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Retrospective: Radiation damage and its associated "Information Limitations"

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ABSTRACT

The fact that radiation damage would limit the usefulness of electron microscopy with biological specimens was a concern in the earliest days of the field. Good estimates of what that limitation must be can be made by using Rose's empirical relationship between the inherent image contrast, the exposure used to record an image, and the smallest feature size that is detectable. Such estimates show that it is necessary to average many images in order to obtain statistically well-defined data at high resolution. Structures are now routinely obtained by averaging large numbers of shot-noise limited images, and some of these extend to atomic resolution. The signal level in current images is nevertheless far below what physics would allow it to be. A possible explanation is that beam-induced movement limits the quality of images recorded by electron microscopy. For specimens embedded in vitreous ice, beam-induced movement can even be severe enough to limit the resolution achieved during tomographic reconstruction. The fact that very high-quality images can nevertheless be obtained, although only unpredictably, suggests that it may be possible to devise new techniques of specimen preparation and/or data collection that at least partially overcome beam-induced movement. If so, the need for image averaging would be correspondingly reduced.

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1. Having fun in the lab proved to be a serious business

During the period 1967–68, I spent many days in the lab, playing with a newly published experimental technique for producing small-angle electron diffraction patterns (Ferrier and Murray, 1966). I did this for little good reason other than it was exciting and fun. This playful experimentation suddenly made me recognize that radiation damage makes it impossible to achieve high-resolution images of biological specimens. Although physicists realized this as soon as the idea of an electron microscope was conceived, such a limitation seemed to have been dismissed in the years that followed, i.e. during a period when microscope performance and sample preparation advanced hand in hand. Thus, while it was never forgotten that electrons are a form of short-wavelength radiation that can be focused, what seemed to have become irrelevant in this period was the fact that electrons are also a form of ionizing radiation, and as such they rapidly destroy the biological specimens that one hopes to characterize.

This retrospective of my 1971 paper in Journal of Ultrastructure Research (Glaeser, 1971), and some of the work that followed, relates how diffraction experiments, the classic manifestation of the wave nature of electrons, show us that radiation damage places unforgiving limitations on the resolution that is achievable in electron microscopy of organic macromolecules. Ironically, diffraction experiments themselves overcome the same limitations by averaging over many identical copies of molecules, all presented with the same view.

My involvement with the issue of radiation damage was not based on logic, knowledge of the literature, or the intent to test hypothesis. Instead, lacking X-ray diffraction facilities suitable for studying ordered biological materials with large "unit-cell" dimensions, I took the advice of Cornelius Tobias, a colleague in my department who was a constant source of "outside the box" ideas, that I consider trying instead the "long camera length" electron diffraction methods published by the laboratory of Robert Ferrier (Ferrier and Murray, 1966). When these methods were applied to artificial test specimens such as rafts of 88 nm polystyrene spheres or plastic sections of skeletal muscle and the stacked membranes in retinal rods, it was quite easy to see diffraction patterns produced by these large-spacing specimens (Glaeser and Thomas, 1969). When, just for fun, these methods were applied to test specimens that had very small unit-cell constants, however, such as evaporated gold, no diffraction could be seen because the camera length was so long that the first allowed reflections lay out beyond the edge of the viewing screen.

Upon switching to the conventional technique for observing electron diffraction patterns, in which one first carefully focuses the image that is produced at the "selected area diffraction" plane, many orders of diffraction could be seen in the "powder patterns" produced by evaporated gold. Still clueless about radiation damage, however, I became deeply frustrated by the fact that crystals of valine never showed any diffraction by the same technique,

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and I effectively gave up on trying to understand why these specimens "must not be crystalline". Nevertheless, because of a nagging disbelief that just would not go away, I tried one day to see whether specimens of valine, which certainly looked like crystals, would produce diffraction patterns if I used one of Ferrier's long camera length methods. By great luck, the first allowed reflections produced diffraction spots that were just barely at the edge of the viewing screen. What then immediately happened, and resulted in a career-changing epiphany, was that these spots faded within seconds of bringing a new area of the specimen into the beam. I finally understood that I could now see diffraction from valine crystals, but not before, because the highly collimated electron beam produced by Ferrier's method is inherently much less intense than the beam that is conventionally used for the selected area diffraction method. It thus became clear that the usual level of electron exposure destroyed the crystallinity of the sample. Furthermore, if this was happening to amino acids, then it would happen just as easily to proteins.

