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Specimen Preparation for Electron Diffraction of Thin Crystals

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Abstract

Electron crystallography has become a powerful approach for structural characterization of two-dimensional (2-D) protein crystals. The crystallographic approach provides the simplest route to the type of averaging that is essential for obtaining high resolution structural information from radiation-sensitive samples such as organic molecules. Several atomic or near atomic resolution protein structures have been solved by using cryo-electron crystallography, and most of them involved using both image and electron diffraction data. An essential step in either type of work is preparation of specimens suitable for electron microscopy which retain their native state and high degree of order. Methods for preserving samples in a near-native, hydrated state have been developed, with minor variations for different specimens. The major challenge of collecting electron diffraction data particularly at high tilt angle is the blurring of diffraction spots due to imperfect flatness of the crystals. This paper discusses specimen preparation methods for electron crystallographic data collection of 2-D protein crystals with particular emphasis on the factors which affect the flatness of crystals. We also discuss some of the aspects of the data collection protocols which are particular to work with crystals.

Keywords

electron crystallography; diffraction; specimen preparation; protein structure

1. Introduction

Electron diffraction has played an important role in the structural characterization of inorganic and organic thin crystals since the Davisson-Germer experiments in 1927 (Davisson and Germer, 1927). Numerous applications with small organic molecules have been described, where the small unit cells allow working with small crystals and computational methods have been developed that allow reliable structure determination directly from diffraction data (Dorset, 1995). Application to protein structures has proven to be more demanding for a number of reasons. Much of the methodology for processing and combining image data to obtain three dimensional (3-D) structures by electron crystallography was worked out in moderate-resolution studies of stained specimens such as bacterial S-layers (Amos et al., 1982). Henderson and Unwin obtained the first 3-D electron microscopic structure of an unstained membrane protein by electron crystallography, which for the first time visualized the membrane-traversing alpha-helices in bacteriorhodopsin (bR) (Henderson and Unwin, 1975). This breakthrough success required development of glucose-embedding to preserve hydration, refinement of low dose imaging techniques, and extension of diffraction data processing methodology from x-ray crystallography. The use of cryo-technology and development of lattice “unbending” to correct images for distortion of the crystal lattice image led to extension of resolution to 3.5 Å in projection images of bR

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(Henderson et al., 1986). Subsequently, the resolution was improved in three dimensions to the point where an atomic model of bR could be constructed—the first atomic structure of a protein solved by electron crystallography (Henderson et al., 1990). Since then more atomic or near atomic resolution protein crystal structures have been solved by electron crystallography, including the plant light harvesting complex (Kühlbrandt et al., 1994), tubulin (Nogales et al., 1998; Lowe et al., 2001), aquaporins (Murata et al., 2000; Ren et al., 2001; Gonen et al., 2004; Gonen et al., 2005; Hiroaki et al., 2006) and other membrane proteins (Holm et al., 2006; Jegerschold et al., 2008). The most stunning example is the 1.9 Å resolution structure of the aquaporin AQP0, which provided a detailed visualization of nonspecific lipid-protein interactions as well as the highest resolution achieved to date in a protein structure by electron crystallography (Gonen et al., 2005). Continuing developments in specimen preparation have contributed substantially to the evolution and progress in the field.

In the context of crystallography, the resolution of the data or resultant structure is generally considered to be the highest resolution for which there is significant data, i.e. the highest resolution diffraction spot. This can be a substantial overestimate of the actual resolution. However, experience has shown that with a nominal resolution of about 3.5 Å there is sufficient definition in the density map to allow reliable *de novo* chain tracing, structure building and refinement. Obtaining data at this level requires optimized conditions for both crystallization and specimen preparation. Because monolayer crystals are so flexible and delicate, these issues can present serious limitations. As described below, methods have been developed to overcome these limitations.

One of the most significant advantages of electron crystallography is the ability to obtain structure factor phases directly from images. The Fourier transform of an image provides the complex values—amplitude and phase—for a plane in reciprocal space at an angle corresponding to the crystal orientation. However, image data are subject to several influences that often degrade quality. Specimen movement along with the focus-dependent contrast transfer and envelope functions of the electron microscope can alter both the amplitude and phase. To some extent these effects can be systematically compensated. Electron diffraction patterns, though, are not affected by these factors, and they usually can provide higher resolution and more accurate amplitudes than images. Good diffraction is in fact generally a prerequisite for good images, with the exception that it is possible to obtain good image data from crystals that are too small to produce good diffraction patterns. All the atomic and near-atomic resolution electron crystallographic structures resolved to date have used data from electron diffraction patterns in addition to the images.

