

# Advanced Transmission Electron Microscopy in Drug Development

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## INTRODUCTION

Increasingly, pharmaceutical research and development is focused on understanding and manipulating the molecular mechanisms that ultimately constitute the vastly complex machinery of all living systems. Transmission electron microscopy (TEM), with its ability to directly image biological structures at the molecular scale, promises to play a crucial role in the future of drug discovery and development.

As Richard Feynman wryly observed in his famous lecture, "There's Plenty of Room at the Bottom, a lecture that arguably created the field of nanotechnology: 'It is very easy to answer many of these fundamental biological questions; you just look at the thing!' He continued by noting that, in order to do so, we really need an electron microscope 100 times more powerful than those available at the time (1959). In the nearly 50 years since, microscope manufacturers have made great progress in both the power and usability of their instruments.

In certain applications, the latest generation of TEMs can resolve features as small as half an Ångstrom ( $1 \text{ \AA} = 0.1$  nanometer; a carbon atom is approximately  $2 \text{ \AA}$  in diameter and the DNA helix is about  $20 \text{ \AA}$  wide), nearly achieving the 100x improvement Feynman desired.

Advanced automation and control technologies have reduced training requirements for routine operations from PhD to technician level. However, practical limitations related to the nature of biological samples limits resolution in most biological applications to a nanometer or more, and the most sophisticated techniques still require considerable experience and expertise.

In addition to the technical limitations of the microscope itself, the use of TEM in drug development must also satisfy requirements imposed by the business and regulatory environment of the pharmaceutical industry. These include timely, reliable execution; tightly controlled, well-documented

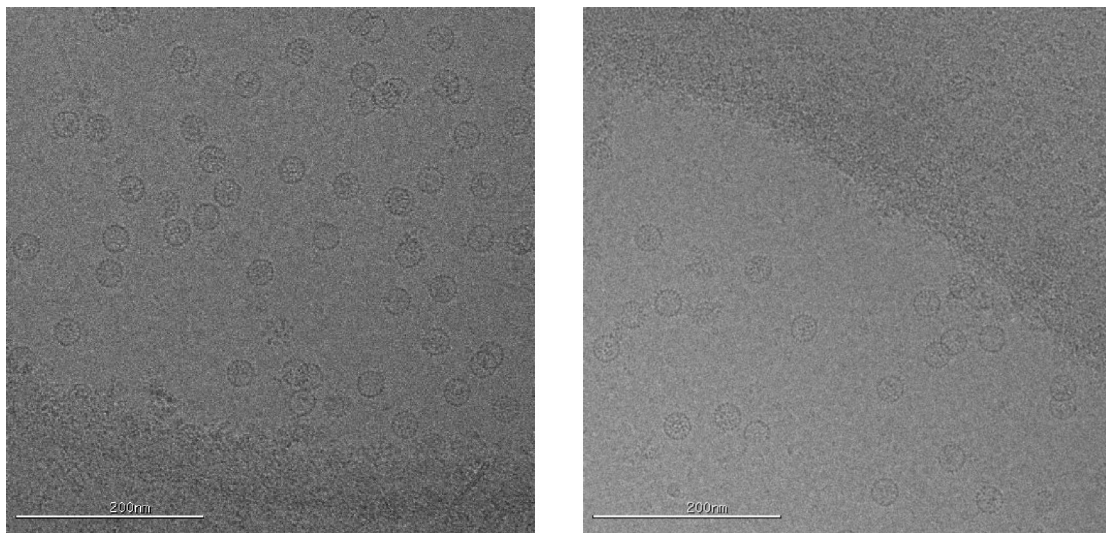


Figure 1: CryoTEM images, such as these of the hepatitis B virus capsid, contain high-resolution information, but exposure limits that are required to prevent radiation damage also limit the contrast and signal-to-noise ratio available in a single image. Courtesy, The Scripps Research Institute

procedures; repeatable, statistically valid results; secure, accessible data storage, and clear ownership of intellectual property. While technical requirements have, until now, limited the availability of advanced TEM analysis to large institutional and academic laboratories, its growing importance in drug development applications may be better served by a commercial service model free from the sometimes conflicting demands of a busy research organisation.

