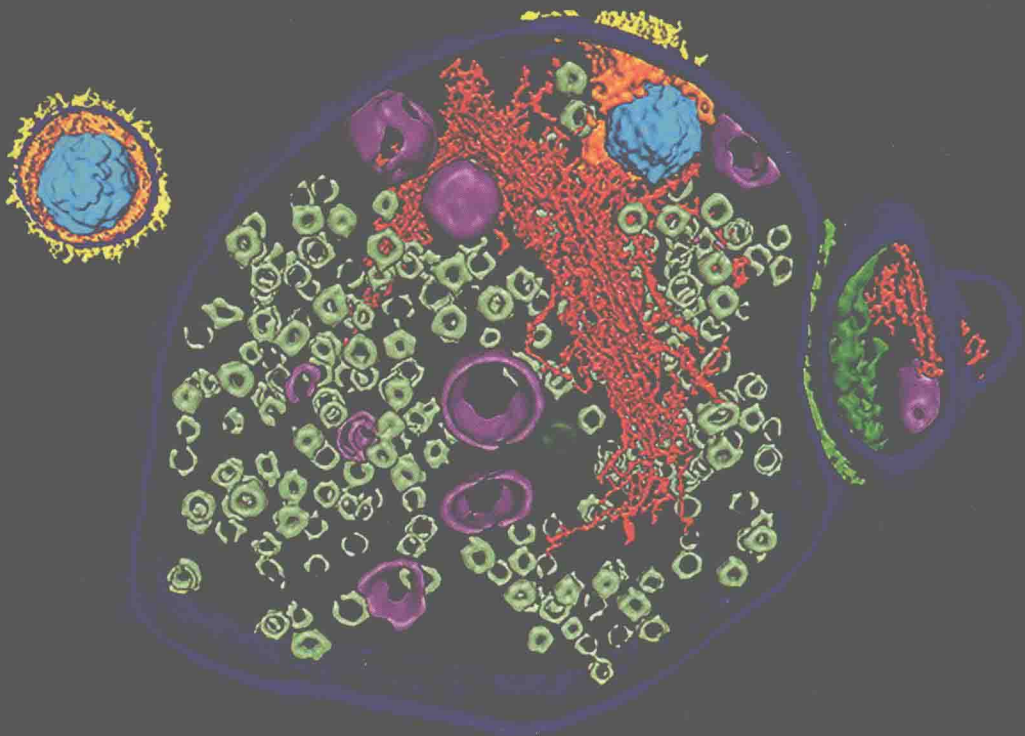
# Emerging Topics in Physical Virology



Peter G Stockley • Reidun Twarock

*editors*

Imperial College Press

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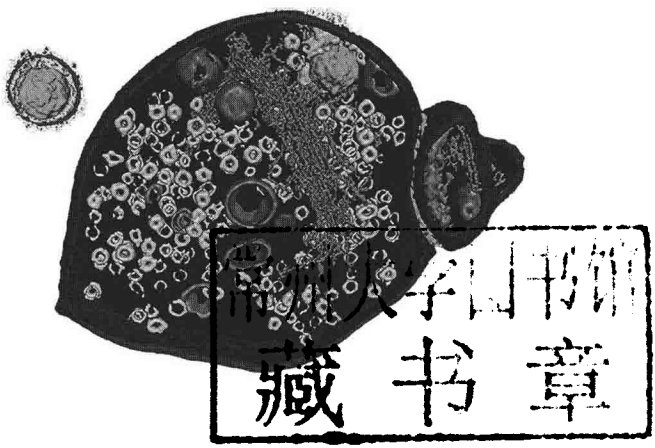



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# Preface

*Emerging Topics in Physical Virology* is a state-of-the-art account of recent developments in the analysis and modelling of virus structure, function and dynamics. It pays tribute to the importance of interdisciplinary research by integrating an exposition of experimental techniques such as cryo-electron microscopy, atomic force microscopy and mass spectrometry with mathematical and biophysical modelling techniques. The number of chapters co-authored by experimentalists and theoreticians is testimony to the importance of interdisciplinarity in tackling some of the most challenging and exciting research problems in this area.

