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Review

Advances in electron microscopy: A qualitative view of instrumentation development for macromolecular imaging and tomography

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ABSTRACT

Macromolecular imaging and tomography of ice embedded samples has developed into a mature imaging technology, in structural biology today widely referred to simply as cryo electron microscopy.[1] While the pioneers of the technique struggled with ill-suited instruments, state-of-the-art cryo microscopes are now readily available and an increasing number of groups are producing excellent high-resolution structural data of macromolecular complexes, of cellular organelles, or the morphology of whole cells. Instrumentation developers, however, are offering yet more novel electron optical devices, such as energy filters and monochromators, aberration correctors or physical phase plates. Here we discuss how current instrumentation has already changed cryo EM, and how newly available instrumentation – often developed in other fields of electron microscopy – may further develop the use and applicability of cryo EM to the imaging of single isolated macromolecules of smaller size or molecules embedded in a crowded cellular environment.

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Introduction

The number of published structural cryo EM single particle and tomography studies with high-resolution molecular models of macromolecular complexes together with the number of reviews discussing the important role of cryo EM in structural biology clearly shows that this technology has finally come of age. More and more work is presented at a level of resolution and functional detail which has in the past only been expected from protein crystallographic studies. Dynamic studies [1] and the ability to analyse heterogeneous data sets [2,3] has pushed the technique forward and has established microscopy as standard tool for structural studies.

Looking at the early history of TEM its importance has always been recognised, but it was also obvious, that materials science-centred research based its activities at a much larger scale on imaging methods. It appears to be easier to image single atoms in their solid state environment than in a suspension of soft matter in water. Even though it took the materials science community still almost 60 years to finally visualise individual atoms at a resolution better than 1 Å [4]. Thus in our biomedical field, with a very beam sensitive sample, it is not surprising, that the way from early negative stain studies [5] to first 3D models [6], a first real molecular 3D model in a 2D crystal [7], to quasi molecular models of biopolymers [8–10] (the author apologizes for this very incomplete and subjective selection of examples) to the newest studies with highest spatial resolution we will discuss here have taken even longer – and are still behind in resolution, maybe always will be.

The scope of review is to take a step back and to discuss how this progress has been coupled to technical innovation and advances in electron optical engineering. While researchers in earlier studies had to use ill suited instruments, today we have assembled enough know-how to define our state-of-the-art instrument. This does not mean, however, that development of new technologies will end, but the discussion here intends to help defining the decisive stepping stones of the recent years and to point out novel ideas, which could help to improve cryo EM even more.

The early years of cryo-microscopy and initial success

When Henderson and co-workers [7] or Unwin [11] published their membrane protein models the microscopes they used provided unique technologies for obtaining high-resolution images. Both groups used liquid Helium temperature stages as it had been shown, that lowering sample temperature somehow preserved sample structure. It was common to record a huge number of

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[1] In the following I will strictly use the phrases 'cryo electron microscopy' and 'cryo electron tomography', even though I am well aware that there are no 'cryo electrons', and also that other authors use a different wording. Terms and abbreviations such as EM and ET have been in use for decades and it seemed to me more natural to add 'cryo' than to ignore the historically accurate ancestry of the techniques.

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micrographs on sheet film material and to select only the few best images. No automated microscope operation was available and computing workflows were adjusted to the computing power at hand. Many obstacles had been defined, such as e.g. charging [12,13] or the lack of high-sensitivity online-detectors. But with time such problems were solved and even cryo ET on whole cells was finally achieved [14].

In hindsight one has to ask now, which of the steps taken in such studies have really been decisive? One example is the sample temperature: The most recent studies reached highest resolution without liquid Helium stages, in general very few groups are still working at liquid Helium temperatures. Was it – maybe – more important that such microscopes and sample stages showed less vibrations and drift, simply since their design for liquid Helium asked for less coupling to the outside environment? And thus the microscope got so stable, that any resolution damping mechanical vibration was eliminated?

Driven by the needs in materials science we have seen better and better microscopes being commercially available now, and today's state-of-the-art cryo TEMs comprise a basic liquid nitrogen temperature instrument, which is capable of imaging in a typical resolution range of better than 1.5–2 Å, very stable stages with very little drift, high brightness field emission guns, which allow a very coherent illumination of large fields of view, low hysteresis electron optics, which allows rapid switching between imaging modes and thus a high level of microscope automation, and reasonable online image recording by very efficient CCD cameras. Any institution being seriously interested in setting up a functioning cryo EM lab can today acquire such a basic instrument.

