Alignment Error Envelopes for Single Particle Analysis

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To determine the structure of a biological particle to high resolution by electron microscopy, image averaging is required to combine information from different views and to increase the signal-to-noise ratio. Starting from the number of noiseless views necessary to resolve features of a given size, four general factors are considered that increase the number of images actually needed: (1) the physics of electron scattering introduces shot noise, (2) thermal motion and particle inhomogeneity cause the scattered electrons to describe a mixture of structures, (3) the microscope system fails to usefully record all the information carried by the scattered electrons, and (4) image misalignment leads to information loss through incoherent averaging. The compound effect of factors 2–4 is approximated by the product of envelope functions. The problem of incoherent image averaging is developed in detail through derivation of five envelope functions that account for small errors in 11 “alignment” parameters describing particle location, orientation, defocus, magnification, and beam tilt. The analysis provides target error tolerances for single particle analysis to near-atomic (3.5 Å) resolution, and this prospect is shown to depend critically on image quality, defocus determination, and microscope alignment.

Key Words: alignment; electron microscopy; envelope functions; image processing; resolution; single particle analysis.

INTRODUCTION

High-resolution structure determination of biological particles by electron microscopy (EM) requires image averaging for two fundamental reasons. First, because EM images are projections, multiple views are required to reveal the distribution of mass in three dimensions. Second, for every elastic electron scattering event, approximately three inelastic events occur (Angert et al., 1996; Langmore and Smith, 1992), which gradually destroy the particle. The useful dose per particle is therefore limited, image signal-to-noise ratios (SNR) are low, and the full dose required to statistically define the particle structure must be distributed over a set of identical particles.

Image averaging requires estimation of at least 11 “alignment” parameters: 2 translational parameters (x, y) locating the particle center, 3 rotational parameters (α, β, γ) describing particle orientation, a magnification parameter (m), 3 parameters (Δz, za, and td) specifying defocus and astigmatism, and 2 parameters (tx, ty) giving the magnitude and direction of beam tilt. The word “alignment” is used broadly here to describe all the computational operations required to make images directly comparable and prepare them for averaging.

It has been argued that ideal images of particles as small as 38 kDa should contain enough signal to be aligned precisely enough for near-atomic resolution reconstructions, but that under current experimental conditions particles must be about 100-fold larger (Glaeser, 1999; Henderson, 1995). Experiments have shown that real particle images can in fact be aligned well enough to obtain near-atomic resolution structures when the particles assemble into ordered aggregates and their combined signal can be used to estimate the necessary parameters: large, well-ordered two-dimensional crystals have yielded atomic models (Henderson et al., 1990; Kimura et al., 1997; Kühlbrandt et al., 1994; Nogales et al., 1998), reconstructions of helical crystals have reached below 5 Å (Miyazawa et al., 1999), and icosahedral viruses have revealed protein secondary structure (Bottcher et al., 1997; Conway et al., 1997; Trus et al., 1997; Zhou et al., 2000). In contrast, reconstructions of unordered, asymmetric particles have been limited to lower resolutions (Gabashvili et al., 2000; Matadeen et al., 1999).

While many factors combine to limit resolution in these studies, the clear correlation between crystallinity and resolution suggests that alignment errors,
which grow as the size of the ordered unit decreases, are currently key resolution limitations in single particle analysis. A comparison of three recently published reconstructions, all done on high-end microscopes with similar numbers of particles embedded in vitreous ice over holey carbon film at similar defoc is visible, highlights the pattern: reconstructions of 80 000 nicotinic acetylcholine receptors arranged as helical crystals agreed to approximately 5 Å resolution (Miyazawa et al., 1999); 35 000 asymmetric units of the herpes simplex virus capsid arranged in 60-member icosahedral "crystals" yielded 8.5 Å resolution (Zhou et al., 2000); and 74 000 ribosomes arranged as true single particles yielded 11.5 Å resolution (Gabashvili et al., 2000). Several avenues exist to improve image alignments: better algorithms could be developed, image quality could be improved, particle orientations could be limited through specimen preparation (through crystallization or specific attachment to a support, for instance), or particle visibility could be enhanced. To this latter end the author has proposed rigidly fixing multiple heavy atom clusters to particle surfaces as alignment markers (Jensen and Kornberg, 1998).

While the severity of some alignment errors can be estimated rapidly by calculating the phase errors introduced at a resolution of interest, such estimates do not reveal their compound effect in the context of the other resolution limitations or predict how many more particles must be included in an average to overcome them. This paper addresses these issues by deriving equations quantifying the relative contribution of alignment errors to the other resolution limitations involved in single particle analysis. In Part 1, alignment errors are placed in the context of the theoretical minimum number of images needed to determine a structure and four experimental factors that increase that number. In Part 2, the impact of error in each alignment parameter is considered in detail through derivation of envelope functions. Finally, in Part 3, target alignment errors for single particle analysis to near-atomic resolution are estimated.

\begin{equation}
\text{N}_{\text{images}} = \left( \frac{\text{SNR}_{\text{required}}}{\text{SNR}_{\text{ideal image}} \cdot \text{E}_{\text{particle disorder}} \cdot \text{E}_{\text{image degradation}} \cdot \text{E}_{\text{incoherent averaging}}} \right)^2
\end{equation}

where $\text{SNR}_{\text{ideal image}}$ is the SNR of an ideal image limited only by shot noise and the factors $E$ represent envelopes of signal decay that arise from factors 2–4 described above. More rigorously, the factors $E$ should be envelopes of SNR decay rather than signal decay alone, but the difference is negligibly small, as discussed later. Rich analyses of each term in Eq. (2) besides $E_{\text{incoherent averaging}}$ are already available in the literature. In the following paragraphs, these references are collected and briefly summarized to place alignment errors in their proper context and to highlight the analogy of their treatment, as developed for the first time in this paper, to the related limitations.

