Comparison of phase contrast transmission electron microscopy with optimized scanning transmission annular dark field imaging for protein imaging

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Received 4 June 2002; received in revised form 8 November 2002

Abstract

Henderson has already shown that electron microscopy should be superior to X-ray and neutron diffraction for determining protein structure with minimum radiation damage. Since the contrast for a molecule embedded in vitreous ice is very low, it is conceivable that dark field imaging would be superior to bright field phase contrast microscopy. A detailed analysis of contrast and signal/noise for both imaging modes is presented. Annular dark field scanning transmission microscopy gives improved contrast and equivalent signal/noise to phase contrast TEM when the molecule is the same thickness as a vitreous ice embedding medium. For a constant embedding medium thickness of 200 Å the contrast is equivalent to phase contrast TEM but the signal/noise is 5 times worse. Even with an efficient detector that only excludes scattering less than 5 mrad there is insufficient signal at a dose of 5 electrons/Å² to produce an image with more than 1 electron/per pixel. For larger molecules (> 100 Å thick which corresponds to 420 kDa for spherical molecules) the weak phase object approximation used to analyse a phase contrast image no longer applies at 100 kV. This limit could be extended to about 200 Å (about 3 MDa) if a 400 kV microscope were used.

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Keywords: Protein imaging; STEM ADF; Phase contrast

1. Introduction

Understanding the three-dimensional structure of proteins is critical in determining how they perform their biological functions. Although in principle the sequence of the approximately 30K proteins encoded by the human genome is known, the number of proteins whose structure has been determined is still less than about 2000 [1]. The technique of choice is usually X-ray diffraction, but this requires perfect crystals with approximately $10^{12}$ molecules, limiting it to proteins that crystallize easily. Electron microscopy and quantitative diffraction have been successfully used to determine the structure of membrane proteins, the best-known example being purple membrane [2,3].

In a comprehensive and definitive review, Henderson [4] showed that electron microscopy should be superior to both X-ray and neutron diffraction in terms of resolution for a given dose. He only considered bright field phase contrast microscopy.
microscopy. He argued that for weak scattering that is a fraction, $f$, of the incident beam, the phase contrast signal is linear in $f$, while any amplitude scattering has to be proportional to $f^2$. For specimens where the protein is embedded in vitreous ice, as is the standard cryomicroscopy practice, the contrast must be very low. It would be expected that any dark field technique would give superior contrast for a weakly scattering specimen because the large background from the unscattered (or more correctly forward scattered) electrons is absent.

The most efficient way to perform dark field microscopy is to use an annular detector in STEM. Annular dark field (ADF) STEM is often used for determining molecular weights [5], though doses can be high as a large inner cut-off angle is used (40 mrad on Brookhaven instrument). Before ADF STEM became widely available early work by Ottensmeyer showed that structural information on DNA and polylysine molecules could be obtained with a displaced aperture dark field microscopy [6]. More recently the structure of Signal Sequence Binding Protein SRP54 has been determined by Czarnota et al. [7], using ADF STEM combining signals from the high angle (40–200 mrad) and lower angle (15–40 mrad) detectors.

To be competitive STEM must be made as efficient as possible and, contrary to popular belief, high inner cut-off angles are not necessary to discriminate between different elements. The inner cut-off angle can be reduced to about 5 mrad. Cross sections for ADF scattering are compared to scattering factors for phase contrast, and it is shown that in both cases most of the contrast arises from a combination of the differences in composition and density between the vitreous ice embedding medium and the protein. A theoretical analysis of contrast and signal/noise is given for both bright field phase contrast and ADF STEM. Following Henderson [4], results are tabulated for different sizes of protein macromolecule. If the embedding medium is the same thickness as the molecule ADF STEM always gives better contrast with approximately the same signal/noise as phase contrast TEM. Making a more realistic assumption that the embedding vitreous ice is about 200 Å thick, the contrast is about the same as phase contrast TEM, but the signal to noise is 5 times lower.