It should be noted, however, that to obtain the excellent results seen more and more often published now, more technology than just a basic microscope is needed. Here we will not discuss sample preparation in detail nor any of the modern image processing approaches, without those none of the high-resolution results would have happened. Instead we will focus on a few novel technologies, which are about to add momentum to cryo EM or have started to have a large impact already.

The game changers for high-resolution imaging of cryo samples

The breakthrough of cryo electron microscopy in structural biology has been strongly related to major advances in software development for microscope control and automation and also to advances in electron image recording.

From the discussion of the historical development of cryo EM it is apparent that low-dose image recording is one key element for high resolution structural imaging. Therefore online image recording, such as was first realised with TV – and later with CCD – cameras, allowed computer-controlled alignment and set-up of the microscope for optimal imaging conditions at minimum cost of electron dose. Additional automation of the recording process today allows the collection of large datasets at relative ease of use. Examples of such software suites are LEGINON [15], TOM Toolbox [16], or several commercially available packages.

Another key to success were advances in the processing of the recorded images and their 3-D reconstruction, in particular novel mathematical concepts combined with the availability of large computing power (for reviews on these topics we like to cross reference to other articles in this volume).

However, what improved the resulting 3-D reconstructions most in the last few years was the development of direct electron detection (DED) devices. Combined with stable high-resolution TEMs, typically at liquid nitrogen temperature, DED cameras provide raw image data with typical intrinsic imaging resolution of better than 3 Å. In many cases such raw data have then been processed to about 3–4 Å resolution [17,10].

DEDs make use of advanced and radiation hardy semiconductor circuit technology. As detection signal they collect directly secondary charges produced as electron–hole pairs by the incident imaging electron (cf Fig. 1). These secondary charges are then collected in confined electronic circuits defining individual image pixels. This direct detection mechanism eliminates electron-to-photon conversion steps, which usually add noise and thus decrease detection quantum efficiency (DQE). In fact, the detection mechanism is more equal to the process in negative sheet film, where energy deposited in the emulsion also directly initiates the chemical detection process.

As is indicated in Fig. 1b unwanted detection events are those, which are derived from incident electrons scattered to the side or even upwards to the top detection layer. Such backscattering events blur the localization of the detected electron over many pixels and result in a drop of the modulation transfer function (MTF), i.e. a loss in recordable spatial resolution. While DEDs are already thin compared to conventional detectors (Fig. 1a) the MTF of DEDs can be further improved by making the supporting layer as thin as possible. Back-thinning (as marked in Fig. 1b, [18]) reduces the probability of backscattered electrons and thus improves the MTF further. Comprehensive discussion of the detection properties for different designs of DEDs were recently published by a number of groups [19,20].

The decisive difference between negative sheet film and direct electron detection is the very short detection time realised by DEDs. Frame rates for images of up to 4K×4K pixels are in the order of ten to hundreds of frames per second (fps), at a current maximum of 400 fps in rolling shutter mode, (GATAN Inc., FEI Inc., Direct Electron Inc., [19]).

This movie detection can now be used to analyse specimen movement frame by frame. Such studies have been published recently [3,17,21,22], and they all document an extensive movement of the recorded objects. Fig. 2 illustrates nicely such a study and how sample movement and the effect of its correction are reflected in the Power spectra of the recordings [20] and what astonishing result can be achieved when correcting the data (Fig. 3, [21]).

At this point we do not have a conclusive physical model for the observed movement, which is often not uniform, even within a small field of view. Reference [3] describes the example of ribosome movement in the embedding ice layer, illustrating a seemingly chaotic behaviour. In addition it has been discussed that the observed object drift in the images could also result from a vertical movement of the embedding ice layer in the direction of the incident beam [21]. In contrast to these findings recent work by Russo and Passmore (Fig. 4, [23]), reduced sample movement considerably simply by replacing carbon film by a 50 nm thick gold film with regular holes. Further experiments may be needed to clarify the physical mechanism for this remedy, most likely explanations could be a different charge balance for carbon film vs. gold film, as secondary electron yield will certainly be different for the different materials.

There is no question, that movie recording and movie processing has resulted in a "revolutionary" increase of obtained structural resolution [24]. At this point we may therefore conclude that the combination of novel sample support and movie recording will be the new standard for sample preparation and data recording.

Together with the state-of-the-art cryo TEM we therefore have an excellent technology for a wide range of macromolecules and macromolecular complexes. It remains to be seen in future studies, how far down in molecule size one can go now, when sample movement is directly suppressed and thus alignment and signal integration of individual DED frames should be further improved.

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