\textbf{Ideal Images}

Two considerations limit the SNR of an ideal image. First, the cross section for elastic scattering of electrons by carbonaceous materials is small, so that an ideal phase contrast image of a single carbon atom has an intensity only a few percent lower than that of the surrounding background (Chiu and Glaeser, 1975). Second, high-energy electrons gradually destroy biological specimens, so the useful dose per particle is small and significant shot noise is inevitable. Two assessments of $\text{SNR}_{\text{ideal image}}$ and $\text{SNR}_{\text{required}}$ appear in the literature (Glaeser, 1999; Henderson, 1995) and allow the minimum number of images...
(N_{\text{min}}) required to overcome shot noise in the ideal case in which $E_{\text{particle disorder}}$, $E_{\text{image degradation}}$, and $E_{\text{incoherent averaging}}$ of Eq. (2) are all unity, to be estimated. Henderson’s treatment gives this minimum number as a function of spatial frequency:

$$N_{\text{min}} = N_{\text{views}} \left( \frac{\text{SNR}_{\text{required}}}{\text{SNR}_{\text{ideal image}}} \right)^2 = 38,000 \text{ Å} \cdot s \quad (3)$$

(Table 2 in Henderson, 1995). In the specific case of 3-Å resolution, Glaeser concludes that $N_{\text{min}}$ is about nine times lower.

**Particle Disorder**

In the assessments of $N_{\text{min}}$ cited above, it was assumed that the particles were rigid and identical. $E_{\text{particle disorder}}$ quantifies how thermal motion and particle inhomogeneity within a real population of particles reduce the SNR of a reconstruction. The effects of particle disorder on structural models have long been understood and described through the crystallographic temperature, or B factor, which specifies the decay of structure factor amplitudes. Thus,

$$E_{\text{particle disorder}} = \frac{f_{\text{exp}}(s)}{f_{\text{ideal}}(s)} \approx e^{-B_{\text{temp}}s^2}, \quad (4)$$

where $f_{\text{exp}}(s)$ is the average experimentally observed structure factor amplitude at spatial frequency $s$, $f_{\text{ideal}}(s)$ is the ideal value calculated from the number and types of atoms present in the particle under the assumption that the particles are perfectly rigid and homogeneous, and $B_{\text{temp}}$ is the temperature factor. It should be noted that in much of the X-ray literature, the envelope of decay in structure factor amplitudes is described as a function of $\sin^2(\theta_B)/\lambda$, rather than $2\sin(\theta_B)/\lambda$ (where $\theta_B$ is the Bragg angle and $\lambda$ is the X-ray wavelength) as appears here, and therefore the B factors here and in much of the electron microscopy literature are a factor of 4 smaller than those in the alternative convention (Drenth, 1999; p. 94).

**Image Degradation**

Henderson extended his treatment of the theoretical minimum number of particles needed to include the imperfect imaging system. In analogy to the X-ray temperature factor, he considered the resolution-dependent ratio of the amplitudes of reflections in the Fourier transform of an experimental image to those in an ideal image and referred to this as “contrast” (Henderson and Glaeser, 1985). $E_{\text{image degradation}}$ here is defined as the same ratio, but the terminology of Thuman-Commike et al. (1999) is preferred, since “contrast” is often understood as the absolute difference in intensity between pixels (Zhu et al., 1997), which can be scaled readily, as on a graphics display, without altering the information content.

Many factors impair image quality, including spatial (Frank, 1973) and temporal (Hanszen and Trepte, 1971; Wade and Frank, 1977) incoherence of the electron beam, specimen movement (Frank, 1969), specimen charging (Brink et al., 1998), the physical processes of image capture and digitization (Downing and Grano, 1982; Downing and Hendrickson, 1999; Mitsuoka et al., 1997; Sherman et al., 1996), noise from a radiation-damaged specimen (Brink and Chiu, 1991; Glaeser and Taylor, 1978), noise from specimen supports such as water or carbon films (Thuman-Commike et al., 1999), movement of the cryoholder (Downing and Chiu, 1990; Henderson and Faruqi, 1995), etc. The impact of each factor can be approximated by an envelope function in reciprocal space, and many have been theoretically derived and/or experimentally measured.

Thus,

$$E_{\text{image degradation}}(s) = \frac{f_{\text{images}}(s)}{f_{\text{diff}}(s)} \approx \prod_i E_i(s), \quad (5)$$

where $f_{\text{images}}(s)$ represents the average structure factor amplitude in an experimental image at spatial frequency $s$; $f_{\text{diff}}(s)$ represents the corresponding ideal value that would have been recorded by the perfect microscope of Eq. (3) (estimable through directly measured electron diffraction intensities); and the $E_i(s)$’s describe the individual envelope functions.

**Incoherent Averaging**

After images are digitized, signal is lost due to small alignment errors in the process of image averaging. Thus in analogy to $E_{\text{particle disorder}}$ and $E_{\text{image degradation}}$, $E_{\text{incoherent averaging}}$ is defined as

$$E_{\text{incoherent averaging}}(s) = \frac{f_{\text{with errors}}(s)}{f_{\text{without errors}}(s)} = \prod_j E_j(s). \quad (6)$$

In Part 2, five envelopes ($E_{\text{trans}}, E_{\text{rot}}, E_{\text{mag}}, E_{\text{def}}, E_{\text{vib}}$) are derived to account for small errors in the 11 named alignment parameters. A further envelope function ($E_{\text{alg}}$) is required to describe the information losses inherent in the interpolations and algorithms used in the averaging process itself. In Part 3
it is verified through numerical tests that their net effect can in fact be described by their product. Thus,

\[ E_{\text{incoherent averaging}} = E_{\text{trans}} E_{\text{rot}} E_{\text{mag}} E_{\text{def}} E_{\text{til}} E_{\text{alig}} \]  

