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RESTORATION OF WEAK PHASE-CONTRAST IMAGES RECORDED WITH A HIGH DEGREE OF DEFOCUS: THE "TWIN IMAGE" PROBLEM ASSOCIATED WITH CTF CORRECTION

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ABSTRACT

Relatively large values of objective-lens defocus must normally be used to produce detectable levels of image contrast for unstained biological specimens, which are generally weak phase objects. As a result, a subsequent restoration operation must be used to correct for oscillations in the contrast transfer function (CTF) at higher resolution. Currently used methods of CTF-correction assume the ideal case in which Friedel mates in the scattered wave have contributed pairs of Fourier components that overlap with one another in the image plane. This "ideal" situation may be only poorly satisfied, or not satisfied at all, as the particle size gets smaller, the defocus value gets larger, and the resolution gets higher. We have therefore investigated whether currently used methods of CTF correction are also effective in restoring the single-sideband image information that becomes displaced (delocalized) by half (or more) the diameter of a particle of finite size. Computer simulations are used to show that restoration either by "phase flipping" or by multiplying by the CTF recovers only about half of the delocalized information. The other half of the delocalized information goes into a doubly defocused "twin" image of the type produced during optical reconstruction of an in-line hologram. Restoration with a Wiener filter is effective in recovering the delocalized information only when the signal-to-noise ratio (S/N) is orders of magnitude higher than that which exists in low-dose images of biological specimens, in which case the Wiener filter approaches division by the CTF (i.e. the formal inverse). For realistic values of the S/N, however, the "twin image" problem seen with a Wiener filter is very similar to that seen when either phase flipping or multiplying by the CTF are used for restoration. The results of these simulations suggest that CTF correction is a poor alternative to using a Zernike-type

phase plate when imaging biological specimens, in which case the images can be recorded in a close-to-focus condition, and delocalization of high-resolution information is thus minimized.

INTRODUCTION

Unstained biological specimens are often well approximated as being weak phase objects. As Zernike emphasized in his Nobel lecture, images of phase objects show no contrast in a perfectly corrected microscope [1]. In order to overcome this problem, the objective lens is normally defocused by an amount that is large enough to produce sufficient contrast to see the specimen. As an example, a defocus value of 1 μm or more might be used in order to see particles with a molecular weight of ~1 MDa.

Although the low-resolution features of a phase object are made visible by introducing a substantial amount of defocus, the higher-resolution features then become badly corrupted due to oscillations in the contrast transfer function (CTF). This adverse consequence of using high amounts of defocus can be overcome to a substantial degree by computational "image restoration". Applying a computational CTF correction to an out-of-focus image is, in fact, not unlike Gabor's original concept of optical restoration of the object from an in-line hologram – which is nothing other than a highly defocused image [2].

It is well known that optical reconstruction of an object from an in-line hologram suffers from a substantial artifact, however, an effect that is referred to as the "twin image problem". As Gabor explained in his Nobel acceptance speech [3], optical reconstruction of an in-line hologram produces two images superimposed on each other, one of which is in sharp focus and the second of which is defocused by twice the amount of that in the original hologram. The issue that is addressed here, therefore, is the extent to which computational CTF correction also suffers from a similar "twin image" problem.

Our original purpose in simulating CTF correction was to understand how effectively it deals with the fact that a portion of the scattered wave produces an interference pattern in the region of the image that is adjacent to, but outside the geometric shadow of a small particle, for example a multiprotein complex. As is explained below, this delocalized information is not modulated by the usual CTF. Instead, the delocalized information can be described as a sum of interference fringes, each with a different spatial frequency, that are shifted in phase by an amount proportional to the product of the defocus and the spatial frequency.

We have investigated this question by applying three commonly used restoration techniques to the Fourier transforms of various simulated images. The results show that "phase flipping" and multiplying by the CTF both restore only about half of the original signal, the second half going into a doubly-defocused twin image (background). Although the results obtained by these two methods are similar, phase flipping results in a slightly better restoration of signal than does multiplying by the CTF. In light of these first results, it is not surprising that we also found that the ability of a Wiener filter to restore the object depends upon the value of the parameter that is used to estimate the signal-to-noise ratio (SNR). When the SNR is high, using a Wiener filter approaches the operation of dividing by the CTF, which is algebraically guaranteed to produce perfect restoration (but only in the absence of noise-amplification at the zeros of the CTF). When the SNR is low, however, as it is in low-dose cryo-EM images, use of a Wiener filter approaches the operation of multiplying by the CTF.

One of the advantages of recording images of weak phase objects with a phase-contrast aperture [4-8] is that defocus is no longer required in order to produce adequate contrast, and thus no information-delocalization occurs. On the other hand, this advantage would not be as important as it first sounds, if it were also true that no information-delocalization remained after the appropriate CTF correction had been applied. Since both numerical simulations and analytical theory show that CTF correction can be only partially effective in restoring the initially delocalized information, however, we conclude that CTF correction is a poor alternative to the use of in-focus phase-contrast imaging.