The aim of this book is to introduce the reader to recent developments in the field, and to provide a comprehensive review of the results that prompted them. It is therefore not only a primer for researchers working in the analysis and modelling of viruses, but also serves as an introduction for non-experts into this tantalising field of research. The book starts with a description of cryo-electron microscopy by Neil Ranson and Peter Stockley and demonstrates its power in determining the structure and dynamics of viruses. Structural insights gained from X-ray crystallography have revealed an intriguing phenomenon: there is a striking conservation in the topologies of the capsid proteins that form the containers encapsulating viral genomes. This has prompted Dennis Bamford, David Stuart and collaborators to classify viral families into lineages based on the concept of the viral 'self'. An important feature of this conservation of capsid protein folds is that it appears to be non-sequence-specific, i.e. the chemical structures of proteins with homologous folds can often be very different. This suggests that there must be a guiding principle for the formation of the capsid proteins that is independent of their sequences. Thomas Keef and Reidun Twarock suggest that the icosahedral symmetry of many viruses may provide such a guiding principle, and they introduce novel group theoretical techniques to model this effect. Their approach implies that a wide spectrum of viral features can be predicted based on symmetry, and that perhaps the limited number of structural folds is a

consequence of the fact that only a limited number of layouts are possible for viruses with such symmetry.

Atomic force microscopy provides important insights into the mechanical properties of viruses as detailed in the chapter by Wouter Roos and Gijs Wuite. The authors discuss capsid shell structure, presence of encapsidated material, capsid failure, maturation and capsid protein mutations in relation to viral material properties and highlight similarities and differences for different types of viruses. Another important experimental technique in the study of viruses is mass spectrometry. Eric Monroe and Peter Prevelige show how this technique can be used to gain invaluable information on viral proteins. Mass spectrometry can also play a crucial role in the study of virus assembly, i.e. the process of formation of viral particles from their protein building blocks and genomic material. An overview of capsid assembly kinetics is provided by one of the pioneers in this area, Adam Zlotnick, and his collaborator Zachary Porterfield. Their chapter covers both modelling and experimental techniques and provides a comprehensive overview of viral capsid kinetics. An important factor in virus assembly and disassembly is the mechanical stress on different components of the viral capsid. A beautiful account of how stress distributions impact on the assembly and disassembly of viral capsids formed from pentamers and hexamers is provided by Robijn Bruinsma and collaborators.

Viruses may package genomic material in the form of DNA or RNA. An important question concerning the formation of RNA viruses is 'what determines the size of an RNA virus?' It is addressed by Chuck Knobler and Bill Gelbart in their discussion of the correlation between capsid and genome sizes. The impact of genome length versus capsid size on the physics of viral infectivity is also discussed with respect to double-stranded DNA (dsDNA) phages by Alex Evilevitch and Martin Castelnovo. Another intriguing feature of packaged DNA genomic material is its topology. Together with their experimental collaborator Joaquim Roca, Jarvier Arsuaga and De Witt Sumners provide a comprehensive account of the mathematical and experimental analysis of the topology of viral DNA.

A volume on emerging topics in physical virology would not be complete without a discussion of the fascinating applications of viruses and virus-like particles in biomedical nanotechnology that are opened up by

these recent theoretical and experimental approaches. We therefore conclude with a chapter by Kristopher Koudelka and Marianne Manchester that discusses the state of the art in this area.

We would like to express our special thanks to the authors of these fascinating chapters for making this volume possible by sharing their exciting research with us.

