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Basics of Biological Cryo-TEM

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There is a wide variety of techniques in TEM that involve low-temperature preparation, but do not require that the specimen be examined in the TEM at low temperature. The discussion here relates only to techniques that involve frozen-hydrated specimens, which must be kept below about -138°C at all times. The advantage of frozen-hydrated specimens is that they offer a means to study ultrastructure without chemical fixatives, dehydration, or staining. The disadvantages are that the specimens are easily damaged by electron irradiation, and have low contrast.

The water in frozen-hydrated specimens must be in "vitreous" (glass-like) form. If ice crystals are formed, the ultrastructure is disrupted. Fortunately, it was discovered that when an EM grid carrying a thin layer of an aqueous solution is plunged into a suitable cryogen, the water freezes rapidly enough to produce the vitreous state [1]. The maximum specimen thickness for the plunging technique is about 1µm. The method is used to freeze small cells, organelles, and macromolecules. The cryogen of choice is liquid ethane, cooled by liquid nitrogen. The desired water layer thickness is achieved by blotting the grid after application of a drop of solution. Filter paper type, blotting time, and humidity of the air surrounding the grid, all affect the thickness of the frozen water layer..

When tissue or large cells need to be examined, high-pressure freezing [2] is followed by cryo-ultramicrotomy [3]. Slam freezing [4] is a less-expensive alternative to high-pressure freezing, but it is rarely used today because the volume of distortion-free good freezing is only several micrometers deep, compared to at least 100 µm deep for high-pressure freezing. After freezing, the specimen is transferred under liquid nitrogen to the microtome, and sections are cut at -160 to -140°C. The specimen must remain below about -138°C, otherwise devitrification will occur and ice crystals will damage the ultrastructure. Cryo-ultramicrotomes and cryo-diamond knives have reached an advanced state of development, and sectioning is not particularly difficult, although collecting the "dry" sections and firmly mounting them on grids is still a challenge [5].

Grids with frozen-hydrated specimens must be transferred to the TEM without frosting, and kept cold, and without contamination, in the EM. A cryo-transfer specimen holder is required. The distinguishing feature of a cryo-transfer holder is a moveable shutter that covers the specimen. The shutter protects the specimen from frost that forms during the brief time the cold specimen holder passes through room-temperature air. In a special workstation, the grid is loaded into the specimen holder under liquid nitrogen or cold nitrogen gas. The shutter is then closed. The workstation is brought to the TEM, and the specimen is quickly removed from the workstation and put into the EM airlock. After pre-pumping, which often takes longer than for a room-temperature holder, the holder is moved into place in the EM. After about 20 min, the EM vacuum and specimen temperature will stabilize, and the shutter may be opened. Water vapor slowly sublimates from the specimen in the EM, and gasses may evolve during prolonged or intense electron irradiation. An anticontaminator that is colder than the specimen is needed, so that water vapor, and any other contaminants in the EM column, do not condense on the specimen. In some TEMs, an accessory dual-blade anticontaminator must supplement or replace the standard anticontaminator.

Radiation damage is an important issue with frozen-hydrated specimens, so the electron dose must be known. The best way to determine the dose is to measure the total incident beam current with a Faraday cage, in order to calibrate the screen current (exposure) meter, CCD camera, or photographic film. Some room-temperature specimen holders have a built-in Faraday cage. An alternative is to use sensitivity data from the film manufacturer, if data for the accelerating voltage to be used is available. Images should be recorded with minimal pre-irradiation. Surveying the grid at low magnification is usually unavoidable, but critical high-magnification focusing must take place adjacent to--not at--the area to be photographed. Most TEMs have built-in "low dose" software to quickly access a sequence of preset conditions for searching, focusing, and recording the image. For electron tomography, EM control software combines the low-dose sequence with automatic tilting, specimen tracking, and re-focusing [6]. Automated tomography requires a CCD camera, since image data is needed for focus and tracking. For single cryo-EM images, an electron dose of 10-30$e^-/Å^2$ is commonly used. For the highest resolution, the dose must be kept to the lower end of this range. For electron tomography, the total dose of all the images in a tilt series must be kept to between 30 and 100$e^-/Å^2$, so the dose for each image will be 1 $e^-/Å^2$ or less.

Because they lack heavy metal stains, frozen-hydrated specimens are imaged using phase contrast, which is normally generated by strongly underfocusing the objective lens. It is important to maximize the signal-to-noise ratio, since the electron dose needs to be kept as low as possible. Thus, for tomography, the underfocus value should be set to give maximum contrast at the resolution expected for the experiment at hand [7]. Zero-loss energy filtering is especially useful for maximizing the signal-to-noise ratio, since it removes inelastically scattered electrons that add blur to the image [8]. For 3-D reconstruction, the resolution can be roughly estimated from $d=\pi D/N$, where d is resolution in nm, D is the size of an object in nm, and N is the number of evenly-spaced views (over a 180-degree angular range) that contribute to the reconstruction [9]. In tomography, D can be roughly equated with the thickness of the specimen. In tomography, this gives a best-case value of resolution, so use of an underfocus value that emphasizes a higher resolution would needlessly reduce the contrast and thus require excess electron dose. Since in tomography around 100 views must be recorded from the same object, electron dose sets the ultimate resolution limit, because camera sensitivity limits the minimum dose per image [7]. In single-particle reconstruction [10], all particles may be assumed to be identical and randomly oriented, thus each particle need only be photographed once, and a relatively higher dose may be used for each image. The resolution is much higher than in tomography, since views of thousands of particles are combined in the reconstruction. [11]

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