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Progress towards an optimal specimen support for electron cryomicroscopy

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The physical principles of electron–specimen interaction govern the design of specimen supports for electron cryomicroscopy (cryo-EM). Supports are constructed to suspend biological samples within the vacuum of the electron microscope in a way that maximises image contrast. Although the problem of specimen motion during imaging has been known since cryo-EM was first developed, the role of the support in this movement has only been recently identified. Here we review the key technological advances in specimen supports for cryo-EM. This includes the use of graphene as a surface for the adsorption of proteins and the design of an ultrastable, all-gold substrate that reduces the motion of molecules during electron irradiation. We discuss the implications of these and other recent improvements in specimen supports on resolution, and place them in the context of important developments in structure determination by cryo-EM.

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Introduction

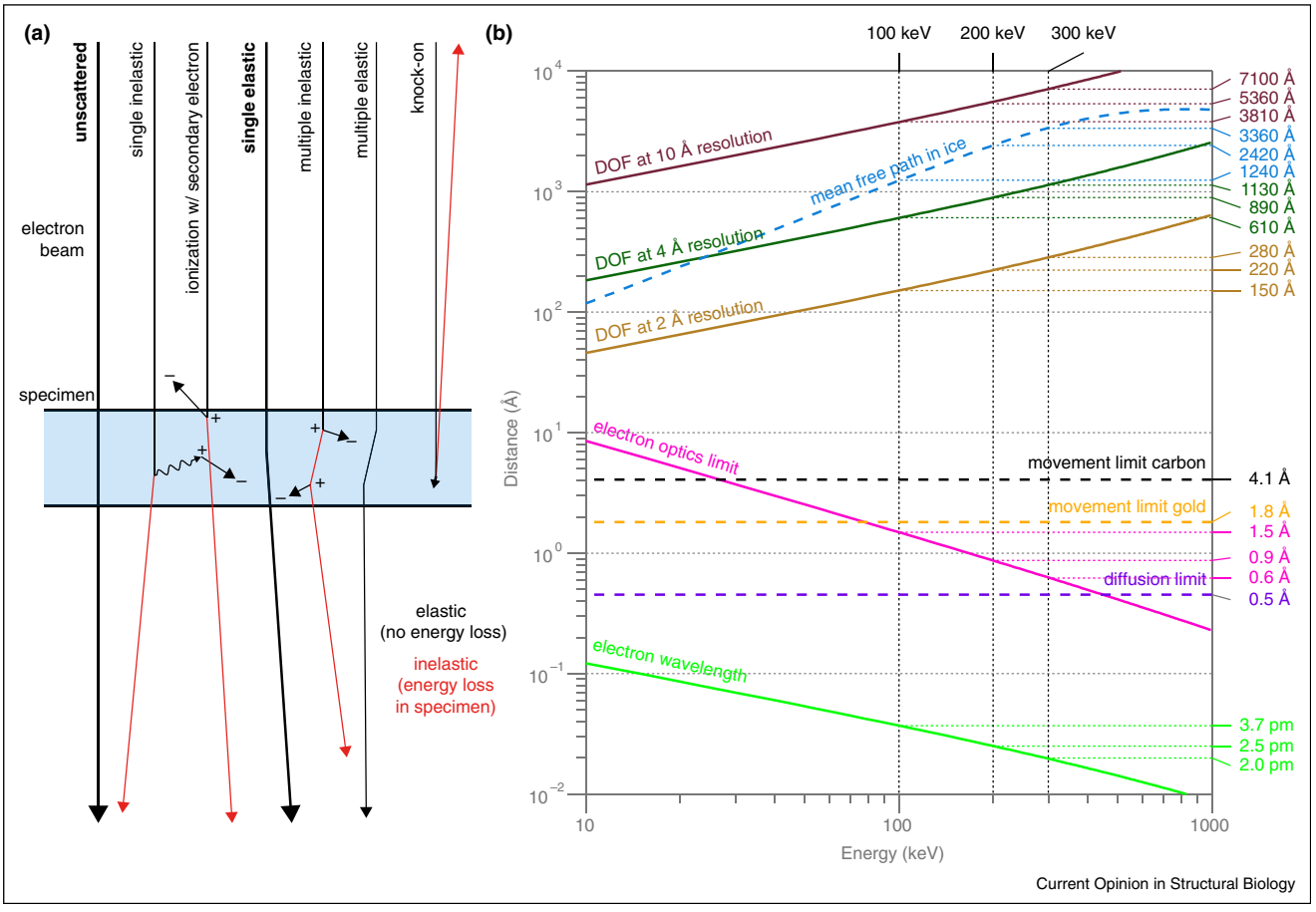
After important studies of the damage caused by high-energy electrons to biological specimens [1,2] and development of methods to compute 3D density maps from 2D projection images [3–5], the key technological advance that underpins the field of cryo-EM is the vitrification of water [6••]. Vitrification rapidly freezes proteins in thin layers of water ice, thus preserving their structures in a native environment for imaging. The device most often used to support thin layers of ice comprises an amorphous carbon foil suspended across a metal mesh grid [6••]. The carbon foil is perforated with holes of order one micrometer in diameter. Biological specimens suspended across the holes are frozen such that the water surrounding them enters an amorphous solid phase, nearly identical to motionless liquid water, which preserves the arrangement of the molecules as they were just before freezing [7••].