Peter Stockley and Reidun Twarock
March 2009

# Contents

*Chapter 1*

# Cryo-Electron Microscopy of Viruses

Neil A. Ranson*,† and Peter G. Stockley*,‡

Cryo-electron microscopy (cryo-EM) is a structural technique that images biological macromolecules in native-like conditions, and has been widely applied to the study of viruses. Virus structures have been determined by cryo-EM at resolutions ranging from molecular (~30–50 Å) to near-atomic (~4 Å). Here we introduce cryo-EM of virus particles for the non-expert reader and review how some of the key cryo-EM studies have advanced our understanding of virus biology. We also describe the latest advances in cryo-EM. These advances are on the one hand driving cryo-EM studies of symmetric viruses towards atomic resolution. On the other, they are developing structural methods that allow the study of individual, pleiomorphic virus particles and the interactions they make with cellular machinery.

## 1. Introduction

The molecular details of how viruses infect and hijack the cellular processes of their host cells are of critical importance in biology and medicine. It is only through a precise understanding of such events that new anti-viral therapies will be developed. One of the key elements of this growing understanding of the viral life cycle has been an increased understanding of the structure of viruses, and in particular their dynamic properties. Together with X-ray crystallography, cryo-electron microscopy (cryo-EM) techniques have played a central role in studying virus structure. At the same time, viruses have played an equally important role in the development of cryo-EM and single-particle image processing as techniques in modern structural biology. In this chapter we will illustrate these developments for non-expert readers with selected examples.

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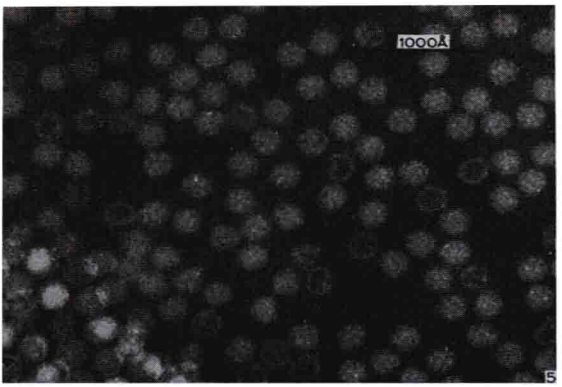


**Fig. 1.** Electron micrograph of Turnip Yellow Mosaic Virus (TYMV) negatively stained with 1% phosphotungstic acid. From Brenner & Horne (1959). *Biochem. Biophys. Acta.* **34**, p. 103.

Electron microscopy has been used to image viruses for more than 50 years. Initial studies involved the use of negative staining, in which the virus is placed on a continuous carbon film, coated in a heavy metal salt such as uranyl acetate, ammonium molybdate or phosphotungstic acid, and then dried before imaging (Fig. 1). Such treatment embeds the virus in a cast of the heavy metal salt and it is this cast, rather than the biological macromolecule itself, that gives rise to contrast in the images; hence 'negative' staining. Staining methods are rapid, generate high image contrast, and allow relatively large electron doses to be used, producing readily interpretable relatively low-resolution images of virus particles. However, staining also has major disadvantages. Firstly, penetration of stain into the interior of macromolecules is limited by both the structure of those macromolecules and by the size of the stain crystals. In practice this means that negative staining typically reveals only the external envelope of a macromolecule rather than any internal features. Secondly, the drying process can significantly distort the structure being imaged, as specimens are typically flattened onto the carbon support film as they dehydrate (see Fig. 2a). Such structural deformations are exacerbated by the pH and ionic strength of the stain solution, which places the biological macromolecule in a profoundly non-native environment. In summary, negatively stained images of viruses therefore are excellent at identifying the presence of viral particles in both purified and cellular samples, and this remains a major tool in the search for the presence of novel viruses in tissues.

