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Single electron imaging vs. coherent electron beam diffraction: Optimization of image contrast in cryo-EM

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Cryo-transmission electron microscopy (cryo-EM) of frozen hydrated specimens is an efficient method for the structural analysis of purified biological molecules [1]. In cryo-EM, thin preparations of free-standing 100 nm thin layers of vitrified sample solution are imaged at liquid nitrogen temperature with the electron microscope to record projection images of the samples. These images have a very low signal-to-noise ratio (SNR), so that only protein particles larger than a certain size, typically > 50 kDa weight, can be localized in the foggy images. If imaged particles are sufficiently large and homogeneous in their shape and conformation, then computer image processing can be used to combine the image signal from hundreds of thousands of "single particles" into a three-dimensional (3D) reconstruction. With suitable protein preparations, this method can reveal the 3D structure of the protein at resolutions reaching 1.1 Å, so that the positions of almost all atoms in the protein can be determined in 3D [2]. This method does not require any prior sample-altering preparation, such as crystallization or fixation or labeling. It reports the structure of the proteins in (vitrified) water, therefore under near-native conditions. Cryo-EM was recognized by the Nobel prize in Chemistry in 2017 [3], awarded to Henderson, Frank and Dubochet. Jacques Dubochet is from Switzerland, his team had developed the freezing method that enabled the vitrification of biological specimens [1].

Cryo-electron tomography (cryo-ET) is an extension of cryo-EM. In cryo-ET, specific locations in frozen hydrated sections of biological tissue are imaged by cryo-EM, while tilting the sample to different tilt angles. Computer image processing later allows the reconstruction of the 3D structure of that tissue section at nanometer resolution. For this method to work, the tissue section must be thinner than a few hundred nm. Preparation of such thin "lamellae" of biological tissue is a very difficult task, which is currently being optimized in several laboratories [4]. A reconstructed 3D structure of a section from high-pressure frozen tissue can reveal the cellular structure at high resolution in its near-native state [5]. And if many identical particles of sufficient size are present in that tissue section, then cryo-ET combined with sub-volume averaging in a few ideal cases has already allowed the reconstruction of the high-resolution structure of those particles at amino-acid resolving resolution [6]. This method thereby revealed the atomic structure of those proteins directly within the cellular context.

Both methods, cryo-EM and cryo-ET, are limited by their very low SNR. Only the larger protein particles can be analyzed with these methods at highest resolution. The low SNR of conventional cryo-electron microscopy stems primarily from the sensitivity of the samples to the electron beam, forcing the operator to use only a very low number of electrons for imaging. The SNR is further limited by the image formation mechanisms in the electron microscope.

Progress in improving the SNR in cryo-EM is desperately needed. Intensive research is ongoing in several laboratories to find ways to optimize the thin samples, including attempts to reduce the primary electron beam damage onto the sample, or to boost the recovery of phase contrast signal from electrons that have interacted with the fragile sample. The primary beam damage can be reduced, if a suitable acceleration voltage for the electron beam is chosen [7].

An alternative strategy to reduce beam damage appears to be the stroboscopic imaging with single, isolated electrons that meet the sample at precise time points in at nanosecond periodicity. Such single electrons might cause less damage than an equally intensive stream of electrons that arrive at random time points. If an electron traverses the frozen specimen, it can interact with the specimen in various ways, including elastic or inelastic scattering. Electron scattering in the sample among many other effects can cause phonons in the samples, which have lifetimes significantly shorter than nanoseconds. It is not clear, what happens if more than one electron from the electron beam reaches the sample within that short time span. When considering de Broglie's wave nature of the electron beam, the continuous wave of a random-in-time arriving electron of a low energy spread and therefore longer coherence length might be different from that of one single electron. Stroboscopic single-electron illumination at nanosecond repetition rate might therefore have a less damaging effect on the sample than a random-in-time electron illumination, because each arriving electron would never encounter the specimen still in a phonon-excited state from the previous electron. Beam-damage reduction on materials sciences samples from such electron illumination has been reported [8–10]. We have implemented a 300 kV Titan Krios Transmission Electron Microscope that is equipped with a DrX.works RF cavity (Figure 1). This allows to send

