which monocular depth cues were used to compare 2-D and 3-D metrics. For example, the optimal interval between frames is greater for objects when they appear to lie at different depths (*13*), and minimum frame duration for equal angular rotations in the frontal and depth planes is similar (*14*). It therefore seems likely that both monocular and disparity cues can be used to compute 3-D proximity.

Our results suggest that correspondence matching makes use of a 3-D spatial representation and that depth, or at least disparity, must be determined before motion matching is performed. This conclusion seems to hold for computer as well as biological vision. Correspondence-matching algorithms can also be improved by using disparity to assign depth (*15*).

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8. The disks all appeared the same size. There was no obvious effect of size constancy.
9. One pair of disks was always in front of the fixation plane and the other behind. Since fixation was at zero disparity, one pair was in crossed disparity while the other was uncrossed. Similar effects could be obtained when both pairs were at different crossed or uncrossed disparities. Relative rather than absolute disparities seemed to be important.
10. Observers differed somewhat in their sensitivity to frame duration. The 167-msec duration produced the best motion for J.V.O. and the poorest for K.C. At this speed, K.C., who had never seen stereograms before, found that the disks sometimes dissolved into the background. We believe that J.V.O., being experienced in viewing stereograms, was better at maintaining fusion. When extra fusion cues were provided by darkening the disks, K.C.'s performance was 93 to 100% at all frame rates.
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Technical Comments

Nucleosome Structure

Harauz and Ottensmeyer (*1*) present a structural model for the nucleosome core produced from electron energy loss (EEL) imaging and a new technique for three-dimensional (3-D) reconstruction. Their work can be criticized on two grounds. First, the EEL imaging required doses of electron irradiation that are known to destroy the high-resolution structure that was being imaged. Second, their novel reconstruction technique appears to depend largely on subjective judgments for the fit and selection of images. There are no objective criteria for determining the validity of the images or the reconstruction.

The authors' apparent assumption that the fine details in the EEL images reflect the high-resolution structure of the native nucleosome seems unwarranted in view of the very large electron dose required to obtain them—1000 electrons per square angstrom. Loss of high-resolution order has been demonstrated most precisely for crystalline specimens, where doses of one to ten electrons per square angstrom cause fading and loss of the diffraction pattern (*2*). This may only show loss of long-range order, but higher resolution has not been convincingly demonstrated for single protein molecules (*3*).

Perhaps the greatest effect of radiation damage is the loss of 50% of the mass of biological macromolecules, which occurs at a dose of 100 electrons per square angstrom. This has been demonstrated for a variety of model systems (*4*) and would mean that 50% of the organic matter (and an undetermined amount of the phosphorus) in the nucleosomes had been blasted away before the image recording could be completed. It is not clear how the residue from such an incineration could reorganize into a skeleton that retains the high-resolution structure of the protein and DNA.

If the images are artifacts, how could a 3-D model be reconstructed? I suggest that their reconstruction system should be easily capable of fitting noise into a plausible model. The only data presented are in a single, tiny image area. One can assume that other images are similar: three to six grainy splotches or streaks within the boundary presumed for the particle. With this limited detail, it is not surprising that most images could be rotated to fit at least one helix projection. The authors state that fully half the "images were rejected if the nucleosomes appeared distorted or severely altered by the electron bombardment." This selection, with no objective criteria for determining the goodness of fit or the validity of each image, raises doubts about the reconstruction.

It seems that the authors increased the pitch of the DNA superhelix from 2.8 to 4.0 nm to obtain a reasonable fit. Clearly this implies that the images are not just random noise, because they constrain the model. I suggest, however, that the only nonrandom feature is the spacing of the streaks and splotches. They are about 4 nm apart, which may reflect the transfer function and optical resolution of their image (like the granularity of the carbon film in conventional microscopy). Obviously one could only get a good fit if the model had spacings close to those in the image, so the pitch would have to be increased to 4 nm.

Finally, other work on EEL imaging suggests that some fraction, perhaps large, of the detail in Ottensmeyer's images may be amplitude contrast (*5*). One must be concerned, therefore, that the contrast reflects primarily the mass density and granularity of the specimen and carbon film, with phosphorus making only a small contribution.