SIMULATION METHODS

Image simulations were carried out using scripts written in DigitalMicrograph (Gatan, Inc., Pleasanton, CA). For calculation of single-sideband images, the original image was defined as a complex array so that the full Fourier transform would be computed and one half could then be set to zero. Fourier transforms of the images were modified either by multiplying by an appropriately defined function or by separating the modulus and phase components and modifying these as appropriate.

A modified spoke pattern was generated using the function “sin(n + itheta)” in DigitalMicrograph that makes a full-circle pattern with n spokes. The full-circle pattern was then masked to produce a narrow wedge, after which the test pattern was low-pass filtered to smooth the edges of the pattern.

A two-dimensional, sinusoidal cross-grating pattern was defined in a 512x512 pixel array as the product of one-dimensional sine functions that are parallel to the x-axis and the y-axis, respectively. The strongest Fourier components in this test pattern therefore run diagonally with respect to the x and y axes. This full pattern was masked with a square box whose edge-length was equal to 6.5 cycles of the sine functions.

An image of the 50S (large) subunit of the *E.coli* ribosome was calculated using atomic coordinates given in the PDB file 1VOR [9]. A molecular model was generated using the "copy from pdb" command in SPIDER [10] to calculate a 3-D density at a resolution of 0.1 nm/pixel. Functions in Bsoft [11] were then used to project the density and output a file in TIFF format as a 512x512 array.

Noise was not included in the simulations shown here. We assume that (1) the signal and the electron shot-noise that are present in experimental image intensities are additive, and (2) these two terms remain additive during the CTF-correction operations that are applied during data analysis. It is true that CTF-correction of just the intensity pattern corresponding to the electron shot-noise itself will result in a texture whose amplitude spectrum is no longer "white" but whose phases are still random. Even so, this texture will be uniformly the same within the envelope of a particle and in the area outside the particle. Since an appropriate level (and texture) of "CTF-corrected" noise can be added to the results shown here, there is no loss of generality in computing and displaying only the effects that delocalization and subsequent restoration have on the signal. The purpose of NOT including noise in the simulations is to avoid confusion between the effects that are due to delocalization of the signal and those that are added by noise. In practice, the delocalized signal is largely masked by the noise, but because of the additivity of the signal and the noise, both the delocalization of signal and its partial restoration will be well-described by our noise-free simulations.

BACKGROUND AND THEORY

The contrast transfer function (CTF) that is used for image restoration in cryo-EM is given, in the simplest case, by

$$CTF(s) = \sin\gamma(s),$$
$$\gamma(s) = 2\pi[\frac{C_s\lambda^3}{4}s^4 - \frac{\Delta z}{2}s^2],$$

Equation 1

where

s = the spatial frequency (resolution),

$\gamma(s)$ = the wave aberration associated with spherical aberration and defocus,

C_s = the coefficient of spherical aberration,

λ = the electron wavelength, and

Δz = the defocus of the objective lens.

We have ignored the wave aberration due to spherical aberration in this paper, in order to emphasize solely the effect of defocus. For typical electron microscopes, the wave aberration due to spherical aberration makes a significant contribution to delocalization for only the highest-resolution features, for example Fourier components with a wavelength shorter than ~0.5 nm. The addition of a spherical-aberration term in the simulations shown here would have had no visible effect, and in any case it would not contribute new principles to what is learned from the simulations presented here.

If the specimen is a weak phase object, then the Fourier transform of the experimental image intensity, $\hat{I}_{\exp}(s)$, is related to the Fourier transform of the shielded Coulomb potential of the object, $F(s)$, by

$$\hat{I}_{\exp}(s) = \delta(s) - 2F(s)\, CTF(s) \qquad \textbf{Equation 2}$$

The derivation of equation 2 assumes that the Fourier transform of the object satisfies Friedel's law and that the sinusoidal Fourier components of the object are spatially unbounded, as they are for a two-dimensional crystal [12]. Under these conditions pairs of sinusoidal "fringes" in the image that are produced by interference of one diffracted beam with the unscattered beam and by the interference of its Friedel mate with the unscattered beam are shifted in opposite direction. The amount of their respective phase shifts corresponds to the magnitude of the resolution-dependent phase distortion, $\gamma(s)$. Depending upon the amount of defocus, these individual pairs of fringes thus vary from being in phase with one another to being completely out of phase with one another, and then back to being in phase but with the reversed contrast. When these two intensity fringes are added, the net result is that the phase origin of the resulting sum is *not* shifted but its amplitude is modulated by an amount that varies between 1 and -1 of what it should be, i.e. by the value of the CTF that is given in equation 1.

CTF correction is normally thought of as an operation that, at the very least, reverses the sign of those Fourier components that have been transferred with the wrong sign. Since a change in sign is equivalent to a 180 degree phase shift, this sign change is often referred to as "phase flipping". Depending upon what version of CTF correction is used, the operation may also adjust the magnitude as well as the sign of the amplitude. Multiplication by the CTF, for example, not only corrects the sign of the amplitude but down-weights the contributions of frequencies where the SNR is especially poor (for example, close to the zeros in the CTF).

