inferior linear interpolation scheme used in SIRT (this impediment is shared with WBP1 and WBP2). [The dip of the SIRT fidelity curve at a spatial frequency of 0.42 in Fig. 2.5.6.8(*a*) is due to an inconsistency between the interpolation methods used internally in SIRT to generate intermediate projections of the object to be reconstructed and the Fourier-space-based method used to generate the test data.] In terms of computational efficiency, gridding-based algorithms outperform weighted back-projection algorithms by a small factor while SIRT is approximately ten times slower (depending on the number of iterations used).

2.5.6.7. 3D reconstruction of symmetric objects

Many objects imaged by EM have symmetries; the two types that are often met are helical (phage tails, *F*-actin, microtubules, myosin thick filaments, and bacterial pili and flagella) and point-group symmetries (many macromolecular assemblies, virus capsids). If the object has helical symmetry, it is convenient to use cylindrical coordinates and a dedicated reconstruction algorithm; particularly for the initial analysis of the data that has to be done in Fourier space. Diffraction from such structures with c periodicity and scattering density $\varphi(r, \psi, z)$ is defined by the Fourier–Bessel transform:

$$\begin{aligned}\Phi(R, \Psi, Z) &= \sum_{-\infty}^{+\infty} \exp\left[in\left(\Psi + \frac{\pi}{2}\right)\right] \int_0^\infty \int_0^{2\pi} \int_0^l \varphi(r, \psi, z) \\ &\quad \times J_n(2\pi r R) \exp[-i(n\psi + 2\pi z Z)] r \, \mathrm{d}r \, \mathrm{d}\psi \, \mathrm{d}z \\ &= \sum_n G_n(R, Z) \exp\left[in\left(\Psi + \frac{\pi}{2}\right)\right]. \qquad (2.5.6.46)\end{aligned}$$

The inverse transform has the form

$$\rho(r, \psi, z) = \sum_n \int g_n(r, Z) \exp(in\psi) \exp(2\pi i z Z) \, \mathrm{d}Z, \qquad (2.5.6.47)$$

so that g_n and G_n are the mutual Bessel transforms

$$G_n(R, Z) = \int_0^\infty g_n(r, Z) J_n(2\pi r R) 2\pi r \, \mathrm{d}r \qquad (2.5.6.48)$$

and

$$g_n(r, Z) = \int_0^\infty G_n(R, Z) J_n(2\pi r R) 2\pi R \, \mathrm{d}R. \qquad (2.5.6.49)$$

Owing to helical symmetry, (2.5.6.48) and (2.5.6.49) contain only those of the Bessel functions that satisfy the selection rule (Cochran *et al.*, 1952)

$$l = mp + (nq/N), \qquad (2.5.6.50)$$

where N, q and p are the helix symmetry parameters, $m = 0, \pm 1, \pm 2, \ldots$. Each layer l is practically determined by the single function J_n with the lowest n; the contributions of other functions are neglected. Thus, the Fourier transform of one projection of a helical structure, with an account of symmetry and phases, gives the 3D transform (2.5.6.49). However, biological specimens tend to be flexible and disordered, and exact helical symmetry is rarely observed. A possible approach to dealing with flexibility is to computationally straighten filaments (Egelman, 1986), but this has the potential for introducing artifacts. Another difficulty with helical analysis is that the indexing of a pattern can be ambiguous and the wrong symmetry can be chosen (Egelman & Stasiak, 1988). Further complications exist when the filament does not have a precisely defined helical symmetry, such as *F*-actin, which has a random variable twist (Egelman *et al.*, 1982). To address these problems, Egelman developed a real-space refinement method for the reconstruction of helical filaments that is capable of determining the helical symmetry of an unknown structure (Egelman, 2000). In this approach, the Fourier–Bessel reconstruction algorithm is replaced by a general reconstruction algorithm discussed in Section 2.5.6.6 with the real-space projections supplied as input. Thus, the symmetry is not enforced within the reconstruction algorithm; instead, it is determined and imposed subsequently by real-space averaging.

The presence of point-group symmetry in the structure means that any projection yields as many symmetry-related (but differently oriented) copies of itself as the number of symmetry operations in the group. For example, for three dimensions, each projection enters the reconstruction process at six different projection directions, while for icosahedral symmetry (I) the number is sixty! This high multiplicity makes it possible to write a reconstruction program that explicitly takes into account the symmetry and can perform the task much faster than generic algorithms. Such programs are usually part of dedicated software packages that were specifically designed for the determination of icosahedral structures (Fuller *et al.*, 1996; Lawton & Prasad, 1996; Liang *et al.*, 2002). These icosahedral reconstruction programs are heavily indebted to a set of FORTRAN routines written by R. A. Crowther at the Medical Research Council Laboratory of Molecular Biology (MRC LMB) and perform the 3D reconstruction using the Fourier–Bessel transform strategy outlined above [(2.5.6.46)–(2.5.6.49)] (Crowther, Amos *et al.*, 1970; Crowther & Amos, 1972).