A more serious problem for ADF STEM is the low values of the cross sections, even for a reduced inner cut-off angle of 5 mrad. This results in less than 1 electron per 3 Å pixel at a dose of 5 electrons/Å² that is consistent with preserving the protein structure. Larger molecules thicker than 100 Å (approximately 420 kDa for a spherical molecule) are no longer weak phase objects at 100 kV and it is inappropriate to base structural analysis on this simple model. Increasing the accelerating voltage to 400 kV means that molecules 200 Å thick in the beam direction can be analysed.

2. Theory

We shall assume that the specimen is a protein or some other molecule of lateral size $D$ embedded in vitreous ice of thickness $t$. The thickness of the molecule at a position $r$ is $f(r)t$, where $f(r)$ is a function such that $f(0) = 1$ then the thickest part of the molecule would be the same thickness as the embedding medium. A schematic drawing showing the specimen is given as Fig. 1.

If the average electron scattering potential along the beam direction is $\bar{\mathcal{V}}(r)$ then standard weak phase object imaging theory gives the intensity at position $r$ as

$$ I_I(r) = 1 + \sigma \bar{V}(r)t * s(r), $$

where $\sigma$ is an interaction constant defined by

$$ \sigma = \frac{me^2}{\hbar^2 k}. $$

![Fig. 1. Schematic diagram showing molecule of diameter $D$ embedded in vitreous ice of thickness $t$.](image)
The smearing function \( s(r) \) is the Fourier Transform of the phase contrast transfer function of the electron microscope \( S(u) = \sin(\chi(u)) \) where \( \chi(u) \) is the wavefront aberration function given by

\[
\chi(u) = k \left( \frac{1}{4C_0} \left| \frac{u}{k} \right|^4 - \frac{1}{2} \frac{\Delta}{k} \left| \frac{u}{k} \right|^2 \right)
\]

where \( u \) is a scattering wavevector. The smearing function represents the degradation of the resolution due to the aberrations of the microscope. We shall neglect it in our discussions since we are only considering an approximate expression for the contrast with a resolution greater than the point resolution of the microscope.

Making use of the properties of Fourier Transforms, the average value of the potential in real space is related to the zero scattering wave vector or scattering angle component in reciprocal space.

\[
V(0) = \frac{1}{V_N} \int V(r) \, dr.
\]

In a complete unit cell \( V_N \) would be the cell volume. Since we are dealing with a molecule with no ordered structure we will just work in terms of an average volume per atom \( V_{\text{ave}} \). The Fourier Transform of the potential can be related to the electron scattering factor by

\[
\bar{\psi}_\sigma = \frac{f_{\text{el}}(0) \lambda}{V_{\text{ave}}},
\]

where \( \lambda \) is the electron wavelength. (Note: This analysis could have been performed on a large block of the material \( D \times D \times D \) which would act as a “unit cell” for the molecule. The electron scattering factor would then become a structure factor and \( V_{\text{ave}} \) would be replaced by the “cell” volume \( D^3 \).)

Assuming an average protein composition of \( C_{0.31} \) N_{0.08} O_{0.08} S_{0.005} H_{0.525} \) and a density of 1.3 g/cm\(^3\) allows us define an average electron scattering factor for the molecule \( f_{\text{el}}^{\text{ave}} \)

\[
f_{\text{el}}^{\text{ave}} = \sum_n f_n f_{\text{el}}^n,
\]

where the index \( n \) refers to a particular type with atomic fraction \( f_n \). For water if we neglect hydrogen the average electron scattering factor is

\[
f_{\text{el}}^w^{\text{ave}} = \frac{4}{3} f_{\text{el}}^w.
\]

If the average number of atoms per unit volume is \( N_p \) for the protein and \( N_w \) for water then from Eqs. (1), (5) and (6) the observed intensity is

\[
I_T(r) = J d^2 \left( 1 + (N_p f_{\text{el}}^p(0) - N_w f_{\text{el}}^w(0)) t f(r) \lambda \right),
\]

where \( J \) is the dose in electrons/unit area and \( d \) is the linear pixel dimension. The contrast depends on both a difference in electron scattering factors and a difference in atomic density. The variation of the electron scattering factors for the principle elements present C, N and O is given as Fig. 2. These were calculated from the corresponding X-ray scattering factors using the Mott formula [8]. There is a 15% difference between the carbon and oxygen scattering factors at low resolutions around \( s = 0 \), but the scattering factors converge at \( s = 0.4 \text{ Å}^{-1} \) corresponding to a resolution of about 2.5 Å. For our purposes what matters is the average electron scattering factor defined in Eq. (6a). In Fig. 3 this is plotted for the average protein and water. The average protein has an average scattering factor that is almost twice that of water for small scattering angles corresponding to low resolution.