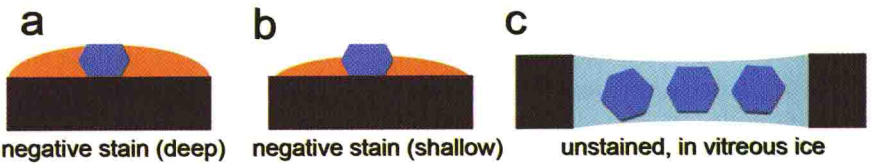


**Fig. 2.** A schematic representation of specimens for EM studies. (a) A negatively-stained virus (blue hexagon) adsorbed to a carbon support film (grey), and embedded in deep stain (orange). Note the flattening of the hexagonal virus onto the support film. (b) A viruss particle partially embedded in shallow stain. The portion of the virus structure not embedded in stain will not contribute to images of the specimen. (c) A cryo-EM specimen. Virus particles are unstained and trapped in a layer of vitreous ice. The ice layer is formed in a hole in the carbon support film.

A further complication of electron microscopy in general is caused by the nature of image formation in the microscope. EM has a depth of field that is significantly larger than most biological specimens. The consequence of this is that the images formed are projections of the three-dimensional (3-D) object onto the plane of the two-dimensional (2-D) recorded image, which greatly complicates interpretation. Various tricks have been developed to reveal information about the third dimension. One technique often used is metal shadowing at defined angles, which produces a pattern of electron dense material around particles of interest. The lengths of shadowed areas are proportional to the height of the object. More sophisticated image processing techniques have also been developed to reconstruct 3-D objects from a series of 2-D projection images, techniques that are very similar to those used in computed tomography (CT) scanning in medicine. Such methods work particularly well for viruses that have highly symmetric capsids. A major advance has been the use of cryogenic freezing of unstained samples (cryo-EM) and techniques for data collection that allow 3-D reconstructions from samples undamaged by excessive exposure to the electron beam (see below). Such techniques yield structures for viruses at resolutions that can rival those of X-ray crystallography, and of course do not require crystallisation of the samples before structure determination. Fortunately it turns out that in most cases to date structures determined by X-ray and cryo-EM methods are very similar, and X-ray structures can often be fitted into cryo-EM density. This implies that crystal structures reflect viral structures in solution but it has also opened up a wonderful synergy between the two techniques, as there are many conditions in which virologists would like to examine viruses

that are unlikely ever to be accessible by single crystal diffraction studies. Recent developments allow 3-D reconstructions to be determined for single virus particles, further extending the power of cryo-EM to interrogate viral life-cycles. Such techniques allow the determination of tomograms of viruses that lack isometric structures, such as the major pathogens of influenza, HIV and herpes. Viral asymmetry is therefore no longer a barrier to structural studies

## 2. The Cryo-EM Technique

As a result of the problems described above with interpreting stained images, structural biologists were keen to move to imaging of unstained specimens. The development of cryo-EM built upon the long established concept that low temperature preserves biological specimens. However at the molecular level, the formation of ice crystals during freezing can disrupt biological structures and withdraws water molecules from their hydration shell causing macromolecules to become partially dehydrated. Furthermore, crystalline ice diffracts the electron beam, preventing useable image formation. Dubochet and colleagues at the EMBL in Heidelberg discovered that if a biological specimen was frozen sufficiently rapidly the formation of ice crystals was prevented, preserving the solution conformation and allowing it to be imaged in a thin layer of vitreous ice. The key to this rapid freezing was the use of liquid ethane or propane, cooled to near liquid nitrogen temperatures, to freeze the samples. These coolants have extremely high thermal conductivity, which allow extremely rapid cooling rates to be achieved. Together with the low mass of an aqueous thin film, cooling rates approaching a million degrees per second can be achieved during vitrification. In the vitreous ice layer that results from such rapid cooling, biological macromolecules remain hydrated, and in a structural state essentially identical to that seen in the liquid phase. Typically, the thin film of ice was formed and frozen in a hole in a carbon support film, ameliorating the effects of a support film that can flatten particles and cause preferred orientations to be observed (see Fig. 2). The first practical results of this new technique were images of viruses: unstained, frozen-hydrated Semliki Forest Virus and bacteriophage T4 (see Fig. 3e). Even in these first cryo-EM images, the potential of the technique for studying the structure of viruses was immediately apparent. The images of SFV immediately settled an ongoing debate about whether SFV had a $T = 3$ or a