### **BIOLOGICAL TEM - CHALLENGES AND SOLUTIONS**

A TEM forms a real, magnified image from electrons transmitted through the sample. Its operation can be compared to a slide projector, though it uses electrons and magnetic lenses rather than light and glass lenses. Because electrons are readily scattered by gas molecules, it must operate in a vacuum. The sample must be thin enough to transmit most of the electrons, ideally with no more than a single scattering event within the sample—typically 100 nm or less.

Although TEM is capable of atomic-scale resolution, its application to biological specimens is limited primarily by its ability to accumulate enough signal to generate sufficient image contrast before the sample is damaged by the high-energy beam electrons. Biological materials, predominantly composed of light elements with similar scattering cross-sections, have inherently low contrast. Furthermore, the molecular bonds that constitute biological structures are readily disrupted by the ionising radiation of the electron beam.

Additional difficulties arise from sample preparation procedures required to make the sample compatible with the

vacuum environment of the TEM. Most biological material exists naturally in a fully hydrated state. Conventional procedures that involve drying, chemical fixing and embedding necessarily disrupt the native structure. Though they are often useful on a larger scale, the changes they introduce limit their utility at the molecular and supra-molecular scale. Likewise, conventional chemical fixation techniques, which add heavy elements with selective affinity for targeted features in order to enhance image contrast, have limited application at this scale.

Finally, biological TEM is limited at the molecular scale by the superposition of many molecules in the projected image of even the thinnest samples. The native environment of biological systems is densely populated at the molecular level with components that are exquisitely detailed but difficult to distinguish. It can be compared to looking into a barrel of marbles.

Fortunately, a number of techniques have been developed to address these challenges. Negative staining provides a fast and relatively easy way to evaluate the morphology of dispersed particulate samples such as viruses and virus-like particles. The particles, supported on a thin, transparent substrate (typically a carbon film) are thinly covered by a layer of higher-density staining material. The high-density stain is readily imaged in the TEM and its image is a negative image of the particle. Though less disruptive than conventional chemical stains, negative staining can induce forces strong enough to distort or collapse delicate structures, and its use should be qualified by recognition and evaluation of this potential.

Cryo techniques, in which the sample is suspended in a thin film of aqueous material and quickly frozen, offer many advantages in biological TEM (see Figure 1). At cryogenic temperatures, such samples can be sustained in the TEM for many hours without noticeable evaporation. In the frozen state, beam electrons may still break bonds and ionise atoms, but the molecular fragments thus created remain in place, preserving the essential structure and significantly increasing the radiation dose the sample can tolerate.

The key to success is the speed of the freezing process. At slow speeds, nucleation and crystallisation remove water molecules from the biological structures and push larger molecules like proteins to the boundaries between ice crystals. At high freezing rates, the water forms an amorphous solid (vitreous ice) that immobilises the structure in its native, fully hydrated state. Vitrification is usually accomplished by plunging the sample into liquid ethane at cryogenic temperature. Ethane permits faster heat transfer than liquid nitrogen because it avoids the formation of a

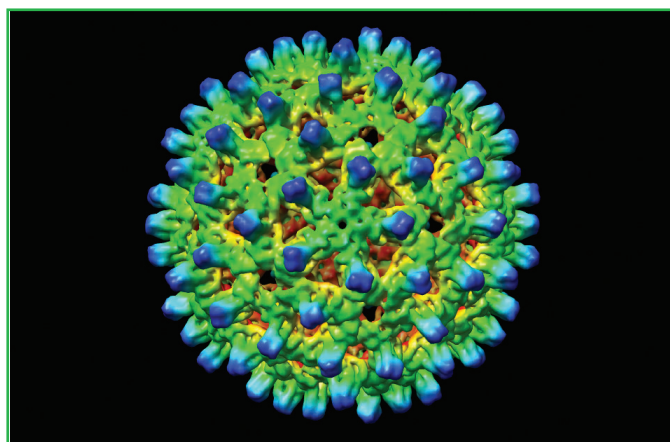


Figure 2: Single-particle analysis automatically acquires, sorts and averages thousands of images of identical particles to reconstruct a detailed three-dimensional model. This model of the hepatitis B virus capsid was constructed from images such as those shown in Figure 1. Courtesy, The Scripps Research Institute

vapour layer adjacent to the sample surface. Its evaporation temperature is low enough to permit its evaporation from the sample surface without allowing the vitreous ice to crystallise. Robotic vitrification systems provide easy operation and highly repeatable results.