Electron crystallography studies of 2-D crystals require imaging or diffraction data collection at tilt angles up to 60 or even 70° in order to achieve sufficient resolution in the direction perpendicular to the crystal plane. The productivity of good diffraction patterns at high tilt angle, i.e. the fraction which show high resolution spots along every direction when the specimen is highly tilted, is often very low due to factors that cause imperfect flatness of the crystals. The success rate for obtaining good images is even lower, due to additional problems with specimen and image movement. In fact, the most challenging part in electron crystallographic data collection is the preparation of samples that are sufficiently flat to diffract well at high tilt angles. When the grid is highly tilted, the resolution of the reflections perpendicular to the tilt axis is frequently much lower than that along the tilt axis, due to the non-flatness of the crystal. When recording images, beam- or charging-induced specimen and image movements can cause even further data degradation. By careful materials selection and specimen preparation, these problems can be substantially mitigated and the productivity can be greatly improved. Here we discuss methods that have led to successful specimen preparation for electron diffraction and imaging experiments.

2. Specimen preparation for electron crystallographic data collection

For electron crystallographic studies of 2-D protein crystals, the most difficult part is to collect data at high tilt angles. The diffraction patterns are often quite symmetric with high resolution spots in all directions for untilted crystals, but the sharpness of the spots from highly tilted crystals tends to decrease in the direction perpendicular to the tilt axis. This loss of diffraction quality is a major limitation for obtaining high resolution structure of 2-D protein crystals by electron crystallography. The effect is attributable to variations of tilt within the crystal. Figure 1 illustrates the geometric cause of blurring, which can increase rapidly as the tilt angle increases. The diffraction spots will appear at a radius corresponding to g_1 in fig. 1 when the crystal tilt angle is θ , of a radius of g_2 when the angle is $\theta + \delta$. Variation of tilt from θ to $\theta + \delta$ causes the spot to be blurred from g_1 to g_2 . For small tilts, where the magnitude of g varies only slowly with θ , there is little effect from non-flatness. However, at high tilts, the demands for flatness are actually quite stringent. For a crystal with a unit cell around 50 Å tilted to 60°, variation of the tilt angle of 1° over an distance of 1 µm will result in diffraction spots blurring together at about 4 Å resolution, and would cause significant loss of signal in images and diffraction patterns at much lower resolution (Glaeser et al., 1991). It is easy to show that for such a crystal thermal energy would produce bending with a radius of curvature around 3.5 µm, corresponding to a variation of angle greater than 15°. Thus, there is a natural tendency of protein crystals to deviate from flatness far in excess of what is tolerable, and it is essential to overcome this tendency.

Figure 2 illustrates some of the characteristics of diffraction patterns frequently encountered with monolayer protein crystals. Figures 2a and 2b are from a nominally untilted crystal of tubulin, and show good diffraction with measurable intensities to at least 3.5 Å resolution. The pattern in fig. 2c is from a highly tilted crystal, and although the intensities could be determined accurately for spots along the tilt axis, in the direction perpendicular to the axis there is an undesirable amount of blurring and loss of intensity.

Specimen flatness has been found to be dependent on a number of factors including roughness of the film supporting the crystals as well as how the crystals contact the support film. In some cases it may be preferable to avoid contact with a support film and prepare crystals in vitreous ice on a holey film. While this approach has met with some success, it appears to be even more difficult to obtain sufficient flatness, presumably because the crystals are more free to flex under thermal excitation than when associated with a flat support. To make well preserved and perfectly flat crystals on cryo-grids, factors including support films, grid material, embedding medium, and embedding method need to be considered.