(7)

**PART 2: DERIVATION OF ENVELOPE FUNCTIONS**

Translational Alignment (E\text{trans})

In the transformation of an ideal single particle image marred only by shot noise, each complex value can be decomposed into a “signal” component equal in magnitude and phase to the true structure factor of the particle at that spatial frequency and a remaining “noise” component. In an experimental reconstruction, the positions of the particle centers cannot be determined exactly, and because amplitudes are invariant with respect to translation, such errors persist only on the phases of the image transform. An envelope \( E_{\text{trans}} \) was defined as the fraction of signal retained in the average using the erroneous alignment parameters compared to the signal that would have resulted had there been no alignment errors, given by the projection of the vector sum of pixels at a given spatial frequency in the first case onto the corresponding vector sum in the error-free case. In the average of \( N \) misaligned images, the envelope of signal decay at frequency \( \mathbf{s} \) is then

\[ E_{\text{trans}}(\mathbf{s}) = \frac{\sum_{i=1}^{N} A(\mathbf{s}) \cdot \cos(2\pi \mathbf{s} \cdot \mathbf{f}_i)}{N \cdot A(\mathbf{s})}, \]

(8)

where \( A(\mathbf{s}) \) is the true structure factor amplitude, and \( \mathbf{f}_i = (x_i, y_i) \) is the translational error for image \( \mathbf{s} \). This definition of \( E_{\text{trans}} \) could be negative for certain improbable distributions of error, but this is not experimentally relevant. Assuming a radially symmetric, Gaussian distribution of errors where the errors in any arbitrary direction \( n(x) \) have a standard deviation \( \sigma_{\text{trans}} \)

\[ n(x) = \frac{N}{\sigma_{\text{trans}} \sqrt{2\pi}} e^{-x^2/(2\sigma_{\text{trans}}^2)}, \]

(9)

the sum can be replaced by an integral, and the direction of \( \mathbf{s} \) is no longer important. Equation (8) then simplifies to

\[ E_{\text{trans}}(\sigma_{\text{trans}}, \mathbf{s}) = \frac{1}{N} \int n(x) \cos(2\pi sx) dx = e^{-2\pi^2 \sigma_{\text{trans}}^2} = e^{-B_{\text{trans}}x^2}. \]

(10)

\( B_{\text{trans}} \) is independent of particle size, and the result is seen to be analogous to the Debye–Waller temperature factor (Woolfson, 1997). The same result was also obtained more directly by convoluting a function representing the specimen with the Gaussian distribution of translational errors, so that in reciprocal space the translational errors produce a multiplicative, Gaussian envelope given by the transform of Eq. (9). The first derivation was presented because its pattern is conceptually useful in the derivations that follow.

Rotational Misalignment (E\text{rot})

Three rotation angles must be determined for each particle. Errors in rotation angles decrease the SNR in reconstructions in the same fundamental way as translational errors, in that the position in the micrograph used to reconstruct a specimen feature is slightly displaced from its actual image. Consider an atom of a particle at radius \( R \), rotated randomly and imaged onto a micrograph. In the Euler angle convention of Frank (1996), uncertainty in \( \phi \) is manifest in an arc of possible positions. Uncertainty in \( \theta \) is generally manifest as a spreading of the arc in a second direction. Uncertainty in \( \psi \) gives further spreading. The exact distribution of possible positions and their likelihoods depend critically on the position of the atom on its sphere of rotation before projection. Assuming for simplicity a Gaussian distribution of errors with standard deviation \( \sigma_{\text{rot}} \) in the determination of each rotation angle, the distribution of possible projected positions can be roughly approximated as a two-dimensional Gaussian, where the standard deviations of errors in each direction are simply the standard deviations of angular errors multiplied by the radius

\[ \sigma_{\text{trans}} = \frac{\pi R}{4} \sigma_{\text{rot}}, \]

(11)

where the radius used is the average projected radius over all rotations, \( \pi R/4 \). Inserting this into Eq. (10) leads to

\[ B_{\text{rot}} = 2\pi^2 \left( \frac{\pi R}{4} \sigma_{\text{rot}} \right)^2 = \frac{\pi^4}{8} R^2 \sigma_{\text{rot}}^2. \]

(12)

A more precise expression was found by simulation. A point on the surface of a unit sphere was chosen randomly, rotated by a set of three random rotation angles, and projected onto a plane. The three rotation angles were then perturbed by addition of small Gaussian errors and used again to rotate the original point, and the process was repeated enough times to reveal the pattern of uncertainty in the atom’s projected position. Typical patterns were highly eccentric, with approximate Gaussian decays away from the center. The recon-
The estimate of Eq. (12) is found to be about 10% too optimistic.

**Magnification Variation ($E_{mag}$)**

The actual magnification within and between images recorded in the electron microscope varies due to lens hysteresis, lens instabilities, defocus, and image distortion. Magnification differences between micrographs of a few percent and differences within micrographs as large as 2% have been measured (Aldroubi et al., 1992; Trus et al., 1997). Magnification variances within individual particle images on a micrograph, however, should be negligible, so that determination of a single magnification parameter for each particle is adequate. Under good conditions a single value would apply to an entire micrograph. Undetected variation, however, causes incoherent image averaging. As for translational and rotational errors, magnification errors decrease the SNR because the position in the micrograph used to reconstruct a specimen feature is slightly displaced from its actual image. Thus the derivation strategy is again to relate magnification errors to translation errors.