An even better restoration is provided by the Wiener filter [13], expressed in the form that is appropriate for deconvolution of the effects caused by the CTF (http://en.wikipedia.org/wiki/Wiener_deconvolution):

$$w(s) = \frac{CTF(s)}{[CTF(s)]^2 + 1/SNR(s)} \qquad \textbf{Equation 3}$$

where

w(s) = the weighting function that is applied as a filter during image restoration, and

SNR(s) = the "signal-to-noise ratio" at a given spatial frequency, *s*. Note that SNR is defined here as the mean power spectral density of the input signal divided by the mean power spectral density of the noise.

Although the Wiener filter provides a restored image that has the smallest possible mean square error relative to the true structure, it is often not used in practice because no method has been established for determining the experimentally correct value for *SNR(s)*. Even when an estimate of the Wiener filter is used, one often assumes that *SNR(s)* is constant, in effect assuming that the power spectral density of the input signal is constant. This is an approximation that is never satisfied for images of biological macromolecules, however.

When the Fourier components of an object are confined to a small area, as they are for small crystalline patches and for single particles, a given set of fringes is shifted as a small, defined patch. The size and shape of the patch corresponds to the dark-field image that would be formed by the diffracted beam if the unscattered beam were blocked by the objective aperture [14]. The center of the patch is displaced from the center of the particle by an amount equal to the value of (gradient of $\gamma(s)$ divided by 2π) at the spatial frequency of the fringes, *s*."

As theory [14] requires, sinusoidal fringes – which should lie at the positions of crystalline lattice planes, for example – are thereby shifted beyond the edge of a particle. The amount of displacement that can occur before the fringes are no longer visible outside the particle is determined by the spatial coherence of the incident electron beam. Similarly, depending upon the amount by which the patches are shifted (i.e., depending upon the amount of defocus), the area within which the fringes overlap with one another is only a fraction of the patch size. In particular, the area of overlap falls to zero when the amount of shift is one half of the particle size. In the region (if any) where the patches still overlap with one another, the contrast transfer is still described by the usual CTF that is written in equation 1. Outside the region of overlap, however, the fringes behave as "single sideband images" [12]. In this case the amplitude of the fringes is no longer modulated by $sin(\gamma)$; instead, their phases are shifted by $\gamma(s)$.

The EM community is generally aware that one must apply CTF correction to an area surrounding a particle and not just the particle itself (i.e. one must not mask the particle too tightly). It is also clear that dividing the Fourier transform of a defocused image by the CTF is the mathematical inverse of the multiplication by the CTF that is shown in equation 2. Although dividing by the CTF thus restores the object perfectly for noise-free images, the images obtained in cryo-EM are so noisy that dividing by the CTF is not a viable operation. What has not been discussed in the EM literature, however, is the extent to which alternative methods of CTF correction are successful in restoring the portion of the delocalized signal that is transferred as single-sideband fringes (rather than as CTF-modulated fringes).

RESULTS

The signal-delocalization effect is first illustrated with the help of two relatively simple test-objects, a spoke-type resolution-test pattern, and a square cross-grating pattern. A simulated image of the large ribosomal subunit is then used to show the relevance of delocalization in the context of cryo-EM images of macromolecular complexes.

The spoke pattern shown in Figure 1A is informative because it contains a ramp of spatial frequencies that are separated in space along the length of the pattern. This test pattern thus makes it possible to easily visualize the frequency dependence of the delocalization effect. Figure 1B shows the image that would be obtained for this test “object” with a defocus of 2 μm, if we assume that the width of the entire panel is 30 nm. Note that the zeros of the CTF cause zones of little or no contrast at different horizontal positions in the image of this test pattern, while the sign reversals of the CTF cause zones of inverted contrast. Both of these effects are confined to the (vertically defined) middle portion of the defocused image, however. The top and bottom portions of the spoke pattern retain full contrast even where the CTF is zero because the fringes from a given Fourier component no longer overlap with those from its Friedel mate at the edge of a “particle”, due to delocalization. In addition, delocalization causes the appearance that the spokes curve away from the edge of the original test pattern as the spatial frequency increases (i.e. as one moves to the right along the test pattern).

Due to the linearity of image formation for weakly scattering objects, the image in Figure 1B can be described mathematically as the sum of the two single-sideband images that are shown in Figures 1C and 1D. The contrast in single-sideband images is not modulated by zeros in the CTF, and this fact is reflected in these two respective simulations. The phases of Fourier components of the object are, however, shifted by the wave aberration in single-sideband images [12], as we have already said above. This shift in phase accounts for the bending (vertical shifting) of the images of the spokes, which only becomes conspicuous in the far-right portion of the test pattern. The "beating" or interference of shifted fringes that occurs when the images in Figures 1C and 1D are superimposed is what accounts for the zones of no contrast and the zones of inverted contrast that are shown in Figure 1B. Constructive and destructive interference between the two single-sideband images can occur only over the vertical positions where the shifted Fourier components overlap with one another, of course. As a result, the usually invoked effects of the CTF (contrast modulation and phase flipping) apply to a progressively smaller fraction of the signal as the resolution increases, and they no longer apply at all when the amount of delocalization is greater than half the size of the object.