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3. Ottensmeyer has stated that he obtained resolutions better than 1 nm in his previous work with dark-field electron microscopy. This has been criticized by A. Klug [*Chem. Scr.* **14**, 257 (1979)] with objections similar to those raised here. J. Dubochet also presented images similar to Ottensmeyer's myokinase that he found "on carbon films treated only with tap water" (*ibid.*, p. 293).
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Response: Erickson's comments on high-dose imaging are historical theoretical concerns that have been addressed and an-

swered by the results of experiments published over the last 15 years. His second concern stems from the first and so is not answered directly. Because he considers the images (useless) artifacts (my qualifier, since all electron micrographs are artifacts), no criteria can make the images or the reconstructions valid. However, for the reader who finds the compendium of results on high-dose imaging persuasive, we restate the criteria for selection and validity of the images implicit in our report (*1*). Erickson's concerns regarding mass-density effects that introduce nonlinearities in the image intensities resulting from multiple electron scattering and changes in the energy loss spectrum can be easily removed. The nucleosome has a diameter approximately one-tenth the mean free path of the electron at 80 kilovolts. As a consequence, multiple scattering, which would reduce the DNA-phosphorus signal, is on the order of 5% of total scattering. An error this small was not observed even at thicknesses greater than 100 Å (*2*). Similarly, the more exact treatment of the energy loss spectrum by Chang *et al.* (*3*) removes a further error that is on the order of 1.5% as calculated from their data; these effects have a negligible impact.

Direct imaging of noncrystallized and possibly noncrystallizable individual biological macromolecules by electron microscopy at high resolution requires (i) the elimination of resolution-limiting heavy atom contrast agents, (ii) a technique that provides sufficient contrast for visualization without such agents, and (iii) a number of interacting electrons sufficient to identify a high-resolution detail structurally or chemically. Dark-field electron microscopy and electron spectroscopic imaging, techniques that include electron energy loss (EEL) imaging, are two approaches that provide the required contrast directly. However, the electron exposure required to define a 5 Å resolution statistically ranges from 100 electrons per square angstrom past 1000 electrons per square angstrom, depending on the instrument used or on the energy loss measured. Results from both approaches are relevant to Erickson's concern, as indicated by his reference 3.

A potential spatial resolution of 3 Å or better in dark-field images was demonstrated by the direct imaging of individual atoms in fixed beam (*4*) or scanning transmission electron microscopes (*5*) even at electron exposures of tens of thousands of electrons per square angstrom (*6*). Problems with phase effects and selective enhancement of spatial frequencies by microscope transfer functions were not encountered by these techniques, which, like EEL imaging, delineate detail with amplitude contrast and operate predominantly under incoherent imaging conditions. Measurements in EEL imaging yielded potential resolutions of at least 4 to 8 Å at 100 electron volts (eV) and at 350 eV and about 3 to 5 Å and 7 Å at 150 eV (*7*).

The feasibility of using these high-exposure imaging capabilities to obtain high-resolution structural information on biological macromolecules in the face of potentially complete disruption of their three-dimensional atomic arrangement required the testing of two assumptions: (i) that the mass loss known to occur under such conditions is sufficiently proportional to leave a representative projection and (ii) that the lateral movement of atoms, ions, and radicals produced by electron bombardment is sufficiently small during image acquisition to result in an image of the projected mass that retained useful detail. The experimental results that delineated such detail in nucleic acids and proteins progressed through known (*8*) to unknown structures (*9, 10*), including specific heavy atom labeling and signal averaging for verification, and blind tests for even greater objectivity (*11*). Our awareness of the problems even led us to suggest the term microtephroscopy—a close look at the ashes (*9*).

For one unknown protein, protamine, the 5 to 6 Å detail seen was sufficiently definitive for us to propose a three-dimensional atomic model (*12*) whose structure was subsequently verified crystallographically (*13*). The same protein was used to define the exposure at which lateral diffusion finally obscured its definitive detail (*14*). Subsequently, completely defined objective criteria and computer analysis were used to confirm the presence of the alpha-helical structure at 5.4 Å and remnants of the amino acid side chains in images of poly-L-lysine (*15*). Klug's concerns (reference 3 in Erickson's comment) have therefore been fully addressed by experiment. Dubochet, in the same reference, indicated that known structures can also be found in and correlated with pure noise. For our selection of protamine and myokinase, whose structures were not known, this criticism did not apply. Finally, the advent of cheaper computing power has made possible the automatic selection of images with the use of minimal criteria (mass of the molecule), removing the last vestige of subjectivity from the process (*16*).