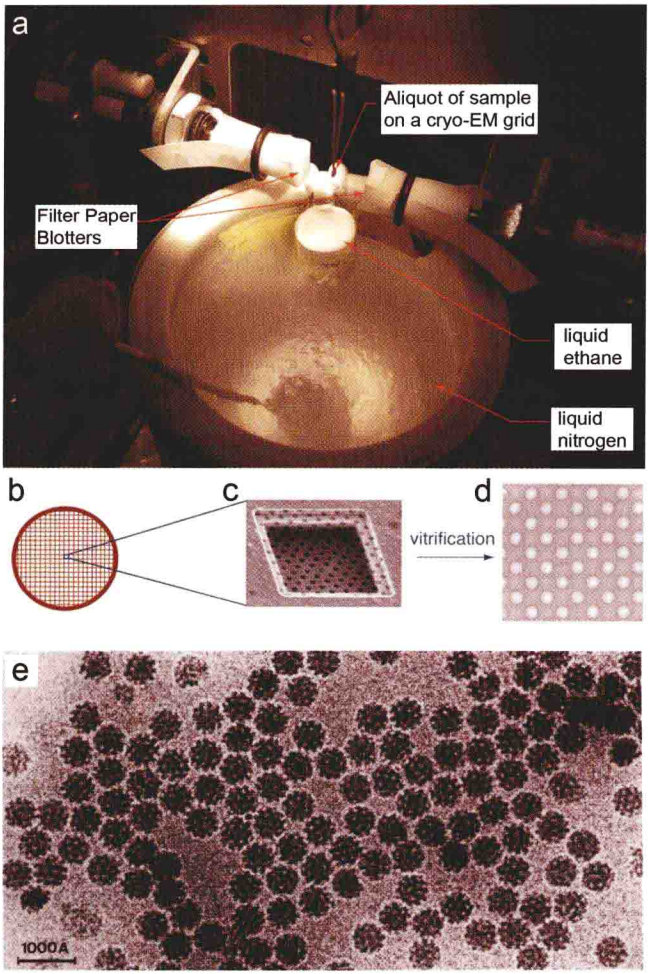


**Fig. 3.** Cryo-EM sample preparation. (a) A cryo-EM freezing apparatus. An EM grid is held in forceps above a reservoir of liquid nitrogen-cooled liquid ethane. Sample in water of buffer is added, excess liquid is blotted away (here by pneumatically-driven blotters) and then the grid is plunged into the liquid ethane reservoir. (b) A typical EM grid The grids are ~3 mm in diameter and consist of a mesh made from a variety of metals, and in various spacings. (c) Grids covered with a support film lithographically etched to contain a regular array of regularly sized holes are commercially available. The support film shown is a Quantifoil R2/2 grid (Quantifoil Microtools, Gmbh), in which the holes are 2 $\mu$m in diameter). (d) A close-up view (~1200x) of such a support film with a thin layer of vitreous ice in the EM is also shown. (e) The first published cryo-EM images of a virus; Semliki Forest virus. From Adrian *et al.*, (1984).

$T = 4$ capsid morphology, unambiguously revealing a $T = 4$ morphology (Adrian *et al.*, 1984).

