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Introduction

Cryo-electron microscopy (cryo-EM) is a method that uses a transmission electron microscope (TEM) to record images of frozen hydrated protein solutions that are prepared as a thin film of approximately 100 nm thickness. In conventional cryo-EM, the TEM is used to illuminate the sample with a broad and parallel beam of electrons. The electrons traverse the thin specimen, and lenses in the TEM are used to form a phase-contrast image behind the specimen, which is recorded with a large area camera (Figures 1 and 2) [1]. Important parameters for high-resolution cryo-EM are not only the choice of the instrument hardware including its camera, but also the quality of the environmental conditions of the instrument setup and the instrument alignment. In cryo-EM of frozen preparations of a homogeneous preparation of biological particles, the so-called single particle analysis (SPA), a large number of images are recorded with the help of automation softwares. Typical recording times for a full dataset range from hours to days and reach up to 20'000 or more dose-fractionated movie recordings for one sample. Recorded images typically show projection maps of protein particles at very low signal to noise ratio. However, if the signal from hundreds of thousands of such protein particle images is computationally combined, a high-resolution 3D reconstruction of the average protein map can be obtained. Such SPA processing requires that the majority of the particles are the same. If more than one type of protein particles were present, then classification algorithms can be used to reconstruct these. Typically, up to 10 different particle types, or particle conformations can be recognized. Once a 3D map of a protein has been reconstructed at a resolution better than 3.5 Å, an atomic model for the protein can be built into the 3D map (Figure 1). Cryo-EM is an extremely productive tool to study the atomic resolution structure of proteins, as long as the proteins are large enough to be visible in the noisy images, and as long as they are available in sufficiently homogeneous preparations. If strong heterogeneity is present in the ensemble of particles, then cryo-EM fails. Cryo-EM was recognized with the Nobel prize in Chemistry in 2017, awarded to Richard Henderson, Joachim Frank, and Jacques Dubochet [2]. Jacques Dubochet is an Emeritus Professor of the University of Lausanne, and he had studied physics at the institute that today is the EPFL, which triggered the creation of the Dubochet Center for Imaging with its three sites in Lausanne, Geneva and Bern [3].

Ptychography – A diffraction approach to imaging

Cryo-EM suffers from the low signal-to-noise ratio of the recorded images, and from the resolution limits of the TEM imaging process. An alternative to recording real-space images with the TEM is to exploit the strong interaction of the electron beam with the sample and record electron diffraction patterns. When applied to 3D crystal specimens, this 3D electron diffraction is a powerful method that can determine atomic details about protein structures from 3D crystals that are much smaller than needed for X-ray crystallography [4]. The TEM can also be used with the electron beam focused to a tiny point on the specimen surface, and this sub-Angstrom sized probe is then scanned over the sample, while recording non-scattered and scattered electrons as a function of the probe position, which is called Scanning Transmission Electron Microscopy (STEM). For cryo-EM specimens, integrated Differential Phase Contrast STEM has recently been shown to be able to determine high-resolution details of vitrified biological samples at low electron doses [5].

