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A BRIEF INTRODUCTION TO AIMS AND METHODS FOR IMAGE PROCESSING OF ELECTRON MICROGRAPHS OF BIOLOGICAL SPECIMENS

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Abstract

A common problem with electron micrographs of biological objects is that fine details are usually faint and, moreover, tend to be obscured by background noise from stain and support film. Filtering is a useful way to improve the signal-to-noise ratio and is particularly important when trying to detect small changes (conformational or due to labelling) or when examining frozen hydrated objects, where the statistical definition of the image has been reduced because of the low-dose conditions needed to prevent radiation damage. Filtering can also be used to address the superposition effects that result from the large depth of focus of electron microscopes and is particularly effective when dealing with the Moiré patterns produced by overlapping regular layers as found, for example, in helices or tubes. The alteration of the image by the non-uniform phase contrast transfer function of the microscope can also be compensated for by using image processing in conjuction with electron diffraction. Finally, the essentially two-dimensional nature of the information can be extended to three-dimensions by combining views from different orientations. Fourier-based methods are particularly effective when dealing with regular objects, such as crystals, helices and shell structures such as icosahedral viruses.

Key words: Image processing, resolution, biology, Moiré patterns, helices

Introduction

Although electron microscopy of biological specimens has produced a wealth of information about the structure of cellular components, it has been more difficult to obtain information on their molecular structure or the arrangement of molecules in sub-cellular assemblies. These difficulties often arise from the low contrast and radiation sensitivity of most biological specimens, and so many of the problems encountered with these objects are rather different to those found with materials science specimens and account for the different emphasis often placed on image processing by biologists. Many of the problems intrinsic to electron micrographs of biological specimens can be alleviated by computer image processing. These methods often enable higher resolution structural information than that visible by direct inspection to be extracted and analysed, and so are a powerful adjunct to electron microscopy when studying the structure of biological macromolecules and their arrangement in sub-cellular assemblies. In addition to the series of manuscripts which follow in this volume, there have been a number of in-depth reviews of this field (for example, Aebi *et al.* 1984; Amos *et al.* 1982; Frank *et al.* 1988; Glaeser, 1985; Stewart, 1988*a,b*; Moody, 1990) to which the reader is referred for details of the methods employed and specific examples. My aim here is rather to provide a broad overview of the field, with the objectives of explaining the methods that are used with biological specimens; the problems and difficulties they present; and indicating how the nature of biological structural problems necessitates a somewhat different emphasis to that placed on image processing by physicists and materials scientists.

Although in principle these computer-based image processing methods could be applied to almost any sort of electron micrograph of any biological object, they are generally most powerful in several defined cases where the intrinsic limitations of the specimen are particularly severe or where they inhibit meaningful interpretation of the structural data that can be obtained. Generally, biological objects

have rather low intrinsic contrast and interpretation is complicated by superposition of information from different structural levels and by the inherently two-dimensional nature of the images obtained (unlike light microscopy, the large depth of focus of electron microscopes generally means that it is not possible to focus on different structural levels in the object). The sensitivity of most biological objects to radiation damage and some preparative procedures can cause further difficulties. My aim here is to provide a background for assessing image processing of biological material by identifying some of the major areas in which image processing has been useful and also identifying areas in which the methods might be usefully extended.

It is perhaps worth pausing to think how the biologist's view of the world may be different to that in some other fields of science. Biology studies living things and it is this that distinguishes it from disciplines such as Physics and Chemistry. What differentiates a living system from a mixture of molecules in a test tube is the way molecules are organised. One of the secrets of life is macromolecular organisation and it is primarily for this reason that biologists are interested in structure. But biologists are rarely interested in structure for its own sake. The aim of Structural Biology is usually to relate structure to function. Therefore, one's primary emphasis tends to be to obtain information that can be interpreted usefully in a biological context rather than pure structural information in its own right. Appreciating this perspective can make it easier to understand the emphasis many biologists place on different image processing methods.