## 3. Determining the 3-D Structure of Viruses from EM Data

Like all methods that are in principle suitable for determining the high-resolution structure of biological macromolecules, electron microscopy has both advantages and disadvantages. Perhaps the most notable advantage of an electron microscope might sound trivial but is not; an electron microscope records an image. The process of image formation means that both amplitude and phase information are recorded simultaneously, in contrast to diffraction-based methods where only amplitudes are captured, and phase information has to be derived. However, aberrations in the magnetic lenses used to focus electrons (which allows formation of the image) mean that very small apertures are required in electron microscopes. Small apertures contribute to the large depth of field in EM. Together with the penetrating nature of electrons, this results in the 3-D electron density of the object being projected onto the 2-D plane of the image (c.f. a medical X-ray). The fact that EM generates projection images of biological structures means that it is extremely difficult to interpret individual EM images. However, it also means that true 3-D structural information is accessible. 3-D reconstruction of an object from projections is possible owing to the fact that the Fourier transform of a projection image is a central section of the 3-D Fourier transform of the original object (Fig. 4).

Thus, if all possible views of the object are available, then a full 3-D reconstruction of the object is possible, in principle to the resolution limit of the instrument. For the case of electrons in the 2–300 keV range as used in modern microscopes, this is better than 1 Å resolution. The key problem faced in calculating such a 3-D structure is determining in which orientation the individual projections of the original object were imaged. The orientation of the projected structure defines which central section through the 3-D transform of the object the projection represents. From this it can readily be seen that a full range of all possible orientations leads to the most completely sampled 3-D transform, and hence contributes towards higher resolution. The essence of determining a virus structure

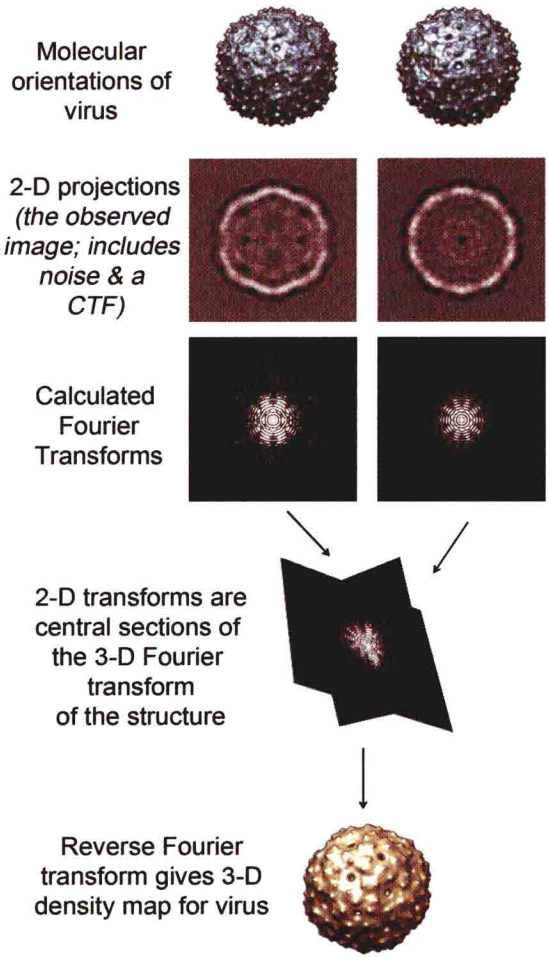


**Fig. 4.** The principle of how to calculate a 3-D structure from 2-D projections. The virus is randomly oriented in vitreous ice layer of a cryo-EM grid. The recorded images, containing noise and distorted by the effects of the microscope CTF are projections of different views of the virus onto the plane in which the image is recorded. The 2-D Fourier transform of those images are central sections through the 3D Fourier transform of the virus. Knowing the orientation of each particle tells you which central section of the 3-D transform that virus particle represents — i.e. how the slices fit together. The degree to which the 3-D transform is populated is one of the limits on resolution in cryo-EM studies; hence the need for randomly distributed orientations and the advantage of high symmetry. The 3-D object is reconstructed by reverse Fourier transformation to give a 3-D electron density map.

by cryo-EM is therefore simply to determine the orientation of each picture of the virus with as high a degree of accuracy as possible. Details of exactly how the orientation of each imaged virus particle is found are beyond the scope of this article. However, in essence a number of different approaches are possible which share the same basic idea that experimental images of the virus are compared to reprojections of a model structure for the virus. For review, see (Baker *et al.*, 1999; van Heel *et al.*, 2000).