The cross-grating test-pattern shown in Figure 2A illustrates the delocalization effect in a complimentary way. For the sake of argument, this object might be considered to be a square particle that is 8.5 nm on edge. The structure within this "particle" consists of the product of two Fourier components that run perpendicular to one another, in directions that are parallel to the edges of the square. In this example, the two sinusoidal components both have a period of 1.3 nm.

Figure 2B shows a simulation of the image that this test object would produce at a defocus of 3 μm. This value of defocus was chosen in order to shift the patch of each set of fringes by half the edge-length.

Figures 2C and 2D show the results produced when two commonly used methods of CTF correction are applied to the defocused image in figure 2B. These results demonstrate that the original object is recovered remarkably well by CTF correction. The contrast restoration is, in fact, somewhat more complete when CTF correction is done by phase flipping rather than by multiplying by the CTF. A quantitative comparison between the contrast in the original object and that obtained after CTF correction demonstrates that 50 % of the delocalized signal is restored by multiplying by the CTF. and 65 % of the delocalized signal is restored by phase flipping. The simulations also show that the remainder of the signal is delocalized once again as a result of both of these procedures. In other words, the twin-image problem observed for optical reconstruction of an in-line hologram also occurs when either phase flipping or multiplying by the CTF is used as the restoration filter

In order to continue this investigation with a test object that is similar to an actual cryo-EM image, we next calculated a noise-free, highly defocused image of the large subunit from the *E. coli* ribosome. Figure 3A shows the image obtained for a defocus of 2 μm and an accelerating voltage of 300 kV. The ribosomal test object, unlike the simpler test objects used in Figures 1 and 2, now consists of a continuous spectrum of spatially superimposed Fourier components. The delocalized information therefore consists of a continuum of overlapping patches, each displaced by an amount that is determined by the gradient of $\gamma(s)$ at that particular spatial frequency.

Figure 3B shows the nearly perfect restoration that is achieved with a Wiener filter when the value of the SNR is assumed to be 900. The simulations themselves are noise-free in all cases, as we have stated previously. The stipulation of a given value of the SNR is used only to see what the effect would be on the signal-component of an image, due to a given value of SNR that is used in the Wiener filter. The restoration produced with SNR = 900 is shown because the Wiener filter in this case is almost equivalent to dividing by the CTF, which in turn is guaranteed to give perfect recovery of the original object from a noise-free image. Nevertheless, even for data with a SNR as high as 900, low-frequency artifacts still remain in the Wiener-filtered restoration, due to the weakness of the filtered amplitudes at low frequencies.

A more realistic simulation of the recovery of delocalized information that can be expected is provided by setting the SNR equal to 0.09 (Figure 3C). In this case the CTF correction lies somewhere between that provided by phase flipping and by multiplying by the CTF, as we show in connection with Figure 4, below. Not at all unexpectedly, based on the simulations shown with the simpler example in Figure 2, the twin-image is once again present when this more realistic Wiener filter is used for CTF correction. In fact, the twin-image artifact persists even when the SNR parameter in the Wiener filter is set to the unrealistically optimistic value of 9 (Figure 3D).

Additional understanding of how various CTF corrections compare to one another is provided by the curves shown in Figure 4, which shows the CTF itself as well as three examples of the product of the CTF and different versions of the Wiener filter. The black curve is the CTF itself (calculated for a defocus of 2 μm and an electron energy of 300 keV), which contains sign reversals between successive zeros. The weighting of CTF-corrected amplitudes (i.e. the resultant "transfer function" for restoration) that is obtained by phase flipping can be envisioned by converting the negative lobes of the CTF curve to identical, positive lobes. Similarly, the weighting of amplitudes that is obtained by multiplying by the CTF can be pictured as a version of the phase-flipped CTF in which the "bell-shaped peaks" are simply more narrow. The blue curve in Figure 4 shows the weighting of amplitudes that would be obtained with a Wiener filter for which SNR = 0.09. Such a Wiener filter is similar to multiplying by the CTF, as we stated previously. The green curve shows the weighting obtained with a Wiener filter when the SNR is set to be 9, as it was for figure 3D. Finally, the red curve shows the near-perfect restoration of amplitudes (except at low frequencies and very close to the zeros in the CTF) that is achieved with a Wiener filter for which SNR = 900.

DISCUSSION

Highly defocused images of a weak phase object can be described from three different, but ultimately equivalent perspectives. From one point of view, one can say that the wave function producing such images is a Fresnel diffraction pattern that is produced by propagation of the exit wave (i.e. the wave function transmitted through the object). The intensity distribution of such a Fresnel diffraction pattern is, in turn (for a weakly scattering object), an in-line hologram of exactly the type that Gabor hoped to use to overcome the limitation associated with the spherical aberration of the objective lens [2]. From another point of view, one can describe these images by the mathematics of linear systems [15], in which case the image wave function is obtained by convoluting the exit wave with the coherent point-spread function of the optical system. From a third perspective, the images can be represented as a sum (integral) of terms contributed by each diffracted beams, i.e. by each Fourier component of the exit wave [14]. This latter perspective, although mathematically cumbersome and requiring the use of a local Taylor-series approximation to provide physical insight, explains very clearly why information at different levels of resolution is delocalized (shifted) by different amounts.