Although I was not yet aware of it at the time, many investigators had already recognized that radiation damage causes extreme chemical changes in organic materials (Reimer, 1965). Crystalline polymers become amorphous, for example (Kobayashi and Sakaoku, 1965), and the production of volatile or diffusible fragments resulted in substantial mass loss within the irradiated area (Stenn and Bahr, 1970a). Some investigators nevertheless took the optimistic view that chemical changes such as the loss of hydrogen atoms and the consequent formation of double bonds would have minimal influence on the positions of non-hydrogen atoms. As a result, it was argued, the high level of radiation damage that is produced by the normally used electron exposures is not necessarily incompatible with imaging meaningful structure at high resolution (Stenn and Bahr, 1970b).

Two further "lucky circumstances" had prepared my mind to look at the situation in a different way, however. First, the research of several faculty members in my academic department was devoted primarily to radiation biology. Although I had no interest in the subject myself, I could not avoid hearing it being regularly discussed by my colleagues. Fortunately, some of the discussion stuck. As a result of my thus having a little familiarity with the subject and expert colleagues to turn to, I could easily read up on topics such as the dose tolerance of proteins, indeed of organic molecules in general, and find typical values of the "yields" of different chemical fragments for a given unit of ionizing radiation absorbed by a specimen. Second, I had the good fortune to attend lectures in a graduate course about scanning electron microscopy (SEM) that was given by Prof. Tom Everhart in the department of Electrical Engineering. I did this just from curiosity, for the fun of learning things I did not yet know, and the advice that I do so from a great friend and departmental colleague, Tom Hayes, who was himself switching his career from TEM to SEM.

The crucial thing that I learned from Everhart's lectures was that Albert Rose had used psychophysical experiments to determine the combination of contrast and feature size at which objects could first be detected by human vision (Rose, 1973). In other words, Rose had effectively quantified the extent to which shot-noise places a limitation on what can be detected by eye. In the case of valine crystals, for example, the electron exposure should be no more than what causes the electron diffraction pattern to disappear, if the goal is to obtain high-resolution images of the crystal structure. As a result, using more sensitive detectors won't improve the images because the fundamental problem is that the image has too few electron counts.

The 1971 paper in the Journal of Ultrastructure Research (Glaeser, 1971) expressed Rose's empirical relationship in a form equivalent to

$$d \cdot C \geqslant \frac{5}{\sqrt{fN}}$$

where the notation is defined as follows:

d	the size of a feature (the resolution)
C	the contrast of that feature, $\frac{I_{\text{object}} - I_{\text{background}}}{I_{\text{average}}}$
f	fraction of incident electrons that contribute to image formation
N	number of electrons per unit area that are incident on the specimen.

Inserting $N = 80$ electrons/nm^2, which I had measured as being the exposure to 80 keV electrons that ultimately destroyed the structure of valine crystals (at room temperature), and taking $C = 0.1$ as a "ballpark" estimate, led me to conclude that features on the size scale smaller than individual protein molecules could never be seen by electron microscopy.