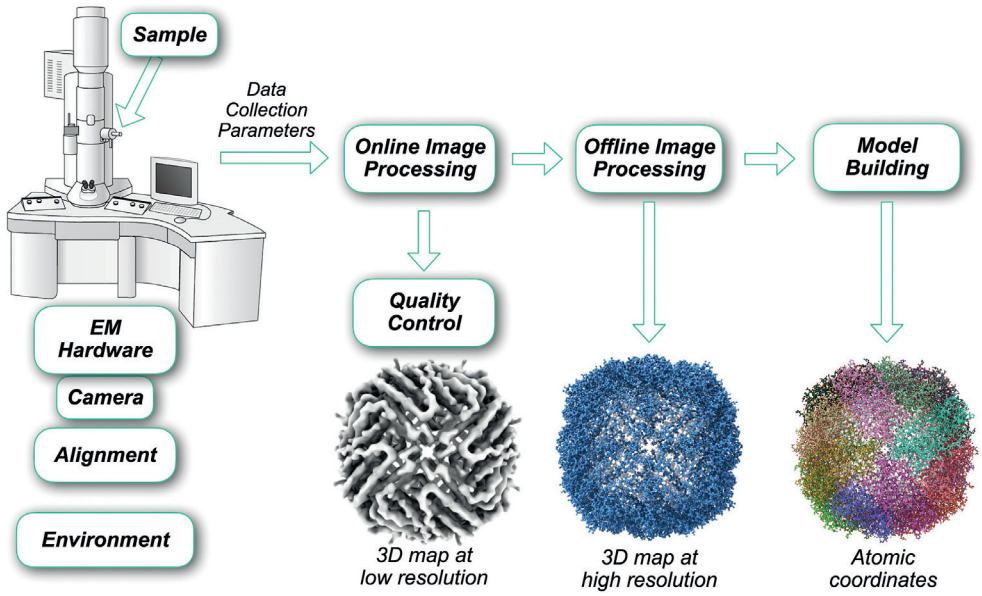

Fig. 1: The single particle cryo-EM workflow. A frozen sample is imaged with a cryo-EM instrument. Important parameters are the instrument hardware, the instrument alignment, and the environmental disturbances of the instrument location. During automated image collection, online data processing is performed as quality control, here resulting in a 3D map of Apoferritin at 7 Å resolution. After data collection, extensive fine tuning of image processing can produce 3D maps at up to 1.09 Å resolution (here shown: EMD-19436). Building of an atomic model into the 3D map can be done manually or with help of computers (here shown: PDB-8RQB).

A hybrid approach that combines real-space and reciprocal space data collection is ptychographic imaging [6]. In ptychography, a beam is focused on a small specimen area, and this beam is then stepped over the surface of the sample in a tight raster, while a camera behind the sample is recording the diffraction patterns from those specimen locations (Figure 2). Computer data

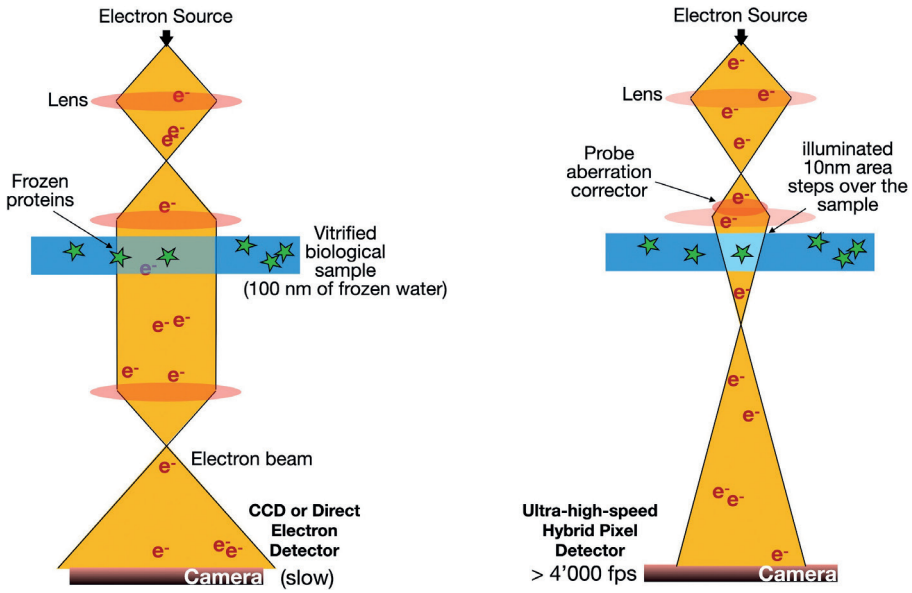

Fig. 2: The electron path in an electron microscope. Left: Conventional (real-space) imaging as it is used in cryo-EM. The beam creates a parallel illumination of micrometer diameter on the sample. A phase-contrast image is created on a camera with (slightly defocused) lenses underneath the sample. A typical exposure time is one second, but the camera may record a few dozen sub-frames during that second if used in movie-mode. Right: In convergent beam electron diffractive imaging, such as in ptychography, the electron beam forms a narrow probe of 10 nm diameter or less. After the sample, in principle no further lenses are required, and the pattern of electron diffraction from the sample is recorded with a hybrid pixel detector. Here, the beam steps over the sample surface at very high speed (e.g., hundreds of positions per second), and at each position the electron diffraction pattern is recorded.

analysis can then reconstruct the 2D or even 3D structure of the sample. In Switzerland, ptychography with an X-ray beam from a synchrotron is performed at the Paul Scherrer Institute [7,8].