For simplicity, like Henderson [4], we shall assume a spherical molecule. The averaged value of the molecule thickness, \( f(r) \) is equal to \( \frac{2}{3} D \), the

![Electron Scattering Factors](image-url)
The contrast is
\[ C = \frac{(N_p f_{el}^p(0) - N_w f_{el}^w(0))^2}{2} D^2 \lambda. \]  
and the signal in a pixel of size \( d \times d \) is \( S = JD^2 C. \)

The background is due to the unscattered (or forward scattered) beam and is \( JD^2 \) per pixel so the noise per pixel is \( \sqrt{JD^2} \). There will also be a noise contribution from random variations in the thickness of the embedding medium. Since the embedding medium is also a weakly scattering object this contribution to the noise will be small compared to shot noise from the constant background.

The signal/noise is therefore
\[ \frac{S}{N} = C \sqrt{JD^2}. \]  
The contrast, number of electrons per pixel contributing to the signal and the signal to noise are all tabulated in Table 1 for the same range of protein molecules as Henderson considered, assuming like Henderson a dose of 5 electrons/Å². To gauge whether the specimen is a weak phase object the phase change across the vitreous ice given by
\[ \Delta \phi = N_w f_{el}^w(0) \lambda t \]  
is also tabulated.

Henderson [4] has a different model for the contrast of a protein that gives a different functional form for the contrast. He assumed that there are \( N_{at} \) atoms at random positions in a spherical molecule diameter \( D \) and that the average electron scattering factor is \( f_{el} \). The structure factor for the average reflection is therefore
\[ F = \frac{1}{tD^2} f_{el} \sqrt{N_{at}} \]  
since all the phases are random. The fraction of the incident amplitude in a given reflection for a thickness \( t \) is then
\[ tF = \frac{\lambda}{D^2} f_{el} \sqrt{N_{at}}. \]  
If the linear pixel size or resolution is \( d \), then a total of
\[ N_r = \left( \frac{\pi D^4}{2t^2} \right) \]  
independent reflections (the \( +g \) and \( -g \) reflections are related to each other) contribute to the image with random phases. The contrast is then
\[ C = \frac{\lambda}{D^2} f_{el} \sqrt{N_{at} 2 \sqrt{N_r}}, \]  
which simplifies to
\[ C = \frac{\lambda}{D^2} f_{el} \sqrt{2\pi N_{at} t}. \]  

Unlike the theory given above, this model is not based on the idea that the contrast arises from difference in scattering between the molecule and the embedding medium. It also predicts a square root dependence on molecule diameter, whereas our model predicts a linear dependence. This suggests that the phase change is related to random changes in apparent thickness or number of atoms. For comparison Henderson’s value of contrast are also given in Table 1. Despite their different assumptions and the approximate nature of both models the values of contrast are similar.

3. STEM imaging

In materials science dark field is often used because the signal is sensitive to atomic number. Although high angle ADF scattering is not exclusively due to Rutherford scattering from the
nucleus [9,10], and therefore does not vary with atomic number precisely as $Z^2$, the signal is still strongly dependent on atomic number. The same ideas have formed the basis of the use of ADF techniques to measure molecular weights in biology. Both materials and biological electron microscopists have implicitly assumed that a large scattering angle is necessary for discriminating between elements. The ADF detector in STEM collects electrons that have been scattered by angles greater than the inner cut-off angle defined by the hole in the detector (see Fig. 4) and less than the outer cut-off angle defined by the detector size. The scattering is weak at high angles, so for practical purposes we shall assume an outer cut-off angle of 100 mrad at 100 kV. The scattering into such a detector can be characterized by an appropriate integrated cross section

$$\sigma = \int_{\theta_1}^{\theta_2} \frac{d\sigma}{d\Omega} d\Omega,$$

(15)

where $\theta_1$ and $\theta_2$ are the inner and outer cut-off angles, respectively. For a material with random atomic positions the differential scattering cross section is given by the square of the electron scattering factor

$$\frac{d\sigma}{d\Omega} = f^2(\theta).$$

(16)

