP) and the RNA polymerase to make ribonucleoprotein (RNP) complexes. Beyond these similarities, influenza virus particles are highly pleomorphic in size and shape, ranging from spherical virions of approximately 100 nm in diameter to filaments reaching many microns in length (Figure 2A-D). Filamentous strains of influenza have frequently been overlooked in virus research; because laboratory-passaged viruses tend to be spherical (with a filamentous morphology more typical of clinical isolates) and also because ultracentrifugation and other purification and storage procedures tend to damage filaments [34]. Filamentous virus structure is of interest as it has been linked to increased pathogenicity, cell to cell transport, resistance to neutralizing antibodies and penetration through host mucus barriers, as well as offering insights into virus assembly and mechanisms of infection [reviewed in 35].

Attempts to incorporate genetically encoded fluorescent proteins into the relatively small influenza genome have had limited success, however a fascinating study published in 2018 overcame this obstacle by developing a virus strain that harbored small (~10 amino acid) tags couples to small-molecule fluorophores on each of the virus's most abundant structural proteins (HA, NA, M1, M2, and NP) that permitted high-efficiency labeling of individual viral proteins [36]. This approach revealed that influenza produces virions with broad variations in size and protein composition; and suggested that such phenotypic variability may contribute to virus survival under stress conditions such as in the presence of antiviral NA inhibitor drugs. STORM imaging of the tagged fluorescent viruses showed an asymmetric distribution of HA and NA, which was implicated in directional diffusion of filaments, perhaps suggesting a mechanism for viruses to penetrate mucosal barriers [37].

In recent work, we have combined STORM imaging using antibody and FISH labelling of virion components with rapid automated analysis software to carry out a high throughput and high-resolution analysis of thousands of influenza virions at a time [35]. We found that length analysis, but not axial

ratio, provides a useful way of characterizing virions: filaments longer than ~ 230 nm formed a broad size distribution, while smaller particles formed two distinct populations, likely corresponding to spherical and ellipsoidal virions. The broad phenotypic variability in filament size, also supported by previous observations [36], suggests a model of virus particle assembly where membrane scission and the release of progeny filaments from cells may occur in a stochastic way.

Multiple studies have been carried out to understand the spatial organization of viral proteins within influenza filaments. The distributions of the surface proteins of influenza are not entirely random; with several EM studies showing that small clusters of NA are formed within the more abundant HA [reviewed in 35, and Figure 2E]. Fourier transform analysis of STORM images demonstrated that no generalized common spatial frequency patterning of HA or NA on the virion surface occurs [35], although individual filaments may have patterning at a certain spatial frequency. A separate study used STORM microscopy to localize the viral M2 protein to the base of budding filaments in virus-infected cells (closest end to the cell membrane) [38], consistent with the proposed role of M2 in membrane scission. Finally, some filamentous virions have been observed to have large bulges at one end, known as Archetti bodies [39]; previous electron microscopy images have indicated that these bulges do not appear to contain RNPs [40] and their exact purpose in viral replication remains unknown. Combined 3D STORM and scanning electron microscopy revealed RNPs located at the proximal ends of budding filaments (furthest from the cell membrane) [41], and a combination of STORM imaging and smFISH was used to show that in long filaments, viral RNP complexes are located preferentially towards the filament pole within Archetti bodies, compared to those without it [35]. Further work on these structures may lend support to the hypothesis that Archetti bodies may play a role in virus transmission.