The mathematical description of image formation that is based on linear-systems theory provides the simplest explanation for why the delocalized information is perfectly restored when the Fourier transform of an image of a weakly scattering object is divided by the contrast transfer function (CTF) [15]. In practice, however, division by the CTF is not possible at the zeros of the CTF. For noisy images of the type that can be recorded by cryo-EM of radiation-sensitive specimens, division by the CTF may even cause severe noise-amplification at most spatial frequencies, i.e. not just those close to the zeros of the CTF.

Data restoration that is achieved by multiplying by the CTF, or even by the simpler operation of phase flipping, gives a result that does not differ greatly from using a Wiener filter when the SNR is low. In fact, phase flipping and CTF multiplication produce a remarkable recovery of the delocalized information, as can be seen most easily with the simplified test object used in Figure 2. Although phase flipping restores a slightly higher fraction of the delocalized signal, multiplication by the CTF is to be recommended as an easy way to give reduced weight to Fourier components with a poor SNR. The use of a

Wiener filter for CTF correction, on the other hand, represents the optimal approach for data recovery at any level of noise.

The simulations presented here demonstrate that full recovery of delocalized information is generally not possible when CTF correction is applied to a defocused image. Instead, approximately half of the signal, which is referred to as the “twin image” in holography, is superimposed on the restored image, effectively being defocused by twice the original amount. This added background is generally not recognizable as being structurally related to the object (i.e. to the restored image), and the twin image thus acts effectively as unwanted noise in the reconstruction.

Yonekura and Toyoshima have investigated the effect that applying solvent flattening after CTF correction, followed by merging of data from images recorded at different defocus values, has on mitigating the “twin-image” noise that intrudes into the area of a given particle from closely adjacent, foreign particles in the specimen. These foreign particles were modeled as rectangles of different width and height, and they were regarded as a form of noise [16]. While solvent flattening clearly is effective in smoothing the background, and averaging is effective in “canceling” the intrusion of twin-image noise that has entered into the volume of a particle, neither procedure is able to capture and return the half of the signal, derived from the particle itself, that is doubly delocalized outside the envelope of the particle during CTF correction.

The simulations presented here show that partial restoration of delocalized information occurs to a similar extent when CTF correction is carried out (1) with a Wiener filter (assuming a realistic value of the signal-to-noise ratio), (2) by multiplying with the CTF, or (3) by phase flipping. This empirical result is not intuitively obvious, and in fact it is not immediately obvious why phase flipping or multiplying by the CTF should restore *any* of the delocalized information. That these operations nevertheless do shift some of the delocalized information back to the area of the particle can be understood in terms of the mathematical approximation used by Budinger and Glaeser [14] to explain, on the basis of Fourier optics, why individual Fourier components of an object are shifted out of the area of a particle to begin with.

Briefly, the Fourier transform of a small object that contains a single, bounded Fourier component is the convolution of a Dirac delta function – at the spatial frequency of that Fourier component – and the Fourier transform of a function that describes the boundary and the position of the particle. According to the Fourier shift theorem, application of a linear phase ramp to such a Fourier transform causes the bounded Fourier component to shift away from the original position of the particle. Defocusing an image involves application of a quadratic phase shift to the Fourier transform of the object, but locally (e.g. at the position of the Dirac delta function) this wave aberration can be expanded in a Taylor series, the second term of which is a linear phase ramp. This linear term thus explains the shift (delocalization) of the Fourier component, while higher-order terms produce additional distortions of the image. By extension, restoration (shifting back) of some of the delocalized Fourier component must also involve the application of a linear phase ramp, this time to the Fourier transform of the defocused image intensity.

Although it may seem contrived to say so, phase flipping can, in fact, be approximated by a linear phase ramp plus higher-order terms. One normally thinks of phase flipping as a function that alternates between π and 0 for successive intervals of the spatial frequency. Formally, however, the mathematical consequences of applying such a function are indistinguishable from applying a staircase function that increases in steps of π. This staircase can, in turn, be represented as a linear phase ramp plus higher-order deviations above and below the ramp. Since the slope of the ramp can be either positive or negative, it is not surprising to find that phase flipping simultaneously relocalizes about half of the delocalized information and further delocalizes the other half.

Multiplying by the CTF is to some extent similar to phase flipping, of course. As with phase flipping, the operation can again be described in terms of applying a linear phase ramp, but in this case accounting for the bell-shaped amplitude changes in addition to the steps in phase. Finally, even application of the Wiener filter can be thought of as being similar to phase flipping, since it again reverses the sign-change that occurs in successive zones of the CTF.

CONCLUSIONS

The fact that CTF correction of defocused images suffers from a "twin image" problem similar to that which occurs in optical reconstruction of in-line holograms suggests that an approximate doubling of high-resolution signal should result from the use of in-focus phase contrast images rather than defocused, CTF-corrected images for single-particle cryo-EM. The ability to record in-focus phase contrast images would also overcome other limitations of defocus-based phase contrast that have not been discussed here. One of these limitations is the reduction of structure-factor amplitudes at high-resolution that occurs in highly defocused images due to the envelope function for spatial coherence. A second limitation is caused by the increased noise that is included when it is necessary to process data (during CTF correction) that come from an area that is much larger than the size of the particle itself. It thus is apparent that the use of defocused phase contrast is a poor alternative to recording images with in-focus phase contrast.