The realization that imaging single protein molecules at high resolution is physically impossible forces one to ask "What is the fall back plan; how can one get around this problem"? The answer is obvious to most biophysicists, who are taught that averaging of repeated measurements makes it possible to pull out an invisibly small signal from inherently noisy data. Since averaging repeated, "phase-locked" measurements was a commonly used tool in neuroscience and certain spectroscopies, it did not take much to think of generalizing the approach to spatially varying, rather then temporally varying, shot-noise limited measurements (Glaeser, 1971; Glaeser et al., 1971; Kuo and Glaeser, 1975). One way to circumvent radiation damage and stop worrying about the problem, therefore, would be to image thin crystals at a "safe-but-noisy" electron exposure, knowing that the pre-existing alignment of identical molecules within a crystal makes it quite easy to do the required averaging. In order to apply this insight to achieve high-resolution images of protein structure, however, a second problem had to be solved: the native, hydrated structure itself had to first be preserved in the vacuum of the electron microscope (Taylor and Glaeser, 2008).

Alternative approaches also had to be investigated in order to determine whether physical (or chemical) conditions existed under which biological materials could tolerate much higher exposures to ionizing radiation. The options were limited, of course, and included varying the dose rate, the energy of the incident electrons, and the temperature of the specimen. No obvious difference in the "total dose" that destroyed electron diffraction patterns was found for exposure times that varied from seconds to minutes. Although specimens could tolerate about twice the exposure at high voltage as they did at 100 kV (Glaeser, 1971), the signal-to-noise ratio in low-dose images has almost no dependence upon the energy of the incident electrons, provided that samples are irradiated to the same "damage endpoint", and provided that the detector that one is using performs equally well at all electron energies. Cooling samples to −90 °C or lower, on the other hand, was found to increase the allowed exposure by a factor of 5–7 (Glaeser and Taylor, 1978; Hayward and Glaeser, 1979). Irradiating negatively stained catalase crystals at helium temperature, on the other hand, resulted in much more severe radiation damage than occurs at room temperature (Glaeser and Hobbs, 1975) or at nitrogen temperature.

The benefit of cooling with liquid nitrogen is most likely due to the "caging" of products of radiolysis by immobile, neighboring molecules rather than any temperature-dependent reduction in primary radiolysis and bond rupture. The benefit of caging is 2-fold: (1) Much as had been argued by Stenn and Bahr (1970b), the atoms in the "daughter" fragments probably do not move very far from their "parent" positions when only a few chemical bonds are broken, and thus the resolution of the damaged structure

initially remains relatively high. (2) The immobilized fragments have limited opportunity to undergo secondary chemical reactions that would otherwise cause further damage following the primary event. Nevertheless, damage continues relentlessly to accumulate as radiolysis progresses, and ultimately, when the concentration of trapped (caged) free radicals becomes high enough, even secondary chemical reactions become inevitable at any temperature, since there is no activation barrier for the reaction of two adjacent radicals.

2. The intervening years

2.1. Signal averaging has led to "atomic-resolution" images for many specimens

The idea of averaging images of many unit cells in a crystal was first implemented by Unwin and Henderson (1975), who had discovered that embedding protein crystals in an air-dried film of glucose preserves the structure as if in a hydrated state. The advantage of this technique was that it did not require the development of a stable cold stage in order to proceed immediately to high-resolution imaging. Subsequently, of course, Hayward showed that even with glucose-embedded specimens it is an advantage to record data at low temperatures (Hayward and Glaeser, 1979). In addition, the contrast matching that occurs between proteins and the glucose embedment makes it impractical to use this method for smaller particles such as helices, icosahedral viruses, and multiprotein complexes.

The path to achieving high-resolution density maps was not as simple as one first thought it would be, however. Glucose-embedded specimens turned out to have rather poor long-range order, thus requiring computational "unbending" (equivalent to real-space averaging) before computing the high-resolution crystal structure factors (Henderson et al., 1986). Computational corrections for imperfect beam alignment were also required for successful averaging at high resolution (Henderson et al., 1986). With these sophisticated tools finally in place, electron crystallography (Glaeser et al., 2007) has been able to provide chain-trace models of a number of 2-D crystals, of which tubulin (Nogales et al., 1998) is arguably the protein of greatest interest in cell biology. Averaging the high-resolution images of a large number of identical molecules has even been successful for well-ordered helical assemblies such as the bacterial flagellum (Yonekura et al., 2003) and tubular vesicles of the nicotinic acetylcholine receptor (Miyazawa et al., 2003).