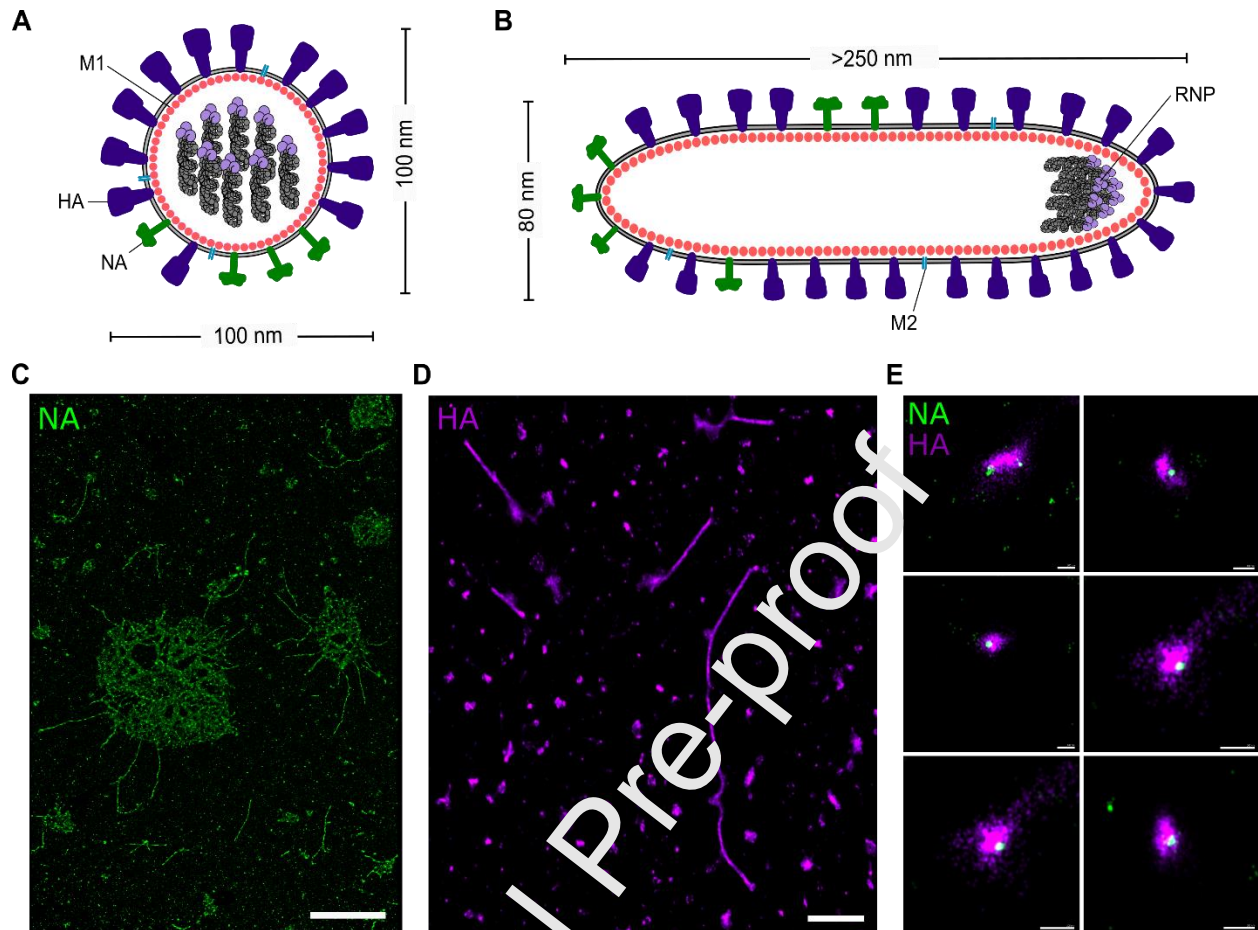


Figure 2. Influenza virus structure. A&B) Schematic diagrams of a spherical and filamentous influenza virus. HA – Hemagglutinin, NA – Neuraminidase, M1 – Matrix protein 1, M2 – Matrix protein 2 (ion channel), RNP – Ribonucleoprotein. C) STORM image of MDCK cells infected with A/Udorn/72 virus and stained for NA, showing long viral filaments emerging from the cells. Scale bar 10 μm . D) STORM image of purified A/Udorn/72 filaments and spheres, stained for HA. Scale bar 2 μm . E) STORM images of individual A/Udorn/72 spherical virions, stained for NA in green and HA in red. Scale bars 100 nm. STORM images credit: Andrew McMahon, University of Oxford.

SRM techniques can also be combined with artificial intelligence to quantify virus populations. Previously, TIRF-SIM was used in combination with machine learning to develop a high-throughput methodology capable of classifying influenza viruses based on their shape [42]. In more recent work, we have used a convolutional neural network to distinguish between microscopy images of single intact particles of different viruses; achieving labeling, imaging and virus identification in less than five minutes [22]. The trained neural network was able to differentiate influenza from negative clinical samples, as well as from other respiratory pathogens such as SARS-CoV-2 and seasonal human coronaviruses, with high accuracy. Single-particle imaging combined with deep learning therefore offers a promising alternative to traditional viral diagnostic methods, and has the potential for significant impact.