Biological Specimens

There are a range of methods generally used to prepare biological specimens for higher resolution electron microscopy. Although material can be observed unstained (usually embedded in a thin film of glucose or vitrified water), it is more usually prepared by negative staining or shadowing. For negative staining, specimens are attached to grids and then washed with a heavy metal salt (such as uranyl acetate) and allowed to dry, so embedding the object in a thin layer of stain. Because the stain is more dense than biological material, the specimen appears light against a darker background (the staining is called "negative" because it results from the exclusion of stain by the object). For shadowing, a heavy metal such as platinum is evaporated at an angle. A flat carbon film would be uniformly coated under these circumstances, but any samples on it will prevent metal from coating an area close to the side opposite to the source of evaporation, and so produce a shadow that is related to the topography of the object (the object will also become partially coated).

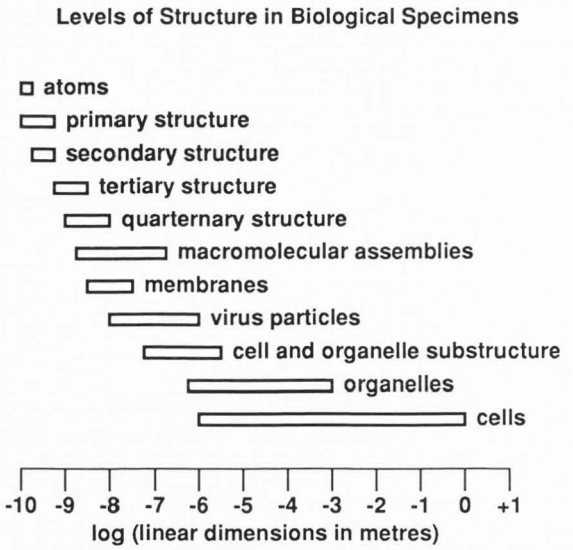


Figure 1. Levels of structure in biological material. Reproduced from Stewart (1990).

Resolution

Although most often image processing tends to be discussed in terms of resolution, a very large number of biological images are limited by detection efficiency rather than the inherent instrumental resolution of the microscope or the resolution to which structural information has been preserved in the specimen. It is very frequently the case that the features of interest are not located next to one another, and so simply detecting their presence can be extremely rewarding. Therefore, the ability to detect a particular structural feature may depend more on contrast than on its actual size. However, although high contrast can aid in detection, ultimately a certain level of spatial frequency information will usually be required for unequivocal recognition. In this context, it is usually helpful to think in terms of levels of structure within biological specimens. Proteins, for example, have a primary structure (their amino-acid sequence); a secondary structure (how the linear sequence folds to form simple elements such as α-helices, β-sheets and turns); a tertiary structure (how the elements of secondary structure are arranged to produce domains) and often a quarternary structure (how molecules or chains interact to form a macromolecular complex). Higher-order structures, such as virus particles, ribosomes, nuclear pores and filaments, are formed from assemblies of molecules. Generally it is necessary to cross particular resolution thresholds in order to detect particular levels of structure and it can be very helpful to have an idea of roughly where these thresholds are likely to be. Figure 1 gives an indication of the likely thresholds for different levels of structure and it is worth noting where these lie in respect to the resolution it is easy to obtain by electron microscopy. Therefore, because it is

frequently possible to obtain resolutions of the order of 20-30Å on negatively-stained biological specimens, electron microscopy is a powerful method for investigating the overall shape and, most particularly, the arrangement of molecules in macromolecular assemblies. However, for the great majority of biological specimens, there is only a very small gain in useful structural information for resolutions better than about 20 Å until it becomes possible to detect secondary structure reliably at about 7 Å resolution (there are, of course, a few specific exceptions to this generalisation). It is for this reason that the often heroic efforts to extend resolution below about 20 Å do not in many instances increase one's understanding of the biological system under investigation. Also, it may be possible to cross a resolution threshold (and so solve a specific question) using comparatively crude and unsophisticated image processing methods (which can also be much more robust as well as cheaper and easier to implement) and this can save a great deal of work while still yielding a reliable answer to a biological problem.