The highest-resolution cryo-EM work, both on 2-D crystals (Mitsuoka et al., 1999; Grigorieff et al., 1995; Gonen et al., 2005) and on well-ordered helices (Yonekura et al., 2003; Miyazawa et al., 2003), has been done on microscopes fitted with cryo-stages that are cooled by liquid helium. It is nevertheless hard to defend the conclusion that these successes are due to a reduced level of radiation sensitivity at helium temperature relative to nitrogen temperature. Some other factors that are more likely to explain the successes achieved with these microscopes include the performance of the instrumentation itself, the high level of skill and persistence of scientists who did the work, and the use of excellent sample-preparation techniques—especially those developed in the Fujiyoshi laboratory. The high resolution of the electron diffraction work on aquaporin-0 (Gonen et al., 2005), for example, required only that well-diffracting crystals be preserved on the EM grids with little "wrinkling" of the specimens. The thought that reduced radiation damage made it possible to record weak diffraction spots would have to be confirmed by quantitative comparisons of the fading rates at helium and nitrogen temperatures. Even better would be to distribute identical samples and perform such measurements at multiple laboratories that have microscopes capable of operating at both helium and nitrogen temperature, much as was done for by an international study group in the mid-1980's (Chiu et al., 1986).

What still remains a challenge, however, is to merge data from images of randomly dispersed, "single" molecules (Frank, 2006) with the accuracy required to achieve "atomic-resolution" maps, and to do so with the large number of particles that are required (Henderson, 1995; Glaeser, 1999). Work in that direction includes the 3-D reconstruction of GroEL at a resolution high enough to build a reliable "ribbon" model of the secondary and tertiary structure (Ludtke et al., 2008) and a similar reconstruction for the epsilon15 virus (Jiang et al., 2008). An even higher-resolution structure of rotavirus has been obtained, one that allows the amino acid sequence of the constituent proteins to be built into the density map (Zhang et al., 2008). The averaging of images of particles with high symmetry provides an important intermediate step, since it employs the same computational tools that are used for large, asymmetric protein complexes, i.e. translational alignment, assignment of Euler angles, and possibly even assignment of particles to subsets of images that correspond to distinct conformational states. At the same time, symmetric structures offer the advantage that alignment of one particle immediately provides data for multiple identical copies of the constituent protein(s). In the case of rotavirus this number was actually 780 identical copies because of its $T = 13$ icosahedral symmetry. The 780-fold "amplification" factor in the number of molecules that are averaged thus made it practical to merge image data from over 6 million copies of the protein, a number that may be somewhat more than but nevertheless similar to what is used when merging high-resolution data from 2-D crystals.

2.2. Radiation-induced movement has emerged as another limitation

Data processing of images of 2-D crystals revealed the fact that the short-range disorder in images (characterized by a Gaussian smoothing of the averaged image) is far worse than in the crystals themselves. Quantitative estimation of the contributions that could be made to the Gaussian smoothing parameter (or B-factor) by "instrumental" factors led to the conclusion that either the samples themselves or their images (or both) move in an essentially chaotic way during irradiation (Henderson and Glaeser, 1985). In any event, "beam-induced movement" causes the high-resolution crystal structure factors to rarely be as strong as 10 percent of what they "should be". Indeed, high-resolution structure factors are almost always just a few percent, or less, of what they are in electron diffraction patterns. As Henderson has pointed out, this makes it necessary to average data from 100 times as many images, or more, than would be required if beam-induced movement did not occur (Henderson, 1995).

