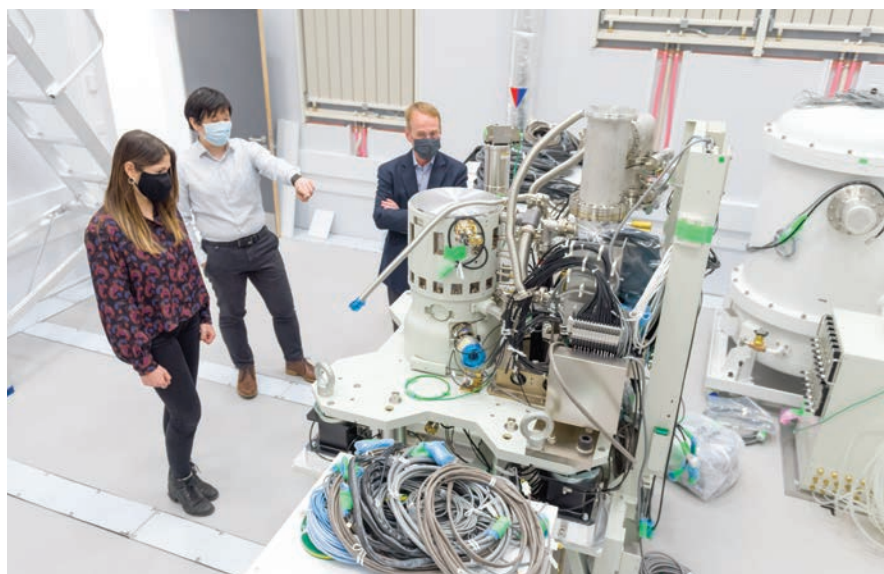# How can advances in time-resolved electron microscopy improve imaging of biological samples? Working on the edge of discovery at the Rosalind Franklin Institute

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Scanning Transmission Electron Microscopy (STEM) has long been an essential technology for understanding the structure of materials in the physical and life sciences. In the past decade, STEM technologies have advanced almost separately in the study of inorganic and biological materials; however, new methods for investigating life are emerging from the overlap between these fields. One such technology advance is high resolution imaging using electrons.

With its remit of transforming life science through interdisciplinary research and technology development, the Rosalind Franklin Institute at Harwell Campus in Oxfordshire is driving research at this overlap, with a pioneering STEM instrument for imaging biological materials conceived and developed through its Correlated Imaging Theme.

Ruska is the first of three instruments being developed at the Franklin, uniquely designed to deliver unprecedented spatial and temporal resolution imaging of cryogenically frozen biomaterials and biological samples in liquid.

The Correlated Imaging team of the Rosalind Franklin Institute checking on the installation of Ruska. Dr Emanuela Liberti, Dr Chen Huang, Professor Angus Kirkland (left to right). Photo credit: Ryan Cowan.

Pulsed Illumination for unprecedented temporal and spatial resolution

Structural damage of biological specimens is the main factor currently limiting spatial and time resolution in STEM [1]. Electrons damage biological matter depending on the energy transferred to the sample during irradiation (i.e. electron dose). The damage occurs as a function of the primary electron energy, imparted by the accelerating voltage of the microscope. Ruska can operate across a wide range of accelerating voltages, from 300 kV to 40 kV, to allow maximum flexibility on the choice of the primary electron energy.

The microscope also has variable pulsed illumination. At the electron source, a fast beam blanker (or electrostatic dose modulator) distributes electrons onto the sample in 'pulses' of adjustable duration from μs to ms. The number of electrons in each pulse is also adjustable, allowing precise control of the dose. This ability to regulate the illumination improves spatial and time resolution because the sample is imaged over short periods of time while damage is controlled [2]. Pulsed illumination particularly benefits imaging in liquids in which minimising damage is essential to avoid beam-driven dynamics.

Ruska also has electrostatic optics for subframe recording. This technology rapidly deflects the beam onto different areas of a high speed camera in a programmable sequence [3]. In this way, the recording process is only partially limited by the speed of the camera, improving the time resolution to potentially hundreds of thousands of frames per second.
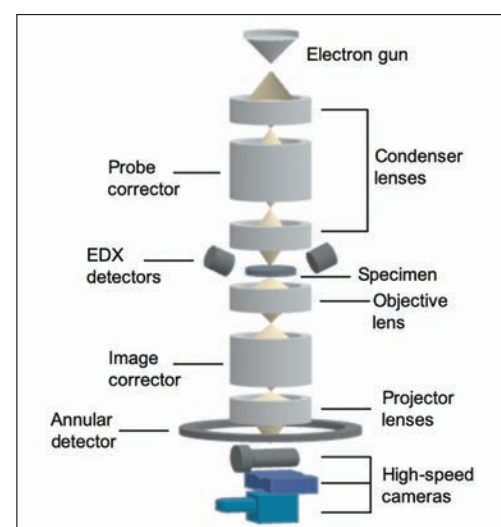

Figure 1. A typical double-corrected scanning transmission electron microscope. Ruska improves on the standard design due to high-speed electrostatic blankers, one mounted after the electron gun and one before the cameras (not shown). Ruska has annular detectors for conventional STEM imaging (only one shown here) but also high-speed cameras for improved time resolution. Annular detectors integrate the scattered intensity to an annular range of scattering angles while high-speed cameras collect the whole range of scattering angles.