It is also important to identify at which level of structure the answer to a particular problem lies. A great deal of frustration can result from addressing a problem at an inappropriate level of structure. We are all familar with the Molecular Biologist who cannot appreciate that the answer to his problem lies in the tertiary or quarternary structure of his material and that a band on a gel or a cDNA sequence is not really telling him much. But we should sometimes ask if we too may not be addressing an inappropriate structural level just because it is easy to investigate.

Generally biological electron microscopy can be divided into studies that aim to examine the internal structure of molecules (the detailed pattern of chain folding in terms of at least secondary structure) and those that concentrate on the overall shape of molecules and their interactions in assemblies. The aims and methods used to process images are very different in each case. Whereas rather simple and straightforward methods, such as filtering, are often adequate when examining overall molecular shape (Moody, 1990; Stewart, 1988*a*), much more intensive analysis is necessary when attempting to examine internal structure (see Henderson *et al.* 1990).

Signal-to-noise ratio

Structural information in many electron micrographs of biological material is often masked because of poor signal-to-noise ratio (Figure 2). It is important to remember that there are two components of this problem: the low intrinsic contrast of most biological specimens (particularly at higher spatial frequencies); and the rather high level of the background, due to stain granularity and support film. There can be an additional problem of poor statistical definition (see below) if low-dose conditions are employed to minimise radiation damage to the specimen.

Biological specimens are generally composed of elements of low atomic number which have low scattering factors and so are essentially weak phase objects which have low contrast, similar to that of the supporting carbon substrate. Consequently, stains containing elements of high atomic number are often employed to enhance image contrast. Negative staining has been particularly successful in delineating the surface contours of molecules. However, negative stain usually has some underlying structure as a result of its forming grains, and so is usually unable to delineate surface features below its grain size. Even for uranyl acetate, this grain size is of the order of 20 Å. Shadowing, which produces much higher contrast than negative staining, is even more limited with grain size usually being limited to about 30 Å except in some special applications (Bachmann *et al.* 1985). Moreover, the ability of such contrasting agents to outline features only slightly larger than their grain size will be somewhat compromised and so, even in the presence of negative stain, most higher resolution structural features are usually rather faint. Additionally, most negative stains tend to attach preferentially to particular groups on the surface of macromolecules and this "positive staining" can produce an artefactual impression of a surface depression. An extreme example would be fibrous systems such as collagen, where positive staining gives a pattern of fine striations perpendicular to the fibre axis which, if interpreted conventionally, would indicate a series of discontinuities along the molecule. Although positive staining is probably less important with globular proteins, it is still likely to introduce artefactual contrast at higher resolutions and features below about 20 Å should probably be assumed to derive from this source until proven otherwise. Thus, the combination of grain size and positive staining indicates that it is seldom productive to extend analysis of negatively-stained specimens below a resolution of about 20 Å. Fortunately, most proteins do not appear to have a great deal of structural information present below this resolution until the level of secondary structure is reached, and so this is not a major impediment to investigating macromolecular structure and assembly by electron microscopy. However, the faint nature of contrast in the 20-40 Å range, coupled with the way in which this information tends to be obscured by the granularity of the stain and support film, does inhibit structural investigations severely and so image processing methods such as filtering are often extremely useful in these instances.

An alternate method of examining biological specimens is to suspend them in a thin film of vitrified water (reviewed by Dubochet *et al.* 1988; Stewart & Vigers, 1986). This can result in outstanding preservation of the material, but contrast is very low, being derived from the density difference between protein and water. Large defocus values can be used to enhance phase contrast over a limited range of spatial frequencies, but the sensitivity of

vitrified specimens to radiation damage limits the dose that can be used to record images (bubbling and other gross damage is often seen at about one tenth the dose routinely used to record images of negatively-stained material). Consequently, electron micrographs of vitrified specimens are usually not only faint but have rather poor statistical definition as well.

Elements of secondary structure or individual amino-acid side chains are contrasted from density variations within the actual protein molecule, and so cannot be enhanced by stains. For reasons that are only partially understood (see, for example, Henderson *et al.* 1990), electron microscopes have rather limited efficiency in recording this information in images, although it is generally well preserved in electron diffraction patterns. Much more sophisticated processing, to take account of lattice disorder and defocus, are necessary to obtain reliable information at high resolution, especially with tilted specimens (see Henderson *et al.* 1986, 1990).