5. Other virus examples

Whilst not extensively studied using SRM, the application of these techniques to elucidating the architecture of a number of other viruses is worth noting. In Herpes Simplex Virus 1 (HSV-1) the processes of cell attachment and membrane fusion involve many different envelope glycoproteins (gB,

gC, gD, gH, and gL) [8]. STED microscopy was used to show the differing patterns of localization of gD between cell-free and cell-bound viruses, indicating that gD can be reorganized on the viral envelope following either maturation of the viral particle or its adsorption to the cell, possibly leading to membrane fusion [43]. STORM, in combination with a model-based analysis of single-molecule localization data, has also been used to determine the relative positions of protein layers within individual HSV-1 particles and discriminate envelope-anchored glycoproteins from tegument proteins [44]. Finally, a new hydrogel expansion microscopy method was shown to better preserve the spherical shape of HSV-1 virions than traditional 'tetra-gel' methods, resulting in a smaller error in localizing the viral envelope layer than earlier versions of ExM [45].

Vaccinia virus (VACV) is a large, enveloped virus belonging to the poxvirus family. The first report of the successful resolution of individual poxvirus particle morphology using light microscopy used 3D-SIM to analyze fluorescent recombinant VACV viruses [46]. This was followed by the introduction of VirusMapper, an ImageJ-based software package that allows for automatic statistical mapping of conserved multi-molecular structures, such as viral substructures or intact viruses [47]. VirusMapper was applied to SIM and STED images of vaccinia virus in isolation and when engaged with host cells, allowing the generation of virion models and detection of nanoscale changes in viral architecture.

Hendra virus (HeV) is a bat-borne Paramyxovirus virus that is associated with a highly fatal infection in horses and humans. 3D-SIM was used to achieve sub-virion imaging of paramyxovirus proteins and ascertain the respective locations of HeV glycoprotein (G), matrix (M) and nucleoprotein (N) within virions [48]. HeV particles were clearly resolved as spheres of approximately 300 nm, with the surface G protein forming a round circle at the particle exterior while the N protein was detected within the spherical structures, consistent with its role of coating the viral genome. Imaging of virions co-labelled for the presence of M, N and G proteins provided a first indication that the M protein appeared to be associated with the RNP rather than the virion envelope.

No review on viral architecture would be complete without consideration of SARS-CoV-2, the causative agent of Covid-19; SRM methods offer an important tool in our ability to study the biology of this novel human coronavirus [10]. SARS-CoV-2 forms spherical virions ranging from 50-200 nm in diameter that contain four structural proteins, namely the spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Figure 3A&B). S is a ~180 kDa glycoprotein that protrudes as a homotrimer from the viral surface (Figure 3C) and mediates viral binding to the cell membrane receptor ACE2. Upon SARS-CoV-2 infection, the virus triggers the biogenesis of replication organelles (ROs) such as double-membrane vesicles (DMVs) in the infected cell, which protect the viral RNA from degradation during replication. The assembly of viral virions occurs in the endoplasmic reticulum (ER) to Golgi intermediate compartment (ERGIC), followed by traffic to lysosomes and egress by Arl8b-dependent lysosomal exocytosis into the extracellular environment [49]. STORM imaging has been used to investigate the size of single virions during their entry and egress by focusing on the basal membrane plane of infected cells, and demonstrated the role of clathrin vesicles, rather than caveolin-1, as major carriers of the virus from the cell surface to the early endosome [50]. A combination of ExM and light sheet microscopy was used to investigate the expression kinetics and spatial arrangement of the four structural SARS-CoV-2 proteins, demonstrating an association of the N protein with ROs and allowing the visualisation of virus-induced morphological changes of host cell structures [51].