Ptychography can also be done with an electron beam. In electron ptychography, the electron beam in the TEM is focused to a narrow beam that can be parallel or convergent. This electron "probe" of merely a few nanometers in diameter is stepped in a 2D raster over the sample, which diffracts the electrons. The diffraction patterns from each probe position are recorded with a 2D camera that is capable of accepting the very high beam intensity often found in the center of the diffraction pattern. This combination of a 2D stepping raster with a 2D detector for data collection in scanning transmission electron microscopy gives this method the name "4D STEM". Hybrid pixel detectors are ideal cameras for this purpose due to their high dynamic range and speed [9]. Recorded diffraction patterns correspond to a dataset in reciprocal space. The diffraction patterns have a high signal-to-noise ratio and offer in principle access to structural knowledge about the sample at a resolution much higher than any aperture-limited real-space imaging method. But the diffraction patterns only contain amplitude information about the specimen. The phase information about the specimen is missing and has to be obtained with additional methods.

The diffraction patterns recorded in the ptychography process still need to be combined with phase information in order to be able to determine the structure of the frozen proteins. This can be done computationally by one of various algorithms. The ptychography algorithm exploits the knowledge of the partially overlapping positions of the electron beam on the sample. An iterative algorithm cycles the recorded dataset between reciprocal (Fourier) space and real (Image) space via Fourier transforms, while applying constraints onto the dataset in the two spaces. In reciprocal space, a trivial constraint is to insist on the known amplitudes from the recorded diffraction patterns. In real space, a simple constraint is to average the image signal from image segments, at which two or more electron probes were overlapping. Additional constraints can be found and applied in both spaces. This hybrid input output algorithm [10,11] converges under suitable conditions to a solution that satisfies

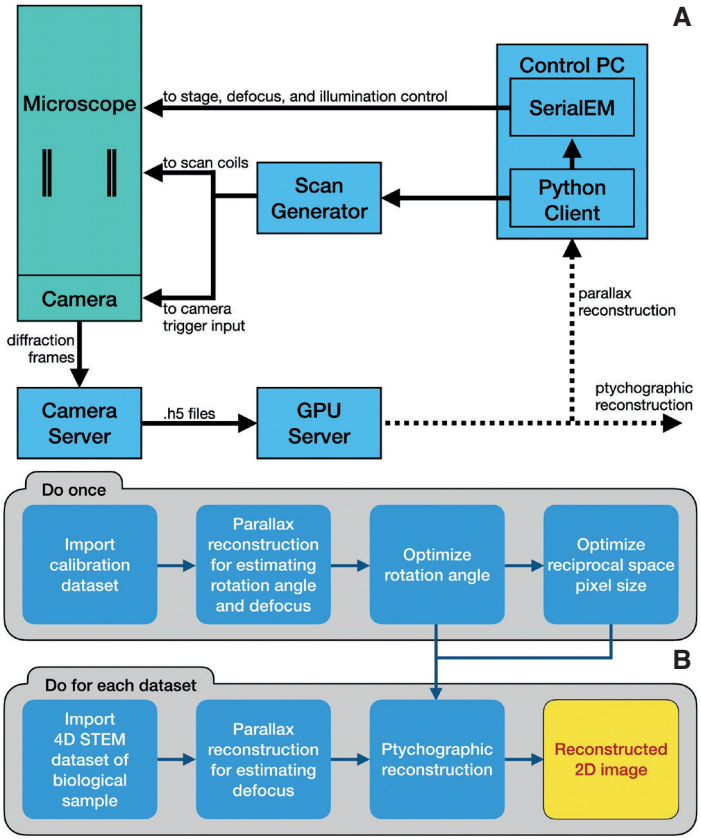

Fig. 3: The cryo-electron ptychography workflow. (A) Experimental setup. The pixelated camera is mounted below the electron microscope which collects the diffraction images. The microscope and an external scan generator are controlled by an PC. Once an acquisition is started, a signal is sent to the external scan generator, which controls the scan coils, which in turn move the electron beam across the sample in a specific 2D raster. For each beam position a trigger signal is sent to the camera. After acquisition, the images are moved to a GPU server for further processing and image feedback to the control PC. (B) Processing pipeline. For a given dataset one calibration image is acquired. This image is used to estimate beam parameters and determine the pixel size of the final images. The individually acquired datasets are processed to determine the defocus value for each, and then a final image is reconstructed, which is used for further processing in a conventional single particle cryo-EM image processing pipeline as shown in Figure 1.

all constraints and then not only delivers the high-resolution projection image of the sample, but also reconstructs the

shape and profile of the electron beam that was stepping over the sample.

In materials sciences, electron ptychography has been used with high electron doses, exposing the samples with millions of electrons per square Angstrom ($e^-/Å^2$) of specimen surface, so that the ptychography algorithm can produce highly contrasted and almost resolution-unlimited images of the sample. The Muller group, for example, studied a crystalline $PrScO_3$ sample by electron ptychography, reporting 0.16 Å resolution maps that are merely limited in resolution by atomic lattice vibrations [12].