Let the position of a particular atom at radius $R$ in a randomly rotated particle be described by coordinates $(x, y, z)$ with respect to a fixed lab frame whose origin is the particle center and whose $z$ axis is parallel to the microscope optical axis. If an image is thought to have a magnification $M$, but the magnification is actually $M(1 + m)$, the atom’s $x$ coordinate in the micrograph will be mistaken by $xmM$. After that distance is divided by the presumed image magnification $M$, the error in the interpreted distance of the atom from the particle center in the $x$ direction will be $xm$. In a randomly rotated particle, each value of $x$ between $-R$ and $R$ is equally likely. Assuming a Gaussian distribution of fractional errors $m$ in the determination of magnification with standard deviation $\sigma_{mag}$ and adapting Eq. (10) above, the envelope of signal loss in the average of an infinite number of images becomes

$$E_{mag}(\sigma_{mag} R, s) = \int_{x=-R}^{x=+R} \int_{m=-\sigma_{mag}}^{m=+\sigma_{mag}} \frac{1}{\sqrt{2\pi\sigma_{mag}}} e^{-m^2/2\sigma_{mag}^2} \cos(2\pi xm) dm dx,$$  

(14)

Numerical solutions of this integral are presented graphically in Fig. 2. In all regions of experimental interest, where $E_{mag} > 0.5$, this envelope can be closely approximated by the general formula

$$E_{mag}(\sigma_{mag} R, s) \approx e^{-5.05\sigma_{mag}^2 s^2} = e^{-B_{mag} s^2}.$$

(15)

Aldroubi et al. (1992) derived a related envelope function that results from averaging two images with slightly different magnifications. Because only two images are considered, however, their envelope is not directly comparable, especially where it begins oscillating with increasing spatial frequency.
Defocus Errors ($E_{def}$)

In Eq. (2), by SNR$_{ideal}$ image was meant the SNR that would be recorded by a perfect microscope, which would deliver full contrast at each spatial frequency. This was the assumption used in Henderson’s assessment of the minimum number of images necessary, and this was nearly the case in Glaeser’s real-space assessment because the images considered were at Scherzer focus. In an actual microscope, however, underfocused to enhance particle visibility as is typical for single particle analysis, only a fraction of the contrast is recoverably recorded in the images, and this fraction is given by the contrast transfer function. Only phase contrast is considered below because it is the sinusoidal nature of the phase contrast transfer function (CTF) at higher resolutions in defocused images that fundamentally defines the envelope and because phase contrast dominates images of thin biological samples.

The CTF is a sinusoidal function of defocus $\Delta z$, spatial frequency $s$, the wavelength of the electron beam $\lambda$, and the coefficient of spherical aberration $C_s$:

$$CTF(\Delta z, s) = \sin\left(2\pi\left(-\frac{\lambda s^2 \Delta z}{2} + \frac{\lambda^3 s^4 C_s}{4}\right)\right).$$  \hfill (16)

To restore the relative contributions of each spatial frequency, image transforms can be “CTF-corrected” by multiplication with a Wiener filter $W$ (Goodman, 1996),

$$W(\Delta z, s) = \frac{CTF(\Delta z, s)}{CTF^2(\Delta z, s) + \frac{1}{SNR^2(s)}}.$$  \hfill (17)

where $\Delta z_0$ is the mean defocus and $\theta$ is the azimuthal angle (Frank, 1996). Image “alignment” in this context means correcting for the image-specific weighting function (the CTF) at each spatial frequency so that Fourier components in different images are directly comparable.

Defocus determination is typically done by curve-fitting theoretical CTF to power spectra of incoherently averaged particles or background supports such as carbon films. Error arises from many sources. Noise in the images and signal loss at higher resolutions make the CTF difficult to discern. Particles can be frozen at different depths within the specimen thickness, and the plane of the specimen can lie in a tilted position (Booy and Pawley, 1993), causing particles on one side of the image to be at heights different from those of particles on the other. Slight image astigmatism can go undetected. Residual beam tilt changes the apparent defocus and introduces astigmatism. Finally, for larger particles, the diameter of the particle itself can cause features at the top of the particle to require a defocus correction different from that of features at the bottom (DeRosier, 2000; Jensen and Kornberg, 2000; Skoglund et al., 1996).

While all the envelopes discussed previously in this paper are concerned with signal only, in the present case of defocus errors, an envelope of signal-to-noise ratios was derived because the role of the Wiener filter is seen more clearly. Suppose $N$ evenly distributed particles are frozen in identical orientations within a thin ice layer across a hole in a carbon film that is perpendicular to the electron beam. Suppose in addition that the defocus of the central plane of the field can be determined exactly ($\Delta z$) and that there is no astigmatism. As in typical single particle images, the defocus is several times Scherzer focus and the CTF is essentially sinusoidal at the resolutions of interest (higher than ~10 Å). Each pixel in an ideal image transform will contain a signal component equal to the true structure factor at that spatial frequency, scaled by the CTF, and a noise component. Let each particle

![Figure 2](image-url)
image be CTF-corrected with a Wiener filter appropriate for the defocus of the central plane of the field (Δz), and let the images be averaged.

Two independent losses of SNR with respect to the ideal situation of Eq. (2) occur. First, the CTF reduces the fraction of the signal that is recorded as contrast, but does not diminish the shot noise generated by the recording process. Thus, at nodes in the CTF, image transforms contain no signal, and the reduction in SNR at and around CTF nodes is unrecoverable, as no form of CTF correction can restore the signal without equally amplifying noise.

Second, additional signal is lost during CTF correction. The Wiener filter has the effect of weighting the relative contributions of different spatial frequencies to balance signal restoration against noise amplification, but also serves to invert negative contrast. Because there is inevitably some error in the determination of defocus parameters, however, the presumed positions of the CTF nodes are in error and CTF correction sometimes fails to invert negative contrast or does invert positive contrast. This subtracts signal from the average image while negligibly affecting the noise.