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FIGURE LEGENDS

Figure 1. The wedge-shaped pattern of radial spokes shown here provides an object in which each spatial frequency is localized at one particular position along the horizontal axis. The height of the panel represents 30 nm in these simulations. (A) The original pattern. (B) The pattern when imaged with a defocus of 2 μm and an electron energy of 300 keV. There are clearly resolved zeros and contrast reversals in certain zones of spatial frequency, while at the higher spatial frequencies it is seen that the Fourier components are shifted so much that two sets of spokes separate from each other. (C, D) single sideband images of (A) computed with the same effective defocus as in (B). Here the effects of the wave aberration can be seen as producing vertical shifts of the Fourier components by amounts that are proportional to the frequency of that component. The image in (B) is the sum of those shown in (C) and (D).

Figure 2. Comparison of the restoration of delocalized information that is achieved by phase flipping and by multiplication by the CTF. (A) A spatially bounded cross-grating pattern is formed as the product of two perpendicular sine waves. With the size-scale set to 0.1 nm per pixel, the period is 1.3 nm. (B) The image of the object in (A) that is computed with an effective defocus of 2 μm. (C) Restoration of (B) obtained by “phase flipping” – i.e. inverting the sign of the Fourier transform of (B) in alternate zones of the CTF. (D) Restoration of (B) computed by multiplying the Fourier transform by the original CTF. Insets show a section of the Fourier transform, with the origin near the lower left corner.

Figure 3. Simulation of the delocalization effect in an image of the large ribosomal subunit, and examples of the restoration achieved with a Wiener filter for different levels of the SNR. Images are not shown on the same relative scale of contrast, since the contrast after Wiener filtration depends upon the value of SNR that is used. (A) The initial image that is obtained when phase contrast is produced by using a defocus of 2 μm. (B) Restoration of the original object from the image in (A) is almost perfect when the Wiener filter assumes that *SNR(s)* = 900. Low-frequencies are still not well-represented in the restoration, however, since the CTF asymptotically goes to zero at low resolution. (C) Simulation of image restoration when the Wiener filter assumes that *SNR(s)* = 0.09, a value that is more realistic for cryo-EM images. (D) Restoration already fails to recover all of the delocalized information when the Wiener filter assumes that *SNR(s)* = 9, a value that is still unrealistically high for cryo-EM images.

Figure 4. The phase-contrast CTF for a defocus of 2 μm and electron energy of 300 keV, and the weighting (resultant "transfer function") that is provided when a Weiner filter is used for image restoration. The CTF is shown by the black curve, while the product of the CTF and the Wiener filter is shown as differently colored curves for which the value of the SNR is identified in the insert. See the text for further explanation.