CryoEM preserves native structure and improves contrast by permitting longer exposure to the beam. Further contrast enhancement relies fundamentally on signal averaging, though it may be accomplished in a number of ways. Crystallography provides an instructive analogy. Because molecules are identically oriented within a crystal, their signals sum in the diffraction pattern while the noise averages out. A technique known (somewhat misleadingly) as single-particle analysis (SPA) uses the same principle to enhance contrast in TEM imaging.

SPA begins by acquiring images of a large number of randomly-orientated single particles. The images are then sorted into categories with similar orientation. The images in each category are summed to create a representative composite image and the composite images are combined computationally to reconstruct a high-resolution, high-contrast, three-dimensional model of the particle (see Figures 2-4). The quality of the model can be improved by simply acquiring more images, though the gain is not linear and practical limitations quickly define a point of diminishing returns.

Advances in automation have made the collection and analysis of tens of thousands of images practical, permitting resolution approaching 1 nanometer in the reconstructed model. SPA is best suited to relatively large particles with shapes that are readily orientated from the projected image.

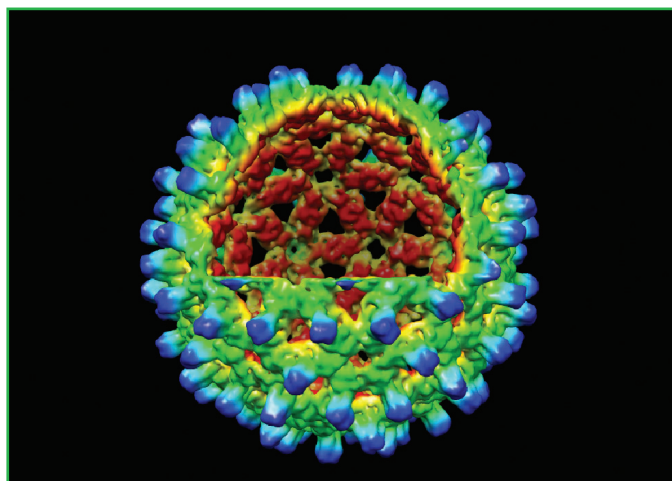


Figure 3: The model can be viewed from any direction and virtually sectioned to reveal internal structure. Courtesy, The Scripps Research Institute

Internal symmetry also facilitates the reconstruction process. Many viruses and virus-like particles are excellent SPA candidates.

Electron tomography (ET) is another technique that enhances contrast and provides three-dimensional reconstructions. Analogous to better-known medical imaging technologies (such as the CT (computed tomography) scan), ET acquires a series of images as the sample is rotated in known increments about an axis perpendicular to the viewing direction, the electron beam. A computer then aligns and combines the different views to construct the three-dimensional model.

Like SPA, the reconstruction procedure averages out the noise while the signals sum coherently. Unlike SPA, ET truly operates on a single particle or, more broadly, a single instance of the imaged structure.

This has the disadvantage of limiting the total amount of the information that can be extracted before the particle is damaged by the beam. It has the advantage of permitting dynamic analysis of flexible particles and particle interactions by capturing snapshots of the structure as it changes shape or interacts. It also addresses the problem of overlapping structures, permitting detailed analysis of structure and interaction in the crowded molecular environment of a natural biological system.

### COMMERCIAL AND REGULATORY CHALLENGES AND SOLUTIONS

As must be apparent from the preceding discussion, advanced TEM techniques still require experience and expertise that would be difficult to justify in any but the largest central laboratories. While academic research labs have played an indispensable role in developing the technology, the priorities of their primary mission, research, do not always align well with the demands for fast turnaround, long-term stability, tight process control and more, which are critical to the success of a commercial development programme. Nor are research operations often well-designed to accommodate the extensive, long-term record keeping and documentation requirements of the pharmaceutical regulatory environment. Finally, even with the best of intentions and the most detailed contractual agreements, it is difficult to avoid contention over the ownership of intellectual property in an arena where discovery is the lifeblood of both the client and the provider.