The general reconstruction methods (algebraic, filtered back-projection, direct Fourier inversion) easily accommodate symmetries in the data. In major single-particle reconstruction packages, reconstruction programs are implemented such that the point-group symmetry is a parameter of the program. The symmetry operation is internally taken into account during calculation of the weighting function and it is also applied to the set of Eulerian angles assigned to each projection so the multiple copies are implicitly created and processed. This approach results in an extended time of calculations, but it is entirely general. In direct Fourier inversion algorithms the numerical inaccuracy of the symmetrization performed in Fourier space will result in nonsymmetric artifacts in real space. Thus, in *SPIDER* (Frank *et al.*, 1996) an additional real-space symmetrization is performed after the reconstruction is completed. Finally, it has to be noted that although it might be tempting to calculate a 3D reconstruction without enforcement of symmetry and to symmetrize the resulting structure, this approach is incorrect. This fact can be seen from the way weighting functions are constructed: weighting functions calculated with symmetries taken into account are not equal to the weighting function calculated for a unique set of projections and subsequently symmetrized.

2.5.7. Single-particle reconstruction

By P. A. Penczek

2.5.7.1. Formation of projection images in single-particle reconstruction

Cryo-electron microscopy (cryo-EM) in combination with the single-particle approach is a new method of structure determination for large macromolecular assemblies. Currently, resolution in the range 10 to 30 Å can be reached routinely, although in a number of pilot studies it has been possible to obtain structures at 4 to 8 Å. Theoretically, electron microscopy can yield data

exceeding atomic resolution, but the difficulties in overcoming the very low signal-to-noise ratio (SNR) and low contrast in the data, combined with the adverse effects of the contrast transfer function (CTF) of the microscope, hamper progress in fulfilling the potential of the technique. However, in recent years, cryo-EM has proven its power in the structure determination of large macromolecular assemblies and machines which are too large and complex for the more traditional techniques of structural biology, *i.e.*, X-ray crystallography and NMR spectroscopy.

Single-particle reconstruction is based on the assumption that a protein exists in solution in multiple copies of the same basic structure. Unlike in crystallography, no ordering of the structure within a crystal grid is required; the enhancement of the SNR is achieved by bringing projection images of different (but structurally identical) proteins into register and averaging them. This is why the technique is sometimes called 'crystallography without crystals'.

Within the linear weak-phase-object approximation of the image formation process in the microscope [see equation (2.5.2.43) in Section 2.5.2], 2D projections represent line integrals of the Coulomb potential of the particle under examination convoluted with the point-spread function of the microscope, s, as introduced in Section 2.5.1. In addition, we have to consider the translation $\mathbf{t}$ of the projection in the plane of micrograph, suppression of high-frequency information by the envelope function E of the microscope, and two additive noises m^{B} and m^{S}. The first one is a coloured background noise, while the second is attributed to the residual scattering by the solvent or the supporting thin layer of carbon, if used, assumed to be white and affected by the transfer function of the microscope in the same way as the imaged protein. In order to have the image formation model correspond more closely to the physical reality of data collection, we write equation (2.5.6.4) from Section 2.5.6 such that the projection operation is always realized in the z direction of the coordinate system (corresponding to the direction of propagation of the electron beam), while the molecule is rotated arbitrarily by three Eulerian angles:

$$d_n(\mathbf{x}) = s_n(\mathbf{x}) * e_n(\mathbf{x}) * \left[\int f(\mathbf{T}_n\mathbf{r})\,\mathrm{d}z + m_n^{\mathrm{S}}(\mathbf{x})\right] + m_n^{\mathrm{B}}(\mathbf{x}), \quad n = 1, \ldots, N. \tag{2.5.7.1}$$

Here $f \in R^{n^3}$ represents the three-dimensional (3D) electron density of the imaged macromolecule and $d \in R^{n^2}$ is the nth observed two-dimensional (2D) projection image. The total number of projection images N depends on the structure determination project, and can vary from a few hundred to hundreds of thousands. Further, e is the inverse Fourier transform of the envelope function, $\mathbf{x} = [x \quad y]^T$ is a vector of coordinates in the plane of projections, $\mathbf{r} = [r_x \quad r_y \quad r_z \quad 1]^T$ is a vector of coordinates associated with nth macromolecule, $\mathbf{T}$ is the 4×4 transformation matrix given by

$$\mathbf{T}(\mathbf{R}, \mathbf{t}) = \begin{bmatrix} \mathbf{R} & \mathbf{t} \\ 0 & 1 \end{bmatrix}, \quad \begin{bmatrix} \mathbf{x} \\ z \\ 1 \end{bmatrix} = \mathbf{T}\mathbf{r}, \tag{2.5.7.2}$$