Improving contrast with phase retrieval electron ptychography methods

Ruska's optical aberration correction is key to the instrument's improved spatial resolution for studying biological materials [4] *(Figure 1)*. Although correctors are commonly used in the physical sciences for studying inorganic materials, their application has been limited in biology. In Ruska, hardware correction of the lens aberrations is applied to both transmission (TEM) and scanning (STEM) optical components. In TEM mode, the image corrector improves the focusing of the sample by the objective lens which results in better information transfer at high spatial frequency. However, the transfer of low frequencies still remains poor, meaning that biological structures can be difficult to discern in individual images. Achieving high contrast is even harder for cryogenically frozen samples or in liquids because the surrounding substrate, which has a similar density to the sample, further degrades the image quality.

The workaround is to exploit the improved resolution of aberration-correction in combination with phase retrieval imaging techniques. These methods exploit the change in the information transfer with illumination setup to improve contrast across a range of spatial frequencies. Phase retrieval methods are applicable in both TEM and STEM modes. The Franklin applied a phase retrieval method in STEM, recently developed for the physical sciences (i.e. electron ptychography) to image biomolecules (e.g. frozen-hydrated rotavirus double-layer nanoparticles) with improved contrast compared to cryo-EM [5]. Better quality images could reduce the currently large number of particles and datasets required to achieve atomic resolution in single particle 3D reconstructions. Phase retrieval methods are also not bound to a homogeneous collection of small particles, but can characterise larger areas (1 μm^2) of heterogeneous specimens for findings on distribution or interaction.

In a STEM phase retrieval technique a convergent electron probe is scanned across the sample to record a 2D array of coherent (interference) electron diffraction patterns which is then fed into a ptychographic iterative engine (ePIE) to recover the object exit wave function resulting from the scattering with the electron beam *(Figure 2)*. ePIE is a computational method used in microscopy to solve the phase of the object function by inverse computation (the so-called 'phase problem') [6]. The bandwidth of spatial frequencies that is transferred in this phase retrieval process depends on the convergence angle of the probe, which can be adjusted to obtain strong phase contrast *(Figure 2)*.

The benefit of probe-correction when Ruska operates in STEM mode comes from the large range of convergence angles available for imaging because focusing of the probe-forming system is improved. For instance, for beam-resistant samples, a convergent probe of sub-Angstrom size provides lateral atomic resolution, and sub-nanometre depth resolution. However, small probes have an extremely high electron flux (more than ten thousand times higher than required) which would completely destroy biological materials. The Franklin is planning to exploit probe-correction for imaging at high spatial resolution of biological materials using sparse scanning geometries, designed to distribute the electron flux in an intelligent way. Sparse scanning methods where the interval between scanning positions is varied in space and time in a geometric or random sequence are already being developed for imaging in inorganic materials in liquid and could provide new imaging methods in biology [7]. For this purpose, Ruska is equipped with an additional random scan generator. This technology can change the scan pattern of the scanning coils to design intelligent subsampling pixel scanning.
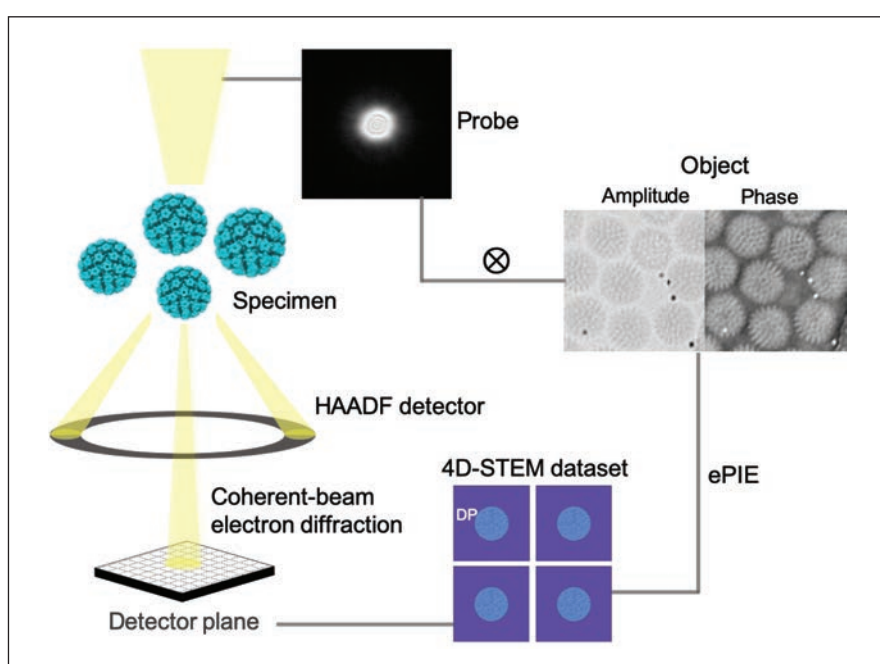

Figure 2. Schematic of an electron ptychography experiment for imaging of biomolecules. As the electron probe scans across the sample, the direct detector collects a coherent-beam electron diffraction pattern (CBED). This 4D STEM data set is fed into an electron Ptychographic Iterative Engine (ePIE) for the recovery of the complex object and probe functions.