Fig. 5 shows the variation of the cross sections for C, N and O as a function of inner cut-off angle at 100 kV. The cross section increases as the inner cut-off angle is reduced, saturating around 5 mrad, though the difference between the cross sections for different elements is also reduced. Clearly there is still discrimination between different elements, even for these very low inner cut-off angles. There is therefore a compromise between maximizing signal and increasing the ability to discriminate between different elements, though the results suggest that an inner cut-off angle between 5 and 10 mrad would be optimal. An averaged cross section

$$\sigma_{\text{ave}}^n = \sum_i^n f_i \sigma_{\text{el}}^n$$

(17)

Table 1
Contrast and signal/noise for phase contrast TEM imaging with a dose of 5 electrons/Å² at 100 kV

<table>
<thead>
<tr>
<th>Weight</th>
<th>Diameter (Å)</th>
<th>Contrast</th>
<th>Number of electrons</th>
<th>Ice phase change radians</th>
<th>Henderson contrast</th>
<th>S/N 2.5 Å</th>
<th>S/N 5 Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large virus</td>
<td>300 MDa</td>
<td>900</td>
<td>1.69</td>
<td>52.94</td>
<td>2.60</td>
<td>0.32</td>
<td>9.47</td>
</tr>
<tr>
<td>Small virus</td>
<td>11 MDa</td>
<td>300</td>
<td>0.56</td>
<td>17.65</td>
<td>0.87</td>
<td>0.19</td>
<td>3.16</td>
</tr>
<tr>
<td>Ribosome</td>
<td>3.3 MDa</td>
<td>200</td>
<td>0.38</td>
<td>11.76</td>
<td>0.58</td>
<td>0.16</td>
<td>2.10</td>
</tr>
<tr>
<td>Enzyme</td>
<td>1.4 MDa</td>
<td>150</td>
<td>0.28</td>
<td>8.82</td>
<td>0.43</td>
<td>0.13</td>
<td>1.58</td>
</tr>
<tr>
<td>Enzyme</td>
<td>420 kDa</td>
<td>100</td>
<td>0.19</td>
<td>5.88</td>
<td>0.29</td>
<td>0.11</td>
<td>1.05</td>
</tr>
<tr>
<td>Enzyme</td>
<td>180 kDa</td>
<td>75</td>
<td>0.14</td>
<td>4.41</td>
<td>0.22</td>
<td>0.09</td>
<td>0.79</td>
</tr>
<tr>
<td>Enzyme</td>
<td>52 kDa</td>
<td>50</td>
<td>0.09</td>
<td>2.94</td>
<td>0.14</td>
<td>0.08</td>
<td>0.53</td>
</tr>
<tr>
<td>Enzyme</td>
<td>18 kDa</td>
<td>35</td>
<td>0.07</td>
<td>2.06</td>
<td>0.10</td>
<td>0.06</td>
<td>0.37</td>
</tr>
<tr>
<td>Enzyme</td>
<td>7 kDa</td>
<td>25</td>
<td>0.05</td>
<td>1.47</td>
<td>0.07</td>
<td>0.05</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Fig. 4. ADF detector in STEM showing inner and outer cut-off angles.
can be defined just as was done for the electron scattering factors.

Fig. 6 shows the scattering cross section for water and a protein as a function of inner cut-off angle using the same composition as assumed above. The effective cross section is 1.6 times greater for the protein than for the water and there is no reason not to maximize the signal using a 5 mrad inner cut-off angle.