2.1 Embedding medium

Electron microscopic structural study of proteins requires the specimen to be preserved in a way that preserves the native and hydrated state during observation in the high vacuum of the microscope. Several methods have been developed to achieve proper preservation, such as freezing in buffer and keeping the specimen at low temperature (Taylor and Glaeser, 1974), or embedding the specimen in a non-volatile material, for example glucose (Unwin and Henderson, 1975), which then allows drying in air. With routine application of these specimen preserving methods, cryo-electron microscopy has become a standard in high resolution studies of biological macromolecules. Keeping the specimen frozen and cooled with liquid nitrogen or even liquid helium overcomes the dehydration problem of specimens in the high vacuum of the microscope as well as reducing some of the effects of beam-induced radiation damage. For cryo-electron crystallography, the protein suspension can be frozen in vitreous ice (Havelka et al., 1993; Ren et al., 2001) or more commonly in media which mimic the effect of water by hydrogen bonding to the polar residues on the protein

surface. Glucose was first introduced as an embedding medium in electron microscopy, which enabled recording of diffraction spots at better than 3.5 Å resolution from native crystalline patches of bR even after extensive drying (Unwin and Henderson, 1975). A number of other sugars were tested in the course of this work, but glucose was the best of those used for bR. Trehalose was later found to be superior for some bacterial porins (Jap et al., 1990) and eventually used for bR under conditions where complete drying was avoided (Hirai et al., 1999). Tannin had been used frequently in preservation of tissues for resin embedding and was shown to be as effective as glucose for preserving catalase crystals (Akey and Edelstein, 1983). Tannin was found to be superior for crystals of the light harvesting chlorophyll a/b-protein complex (Kühlbrandt and Downing, 1989; Wang and Kühlbrandt, 1991) and for tubulin (Nogales et al., 1995). Embedding media that have proven to be productive in the studies that led to atomic resolution structures by electron crystallography include tannin (Kühlbrandt et al., 1994), glucose (Henderson et al., 1990; Gonen et al., 2004), trehalose (Murata et al., 2000; Gonen et al., 2005; Hiroaki et al., 2006; Holm et al., 2006; Jegerschoold et al., 2008), and mixtures of these (Nogales et al., 1998).

The choice of embedding medium may or may not make a significant difference for different protein crystals. Wang and Kühlbrandt tried water, glucose, and tannin for preserving 2-D crystals of the light-harvesting complex and found the success rate for recording high-quality electron diffraction patterns with tannin to be much higher than with the others (Wang and Kühlbrandt, 1991). While several groups had found that glucose was better than trehalose in preparing purple membrane for electron crystallography, Hirai et al. found conditions where trehalose was better than glucose (Hirai et al., 1999). For preservation of 2-D crystals of tubulin for electron crystallography, Nogales et al. found a mixture of glucose and tannin to be better than using either medium alone (Nogales et al., 1995). While both tannin and glucose have been used for preserving catalase, there appears to be insufficient evidence to say which is better, since no direct comparisons have been made at sufficiently high resolution.

The embedding medium, whether water, sugar, or other compound, must surround the protein without producing mechanical deformation such as occurs when water crystallizes. Not only is there a volume change in water as it crystallizes, but proteins and other solutes may be excluded from the advancing crystallization front in a damaging process of phase separation. The best media other than vitreous ice have been found to be those which form a vitreous structure upon drying. It is generally assumed that the medium provides all the hydrogen bonding that would normally be present with water. The stabilizing effect of tannin was suggested to be due to a thin layer of amorphous precipitate that forms on the hydrophilic surface of the 2-D crystals and thus preserves the high resolution structure detail (Wang and Kühlbrandt, 1991). Trehalose is thought to be able to substitute for structural water in a 'hydration shell' around proteins to maintain the integrity of the protein structure during dehydration (Hirai et al., 1999). Studies have also shown a direct effect of sugars or tannin on the carbon film flatness (Koning et al., 2003).

2.2 Support film

The quality and surface properties of the support film appear to be key factors that affect the resolution in cryo-electron crystallography, particularly with respect to specimen flatness. Carbon films are routinely used as support for specimens in electron microscopy for their high transparency, good conductivity and mechanical stability under the electron beam. Carbon films are commonly prepared by evaporation onto freshly cleaved muscovite mica surfaces. It is important to use high grade carbon rods and mica, which are available from most EM supplies vendors.