The effects of beam-induced movement are even worse for images of tilted specimens. In this case the loss of signal (the B-factor associated with imaging) is far greater in the direction perpendicular to the tilt axis, and this effect increases very steeply as the tilt angle of the specimen is increased. Inexplicably, however, there are still occasional instances in which the B-factor in the direction perpendicular to the tilt axis remains small enough that structure factors can be recovered at high resolution. Thus, with persistence, 3-D data sets can be collected from well-ordered 2-D crystals, but the required data collection is frustrating and slow.

Many authors assume that beam-induced movement is primarily due to a deflection of the image that is caused by radiation-induced charging of the specimen. There is reason to question this assumption, however, since the image movement might really be due to physical movement of the specimen itself. In the case of valine crystals, for example, one can easily see rapid changes in the local positions where the tilt angle of the crystal precisely satisfies

Bragg's law, i.e. in the local positions of so-called "bend contours". The sweeping movements of these bend contours occurs at electron exposure levels that are a small fraction of the exposure that causes fading of the electron diffraction pattern. We also know from experiments on photolytically initiated solid-state chemical reactions (McBride et al., 1986) that conversion of parent molecules to daughter products can generate pressures within 3-D crystals that are half the value that can convert graphite to diamond, and that this happens when as little as 5 percent of the parent molecules have undergone photolysis. In the case of thin specimens, this sort of mechanical stress is likely to drive a flexing or bending type of movement, the largest component of which will be perpendicular to the plane of the thin specimen, but some component of which could well be parallel to the plane of the specimen. The hypothesis of radiolysis-induced movement of the specimen is thus a reasonable alternative to the hypothesis of charging-induced movement of the image.

Beam-induced movement has also become recognized as a major factor that can limit image quality in cryo-EM tomography, even at relatively low resolution. One has to expect that the same problems described above could also occur at high tilt angles for thick ("tomographic") samples, although the greater stiffness of a thicker sample should limit how small the radius of curvature of the bend can be. What is rather surprising, however, is the observation that individual particles within "thick", ice-embedded specimens seem to "flow" in erratic directions during the normal series of exposures that is used for tomographic data collection (Wright et al., 2006). Not only does the convection-like movement of fiducial particles interfere with the accurate alignment of successive images in a series, but one has to suppose that it also blurs out features in the tomographic reconstruction. It is not yet known whether the same type of beam-induced movement that is seen for fiducial particles also occurs, unseen, just for individual particles (e.g. ribosomes) or whether whole nano-regions of a vitreous ice specimen drift in a way that is analogous to the movement of tectonic plates.

The question of "protection" against radiation damage at helium vs nitrogen temperature takes on a somewhat different nature at the higher total exposures that are normally used in tomography. The issues in tomography have to do with alignment of successive images, discussed in the paragraph above; the phenomenon of "bubbling"; and other processes that destroy even the coherent, particle-like distribution of mass of an entire protein molecule. Prior to the onset of bubbling, it appears that there is no improvement in the dose at which ordered protein structures such as bacterial S-layers are destroyed (Comolli and Downing, 2005). Comparisons at higher electron exposures (Comolli and Downing, 2005; Iancu et al., 2006) indicate that smaller bubbles are produced at helium temperature than at nitrogen temperature. This observation is consistent with (1) the model in which molecular hydrogen is the gas that accumulates in the bubbles (Leapman and Sun, 1995), and (2) the expectation that diffusion of molecular hydrogen should occur more slowly at helium temperature than at nitrogen temperature. Unexpectedly, however, the bubbling that does occur at helium temperature results in a different structural distribution than it does at nitrogen temperature. Gas accumulates at the site of cell membranes at helium temperature (Iancu et al., 2006), for example, giving the appearance of splitting the membranes in a way that is analogous to what happens in freeze-fracturing.