Again the signal is going to arise from a difference between the scattering of the molecule and the embedding medium and is given by

$$I_s(r) = tf(r)(N_p\sigma_p - N_w\sigma_w)Jd^2,$$  \hspace{1cm} (18)

where $\sigma_p$ and $\sigma_w$ are the cross sections for the protein and water respectively, and the other symbols have the same meaning as in the discussion on weak phase object TEM. The background is the scattering from the embedding medium alone given by

$$I_B = N_w t\sigma_w Jd^2.$$  \hspace{1cm} (19)

The contrast is the signal divided by the background. The noise is the square root of the background signal (Eq. (19)) and thus the signal/noise is

$$\frac{S}{N} = \sqrt{\frac{N_w J}{\sigma_w}}f(r)\left(\frac{N_p}{N_w}\sigma_p - \sigma_w\right).$$  \hspace{1cm} (20)

Note that the dose is the same in both TEM and STEM. In principle a STEM and a TEM can be operated at equivalent dose. To achieve a dose of 5 electrons/Å² a STEM would have to operate with a probe current of 50 pA in a 2.5 Å probe or 0.2 nA in a 5 Å probe, for a time per pixel of 0.1 μs.

The signal, background, contrast, number of electrons per pixel and signal/noise at a dose of 5 electrons/Å² are all shown in Table 2. For small molecules it might not be possible to make the embedding film the same thickness as the molecule diameter. If a fixed thickness of vitreous ice is assumed then the background will be higher and the contrast and signal/noise lower. Table 2 also shows the effects of assuming a 200 Å thick embedding medium. For molecules with thickness greater than 200 Å it is assumed that the embedding medium is the same thickness as the molecule.

4. Results and discussion

From the results shown as Tables 1 and 2, it is apparent that TEM phase contrast microscopy always has better signal/noise than STEM at 100 kV. If the embedding vitreous ice is no thicker than the molecule then the difference is only a factor of two, which can be considered
insignificant given the approximate nature of the theory. If one assumes that the vitreous ice embedding medium is at least 200 Å thick, then the signal/noise in STEM is 5 times less than the signal/noise in phase contrast TEM. Equivalent results for other accelerating voltages can be calculated by multiplying the TEM contrast or phase change and the STEM signal/noise ratio by the \( R \); the product of the Lorentz factor and the electron wavelength, divided by the value of \( R \) at 100 kV:

\[
R = \frac{\lambda}{\sqrt{1 - v^2/c^2}}. \tag{21}
\]

The STEM signal and background that are proportional to the square of electron scattering factors should be multiplied by \( R^2 \). For convenience values of both \( R \) and \( R^2 \) are given in Table 3.

As stated above, there is very little difference between the contrast estimated using Henderson’s model and the contrast given by the model described above, though as expected, the Henderson model gives reduced contrast for larger molecules as it follows a square root dependence on size. The real problem is that the larger molecules are not weak phase objects and should not be analysed using this simple model. A reasonable limit for the weak phase object would be a phase change of 0.1 rad. Looking at the vitreous ice alone this would mean that anything thicker than 35 Å is no longer a weak phase object at 100 kV. This would increase to about 50 Å for 400 kV electrons. Basing the definition of a weak phase object on the thickness of the embedding medium is probably unnecessarily strict, and one should really base it on the difference in scattering between the protein and the vitreous ice. A 0.1 rad change in phase translates into a contrast of 0.2.

Table 1 shows the limit for weak phase object analysis is a 420 kDa enzyme of diameter 100 Å. Raising the accelerating voltage to 400 kV would enable one to treat an embedded Ribosome as a weak phase object (molecular weight of order 3.3 MDa, diameter 200 Å), but the viruses would still be beyond the limit of the approximation.