The texture and flatness of evaporated carbon films had been something of a mystery until studies showed that the surface roughness of carbon films was highly dependent on experimental conditions (Butt et al., 1991). Several variations of the usual evaporating technique have been developed with the aim of preparing strong and flat carbon films. Butt et al. examined the flatness of carbon films by atomic force microscopy and found that the films are much smoother on both the air side and mica-facing sides when they are produced by evaporation in a series of layers (Butt et al., 1991). This approach was initially developed to avoid the degradation of vacuum that generally occurs as the carbon rod and its holder heat up during carbon evaporation. Generally the vacuum should be kept better than 5×10^{-6} Torr throughout evaporation. During evaporation it is common that sparks are emitted from the source as larger chunks of carbon are produced. Several workers have found significantly better flatness when such sparking is avoided (Vonck, 2000). Fujiyoshi et al. developed a pre-evaporation method to achieve spark-less evaporation of carbon (Fujiyoshi, 1998). The freshly cleaved mica is covered in the early stage of carbon evaporation until the carbon stops sparking, then the cover is removed and carbon deposited onto mica surface very slowly without any sparks.

Freshly evaporated carbon is usually somewhat hydrophilic, and sometimes even hydrophobic, but becomes increasingly hydrophobic as it ages over several days to weeks. The mica-facing side of the carbon film is highly hydrophilic, which allows it to float off of the mica, but also gradually becomes hydrophobic. The attractive or repulsive interaction between the specimen and the carbon will influence the number of crystals retained on grids after blotting as well as the flatness of the crystals. The best age of carbon film for cryo-electron crystallography needs to be investigated and may vary among different labs and specimens. Tests of purple membrane specimen preparations made each day following evaporation of a carbon film showed a peak in quality after about ten days (Ceska and Henderson, 1990). We usually use carbon films one to two weeks old for cryo-specimen preparation of tubulin 2-D crystals.

2.3 Grids

Copper grids are most commonly used in electron microscopy. However “cryo-crinkling” of carbon films on copper grids occurs when cooling from room temperature to liquid nitrogen temperature, due to the mismatch between the linear thermal expansion coefficients for copper and amorphous carbon (Booy and Pawley, 1993). The expansion coefficient for amorphous carbon films is expected to be comparable to that for diamond or graphite (6×10^{-6} , $1.2 \times 10^{-6}/^{\circ}\text{C}$, respectively). Molybdenum has about one third the expansion coefficient of copper (5×10^{-6} vs. $16.6 \times 10^{-6}/^{\circ}\text{C}$) and is thus a better match for carbon. It was found that carbon films on molybdenum grids had little or no wrinkling effect on cooling (Booy and Pawley, 1993). Molybdenum grids have also been shown to be able to yield a higher fraction of flat crystals (Vonck, 2000) and have now become standard in cryo-electron crystallography. The surface of some commercially available molybdenum grids is rough and can also cause carbon film to wrinkle. Fujiyoshi et al. designed special molybdenum grids which were manufactured by photochemical etching to preserve a smooth surface especially around the hole edges (Fujiyoshi, 1998). Such high quality grids are now available from EM supplies vendors, for example, Pacific GridTech (San Francisco). Promising new types of grids with near-atomically flat surfaces have recently become available, for example with thin silicon nitride films spanning openings in a thin silicon disk. Whether these will be generally useful for electron crystallography has not yet become clear.

2.4 Cryo-Grid Preparation

Both the number of crystals retained on the grid and their flatness may be strongly affected by surface properties, and the effects may vary with different specimens. Sample adherence to the surface can be quite different in cryo-preparations from stained grids, since stains seem to promote adherence of some crystals. It is often found that a concentration about ten times as high is required for frozen specimens as for stained samples.

The hydrophobic character of the support film surface plays a very large role in the quality of the specimen. The subject of the influence of surface properties has been nicely reviewed (Glaeser et al., 1991), including several models for how the crystals may attach to the surface. Tight adherence to the surface is probably undesirable, since it is likely to cause disordering of the crystal surface. It may thus be fortunate that the binding seems generally to be not tight across the whole crystal. In fact, many proteins and crystals are likely to be more apt to attach to the air-water interface or to a layer of denatured protein that will almost always form at the interface, so deposition onto the support film can be very gentle. Under ideal conditions, surface tension may slightly pull the crystal perimeter and enhance its flatness as the liquid dries. As is the case with negative staining, if the surface is too hydrophobic the embedding layer will be highly non-uniform. A crystal may shed the liquid as it dries or may retain a thick lens, depending on its own properties. It may even be that surface tension of a trapped lens of liquid on top of the crystal may cause compression and distortion as the liquid dries. An appropriately hydrophilic surface will retain a smooth, but not necessarily thick, layer of the embedding medium.