For life sciences cryo-EM samples, only very low electron doses can be used, before the frozen water of the sample melts and the proteins are destroyed from the electron beam. When recording electron ptychography data of vitrified proteins, a dose budget of merely 100 $e^-/Å^2$ can be used. Current ptychography reconstruction algorithms were not designed for such low electron counts in the diffraction patterns, so that the data reconstruction algorithm is difficult to bring to a convergent solution. In practice, the low electron count is the factor that limits the achievable resolution in 4D STEM of biological specimens.

Cryo-electron ptychography of frozen hydrated specimens has been demonstrated by the groups of Wang and Kirkland, when studying vitrified virus-like particles with 4D STEM under low-dose conditions [13]. Their work showed for the first time the outstanding potential for contrast of the method, but left space for further resolution improvements. As discussed below, we have recently shown cryo-electron ptychography of frozen hydrated apoferritin samples, reaching 5.8 Å resolution [14].

There is plenty of room at the bottom to further improve the sensitivity of cryo-electron ptychography of beam sensitive life sciences specimens. Data collection schemes, dose fractionation, computational corrections for slowly accumulating electron beam damage and specimen drift, and additional algorithmic constraints during image processing, all still should be developed for or adapted to cryo-electron ptychography, in order to become able to harvest the full potential of this method also for life sciences samples.

Cryo-electron ptychography to study frozen proteins

We have developed a cryo-electron ptychography setup, based on a 300 kV Titan Krios transmission electron microscope that is equipped with a corrector for spherical lens aberrations (Cs-corrector) above the sample. This Cs-corrector enables the formation of a highly convergent electron beam that is nevertheless fully coherent. The focused electron beam can be stepped over the sample in a custom scan pattern with the help of a TVIPS Universal Scan Generator connected to the instrument, so that for example spiral scan patterns of the beam become possible. Diffraction data are recorded with a Dectris ELA detector at 4'500 frames per second. A much faster data collection will become possible with the Dectris ARINA detector, which in binned operation allows recording diffraction patterns at speeds up to 120'000 frames per second.