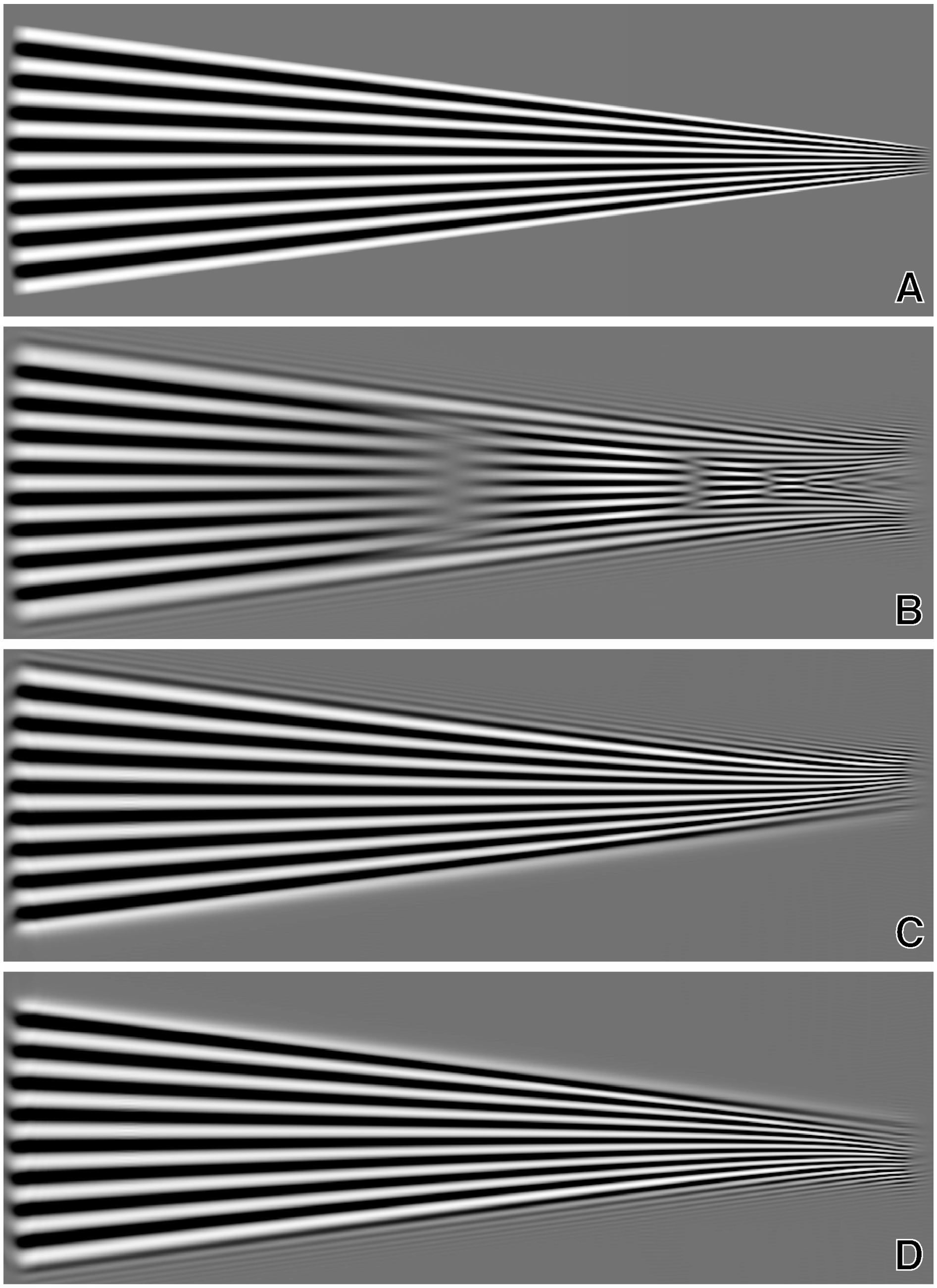

Figure 1

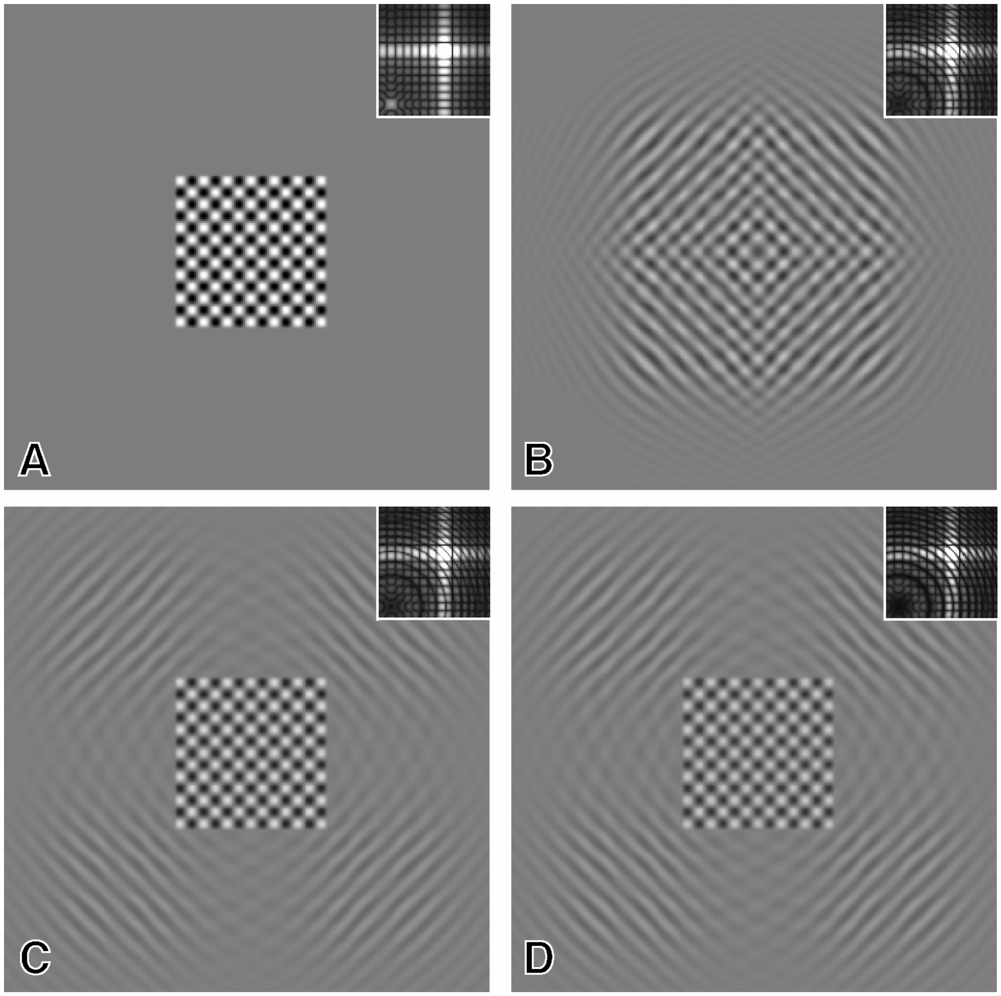

Figure 2

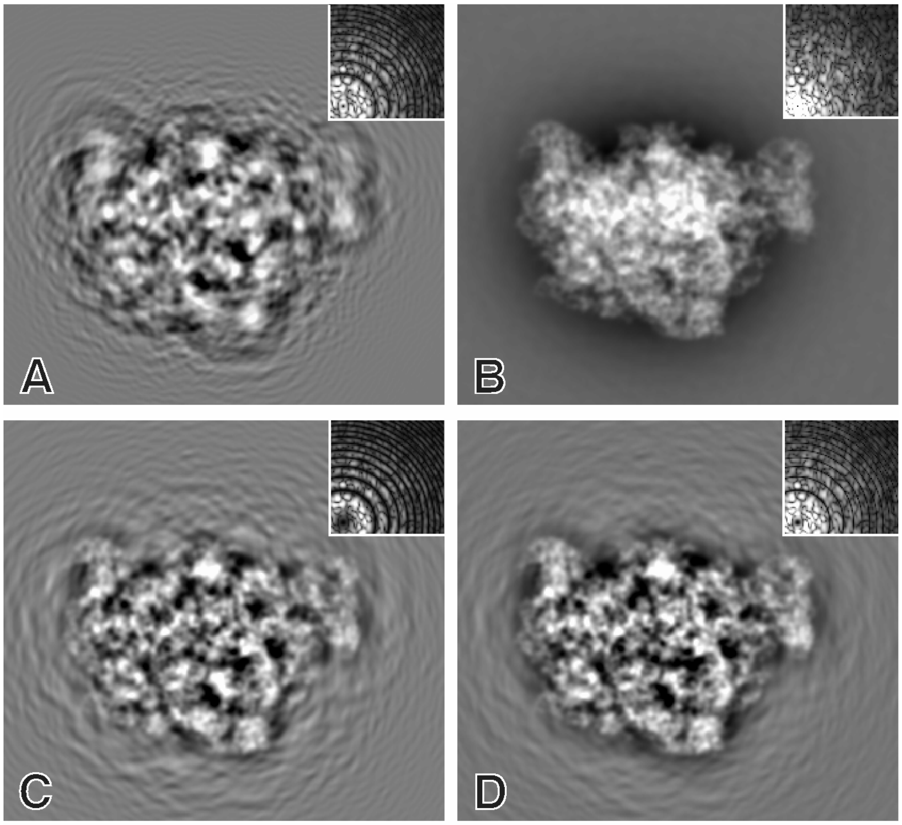

Figure 3