Both SNR losses are naturally accounted for in a single envelope function (E_{def}). Given that a single defocus estimate (Δz) is used to correct each particle, the envelope of SNR loss in the average image due to the CTF and its correction is

\[
E_{\text{def}}(s) = \frac{\text{SNR}_{\text{averaging CTF-corrected images}}}{\text{SNR}_{\text{averaging ideal images}}} = \frac{\sum_{i=1}^{N} A_{\text{true}}(s) \cdot \text{CTF}(\Delta z + z, s) \cdot W(\Delta z, s)}{\sum_{i=1}^{N} \text{Noise}(s) \cdot W(\Delta z, s)},
\]

(19)

where \(A_{\text{true}}(s)\) is the ideal structure factor amplitude of the particle at spatial frequency \(s\), \(z_i\) is the depth of each particle within the ice layer (or equivalently, the error in defocus determination), and \(\text{Noise}(s)\) is the noise vector at \(s\) in image \(i\). Letting \(n(z)\) represent the number of particles with each depth (defocus error) \(z\), and noting that the noise and true amplitude terms cancel and that the directionality of \(s\) is unimportant,

\[
E_{\text{def}}(s) = \frac{W(\Delta z, s)}{|W(\Delta z, s)|} \frac{1}{N} \int n(z) \text{CTF}(\Delta z + z, s) dz.
\]

(20)

The Wiener filter terms simply cause the envelope to be positive when the defocus Δz corresponds to a negative CTF value at \(s\). It is possible, however, that for certain experimentally irrelevant distributions of errors \(z\) and values of \(s\) and Δz, the net signal from images where the contrast was incorrectly inverted can be more than the signal where it was correct and that the value of this envelope could be negative.

It can be seen from Eq. (20) that in the average of a large number of images, when there are no defocus determination errors (\(z\) always equals 0), if the defocus values Δz are spread evenly throughout a range corresponding to at least a half CTF period, \(E_{\text{def}}\) will be the average absolute value of the sine function, or 2/\(\pi\). This corresponds to the first loss of SNR referred to above. Because the depth of field of typical microscopes at near-atomic resolution is only several hundred Angstroms, the distribution of experimental defocus values in a large set of particle images will essentially always fulfill this criteria at high resolution.

The second loss of SNR, that due to errors in defocus determination, depends on the distribution of errors \(n(z)\). Two cases will be considered. First, for the layer of particles described above, a rectangle function is appropriate, and in this case the integral of Eq. (20) can be solved analytically. The result is a sinc function,

\[
E_{\text{def}}(a, s) = \frac{\text{snic}\left(\frac{\pi \lambda a s^2}{2}\right) \cdot \text{CTF}(\Delta z, s) \cdot W(\Delta z, s)}{|W(\Delta z, s)|},
\]

(21)

where \(a\) is the width of the ice layer. Since in any experimental reconstruction many values of Δz will be included, the product of the CTF and the Wiener filter can be replaced with its average absolute value (2/\(\pi\)), so that

\[
E_{\text{def}}(a, s) = \frac{2}{\pi} \text{snic}\left(\frac{\pi \lambda a s^2}{2}\right).
\]

(22)

The second, more likely, situation is that in addition to the variation in particle depth within the ice layer, there is a comparably large error in the determination of the defocus of the central specimen plane and some undetected or misdetected astigmatism. The more general case of a Gaussian distribution of defocus errors with standard deviation \(\sigma_{\text{def}}\) at any particular pixel is therefore considered:

\[
n(z) = \frac{N}{\sigma_{\text{def}} \sqrt{2\pi}} e^{-\left(z - \text{mean}\right)^2/2}.
\]

(23)

While the integral in Eq. (20) can no longer be solved analytically, a closed-form solution that reveals the
functional form of this envelope can be found at the special defocus value \( \Delta z \), where

\[
2\pi \left[ -\frac{\lambda s^2 \Delta z}{2} + \frac{\lambda^3 s^4 C_s}{4} \right] = \frac{\pi}{2}.
\]

(24)

Then the Wiener filter terms cancel,

\[
\text{CTF}(\Delta z + z, s) = \sin\left( \frac{\pi}{2} - \pi \lambda s^2 z \right) = \cos(\pi \lambda s^2 z),
\]

(25)

and

\[
E_{\text{def}}(\sigma_{\text{def}}, s) = e^{-\pi^2 \lambda^2 \sigma_{\text{def}}^2 s^4/2}.
\]

(26)

Equation (26) is the same envelope (expressed in terms of a standard deviation) derived previously in other ways to describe the effects of temporal incoherence and objective lens instabilities, which also produce defocus variations (Hanszen and Trepte, 1971; Reimer, 1997; Wade and Frank, 1977).

In an actual experiment, the distribution of defocus values within an image set will cause the envelope to have values lower than the special case treated. Specifically, the value for random distributions of \( \Delta z, z, \) and \( s \) within experimentally applicable ranges was shown by simulation to be \( 2/\pi \) times the result of Eq. (26) (data not shown). This is recognized as precisely the additional signal loss due to the average absolute value of the CTF discussed earlier, so that the complete envelope in the general case is

\[
E_{\text{def}}(\sigma_{\text{def}}, s) = \frac{2}{\pi} e^{-\pi^2 \lambda^2 \sigma_{\text{def}}^2 s^4/2}.
\]

(27)

Residual Beam Tilt (E_{\text{tilt}})

Beam tilt manifests itself in images in three ways: as a simple shift of the entire image, as an apparent change in defocus and astigmatism, and as a spatial frequency-dependent shift of individual Fourier components. It is detected in images of well-ordered crystals through the perturbations it causes in reflection phases, and the effects can be corrected as well as the magnitude and direction can be determined (Henderson et al., 1986). Because microscope alignment procedures have been developed to minimize beam tilt (Zemlin, 1989) and because it remains constant throughout a microscope session (Henderson et al., 1986), corrections have not always been essential, even to merge data to near-atomic resolution (Nogales et al., 1998). Nevertheless, in the case of single particle images, any residual beam tilt would be difficult to estimate and could be a major resolution-limiting factor in high-resolution reconstructions.