3. Looking ahead: physics still allows that it may be possible to get much better images

Whatever the mechanisms may be for radiation-induced movement, the good news in this otherwise dismal story is that (1) spatial averaging still is able to produce high-resolution structures of biological macromolecules, if one is persistent and simply collects enough data, and (2) images of randomly chosen areas are very occasionally as good as one third or more of what they might be, i.e. the high-resolution Fourier coefficients extracted from images can be as strong as 35 percent of what they are in the diffracted wave (Brink and Chiu, 1991,Typke et al., 2004). The reason why this second point is so encouraging is that it proves that better image quality is not strictly forbidden by some basic physical effect such as secondary-electron emission (charging) or the stress generated by production of radiolytic fragments. Indeed, since rather high image quality can be obtained on some occasions, it should be possible to make it happen essentially all of the time. What we need to do is understand what occasionally goes "right", such that radiation-induced movement remains especially small for at least one particular place on the EM grid. Alternatively, it may be possible to change the technique by which images are recorded, such that the recorded data are not affected by the movement, in spite of the fact that it continues to occur.

The options are currently limited for achieving a consistent reduction in the B-factor associated with imaging (i.e. a consistent reduction in beam-induced movement). Various authors employ a range of techniques for this purpose, most of which are motivated by an attempt to reduce specimen charging. As reported by Glaeser and Downing (2004), however, it seems doubtful that charging can account for a significant amount of image movement, at least not due to electron-optical effects, provided that the specimen is prepared on continuous carbon. As an alternative to preparing samples on continuous carbon films, carbon can be evaporated onto the specimen after it has been prepared (Jakubowski et al., 1989). The approach that is the most effective may be quite different if mechanical movement rather than image movement is the real problem. It seems likely, for example, that bending and shifting of a thin specimen would be reduced, (1) the flatter the support film is to begin with, (2) the thicker it is, (3) the more uniformly it is bonded to the metal bars of the EM grid, and (4) the smaller the holes over which the support film is suspended. The fabrication of grids that can be expected to result in reduced amounts of bending of the thin support is a challenge, however.

4. Do not blink: it may be possible to use exposures that are faster than beam-induced movement

A relatively new alternative that is being discussed is to use ultrafast exposures to overcome the problem of beam-induced specimen movement. The idea here is for the electron exposures to be fast enough to outrun the "sluggish" response of entire regions of a specimen, such as those involved in a bending mode. It is clear that there could be significant improvements both at the level of high-resolution imaging of 2-D crystals and at the level of flow-like movements of vitreous ice that might occur during EM tomography. The idea of using such ultrafast exposures is inspired by the development of pulsed electron guns for applications in the materials sciences (Armstrong et al., 2007).

Space-charge effects cause intractable problems for maintaining the required spatial and temporal coherence when the number of electrons in a pulse is too large, however. As a result, it is still uncertain whether it is technologically possible to produce suitable beam quality for exposures as short as 1 ns, if single pulses must contain as many as 1000 electrons/nm^2, even if the illuminated area would be as small as 0.01 μm^2. In addition, there is no way to know whether an exposure time of 1 ns would be fast enough for the measurement to outrun the hypothesized beam-induced movement; whether even longer exposure times (e.g. 10 or 100 ns) would still be fast enough; or whether much shorter

exposure times would be required. Nevertheless, the issue should be investigated, both experimentally and computationally, whether there is a regime of ultrafast exposures for which (1) data collection substantially outruns much of the beam-induced movement, and (2) the statistical definition of the data that is achievable within a single, ultrafast exposure is good enough to allow alignment (and perhaps unbending, as well), so that data from successive frames can be merged.