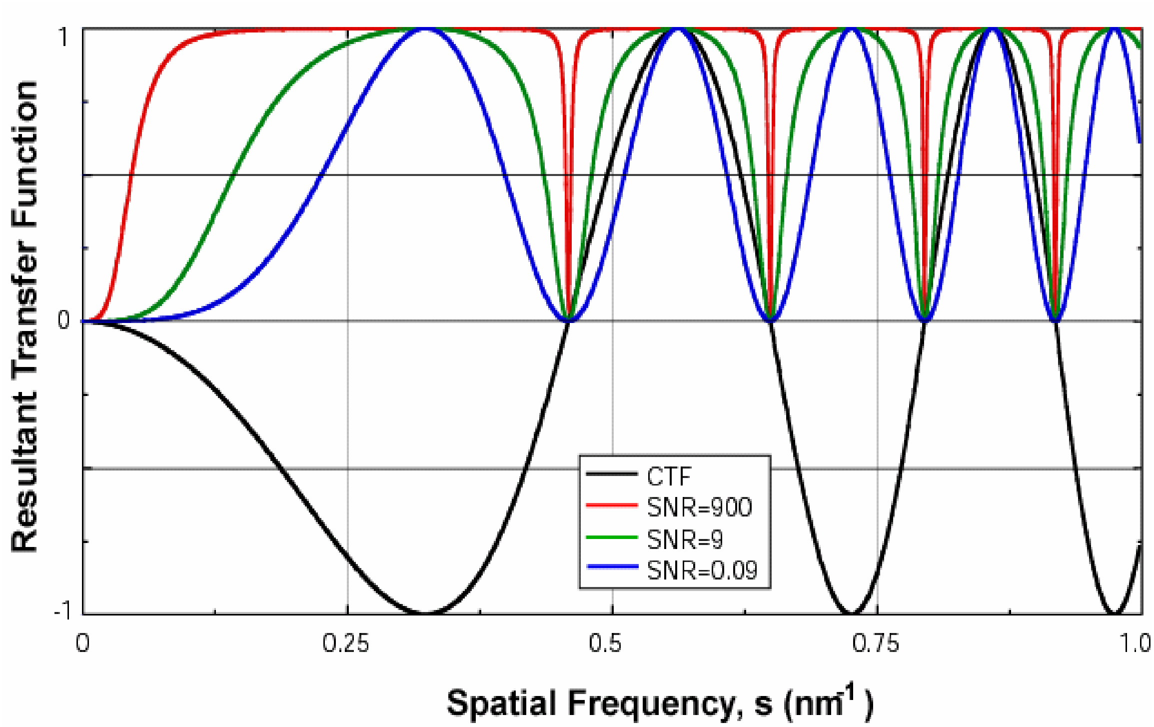
Resultant Transfer Function
1
0
-1
0
0.25
0.5
0.75
1.0
Spatial Frequency, s (nm-1)
CTF
SNR=900
SNR=9
SNR=0.09

Figure 4