Fast Direct Electron Detection for maximum time resolution

Together with the fast blankers, the key technology that makes Ruska a time-resolved instrument is fast direct electron detection. Direct detectors have revolutionised electron microscopy for the past decade [8]. What differentiates these cameras from conventional sensors is their improved recording speed and detector quantum efficiency (DQE). These enhanced capabilities translate into a better signal-to-noise ratio, even when few electrons are used for imaging, which has enormous implications for both life and material sciences. One example is the cryo-EM 'resolution revolution' wherein imaging of protein structures is now possible at atomic resolution using direct detection and single-particle averaging [9]. The advent of direct electron detectors is also revolutionising in-situ imaging. Here the recording speed is vital to capture fast dynamic events, while the detector efficiency is needed because of the low-dose required to minimise the electron beam interaction with the media.

Dr Chen Huang, Professor Angus Kirkland, Dr Emanuela Liberti. Credit: Ryan Cowan

Direct detectors are an essential technology for biological imaging at high spatial and temporal resolution. Ruska will be equipped with three direct electron detectors (high-speed cameras in *Figure 1*). Each one of these cameras performs optimally at different accelerating voltages and imaging modes. Most importantly, these detectors all have very high recording frame rates, of thousands of images per second. The cameras can also be synchronised to the blankers and the scan generator to achieve maximum control over the electron flux, the illumination speed and the recording time.

Liquid Cell Technology

Our ultimate goal is to study biological processes in their native environment as they happen in real-time [2]. This means that imaging in-situ must span a wide range of spatial and temporal resolutions. Ruska will achieve spatial resolution from the microscale to the molecular scale and temporal resolution from milli to microseconds while maintaining the sample in a liquid environment. The microscope will host a newly designed liquid cell holder that can trap a solution of the sample between electron transparent membranes, made either of silicon nitride or graphene foils, protecting the specimen from the vacuum environment of the microscope *(Figure 3)*. This technology will enable the study of a wide range of biological systems, including drug-target interactions, polymer assembly and protein dynamics. Furthermore, Ruska is equipped with a new dual energy dispersive X-ray detectors for chemical mapping. This technology enables collection of chemical data in-situ, expanding the capabilities of the microscope to analytical spectroscopy for biology in liquids. Integrating liquid cell imaging with the high spatial, time and chemical resolution capabilities of Ruska will allow access to, previously hidden, rapid dynamic events in 4D. A greater understanding of the molecular dynamics at play in living systems can be seen as one of the fundamental building blocks to improving our understanding of all biological/medical systems.
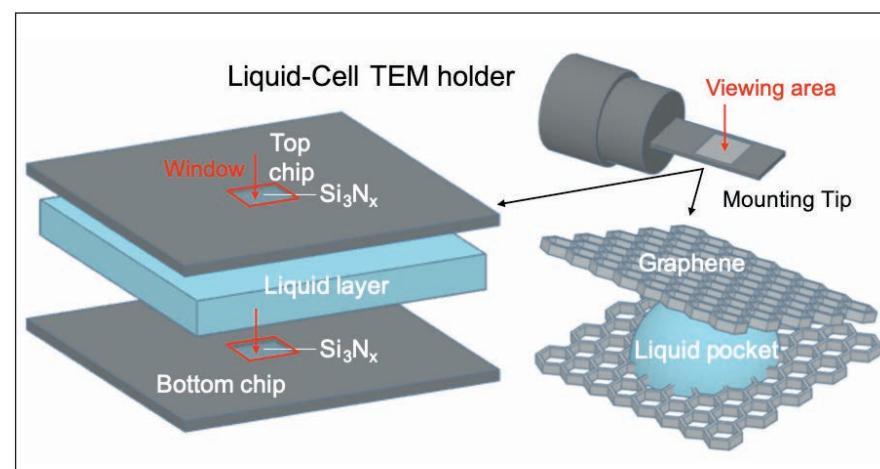

Figure 3. Schematic of liquid-cell TEM holders. LCTEM holders can accommodate chips made of a Si substrate and electron transparent Si3Nx windows, or graphene grids. In both technologies, the sample in solution is encapsulated and hermetically sealed from the ultra-high vacuum in the microscope column.

For more information about the Ruska electron microscope please contact: emanuela.liberti@rfi.ac.uk

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