If the molecule is the same thickness (or even same faction of the thickness) of the embedding medium then the STEM contrast remains a constant, 0.33. Assuming a 200 Å embedding medium reduces the contrast to about the same value as would be obtained in phase contrast microscopy. For STEM the really significant problem is lack of signal. The signal should always contain at least 1 electron (hopefully many more!),

<table>
<thead>
<tr>
<th>Weight</th>
<th>Diameter (Å)</th>
<th>Signal (electrons)</th>
<th>Background (electrons)</th>
<th>Contrast (assuming 200 Å ice)</th>
<th>S/N 2.5 Å probe</th>
<th>S/N 2.5 Å probe, 200 Å ice</th>
<th>S/N 5 Å probe</th>
<th>S/N 5 Å probe, 200 Å ice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large virus</td>
<td>300 MDa</td>
<td>900</td>
<td>2.60</td>
<td>7.86</td>
<td>0.33</td>
<td>0.93</td>
<td>0.93</td>
<td>1.85</td>
</tr>
<tr>
<td>Small virus</td>
<td>11 MDa</td>
<td>300</td>
<td>0.87</td>
<td>2.62</td>
<td>0.33</td>
<td>0.53</td>
<td>0.53</td>
<td>1.07</td>
</tr>
<tr>
<td>Ribosome</td>
<td>3.3 MDa</td>
<td>200</td>
<td>0.58</td>
<td>1.75</td>
<td>0.33</td>
<td>0.44</td>
<td>0.44</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>1.4 MDa</td>
<td>150</td>
<td>0.43</td>
<td>1.31</td>
<td>0.25</td>
<td>0.38</td>
<td>0.33</td>
<td>0.76</td>
</tr>
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<td>420 kDa</td>
<td>100</td>
<td>0.29</td>
<td>0.87</td>
<td>0.17</td>
<td>0.31</td>
<td>0.22</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>180 kDa</td>
<td>75</td>
<td>0.22</td>
<td>0.66</td>
<td>0.12</td>
<td>0.27</td>
<td>0.16</td>
<td>0.53</td>
</tr>
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<td>Protein</td>
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<td>0.14</td>
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<td>0.08</td>
<td>0.22</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>7 kDa</td>
<td>25</td>
<td>0.07</td>
<td>0.22</td>
<td>0.04</td>
<td>0.15</td>
<td>0.05</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Table 2
Contrast and signal/noise for ADF STEM imaging with a dose of 5 electrons/Å² at 100 kV

Table 3
\( R \), the product of Lorentz factor and electron wavelength, for different microscope accelerating voltages

<table>
<thead>
<tr>
<th>Accelerating voltage (kV)</th>
<th>( R )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>200</td>
<td>0.7884</td>
<td>0.6214</td>
</tr>
<tr>
<td>300</td>
<td>0.7060</td>
<td>0.4984</td>
</tr>
<tr>
<td>400</td>
<td>0.6622</td>
<td>0.4385</td>
</tr>
</tbody>
</table>
which is clearly not the case except for very large viruses of order 300 MDa. Comparing to the corresponding column for TEM in Table 1 this is clearly not a problem for TEM. Even with a very small inner cut-off angle that maximizes the effective cross section there is still inadequate signal at low dose. The only solutions are to increase the dose or perform some sort of averaging.

5. Conclusions

Annular dark field STEM is still sensitive to atomic number differences even when the inner cut-off angle is reduced to about 5 mrad as a way of maximizing the signal. If the embedding medium of vitreous ice is the same thickness as the molecule of interest then the signal/noise is half that for phase contrast TEM and the contrast is 0.33, independent of molecule size. If, more realistically, we assume that the embedding ice is at least 200 Å thick then the signal/noise is 5 times lower than phase contrast TEM and the contrast is comparable. However there are insufficient scattered electrons at the low dose of 5 electrons/Å², even with this highly optimized form of ADF STEM.

As Henderson stated, phase contrast TEM is the optimal method for determining protein molecule structure. The main problem is that it would not be appropriate to use the weak phase object approximation for a molecule whose size is greater than 100 Å (about 420 kDa for a spherical molecule) at 100 kV. If a 400 kV microscope were used instead this limit might be extended to about 200 Å corresponding to approximately 3 MDa for a spherical molecule.

Acknowledgements

I should like to thank Dr. Richard Henderson for extensive correspondence and useful discussions. I would also like to thank Dr Richard Leapman for advice on practical limits in biological electron microscopy and Prof J.M. Cowley for discussions on electron scattering theory and phase objects.

References