with $\mathbf{t} = [t_x \quad t_y \quad 1]^T$ being the shift vector of translation of the object (and its projection) in the xy plane (translation in z is irrelevant due to the projection operation) and $\mathbf{R}(\psi, \theta, \varphi)$ is the 3×3 rotation matrix specified by three Eulerian angles. As in Section 2.5.6, two of the angles define the direction of projection $\boldsymbol{\tau}(\theta, \varphi)$, while the third angle ψ results in rotation of the projection image in the plane of the formed image xy; changing this angle does not provide any additional information about the structure f. Both types of noise are assumed to be mutually uncorrelated and independent between projection images (*i.e.*, $\langle m_i^k m_j^l \rangle_{i \neq j} = 0$; k, l = S, B) and also uncorrelated with the signal ($\langle d_i m_i^k \rangle = 0$; k = S, B). Model (2.5.7.1) is semi-empirical in that, unlike in the standard model, we have two contributions to the noise. Although in principle amorphous ice should not be affected by the CTF, so the term m^{S} should be absorbed into m^{B}, in practice the buffer in which the protein is purified is not pure water and it is possible to observe CTF effects by imaging frozen buffer alone. Moreover, if a thin support carbon is used, it will be a source of very strong CTF-affected noise also included in m^{B}.

In Fourier space, (2.5.7.1) is written by taking advantage of the central section theorem [equation (2.5.6.8) of Section 2.5.6]: the Fourier transform of a projection is extracted as a Fourier plane uv of a rotated Fourier transform of a 3D object:

$$D_n(\mathbf{u}) = \mathrm{CTF}(\mathbf{u}; \Delta f_n, q) E_n(\mathbf{u}) \left\{ [F(\mathbf{T}\mathbf{v})]\big|_{u_z=0} + M_n^{\mathrm{S}}(\mathbf{u}) \right\} + M_n^{\mathrm{B}}(\mathbf{u}). \tag{2.5.7.3}$$

The capital letters denote Fourier transforms of objects appearing in (2.5.7.1) while CTF (a Fourier transform of s) depends, among other parameters that are set very accurately (such as the accelerating voltage of the microscope), on the defocus setting Δf_n and the amplitude contrast ratio $0 \leq q < 1$ that reflects the presence of the amplitude contrast that is due to the removal of widely scattered electrons [the real term in (2.5.5.14)]. For the range of frequency considered, q is assumed to be constant and the CTF is written in terms of the phase perturbation function χ [given by equation (2.5.2.33)] as

$$\begin{aligned} \mathrm{CTF}(\mathbf{u}; \Delta f) &= [1 + 2q(q-1)]^{-1/2} \big\{ (1-q) \sin[\chi(|\mathbf{u}|; \Delta f)] \\ &\quad - q \cos[\chi(|\mathbf{u}|; \Delta f)] \big\} \\ &= \sin\big\{ \chi(|\mathbf{u}|; \Delta f) - \arctan[q/(1-q)] \big\}, \end{aligned} \tag{2.5.7.4}$$

where for simplicity we assumed no astigmatism. Finally, the rotationally averaged power spectrum of the observed image, calculated as the expectation value of its squared Fourier intensities (2.5.7.3), is given by

$$P_d(u) = \mathrm{CTF}^2(u)\, E^2(u) \big[P_f(u) + P_{\mathrm{S}}(u) \big] + P_{\mathrm{B}}(u), \tag{2.5.7.5}$$

where $u = |\mathbf{u}|$ is the modulus of spatial frequency.

2.5.7.2. Structure determination in single-particle reconstruction

The goal of single-particle reconstruction is to determine the 3D electron-density map f of a biological macromolecule such that its projections agree in a least-squares sense with a large number of collected 2D electron-microscopy projection images, $d_n \in R^{n^2} (n = 1, 2, \ldots, N)$, of isolated (single) particles with random and unknown orientations. Thus, we seek a least-squares solution to the problem stated by (2.5.7.1) [or, equivalently, in Fourier space, to (2.5.7.3)]. This is formally written as a nonlinear optimization problem (Yang *et al.*, 2005),

$$\begin{aligned} &\min_{\psi_n, \theta_n, \varphi_n, t_{x_n}, t_{y_n}, f, \Delta f_n, q, \ldots} L(\psi_n, \theta_n, \varphi_n, t_{x_n}, t_{y_n}, f, \Delta f_n, q, \ldots) \\ &\quad \equiv \tfrac{1}{2} \sum_{n=1}^{N} \left\| s_n(\mathbf{x}) * e_n(\mathbf{x}) * \int f(\mathbf{T}_n\mathbf{r})\,\mathrm{d}z - d_n(\mathbf{x}) \right\|^2. \end{aligned} \tag{2.5.7.6}$$

The factor of $\frac{1}{2}$ is included merely for convenience. The objective function in (2.5.7.6) is clearly nonlinear due to the coupling