Beam tilt can be specified by a vector \( \mathbf{t} = (t_x, t_y) \) giving the position of the unscattered beam in the back focal plane of the objective lens with respect to the microscope optical axis. To derive the envelope of signal loss due to uncorrected or miscorrected beam tilt, it is assumed that a large number of individual particle images with a radially symmetric, Gaussian distribution of errors in beam tilt estimation are averaged. The contribution of beam tilt to errors in the determination of image centers, defocus, and astigmatism is accounted for in \( E_{\text{trans}} \) and \( E_{\text{def}} \). The spatial frequency-specific phase shift \( \Delta \phi(\mathbf{s}) \) is

\[
\Delta \phi(\mathbf{s}) = -2\pi C_s \lambda^2 s^2 (\mathbf{s} \cdot \mathbf{t})
\]

(28)

(Smith et al., 1983). Specifying the beam tilt estimation errors in any arbitrary direction \( n(t) \) by a standard deviation \( \sigma_{\text{tilt}}, \)

\[
n(t) = \frac{N}{\sigma_{\text{tilt}}} \frac{1}{\sqrt{2\pi}} e^{-\frac{(t-n(t))^2}{2\sigma_{\text{tilt}}^2}},
\]

(29)

the direction of \( \mathbf{s} \) is no longer important, and by analogy to Eq. (10), the envelope due to the phase errors introduced by residual beam tilt becomes

\[
E_{\text{tilt}}(s) = \frac{1}{N} \int n(t) \cos(-2\pi C_s \lambda^2 s^2 t) dt = e^{-2\pi^2 C_s^2 \lambda^2 s^4/\sigma_{\text{tilt}}^2}.
\]

(30)

The situation treated is actually almost exactly analogous to the problem of partial spatial coherence with an assumed Gaussian source distribution, in which a recorded image is in fact the average of a large number of images, each produced by a separate electron wave with variable beam tilt. The corresponding envelope \( E_{\text{pc}}(s) \) was given by Frank (1973),

\[
E_{\text{pc}}(s) = e^{-\frac{q_0^2}{2\sigma_{\text{tilt}}^2} C_s^2 \lambda^2 s^4 + 2\pi C_s \lambda^2 \Delta z \sigma_{\text{tilt}}^2 s^4},
\]

(31)

where \( q_0 \) is the half-width of the distribution, defined in the back focal plane. Converting \( q_0 \) to the equivalent expression \( \sqrt{2\sigma_{\text{tilt}}^2/\lambda} \) in the variables of Eq. (30) and expanding,

\[
E_{\text{pc}}(s) = e^{-2\pi^2 C_s^2 \lambda^2 s^4/\sigma_{\text{tilt}}^2} + 4\pi C_s \lambda^2 \Delta z \sigma_{\text{tilt}}^2 s^4 - 2\pi \Delta z \sigma_{\text{tilt}}^2 s^4/\sigma_{\text{tilt}}^2.
\]

(32)

The first term is seen to be precisely \( E_{\text{tilt}}(s) \) of Eq. (30). Because the image shifts due to beam tilt are given...
by $\sigma_{\text{trans}} = \sigma_{z}$, the last term is seen through Eq. (10) to be their contribution to translational errors. Finally, the middle term’s dependence on $s^{4}$ reveals its correspondence to differences in defocus and astigmatism, as in Eq. (27). The difference in the analogy between recording images with partial spatial coherence and averaging images with variable beam tilt is the opportunity in the latter case for estimation and computational correction of the effects of beam tilt before any averaging is performed.

**PART 3: TARGET ALIGNMENT ERROR TOLERANCES**

One way to identify target error tolerances for the alignment parameters is to find the error that costs a certain fraction of the original SNR. For example, the standard deviations of the alignment errors required to retain at least 90% of the original SNR through the individual steps of the image averaging process at 3.5 and 7.0 Å resolution are listed in Table 1. Only the compound effect of all resolution limitations together, however, is experimentally relevant.

Equation (2) asserts that the combined effect of all resolution limitations can be described by a product of individual envelope functions. This is clearly true for processes that are separate and sequential, such as the signal losses due to imperfect specimens, the imperfect microscope, and incoherent image averaging. At each stage a fraction of the incoming SNR is lost. In contrast, some factors act simultaneously, such as magnification, rotation, and translational misalignments, which combine to cause a single, net displacement in the presumed position of an atom's image in the micrograph. In this case, to the degree that each of the three factors produce Gaussian distributions of displacements, the variance of the net displacement is the sum of the variances of the individual displacements. This fact is expressed equivalently through a product of the individual Gaussian envelopes.

A numerical test showed that the combined effect of the alignment errors is in fact given by the product of the individual envelopes. The combined effect of the alignment errors was calculated with the equation

$$E(s) = \frac{1}{N} \sum_{i=1}^{N} |\text{CTF}(\Delta z, s)| \cos(2\pi s\Delta x + \Delta \phi_i),$$

(33)

which incorporates the effects of translational, rotational, magnification, defocus, and beam tilt errors in the context of a single particle reconstruction as follows. It was assumed that $N$ noiseless particle images all recording the same projection of the particle were available for averaging, but that the alignment parameters describing position, orientation, magnification, defocus, and beam tilt were estimated with Gaussian errors. $E(s)$ is the fraction of signal at spatial frequency $s$ retained in the average using the erroneous alignment parameters and a typical CTF compared to the signal that would have resulted had there been no alignment errors and an ideal CTF. As described initially in Eq. (8), this envelope was defined as the projection of the vector sum of image-transform pixels at that spatial frequency in the first case onto the corresponding vector sum in the error-free case. Each term in the sum represents the appropriate pixel in the transform of each image being averaged. For simplicity, the amplitude of the original pixel of interest (identical in each image) was assumed to be 1 and the phase to be 0. Thus, in the average of $N$ images without align-