Glow discharge has been used since the early days of electron microscopy to make films hydrophilic and improve the distribution and uniformity of samples (Dubochet et al., 1982; Hayat, 2000). Since there are many parameters involved in this process, such as the residual gas pressure and composition, which are not always easy to control, success has often been quite irregular. It has been found for some samples that discharge in water vapor, at the normal pressure around 100 mTorr, provides somewhat more reproducible results (Walian and Jap, 1990; Glaeser et al., 1991). Treatment of the surface by glow discharge probably compensates for most of the effects of aging of the carbon film, but details of the process are generally not understood. Other methods for obtaining surfaces which provide appropriate properties for adsorption of crystals include chemical treatment, for example with alcian blue (Hayat, 2000), but these have not been widely used in the context of electron crystallography, particularly with the development of a method in which the sample is applied to the mica-side of a carbon film.

The method which is currently most commonly used for preparing cryo-grids for electron crystallography has been termed the “back injection method”. This was originally inspired by J. Wall’s description of the “wet film” technique in which a support film was floated onto a water surface, picked up on a grid, and the sample then injected into the water which adhered to the film (Wall et al., 1985). The intent was to use the flat film surface which had been in contact with the substrate surface onto which the film was evaporated and avoid exposing this surface to air and other uncontrolled influences before adding the sample. This method was first applied with purple membrane and then with the light harvesting complex (Kühlbrandt and Downing, 1989) and has now been adopted by most workers in preparing protein crystals for electron microscopy. As pictured in fig. 3, a 3 mm × 3 mm piece of carbon film is floated onto the embedding buffer and picked up by bringing a grid up under the film to lift it off the surface. The grid is turned over and a small amount of the crystal suspension is added to the lens of liquid which adheres to the film and grid. The sample can be gently mixed on the grid and incubated for an appropriate time. The grid is then blotted by pressing the grid onto two layers of filter paper and either completely air-dried or dried for a few seconds before dipping into liquid nitrogen quickly by hand. The cryo-grid can be

loaded onto a pre-cooled cryo-holder for immediate observation or stored in liquid nitrogen for future use.

As described above, there are many options for the embedding medium, and the carbon film can be floated off of the mica directly onto such medium. In the case of tubulin crystals, we generally float the film onto a solution of 1% tannin at pH 5.9, add the crystal suspension, and then add glucose to a final concentration of 0.5–2 %. These grids provide high quality diffraction when air dried and even better diffraction when frozen while still slightly moist.

One of the challenge for sample preparation is to control the thickness of the ice or other embedment, which is dependent on the blotting time, drying time between blotting and freezing, room temperature and humidity, etc. The crystals will become disordered if the specimen is too dry, while too thick vitreous medium will contribute too much to the background and obliterate the diffraction spots. For plunge freezing, automated devices such as the Vitrobot (FEI) can precisely control the chamber temperature, humidity, blotting time, etc. to give reasonably high reproducibility. For the back injection method, reproducible results can usually be achieved with experience, particularly when there is sugar in the embedding medium so that longer blotting times can be used and drying is not so critical.

2.6 Carbon Sandwich Method

While the back injection method provides a substantial improvement in flatness and reproducibility over other methods, it makes an inherently asymmetric specimen that may be subject to forces that cause various movements. The carbon sandwich technique was developed to overcome this difficulty (Koning et al., 2003; Gyobu et al., 2004). This technique is based on the back injection method and involves adding a second carbon film to the exposed side of the droplet on the grid before blotting, as shown in fig. 3. After injecting the sample into the droplet and mixing as required, a second 2 mm × 2 mm piece of carbon film is floated onto the embedding medium or water, picked up with a small loop and placed onto the top of the droplet so the crystals are sandwiched by two pieces of carbon film. The grid is then carefully blotted, for example by touching two pointed filter paper pieces to opposite edges of the grid, and quickly frozen in liquid nitrogen. This carbon sandwich technique can dramatically improve the flatness of the crystals, greatly increasing the quality and productivity of obtaining good diffraction pattern data at high tilted angles (Gonen et al., 2005).