Implicit in the idea that all possible views are required is the fact that structures of highly symmetric particles are more readily calculated, as the symmetry of the particle helps to ensure that all possible views are sampled. Averaging of symmetry-related views also increases the effective size of datasets, meaning that fewer raw images are required for a given resolution than for asymmetric particles. Symmetry averaging does however 'average-away' any asymmetric features of a virus, such as single-copy infectivity or maturation proteins which are commonly essential for viral lifecycles.

The negative-staining method that dominated EM imaging of viruses until the mid-1980s presented major challenges to the application of this approach to 3-D structure determination. Very often biological particles adhere to the carbon support film on the EM grid, deforming the native structure and resulting in preferred particle orientations that make 3-D structure determination difficult because these do not contain enough information. For highly symmetric particles such as isometric viruses, this is not an insurmountable problem, as symmetry ensures an even coverage/sampling of the 3-D transform of the reconstructed density. However, the size of such particles means that obtaining images of viruses fully embedded in stain is extremely difficult, and for larger viruses essentially impossible. Since it is the interaction of electrons with the stain layer that generates contrast, this results in the information from un-embedded portions of the virus not being recorded in the image, i.e. the images represent projections of only part of the structure (see Fig. 2b). Furthermore, owing to the problems associated with staining, only information on the surface envelope of the virus is obtained.

Preservation and imaging of the entire native-like structure is the clear advantage of cryo-EM over staining methods. However, the fact that the specimen is unstained results in a different set of problems for structure determination. Firstly, amplitude contrast in cryo-EM images arises from the scattering power of the atoms found in the specimen, which is related

to their mass. Hence the use of heavy metal salts in negative staining. The masses of atoms typically found in proteins and/or nucleic acids are very similar to those found in the surrounding water and buffer molecules of the vitreous ice layer. This means that although cryo-EM images contain information from the entire macromolecular structure, they typically have extremely low contrast. This problem is made markedly worse by the radiation sensitivity of unstained biological macromolecules, requiring that the electron dose delivered to the specimen be kept low to minimise radiation damage. Together these factors result in the characteristically poor signal to noise ratios (SNR) of cryo-EM images, and necessitate computational averaging of data to improve the SNR to a point where structure determination is possible. In essence, in cryo-EM noisy images of individual molecules have to be explicitly averaged after the data is recorded, whilst in X-ray diffraction averaging is an intrinsic property of the crystal. This averaging creates significant computational challenges that need to be overcome in order to determine cryo-EM structures, especially to high resolution.

To a limited extent, the problem of low amplitude contrast in cryo-EM images can be overcome by tuning the optical properties of the microscope to introduce a second type of contrast into the recorded images: phase contrast. In practice, this is routinely achieved by defocusing the microscope, which introduces low-resolution phase contrast that is often essential to allow relatively small objects, such as virus capsids, to be found in noisy micrographs. It also means that distortion of the observed image by the microscope's contrast transfer function (CTF) becomes significant and problematic (Fig. 5). A CTF is a phenomenon, common to all optical systems, that defines how information is transferred as a function of spatial frequency (i.e. resolution) in Fourier space. The form of this function is of an oscillating sinusoidal variation in information transfer with increasing frequency and decreasing amplitude (Fig. 5f). In real space (i.e. in the observed image) the effects of a CTF are to convolute the image of the object being studied with a point spread function (PSF). A PSF spreads information from each area of the image into surrounding areas and attenuates information at high resolution. The practical consequences of using highly defocused images are therefore that although they have more low-resolution contrast, allowing the particles to be found, the particles themselves have less high resolution information and are profoundly distorted, necessitating computational CTF correction during