Enhancing signal-to-noise ratio can also be vital when attempting to detect small changes in specimens, either as a result of introducing a specific label (such as an antibody or heavy metal cluster) or of a physiologically important conformational change. In these instances it is often necessary to obtain accurate structure factors to enable a difference Fourier to be calculated, and so averaging over a number of areas, correcting for lattice disorder, and compensating for the contrast transfer function become more important.

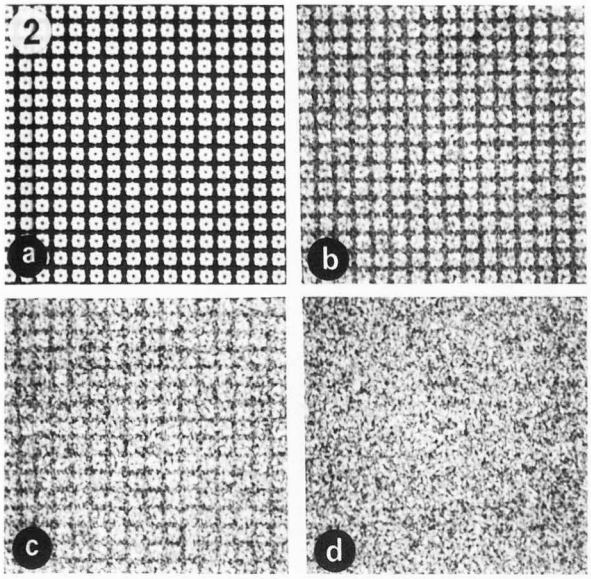
Radiation Damage

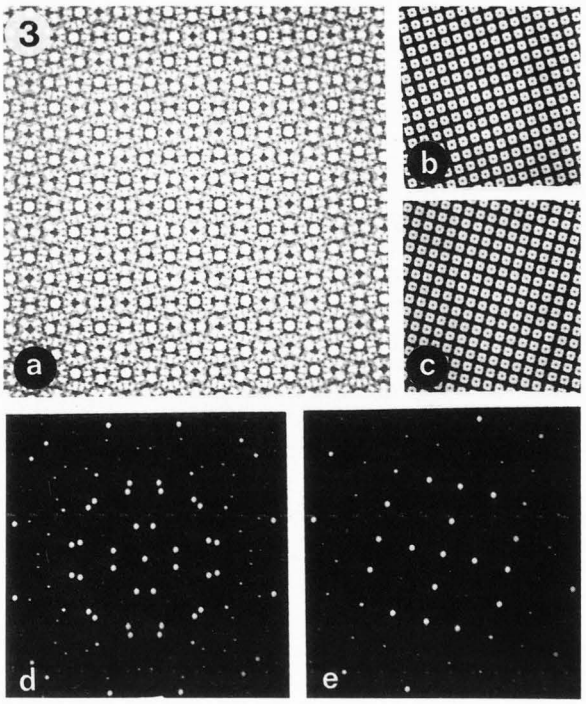
Most biological material is very sensitive to radiation damage and will have been mainly destroyed by the electron doses usually employed to record electron micrographs. Radiation damage is most rapid at high spatial frequencies, and so extremely low doses are needed if information from elements of secondary structure are to be preserved. Negative stain and metal shadow are also subject to radiation damage and can change their distribution during the recording of micrographs (Unwin, 1975). Although the changes in the plane of the specimen often do not appear to be very great, there is usually pronounced shrinkage of the specimen perpendicular to this plane (ie parallel to the microscope axis) as can be seen most easily by the changed position of Laue zones in tilted specimens (Berriman & Leonard, 1986). This shrinkage can have a profound effect on structures recorded using tilt series, especially if low doses are employed, since the rate of shrinkage tends to be greatest for low irradiation doses (of the order of those used to record a single low-dose image). Some of the disordering effects of radiation damage can be reduced by using a spot-scan technique (Henderson & Glaeser, 1985).