A commercial service laboratory, properly staffed and equipped, can address all these concerns. All work performed is simply work-for-hire with ownership of all intellectual

property clearly vested in the client. More importantly the entire organisation can be orientated toward service rather than discovery, with individual and organisational success determined by the ability to cost-effectively satisfy the clients' needs. With that in mind all facilities and procedures can be designed to align with the clients' priorities.

Technology can play an equally important role in meeting the commercial and regulatory requirements of the pharmaceutical industry. Tremendous strides have been made in automating TEM operations. These range from digital control of low-level instrument functions to permit automatic execution of complex set-up and alignment procedures, through fully automated data acquisition and sample manipulation.

Automation is critical in procedures such as SPA that must acquire and process thousands of images. By eliminating human influences, such as operator fatigue and selection bias, automation improves the speed, accuracy and reliability of the analysis. Automation also facilitates the use of low-dose imaging strategies that minimise beam damage. Manual SPA that would take weeks can be performed automatically in as little as a day, including unattended overnight operations.

Data management technology can also play a key role. An online relational database provides immediate and secure access to experimental results, allowing remotely-located investigators to participate in the experiment and share information with colleagues. Archival storage of all images and meta-data supports the provenance and documentation requirements of the regulatory process and provides a basis for comparison in the evaluation of process modifications and long-term quality control.

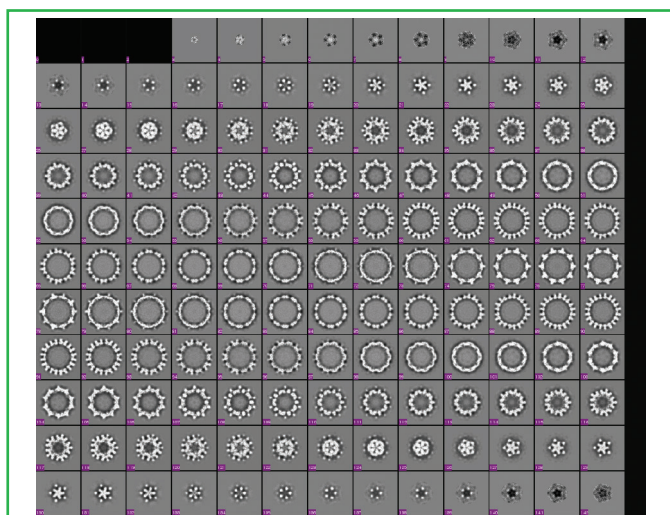


Figure 4: The structure (hepatitis B virus capsid) can also be viewed as a series of slices through the 3D reconstruction. Courtesy, The Scripps Research Institute

## TEM IN THE DRUG DISCOVERY PIPELINE

Direct visualisation with high-resolution TEM imaging can increase confidence and shorten development cycles at nearly every stage of the drug development pipeline. In discovery it provides target validation via compound screening, antibody labelling, and comparison of conformational states. Later in development, it can be used to verify the controlled assembly and conformation of structures such as virus-like particles and lipid vesicles. In regulatory reviews, it provides powerful visual support for conclusions based on less direct assays and analytical data.

## CONCLUSION

We are visual creatures. This assumption was implicit in Feynman's exhortation, '...you just look at the thing!' Although we are not quite to the point where that is as easy as it might sound, TEM has clearly demonstrated its ability to elucidate the molecular mechanisms at the heart of many biological processes, and it promises to play a major role in developing drugs that manipulate those processes. If a picture is worth 1,000 words, a three-dimensional model may very well be priceless. ■

## ABOUT THE AUTHORS

Clint Potter and Bridget Carragher have many years of experience in biological imaging and have published extensively on both TEM imaging technology and the science it enables. In 2007 they founded NanoImaging Services to provide advanced TEM services to the pharmaceutical industry. In addition to their roles as CEO and CSO at NanoImaging Services, Potter and Carragher hold appointments as associate professors at The Scripps Research Institute where they jointly manage the Automated Molecular Imaging research group.

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