In the light of these results the high-dose micrographs of the nucleosomes (*1*) are valid, while the work itself defines the extent of the validity. The selection of images for reconstruction made it imperative to have a uniform population of identical core particles, measured biochemically in solution and densitometrically in the images, to minimize preparation artifacts and to have stigmatic, in-focus images. To maximize the probability of obtaining a more faithful projection of the mass, selection required minimal lateral diffusion of the mass beyond the nucleosome boundary of the first image, as measured by subtraction of the two consecutive images at different energy losses. Distortions and alterations from the first to the second image were easily recognized in the process. All particles that did not meet this criterion were eliminated (*1*). Finally, the phosphorus distribution had to correlate initially with the model of a two-turn DNA supercoil proposed by Finch *et al.* (*17*) in order to define the relative angles of the whole particles, of which the phosphorus distribution is just a part. Optimum angular assignment was determined iteratively by cross-correlation and least-squares difference after initial visual angular assignment from a gallery of orientations. Estimated refinement of angles by this process was to better than 5° even for an initial error of 20° in assignment (*18*).

The final selection criterion can be criticized, because it forced a bias toward the best available model of DNA coiling in the nucleosome (*17*). However, our own results on nucleosomes in chromatin, as opposed to isolated particles, had shown that the DNA-phosphorus distribution could indeed be described by a two-turn supercoil, not only in outline, but also in terms of the relative projected DNA mass distribution (*19*). Measurements on the electron scattering cross section of phosphorus from the nucleosomes had been made, but could not be used as a criterion, since other comparable measurements did not exist. In the meantime it was found that the phosphorus cross section from 50*S* ribosomal subunits (3000 P atoms) and from transfer RNA (about 80 P atoms) agrees with the measurement from those nucleosomes (about 300 P atoms) (*20*).

Nevertheless, Erickson is right, like Dubochet, when he states that even pure noise can be correlated with any model. What appears to have been missed in this case is the mere use of the model to obtain orientations for the nucleosomal protein distribution, about which no assumptions were made. In terms of protein distribution the entire process is self-regulating. For random assignment of angles from random "splotches or streaks within the boundary presumed for the particle," the purported protein distribution averaged for 55 particles in three dimensions should be a uniform, featureless, space-filling mass inside a spherical envelope. Such a result would have made us agree with Erickson's assumption of useless

"image artifacts." The observed, highly structured protein distribution that displayed unforced dyad symmetry and correlated well with biochemical and physicochemical data did not permit that conclusion.

The disagreement of the final reconstruction with the input model structure from Finch *et al.* (*17*) was discussed (*1*). More recent crystallographic data from Richmond *et al.* (*21*) indicate some concordance between the structures in individual components, such as the shape of histone H3, but the major shape difference of oblate versus prolate spheroid remains. We had argued that this difference is due to divergent preparative techniques (*1*); we still think so. However, a much greater similarity in structure and almost identical dimensions can be found in a comparison between our reconstruction and the crystal structure determination of the nucleosome protein core by Burlingame *et al.* (*22*). These authors determined the structure of that particle to be a prolate spheroid 110 Å long, a more slender 65 to 70 Å in diameter due to the absence of DNA, with a proposed pitch of the DNA helix of just less than 40 Å (*23*).

Obviously there is ample room for further definitive experimentation under different conditions, both in high-dose imaging techniques and in the determination of nucleosome structure. In addition, there is room for discussions like the present, which serve to clarify and concentrate thoughts and data that otherwise are scattered over many publications. I would be pleased if this exchange persuaded Erickson to apply his own experimental expertise, in addition to his skeptical interest, to the areas of high-dose imaging, electron energy loss imaging, nucleosome structure determination, or three-dimensional reconstruction.

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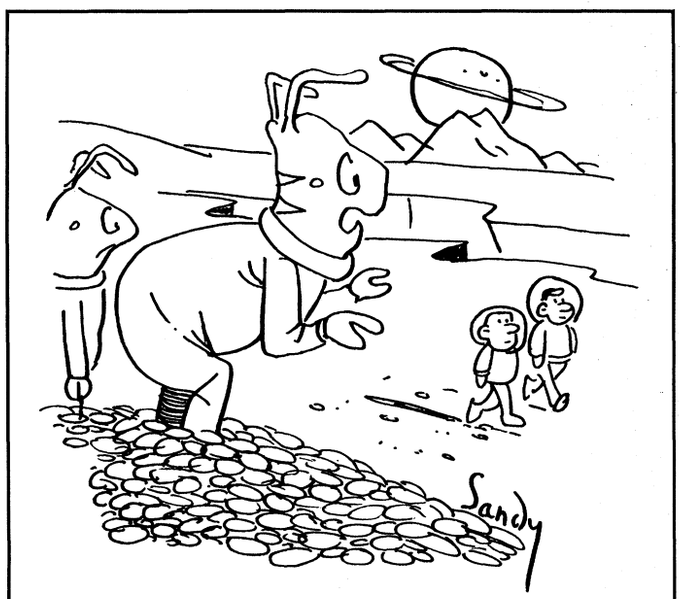

"This looks like the end of civilization, as we know it"