The use of low-dose conditions often results in the density of the image on film being rather low, and so can become partially obscured by the fog level of the film. Moreover, because the number of electrons scattered by most biological macromolecules is small under these conditions, the statistical definition of these images is often low. Both of these effects tend to obscure fine detail in the images and so reliable information can usually only be extracted by using of averaging methods (most often Fourier-based). This consideration has restricted low-dose methods mainly to crystalline objects.

Non-Uniform Contrast Transfer

Contrast in images of biological objects is usually generated in two ways: *amplitude contrast* is generated by the removal of some electrons from the incident beam (for example, by apertures absorbing electrons scattered by the specimen); whereas *phase contrast* results from different areas in the object retarding the incident electron wave to different extents. Phase contrast will only contribute to the image if the difference in retardation of the waves can be visualised by interference. In light microscopy, phase contrast is produced by using a quarter wave plate. Such plates are not available for electron microscopy and instead phase contrast is produced by the phase shift produced by varying the objective lens defocus. However, the phase shift produced in this way varies with spatial frequency, and so not all spatial frequencies in the object are reproduced with equal fidelity in the image. Fortunately, for negatively stained material to resolutions of the order of 20 Å, appropriate defocus causes phase and amplitude contrast to approximately complement one another, and so a roughly constant transfer function can be produced (Erickson & Klug, 1971). Consequently, in appropriately defocused electron micrographs of negatively stained material, the microscope imaging will introduce only minor alterations in image density. However, at higher resolutions (with unstained material, for example) this compensation is not possible, and so it is necessary to correct the images for the effect of the contrast transfer function. The contrast transfer function of the microscope is most easily formulated in terms of the object's Fourier transform. Both the amplitude and phase of the transform are altered. In principle it is possible to correct for these effects by multiplying the image transform by the inverse of the contrast transfer function, but with crystalline objects, it is usually easier to use electron diffraction to obtain amplitudes and Fourier-based image processing to obtain phases (note that the amplitude and phase corrections refer to the complex number used to represent the Fourier transform and are not directly related to amplitude contrast or phase contrast). Special corrections are needed for tilted specimens, where the defocus changes across the specimen (Henderson *et al.* 1990).





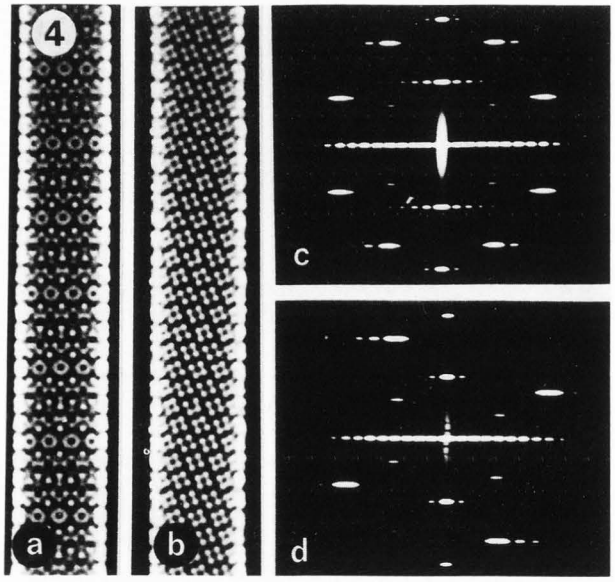


Figure 2. Effect of increasing noise content on the visibility of structural information in images. As the signal-to-noise ratio decreases from 1:1 (a) through 1:10 (b) and 1:20 (c) to finally 1:100 (d), the visibility of fine structural detail decreases. At 1:10 many fine details are still visible, albeit rather faintly, but by 1:20 only the overall particle shape and position are clear and even this is lost by 1:100. (c) is probably fairly typical of many electron micrographs of biological material and so increasing the signal-to-noise ratio by simple filtering, to give something like (b) or even (a) makes fine detail much easier to see. Reproduced from Stewart (1986).