2.4 2-D Crystallization and Screening

Except for rare cases of naturally occurring 2-D crystals, purified protein monomers have to be reconstituted into crystalline sheets for crystallographic studies. A full discussion of the many issues involved in 2-D crystallization of proteins is well beyond the scope of this paper, but several reviews provide insights to the methods in current use (Jap et al., 1992; Hasler et al., 1998). Because of their tendency to exist in the two-dimensional environment of a lipid bilayer, membrane proteins have been by far the most common targets of study by electron crystallography (Fujiyoshi and Unwin, 2008; Raunser and Walz, 2009). Tubulin is a rare case of a soluble protein that spontaneously forms 2-D crystals. The most frequently used approach for membrane protein crystallization is to solubilize the protein in an appropriate non-denaturing detergent along with lipid and then reconstitute the protein into crystals by detergent removal through dialysis or adsorption to beads. The choice of detergent is critical for optimization of protein stability, and much time is often spent exploring the many options now available. The type of lipid used and lipid:protein ratio may be the most important factors in determining the success of crystallization, but even testing variations of these two factors produces a large number of conditions that must be checked. Typically, one grid is prepared for each condition and examined in the electron microscope

for the presence of crystals. Generally, well ordered 2-D protein crystals should be larger than $1\mu\text{m}$ in order to produce measurable spots in electron diffraction patterns, but it is rare to find such large crystals in the early stages of testing crystallization conditions. Thus, negative staining is generally used for fast screening of the crystallization results. Crystal suspensions are embedded in a heavy metal salt solution, most often uranyl acetate, and are enveloped with heavy atoms which provide support against collapse and distortion of the biological structure during dehydration. This approach provides adequate contrast for identifying flat sheets at low magnification as well as for recording images to test for periodic structure. In our lab, we use 2% uranyl acetate aqueous solution as negative stain. Two micro-liters of crystal suspension are applied onto glow-discharged, carbon-coated EM grids, incubated for 30~60 seconds, rinsed with deionized water, stained with 2% uranyl acetate for 30 seconds and blotted to dryness. Negative staining will often provide structural information with a resolution up to 1.5 nm (Bremer et al., 1992), which is generally sufficient for routine documentation of the crystal number, size and crystallinity. In some cases this resolution is all that is required for answering a question of structural biology such as the arrangement of subunits within a complex of known composition. More often, though, an extensive search must be made to find conditions that produce large crystals with very good short and long range order. To enable such searches, automated systems have been developed for handling the large number of EM grids that must be checked and for finding and analyzing crystals on the grids (Cheng et al., 2007; Lefman et al., 2007), some of these incorporating both the crystallization and microscopy tasks (Hu et al.).

3. Data collection protocols

In general data collection protocols for electron diffraction and imaging are similar to those for other low-dose work, except that identifying promising specimen areas can be a substantially greater challenge. To the extent possible, one would like to select crystals initially based on their size and uniformity, but these features tend to be masked by the embedding medium. Appropriate protocols, though, along with microscope control software that allows switching between several defined operating conditions, enable data collection with high efficiency. For diffraction experiments, only two such conditions are needed, the search and record modes. For imaging, an additional mode is needed for focusing.

3.1 Search mode

The basic requirements of such methods are to search for and center target crystals using an exposure rate that allows sufficient time to identify and position the crystal without exposing it to a significant fraction of the total allowable exposure. The maximum exposure that proteins can tolerate is in the range of $10\text{--}20\text{ e}/\text{\AA}^2$ over the usual electron energy range of $100\text{--}300\text{ keV}$, so the intensity in the search mode is usually around $1\text{ e}/\text{\AA}^2\text{-min}$. This low intensity thus requires working at very low magnification, even when using a sensitive video or CCD camera, and also working in a high contrast mode of the microscope. This is achieved by spreading the beam to cover an area of $5\text{--}20\text{ }\mu\text{m}$ diameter and using the diffraction mode but defocusing the diffraction pattern so that the central spot becomes a highly defocused image.