<table>
<thead>
<tr>
<th>Alignment error tolerance</th>
<th>To retain 90% of SNR to 3.5 Å</th>
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<th>To reach 3.5 Å with $10^7$ particles$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translational</td>
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<td>$0.10 \text{ Å}$</td>
</tr>
<tr>
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<td>$0.53^a$</td>
<td>$0.38^a$</td>
</tr>
<tr>
<td>Magnification</td>
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<td>$1.5%$</td>
<td>$0.10%$</td>
</tr>
<tr>
<td>Defocus</td>
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<td>360 Å (not including $2/\pi$ loss)</td>
<td>150 Å (including $2/\pi$ loss)</td>
</tr>
<tr>
<td>Beam tilt</td>
<td>$0.40 \text{ mrad}$</td>
<td>$3.23 \text{ mrad}$</td>
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</tr>
<tr>
<td>$\sigma_{\text{tilt}}$</td>
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<td></td>
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</tr>
</tbody>
</table>

Note. Tolerances for a 1-MDa particle, an electron voltage of 300 kV, and a coefficient of spherical aberration of 2.0 mm.

$^a$ Including estimates for particle disorder, image degradation, and the other alignment errors.

**TABLE I**

Target Alignment Error Tolerances

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$^a$ Including estimates for particle disorder, image degradation, and the other alignment errors.
moment errors and with ideal CTFs, the vector sum would have amplitude $N$ and phase 0. This amplitude appears as a denominator to make $E(s)$ a fractional value. As shown in the section on defocus errors, the two effects of the CTF and its correction with an estimated Wiener filter are first that the signal recorded in the image is unrecoverably diminished by the CTF and second that the contribution from any one image may enter the sum with the wrong sign. For each term in the sum, the first effect was included by choosing a random “true” defocus value $\Delta z_i$ between 0.5 and 2 $\mu$m and using the absolute value of CTF $(\Delta z_i, s)$ for the amplitude rather than 1, as would be the case for an ideal CTF. The second effect was included by choosing a random error $z_i$ in the defocus estimation, calculating the corresponding Wiener filter $W(\Delta z_i + z_i, s)$, and multiplying the term in the sum by $-1$ if the signs of $W(\Delta z_i + z_i, s)$ and CTF $(\Delta z_i, s)$ did not agree. The effects of random errors in rotation angles were incorporated into the envelope by choosing a random point on a sphere, rotating it once by random true rotation angles and then again by the same angles perturbed by the addition of small random errors, and calculating the resultant displacement in projection. The “net” displacement in the x direction $\Delta x_i$ was then obtained by adding random translational errors and scaling the sum by a random magnification error. The resulting phase error is then $2\pi s \Delta x_i$.

A second phase error $\Delta \phi_{s}$ due to errors in beam tilt estimation was added by choosing random x components of beam tilt and using Eq. (28). Finally, the projection of each term onto the error-free vector sum was found as the cosine of the combined phase errors.

Using $N = 10^7$, three sets of standard deviations of alignment errors were tested. The results are shown as symbols in Fig. 3 superimposed on the theoretical envelopes predicted by the product of Eqs. (10), (13), (15), (27), and (30), verifying that the net effect of all the alignment errors considered can in fact be closely approximated by the product of the theoretical envelope functions derived in this paper.

Thus a more useful set of target error tolerances can be obtained by using estimates of some factors to constrain the others. Progress in microscope automation makes it reasonable to expect that while microscopes could automatically record a million particle images within a few days (Oostergetel et al., 1998), more than about $10^7$ particles will still be impractical. Using Glaeser’s value of $N_{\text{min}} = 1400$ at near-atomic resolution (Glaeser, 1999) and Eq. (2), the minimum acceptable value ($E_{\text{min}}$) of $E_{\text{particle disorder}} - E_{\text{image degradation}} - E_{\text{incoherent averaging}}$ is approximately 1%.

In order to identify target alignment error tolerances within this limit, estimates of $E_{\text{particle disorder}} - E_{\text{image degradation}}$ were taken from the literature. Several routes were available. A theoretical expression for $B_{\text{temp}}$ as a function of temperature, applicable to crystals of pure elements, was derived by Debye (Woolfson, 1997). The experimentally measured values from X-ray crystallography are probably more appropriate, however, and range from less than 1 to about 7.5 $\AA^2$ (less than 4 to about 30 $\AA^2$ in common X-ray terminology) (Drenth, 1999). Noting that the effects of selection and stabilization of particles through crystal formation is difficult to judge, for the purpose of identifying target alignment errors, the intermediate value of $B_{\text{temp}} = 4$ $\AA^2$ was selected, which corresponds to $E_{\text{particle disorder}} = 72\%$ at 3.5 $\AA$.

$E_{\text{image degradation}}$ can be estimated as the experimentally measured ratio of image transform amplitudes to those obtained by direct measurement of electron diffraction intensities. Different investigators have obtained values from 4 to 42% at near-atomic (3–4.5 $\AA$) resolution (Brink and Chiu, 1991; Henderson, 1992; Henderson and Glaeser, 1985). As was suggested by Henderson (1992), the largest of these estimates is probably misleading because the specimen used, paraffin, seems less beam-sensitive than most biological particles. Better estimates are...
probably provided by Wilson-like plots from protein crystals, such as Fig. 5 of Henderson (1992), which leads to $E_{\text{image degradation}} = 3.5 \, \text{Å} = 4.5\%$. It is noteworthy, however, that this particular plot came from an image of purple membrane in glucose over continuous carbon, and one of the main points made by the authors is that images of particles in vitreous ice spanning holes in fenestrated carbon will probably have significantly worse image degradation. Nevertheless, recent successes under typical single particle conditions (Miyazawa et al., 1999) justify using this most directly measured value (4.5\%) here.