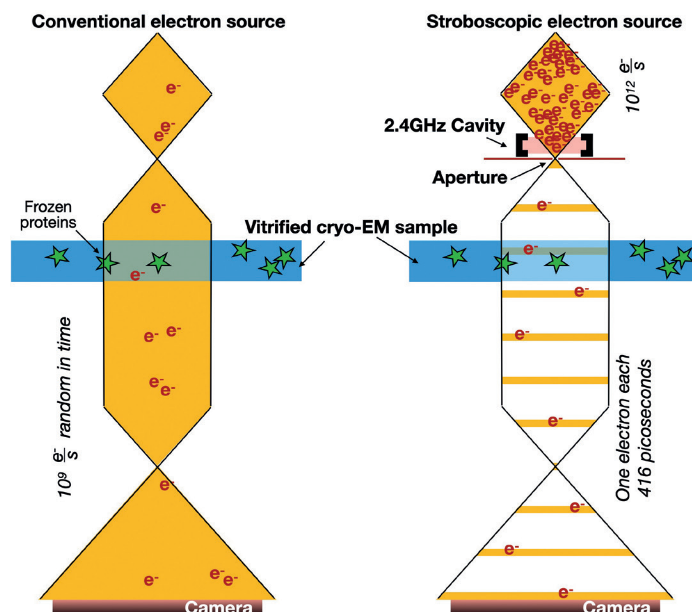

Figure 1: Random-in-time electron illumination vs. single electron illumination. Left: The simplified electron beam path in a conventional electron microscope employs electrons that reach the sample at unknown time points during the recording of the image. Right: A stroboscopic electron source can be realized with an RF cavity in the beam to chop the beam into precisely times pulses, each containing maximally one electron.

single electrons at 75 MHz repetition rate onto the sample. Preliminary experiments using electron diffraction on Paraffin 2D crystals suggest a 40% reduction in beam damage of single electrons vs. conventional random-in-time electron illumination, when all other imaging parameters such as dose rate, exposure time, acceleration voltage, diameter of the exposed specimen area, specimen temperature, and electron recording method remain the same. A clear explanation for this phenomena is still lacking.

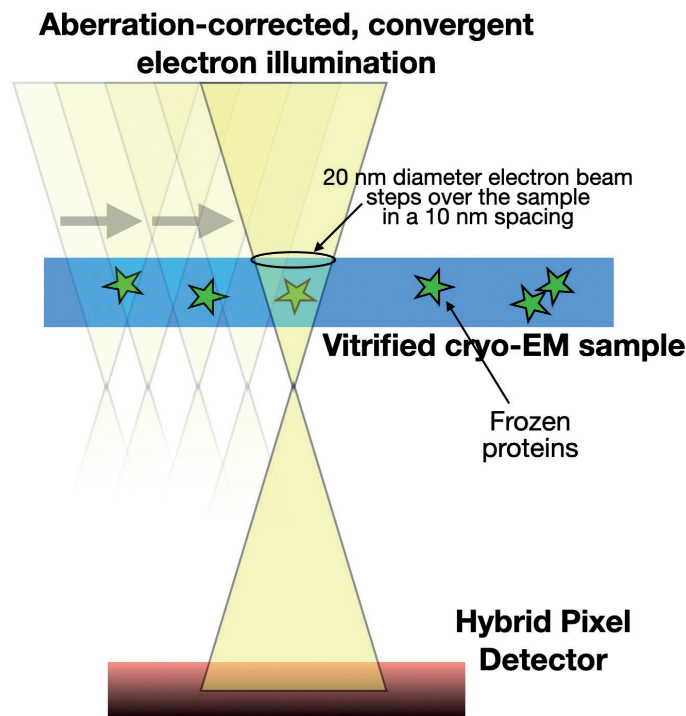

Figure 2: 4D-STEM data collection scheme: The focused electron beam steps over the sample in a 2D raster, while for each electron beam (probe) position, a 2D detector records the electron diffraction pattern.

Progress in improving the SNR in cryo-EM is also expected from optimizing the contrast transfer function (CTF) of the method. The CTF of the electron microscope describes how structural contrast in the sample is transferred into the recorded image. Cryo-EM conventionally offers a CTF that has only weak contrast for low-resolution features of the sample, making smaller particles invisible. This forces the microscope operator to choose between settings that either optimize image contrast or image resolution, but not both. An alternative approach is found in diffractive imaging [11]: When not recording (real-space) images with the electron microscope, but recording (reciprocal space) electron diffraction data, a very different CTF behavior of the method is appearing. Convergent beam electron diffraction, scanning the narrowly focused electron beam in a 2D raster over the cryo-EM sample, while recording for each beam position the 2D electron diffraction pattern (Figure 2), is a method that is also called “4D-STEM”. Such 4D-STEM data can be evaluated with computer algorithms to reconstruct the 2D projection image of the sample, or in some applications even the 3D structure of the sample from a single scan. Employed algorithms include iterative hybrid-input-output algorithms, ptychography algorithms, or direct tilt-corrected bright field / parallax reconstructions [12–15]. Each of these varies in its behavior with respect to CTF, SNR, dose efficiency, and capability of reaching high resolution. For dose-tolerant specimens, 4D-STEM at very high electron doses with electron ptychography analysis has by far surpassed the achievements of conventional transmission electron microscopy [12]. For frozen hydrated life sciences specimens, 4D-STEM is still in its infancy [2,13]. Nevertheless, Feynman’s famous phrase again applies to the currently ongoing revolution in electron microscopy: “There is plenty of room at the bottom” to boost image contrast and resolution in electron microscopy of life sciences specimens. These novel technologies, which still need to be transferred to cryo-EM and cryo-ET, are promising and exciting. Biology, medical research, and pharmaceutical applications stand ready to benefit from it.

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