3.2 Diffraction recording mode

For recording diffraction patterns, the diffraction focus must be carefully set to optimize the spot sharpness (although there may be cases where a slight defocus is desirable to avoid saturating the detector). For crystals of moderate size, the beam should illuminate the whole crystal. Illuminating less than all of the area available will dramatically decrease the spot intensities, which are proportional to the area illuminated, while illuminating a larger area will unnecessarily add to the noise level. While large crystals are almost always very

advantageous, there is an upper limit to the beam diameter that can be used, since lens aberrations prevent obtaining a sharp focus from an area more than some certain diameter. This size may vary widely among different microscopes. Particularly when the beam and crystal size are about the same, it is very important to make sure that the beam is centered on the crystal. This can easily be checked by slightly overfocusing the diffraction pattern, again to produce a strongly defocused image. First off, the beam should expand concentrically as the diffraction focus is changed, indicating that it is properly positioned on the optical axis. If the crystal itself is not visible, one can find some identifiable object like a small speck of dirt that can be used to mark the center as well as the size of the beam after switching to the search mode. The diffraction focus setting for the search and record modes will generally be different. If the microscope software does not allow saving different values of the diffraction focus, one can resort to manually changing the defocus, counting the number of clicks to go from one setting to the other.

The development of slow-scan CCD cameras has had a major impact on diffraction data recording. CCDs offer much greater linearity and dynamic range than photographic film and circumvent the very tedious process of digitizing films, where care must be taken to avoid problems associated with digitizing fine spots on a transparent background (Downing and Li, 2001). One problem that CCDs do suffer is blooming, or spreading of the signal from the very intense central spot region into surrounding areas. The unscattered beam highly oversaturates the CCD, since it is orders of magnitude stronger than diffraction spots from a typical protein crystal. Thus, it is common practice to use a beam stop to block the central beam. Unfortunately, this also blocks a significant portion of the diffraction pattern. The tradeoff of missing part of the pattern due to blooming if the beam stop is not used can be mitigated somewhat by the antiblooming feature of some CCD cameras. This function takes advantage of thermally generated electron-hole pairs within the CCD, but these are only slowly generated under the usual operating conditions. Thus, longer exposure times will better take advantage of the antiblooming capability. Long exposures also reduce the influence of the streak that often shows up from the central beam as the pattern is deflected in and out by the beam blanker. Exposures of 20–60 seconds have generally been found to be suitable. The proper intensity can be achieved with the use of a small condenser aperture and high spot size setting.

To obtain the maximum signal to noise ratio in the diffraction patterns, as well as to calibrate the exposure to be used for imaging, it is very instructive to follow the fading of diffraction spots through a series of shorter exposures. As shown in fig. 4, the higher resolution spots tend to fade substantially faster than those at lower resolution, and the total exposure must be set to capture the most intensity without adding too much noise. The fading is nearly exponential with exposure, and the critical exposure, i.e. the value at which the intensity fades to $1/e$ of the initial value, is a useful parameter for describing the optimal exposure. One can show that the best exposure for diffraction patterns depends on the initial spot strength and can be well over the average critical exposure, while for imaging it is slightly over twice the critical exposure (Downing and Li, 2001).

The use of an energy filter can greatly improve the quality of diffraction data (Downing and Li, 2001; Yonekura et al., 2002). Inelastic scattering contributes most of the intensity at low angles and can be comparable to diffraction spots of average intensity even out to 5–10 Å spacings. Stochastic variations in the intensity of the inelastic scattering contribute to the noise level, and operating the filter in a zero-loss mode, even with a window of 10–30 volts, can improve the statistics of the data substantially. Figure 2d shows an example of the dramatic improvement in appearance of the pattern obtained with an energy filter.

3.3 Imaging

For recording images, one must have a focus mode, where the beam is focused to an area adjacent to the area of interest, as well as the image recording mode itself. These can be set up just as in other low dose work. Spot scan imaging has also been shown to be particularly valuable for imaging crystals, by reducing some of the secondary effects of radiation damage (Downing, 1991). While charging limits the usefulness of spot scan imaging of specimens suspended in ice over holey films, with a continuous support film generally used for crystals charging plays only a minimal role which is itself reduced with spot scan imaging. The beam is focused to a small diameter, from the diffraction limit up to around 100 nm, using a small condenser aperture to limit the convergence angle. Under computer control the beam is then stepped over the crystal in a 2-D raster, with step times in the range of msec. Restricting the illuminated area appears to reduce the overall stress on the specimen that results from radiation damage, and the illuminated area is also strongly constrained by the surrounding area, resulting in significantly better signal strength, resolution and reproducibility. Application of spot scan imaging with tilted specimens has a particular advantage. The beam can be scanned in the direction of the tilt axis and the focus adjusted between scan rows so that the entire image is recorded at the same defocus (Downing, 1992). This procedure avoids the necessity of deconvolving the focus ramp across the image when extracting data from the Fourier transform of the image.