Given $E_{\text{min}} = 1\%$, $E_{\text{particle disorder}} = 72\%$, and $E_{\text{image degradation}} = 4.5\%$, incoherent averaging must be 31\% or more to allow near-atomic resolution single particle analysis. One complete set of target alignment error tolerances that meet this criteria, chosen in the author’s judgement to distribute the alignment challenge equally among the various parameters, is listed in Table 1.

**DISCUSSION**

Just as automatic gene sequencing has caused an explosion in biological knowledge, so would an automatic and generally applicable method of structure determination. Single particle analysis has the potential to become such a technique: miniscule amounts of specimen are needed (only millions of particle images are required), the technique can be applied to any rigid, soluble particle (no crystallization is necessary), the whole process from sample preparation to model calculation is automatable, particles can be imaged in their native concentrations and buffer conditions, images of deformed or substoichiometric particles can be selectively discarded, and multiple conformations can be distinguished and solved independently. Furthermore, the technique may allow in vivo structure determination, as whole cells may one day be frozen and sectioned adequately for high-resolution protein imaging.

Nevertheless, the challenge of precise particle alignment will be a major obstacle. Three fundamentally different methods for aligning particles have been proposed. The first is physically ordering the particles into crystals so that the averaged information from many particles can be used to determine the alignment parameters. This method obviously has the disadvantage of requiring crystals. The second method is to rigidly and specifically label particles with heavy atom clusters, which serve as alignment markers (Jensen and Kornberg, 1998). The third way to align particles is to use the signals from the individual particles themselves. The work presented here suggests that the uncertainty in image alignments and its contribution to the total resolution limitation be estimated and reported in future single particle studies.

Three noteworthy facts emerged from the identification of target alignment tolerances. First, obtaining the highest image quality possible is crucial. The alignment error tolerances vary dramatically with $E_{\text{image degradation}}$. For instance, given $10^7$ images, assuming $E_{\text{particle disorder}} = 72\%$, and noting that $E_{\text{def}}$ can never be larger than $2/\pi$, if $E_{\text{image degradation}}$ is 2\% or less, there is no room for any alignment errors at all and single particle analysis to near-atomic resolution becomes hopeless. In contrast, if $E_{\text{image degradation}}$ can be increased from 4.5 to 10\%, the tolerable translational and rotational errors double (holding all others constant as in Table 1, column 4). Furthermore, better images obviously improve alignment parameter estimation.

Second, defocus errors are particularly costly. If the specimen layer is several hundred Angstroms thick, it becomes important to determine the relative depth of each particle within the ice layer as well as the defocus of the central specimen plane. In one case this has been attempted (Matadeen et al., 1999). Perhaps techniques will be developed to constrain all particles to freeze at the same depth within the ice. High-dose images of heavy-atom clusters attached to particles may allow accurate measurement of defocus through the positions of Fresnel fringes. Perhaps ideal defocus-calibration molecules could be attached to particles, just as magnification-calibration standards have been distributed in other samples. Alternatively, tilted image pairs could reveal the depths of particles within the ice. Reconstructions of particles which themselves are larger than the tolerable defocus determination error can be done with algorithms that allow a defocus gradient correction across the reconstruction (DeRosier, 2000; Jensen and Kornberg, 2000; Skoglund et al., 1996).

The third noteworthy fact is the necessity of precise microscope alignment. In the context of single particle analysis, beam tilt is difficult to detect and therefore may not be computationally correctable. Because the envelope due to beam tilt increases as the sixth power of resolution, even slight beam misalignments (~0.4 mrad) cause drastic losses in signal at near-atomic resolutions.

The convergence between the alignment error envelopes derived here and envelopes derived previously in other contexts is satisfying. Approaching the problems completely from the perspective of averaging images, the envelopes for translational errors, defocus errors, and beam tilt are found to be closely related to the X-ray temperature factor and...
the envelopes for temporal and spatial incoherence, respectively.

The relationship between signal decay envelopes and SNR envelopes deserves mention. Some resolution-limiting factors diminish signal and noise equally, such as the modulation transfer function of a scanner, so that no distinction between the two types of envelope is needed. Others, such as translational misalignments, lose what would otherwise have been useful signal into increased background noise. This additional noise will be only a small perturbation to the shot noise, fog noise, and other sources of noise (Zhu et al., 1997) already present, however, because it adds incoherently and because the signal is, in general, much smaller than the background noise in low-dose images of individual particles at high resolution. As one quantitative example, an average change of 18% in background noise intensities was estimated in diffraction patterns due to random, rigid-body displacements of unit cells in a two-dimensional crystal (Grigorieff and Henderson, 1995).

Finally, the envelope of signal loss due to image averaging underscores the need to restore the amplitudes of higher frequency components relative to lower frequency components in reconstructions to obtain faithful models. If image alignment precision could be estimated accurately during the refinement process, the envelope functions given in this paper could possibly be used to determine the appropriate filter. Ideally, though, an independent measure of the object’s power spectrum, such as by X-ray solution scattering, should be used (Thuman-Commike et al., 1999) to compensate for all the envelopes involved in image recording and averaging simultaneously.

CONCLUSION

The resolution-limiting effects of alignment errors were placed in the context of the number of images averaged, shot noise, particle disorder, and image degradation in Eq. (2). Envelope functions for translational, rotational, magnification, defocus, and beam tilt errors were derived and given in Eqs. (10), (13), (15), (27), and (30), respectively. These envelopes were used to identify alignment error tolerances (Table 1) for near-atomic resolution single particle analysis using estimates from the literature for the theoretical minimum number of images required, particle disorder, and current image quality. The analysis emphasized the importance of improved image quality, precise defocus determination, and good microscope alignment.

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