The question has also been raised whether temporally well-spaced fs electron exposures, consisting of just one or a few electrons per pulse, might produce images that exhibit less radiation damage (Lobastov et al., 2005). The idea would have merit if local pulses of heating, at sites of inelastic scattering, were a significant contributor to radiation damage in biological electron microscopy. To a first approximation, however, one can arrange that the mean time between electrons is the same whether the electrons are emitted from currently used "continuous wave (CW)" sources or they are emitted from intentionally pulsed sources. As a result, it is much easier to provide a suitably long pause between individual inelastic scattering events, for postulated thermal relaxation and "repair" to occur, by simply reducing the intensity from a standard "CW" electron source rather than using an ultrafast, pulsed source. Furthermore, it is incorrect to suggest, as some seem to do, that radiolytic damage itself could be reduced by using pulsed sources rather than CW illumination to deliver the same number of electrons over the same total exposure time. There thus does not seem to be much to be gained from the additional complexity of using a fs pulsed source, other than for work in which pump and probe (i.e. temporally phase-locked) experiments are to be conducted.

It has been calculated that exposure times as short as 10 fs or so are fast enough to inertially "confine" atomic nuclei during an ultrafast period of data collection, even if the target is converted into a plasma early in the course of such an exposure (Neutze et al., 2000, 2004). Thus, when exposures can be made short enough, radiation damage no longer imposes a limitation on how high the dose of ionizing radiation can be. Although space-charge limitations rule out the use of electrons for such short, single-shot exposures, there are no such limitations for X-ray beams. This realization is a major driver for building free-electron lasers that can produce "femtosecond" pulses of hard X-rays, the goal being to produce diffraction patterns from single protein molecules. When the intensity of a continuous scattering pattern is measured at spatial-frequency increments that are half the distance between Bragg reflections (for a hypothetical crystal of the same molecule), the phases of the diffraction pattern can be recovered by an iterative algorithm (Neutze et al., 2000). In other words, the equivalent of "lens-less" images can be generated from the intensities of single-particle diffraction patterns, provided that these intensities are measured accurately enough. Although a nice demonstration of this concept has been produced with a soft-X-ray free-electron laser and a microfabricated target with features on the sub-micrometer scale (Chapman et al., 2006), there is not yet an accepted proposal for how to build a hard-X-ray laser with the brightness that is needed to do the same thing for multiprotein particles.

The idea of averaging large numbers of statistically noisy, single-particle diffraction patterns (Huldt et al., 2003; Spence et al., 2005), reminiscent of the averaging of single-particle cryo-EM images, is thus a reasonable alternative. The first challenge in this case is to produce a beam of single particles that are cloaked in a thin film of aqueous buffer to preserve the native hydrated state. The volume of buffer must be made small enough that its contribution to the diffraction pattern does not overwhelm the scattering from the protein molecule itself. At the same time, the volume of the cloaking buffer must be reduced in a way that does not alter the ionic strength and pH of the medium beyond the point that the protein of interest will tolerate. In addition, averaging noisy diffraction patterns would be made much easier if one could first orient the protein molecules, a goal that is no less ambitious than that of producing streams of hydrated molecules to begin with (Spence et al., 2005; Starodub et al., 2005).

In summary, experimental measurements of the fading of electron diffraction patterns of thin protein crystals have provided quantitative estimates of the maximum electron exposure that can be safely used to record high-resolution images. These "safe" exposures are much too low, however, to record images in which high-resolution features are statistically well defined. The principle of averaging large numbers of shot-noise limited images is thus a standard element of high-resolution electron microscopy of biological macromolecules, regardless of whether the specimens are prepared as ordered crystals, helices, or single particles. Very large numbers of images, much greater than expected from the known amplitude of the scattered-electron wave, must be averaged in order to obtain atomic-resolution structures, however. Radiation damage is again suspected of being the culprit, in this case because it generates stresses that drive beam-induced movement of the specimen. This movement, in turn, causes a much greater decay (as a function of resolution) of signal in images than what one observes in diffraction patterns. The occurrence of almost anecdotal instances in which relatively strong signal was preserved in high-resolution images nevertheless indicates that it must be physically possible to "overcome" the problem of beam-induced movement. The opportunity therefore still exists to improve the quality of high-resolution images of beam-sensitive specimens on a consistent rather than a rare basis, and thus to further advance the usefulness of electron microscopy as a tool in structural biology of molecules and cells.

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