Figure 3. (a) Moiré pattern formed by overlapping two regular arrays (b) and (c) that have been rotated relative to one another. Although the image is confused and cannot be interpreted directly, the lattices from each layer can be separated in Fourier transforms (d) and an image of a single layer (c) reconstructed from its corresponding transform (e) by Fourier inversion. Reproduced from Stewart (1986).

The essentially phase nature of images from vitrified specimens can present special difficulties. Here defocus is usually used to accentuate a comparatively narrow band of spatial frequencies and some care must be taken in interpreting such images directly (see Stewart & Vigers, 1986). The problems are usually least with crystalline objects, but can be severe when dealing with bounded objects such as virus particles or nuclear pores. Additional problems may result from inelastic scattering producing contrast at low spatial frequencies in vitrified material.

Figure 4. Processing of helices. Micrographs of helical particles are usually difficult to interpret directly because of the Moiré pattern (a) that is formed by the superposition of the regular patterns on their top and bottom. The diffraction pattern of a helix (c) consists of a number of layer lines (see Moody, 1990; Stewart, 1988*b*) but these can be analysed to identify those that derive from the top (d) or bottom, and a single-sided image (b) reconstructed. Reproduced from Stewart (1986).

Superposition

For most negatively stained objects, the depth of focus of electron microscopes is large compared with their thickness, and so structure from all levels within the specimen is in focus simultaneously. This superposition of structural information can make interpretation of micrographs extremely difficult (see Figure 3). The most severe problems are caused by the superposition of regular arrays as can happen with crystals, tubes, helices and shells. Often the Moiré patterns produced in this way cannot be interpreted directly and one of the most effective uses of image processing can be to resolve these confused patterns. In this case, filtering is used to remove unwanted signal (from one or more of the superimposed levels) as well as noise. Helical structures (Figure 4) can be treated in a manner analogous to that employed for overlapping crystals and are most easily analysed in terms of a (n,l) plot (see Stewart, 1988*b*). Because they are bounded objects, the Fourier transforms of helices consist of a series of layer lines that can be expressed as a sum of Bessel functions, but it is usually easy to identify the contributions from a single side and so reconstruct an image of the upper or lower half (see Figure 4).

In addition to the problems produced by the Moiré patterns generated by the superposition of regular components of the object (which can be viewed as a nuisance that can be removed by appropriate filtering), the superposition of structural elements from different levels in the object often frustrates detailed interpretation of electron micrographs of biological objects. Many different three-dimesional arrangements and shapes are often consistent with the projected image density observed, and so there is usually considerable ambiguity associated with a single view of an object. These problems generally become more pronounced as resolution is increased. Thus, superposition of elements of secondary structure within a molecule generally makes projection views at high resolution uninterpretable (with the singular exception of the 7 Å map of purple membrane!). To resolve these sorts of superposition problem (that result from the depth of focus of the microscope), it is usually necessary to extend the study to three dimensions.

Three-Dimensional Information.

Because they represent a projection of object density and also because of the superposition of information from different levels in the object, electron micrographs of biological specimens can sometimes be difficult to interpret unequivocally. A wealth of additional structural information can be obtained by generating three-dimensional models. This entails combining information from images of the object in different orientations, relying on the fact that each image will be a projection of the object density in a direction parallel to the direction of view. With helical objects this can often be accomplished with a single view of the particle, exploiting the fact that successive sub-units following the helical paths in the object will be rotated relative to one another. Mathematically such reconstructions are performed most easily in terms of helical waves that produce layer lines that can be analysed as a sum of Bessel function terms in computed Fourier transforms (see Moody, 1990; Stewart, 1988*b*). An analogous method can be employed with particles having icosahedral symmetry.

Three dimensional reconstructions of crystals or even single particles can be produced by combining views from a number of different angles (Amos *et al.* 1982). Ideally these views should sample all possible orientations, but restrictions on tilting in the microscope usually result in a missing cone of data in the Fourier transform. However, although the missing cone does result in a reconstruction in which resolution is anisotropic, it is usually possible to make meaningful interpretations. Combining views becomes particularly complicated when working at high resolution and quite involved image processing is required to compensate for changes in defocus and disorder of the material (see Henderson *et al.* 1990).

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.