When irradiated with the electron beam, vitrified biological specimens move and build up semi-static charge long before they are destroyed by the high energy electrons; this blurs the micrographs and limits their resolution. Although this movement has been known since the early days of cryo-EM and many previous studies contributed to understanding its origin [8••,9–14,15•], it was only with the recent advent of direct electron detectors that we have been able to quantify specimen movement with sufficient accuracy to begin to delineate the physical basis of radiation-induced movement [16•,17•,18,19,20•]. This has revealed that much of the movement is due to the support itself [21••]. In this review, we discuss the physical requirements of cryo-EM specimens and consider how supports have improved since Dubochet and colleagues first demonstrated vitrification. This technological progress has, and will continue to facilitate faster and easier data collection and higher resolution images.

Physical requirements of cryo-EM specimens

The interactions of high-energy electrons with solid materials govern specimen design for transmission electron microscopy (EM). The theory of electron specimen interaction [29] was established long before the technology to prepare native biological specimens was developed [7••]. Since phase contrast is the imaging mechanism that provides the most information from the sample [24••,30], specimens for single particle cryo-EM must be designed to maximise this form of contrast. Specimen design centres on minimising the deleterious effects of inelastic and multiple scattering, which do not contribute to phase contrast and cause damage to the specimen (inelastic), while preserving the elastic and unscattered electrons for the generation of phase contrast (Figure 1). Specimens must be thin because electrons cannot traverse materials that are much thicker than the mean free path of the electron in ice, and the thicker the specimen, the more inelastic and multiple scattering effects will degrade image quality. As shown in Figure 1, the mean free path of electrons in water ice is a few tenths of a micrometer, and increases with energy; it saturates at around 1 MeV due to relativistic effects as the electron approaches the speed of light. Specimen thickness is also limited by the depth of field in the image, which increases with energy (Figure 1). For single-particle EM and electron cryotomography, this limits specimen thickness to less

Figure 1



Physical constraints on specimen design in cryo-EM. Diagram **(a)** of high-energy electron scattering in a thin layer of ice, with types of events shown in order of decreasing probability from left to right. Only the unscattered and single elastic scattering events (bold) contribute to typical phase contrast imaging; the remainder damage the specimen (inelastic) or contribute noise to the image. The relative probability of these events is described by their scattering cross sections, whose sum is closely related to the total mean free path, shown in **(b)**. Several other physical parameters that constrain specimen design in cryo-EM are plotted versus energy in (b). Unlike for light microscopy, neither the electron wavelength (light green line) nor the lens optics (pink line, chromatic aberration) limit resolution. Instead, specimen movement during imaging (black dashed line, information limit for moving particles without motion correction on Quantifoil supports) and information content in the individual images limits practical resolution. High-speed detectors can be used to compensate for specimen motion (to move below black dashed line) and new supports reduce movement (gold dashed line, information limit on all-gold grids). Cryo-EM is now starting to approach the information limits imposed by the optics of the microscope (pink line) and the diffusion of the particles within the vitrified ice (purple dashed line, 1 MDa particles). The thickness of the specimen is limited by the total mean free path in ice (blue dashed line), and the depth of field (DOF) at a particular resolution caused by curvature of the Ewald sphere. Theory after [21••,22,23,24••,25–28]; see Appendix A.

than a micrometer, and for high-resolution even thinner: about 300 Å thick for 2 Å resolution at 300 keV.

Specimens must also be thin for vitrification: water must be cooled to cryogenic temperatures in less than a millisecond to stop the molecules from forming crystals [7••]. At atmospheric pressure this requires a thin layer of liquid that is less than about three micrometers thick. Any thicker, and the thermal conductivity of the water itself will prevent the water from cooling fast enough to enter the amorphous phase. The instability of thin aqueous layers still presents challenges to reliable sample preparation [31].

Since the specimen is damaged by inelastically scattered electrons at a rate that is faster than it is imaged by the elastically scattered ones [24••], it was essential to develop low-dose techniques and supports that minimise irradiation of the specimen. Unfortunately, while low-dose imaging circumvents the fundamental limit of damage to the specimen, it comes at a price: (a) the images become noisy because there are not enough electrons in the image to resolve high resolution features ($\sim$1000 $e^-/Å^2$ are required for atomic resolution but $\sim$10 $e-/Å^2$ destroy the specimen) and (b) when a specimen is first irradiated, it moves (4 Å or more in the first $\sim$10 $e^-/Å^2$ according to our