4. Summary and conclusions

Refinements in specimen preparation methods have enabled significant advances on the application of electron crystallography to protein structure determination. Obtaining crystals is still almost always the major bottleneck, but once crystals are at hand there is a collection of effective options for preparing the crystals.

The most difficult part for electron diffraction of biological 2-D crystals is to collect diffraction patterns from highly tilted crystals. The diffraction spots perpendicular to the tilt axis are often blurred due to imperfect flatness of the crystals. Using molybdenum grids can reduce the problem of wrinkling of carbon films when cooling the specimen to low temperature. The flatness of the carbon film itself is most important and can be achieved by carefully evaporation of carbon onto freshly cleaved high quality mica sheets. Sugar and tannin solutions have been commonly used as embedding media for specimen preparation of biological 2-D crystals. They can preserve the high resolution detail of the protein structures and also benefit the flatness of crystals, but the choice of medium needs to be optimized for each system. The carbon sandwich technique has become the routine method for specimen preparation for collection of high tilt electron diffraction data as it can further improve the crystal flatness. Finally, once good specimens have been obtained there are well established protocols for data collection and subsequent processing.

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Figure 1.

Geometrical representation of diffraction spot blurring and structure-factor variation across the blurred spot caused by imperfect crystal flatness. The horizontal axis represents the $\mathbf{a}^*$, $\mathbf{b}^*$ plane, and the vertical axis represents the $\mathbf{z}^*$ axis in reciprocal space. A single reciprocal lattice line is indicated, running parallel to the $\mathbf{z}^*$ axis and intersecting the $\mathbf{a}^*$, $\mathbf{b}^*$ plane at a distance g_0 from the origin. For a crystal tilt of θ , the diffraction spot will appear at a radius of g . If tilt ranges between the angles θ and $\theta + \delta$, the spots will be blurred to radii from g_1 to g_2 , and will sample values in the region of Δz^* along the lattice line. Redrawn from (Glaeser et al., 1991).

Figure 2.

Diffraction patterns of tubulin crystals. These patterns are selected to illustrate various points made in the text. The tubulin lattice constants are $a=81 \text{ \AA}$, $b=92 \text{ \AA}$. The patterns in (a) was recorded on photographic film and scanned on a PDS1010 densitometer. In the raw pattern, (a), there is a strong background intensity which can be subtracted to improve the visibility of the spots, as shown in (b). Due to the limited dynamic range of the film the center of the pattern is highly oversaturated. Patterns (a) and (b) are from an untilted crystal and show well resolved spots to about $3.5 \text{ \AA}$. The background-subtracted pattern in (c) is from a highly tilted crystal and shows the effects of non-flatness, with blurring and loss of intensity away from the tilt axis direction. This pattern was collected on a slow scan CCD and shows some of the effects of blooming and saturation at the center of the pattern. An energy filter was used to collect the pattern in (d), resulting in great reduction of the diffuse background particularly at low angles, as well as reduction in the overall noise level.

Figure 3.

Back Injection (a, b) and Carbon Sandwich (c, d) techniques for specimen preparation. (a) A $3\text{ mm} \times 3\text{ mm}$ piece of carbon film is floated onto the embedding buffer and picked up by a molybdenum grid. (b) The grid is turned over and a few microliters of crystal suspension is added to the grid from the other side. In the simple back injection method, the grid is then turned over again and blotted by pressing onto filter paper. For the carbon sandwich technique, a second $2\text{ mm} \times 2\text{ mm}$ piece of carbon film is floated onto embedding buffer (c), picked up by a small loop and transferred onto the grid so the crystals are sandwiched by two carbon films. (d) The grid is blotted carefully by touching filter paper to the edges of the grid.

Figure 4.

Diffraction pattern series. Patterns were recorded in sequence (a, b, c, d) from a single crystal of bacteriorhodopsin, each with an exposure of about 5 electrons/Å². Arrows drawn for reference point along symmetry-related rows of spots that are seen to fade away with accumulated exposure. Edge of pattern corresponds to 3.5 Å resolution. For optimal signal-to-noise ratio in the high resolution spots, an exposure of 10-15 e/Å² could be used, while longer exposures would continue to improve the lower resolution spots.