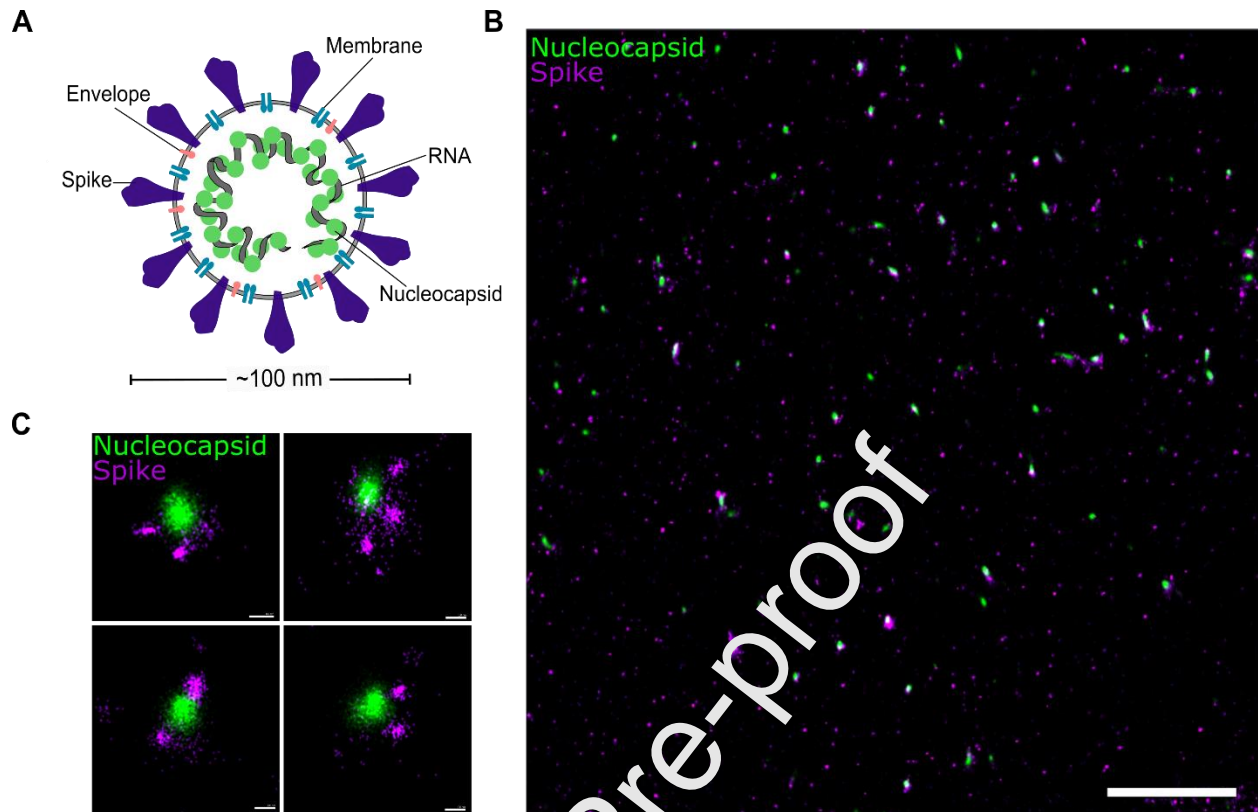


Figure 3. SARS-CoV-2 virus structure. A) Schematic diagram of the SARS-CoV-2 virion. B) STORM image of fixed SARS-CoV-2 particles stained for Nucleocapsid in green and Spike in red. Scale bar 5 μm . C) STORM images of individual virions, stained for Nucleocapsid in green and Spike in red. Scale bars 100 nm. STORM images credit: Andrew McV. *et al.*, University of Oxford.

6. Future directions

A broad range of available SRM techniques have complemented the well-established field of electron microscopy in enabling the architecture of viruses to be visualised with nanometer resolution, offering us valuable insights into their structure, morphology, and biology. SRM methods are under continuous development, constantly breaking new resolution and throughput limits. One such exciting development has been the merger of the STED and SMLM (e.g. STORM) concepts to generate MINimal photon FLUXes (MINIFLUX) microscopy, a technology that has lowered the current resolution barrier from ~ 20 nm to the 1–5 nm size scale of the molecules themselves [52]. All SRM techniques offer unique advantages and disadvantages, and a number of challenges still remain in their application to viral research; the need to image in high containment laboratories, the large and often expensive hardware required for many methods, and difficulties in imaging non-fixed material (thereby limiting studies into dynamic processes) amongst others. The constant development of novel techniques, as well as new developments in the existing SRM methodologies presented here, will no doubt help to reduce these challenges and reveal further exciting details of virus particle architecture.

The urgent rush to understand the immediate threat posed by the SARS-CoV-2 virus makes this the obvious target of much future work, in particular, questions addressing the interactions of SARS-CoV-2 proteins with host cell proteins, SARS-CoV-2 replication dynamics within cells, and the exact sites of SARS-CoV-2 replication within infected cells. The constant threat to humanity from existing and newly emerging viruses has demonstrated the importance of studying how these pathogens are structured,

how they assemble and how they interact with the host cell, knowledge that in turn can guide our strategies for antiviral inhibitors and vaccines.

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Highlights:

- The ultra-small size of viruses makes it challenging to visualize them using traditional fluorescence microscopy.
- Super-resolution microscopy represents a diverse range of techniques aimed at breaking the diffraction limit of optical microscopy, opening up multiple new possibilities for imaging of virus particles.
- Super-resolution microscopy has been crucial for our detailed understanding of the Human Immunodeficiency Virus type 1 (HIV-1) particle architecture and maturation process.
- It has helped to elucidate the importance of influenza virus pleomorphism, giving insights into why virion size and shape may lead to increased pathogenicity.
- Super-resolution microscopy is a field under continuous development; with new advances offering crucial tools in our fight against dangerous new viruses.

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