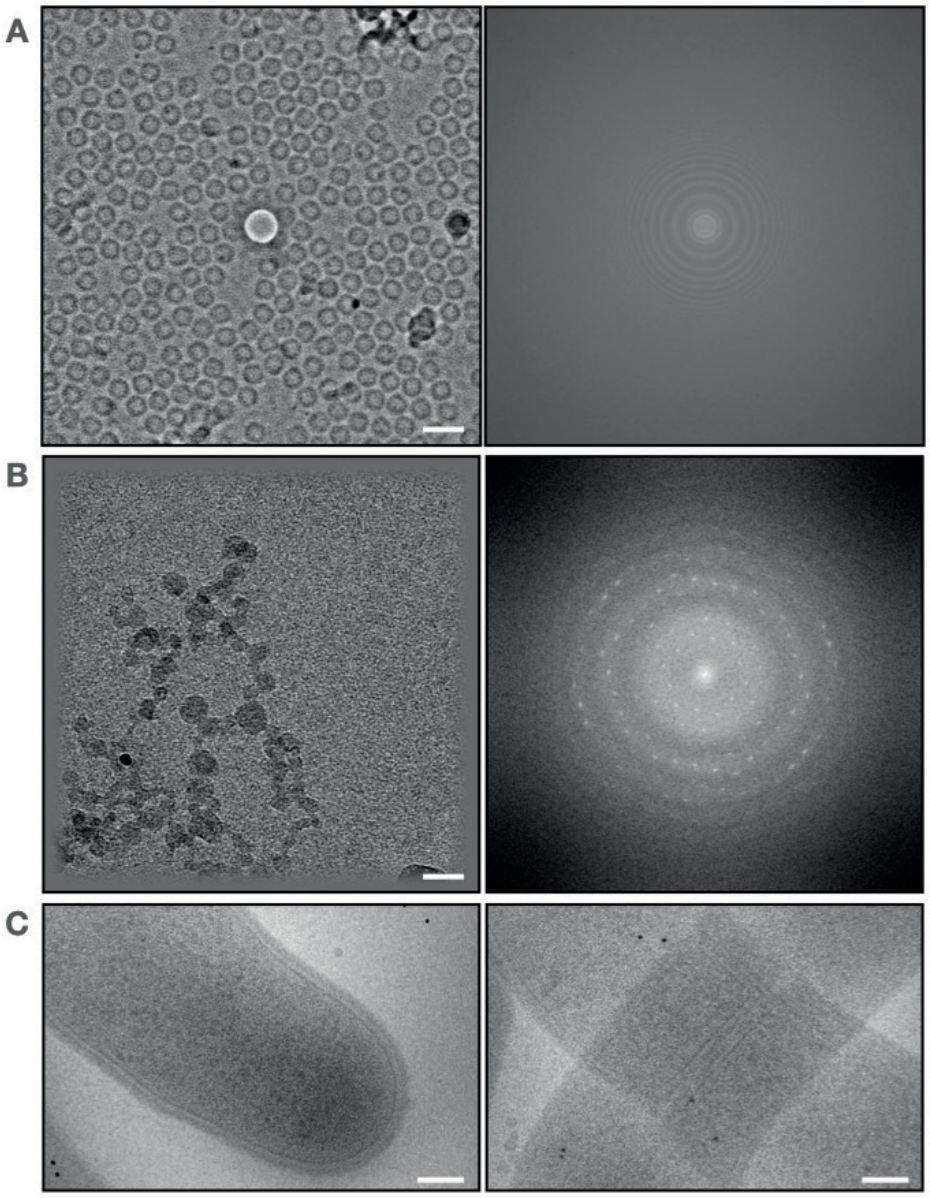

Fig. 4: 4D-STEM of biological cryo-EM specimens.
A, Left: Cryo-electron ptychography reconstruction frozen hydrated Apoferritin sample, allowing the 3D reconstruction at 5.8 Å resolution [14]. Scale bar: 20 nm. Right: Fourier transform of the image.
B, Left: Cryo-4D-STEM parallax reconstruction of a frozen hydrated bacteriorhodopsin 2D crystal sample. The real-space image does not reveal features to the naked eye. Scale bar: 10 nm. Right: The Fourier transform on the same image on the left shows diffraction spots beyond 1 nm resolution.
C, Cryo-electron ptychography image of frozen bacteria. The sample on the left is approximately 600 nanometer thick, the one on the right showing two bacteria overlaid on top of each other has twice that thickness. Both samples could not have been successfully imaged by conventional cryo-EM. Cryo-electron ptychography is especially promising when combined with tomographic acquisition schemes for imaging thicker samples in cell biology. Scale bars: 200 nm.

Recorded cryo-electron ptychography patterns are processed with a multi-GPU Linux computer, using the py4D-STEM software package [15], resulting in highly contrasted two-dimensional real-space images of the specimens (Figure 3).

In this work, we used cryo-electron ptychography to record data of frozen hydrated Apoferritin sample, using a convergence semi angle (CSA) of the electron illumination of 4 mrad, and obtaining a 3D map of the protein at 5.8 Å resolution (Figure 4). Further resolution improvement is expected from the ongoing implementation of collecting data under dose-fractionation, with a higher CSA, and recording much larger datasets with the help of automation.

Conclusion

Electron ptychography in materials sciences holds the world record in resolution. It is a variant of scanning transmission electron microscopy, by stepping the electron beam over the specimen in a 2D pattern, while for each position recording a 2D diffraction pattern, hence the name 4D STEM. We applied cryo-electron ptychography to vitrified protein particles, which allowed a medium-resolution 3D reconstruction of the proteins from a very low number of protein particles.

Further resolution improvements will require transferring several technologies to cryo-electron ptychography that have been developed for conventional cryo-EM, such as recording data under electron dose-fractionation and computationally accounting for accumulating beam effects in the data analysis. Further optimization of the data recording schemes, such as optimizing electron beam convergence angles, stepping patterns, beam defocus and diameter of the illuminated surface area, electron doses, and specimen geometries, promise to strongly boost the achievable resolution, so that the very high contrast of this method can be exploited to its fullest extent.

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Massimo Kube is a PostDoc at the EPFL in LBEM, contributing to the development of 4D-STEM in cryo-EM. He studied biophysics at the Technical University of Munich (TUM) and performed his PhD research at the TUM, studying DNA origami by cryo-electron microscopy.

Henning Stahlberg is a Professor in Physics at the EPFL in LBEM, and Adj. Professor in the Faculty of Biology and Medicine of UNIL. He has studied physics at the Technical University Berlin, performed his PhD research in the Dubochet and Vogel laboratories at UNIL and EPFL, obtained his Habilitation in the Biozentrum of Uni Basel under Prof. Andreas Engel, and has been a biology professor at UC Davis (2003-2009, CA, USA) and at the University of Basel (2009-2020). Since 2020, he is heading the LBEM in Lausanne, where his lab studies neurodegeneration and advances cryo-EM methods.

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