A possible improvement over the 3D projection-matching procedure can be achieved by working in transformed spaces in which the distinction between orientation search and 3D reconstruction is removed: (1) spherical harmonics (Provencher & Vogel, 1988; Vogel & Provencher, 1988), which have found applications exclusively in the determination of icosahedral structures (Yin *et al.*, 2001, 2003); (2) Radon transform (Radermacher, 1994), with selected applications in the determination of asymmetric particles (Ruiz *et al.*, 2003); or (3) Fourier transform, implemented in the *FREALIGN* package (Grigorieff, 2007). In *FREALIGN*, the transformation between the arbitrarily oriented Fourier 2D central section and the 3D Fourier Euclidean grid is implemented using trilinear interpolation that includes *ad hoc* correction for the CTF effects. In high-resolution structure-refinement mode, the program uses a gradient-based Powell optimization algorithm (Powell, 1973), thus overcoming the main deficiency of 3D projection-matching algorithms.

A unified approach to direct minimization of (2.5.7.6) was proposed by Yang *et al.* (2005) and is implemented in the *SPARX* package as the YNP method (Hohn *et al.*, 2007). The premise of the YNP method is that the orientation parameters are approximately known (thus the initial 3D map) and both the orientation parameters and the density map are updated simultaneously in a gradient-based optimization scheme. In the YNP method, the derivatives with respect to the density distribution are calculated analytically and the derivatives with respect to orientation parameters are calculated using finite difference approximations. The YNP method is very efficient and its major advantage is that it avoids many problems associated with approximate solutions inherent in methods that work in transform spaces. The projection/backprojection operations are carried out rapidly using linear interpolation, which due to sufficient oversampling of the data does not have a significant adverse impact on the solution. Moreover, because the density map f is updated simultaneously with the orientation parameters, the computationally demanding separate step of 3D reconstruction is eliminated.

2.5.7.10. Resolution estimation and analysis of errors in single-particle reconstruction

The development of resolution measures in EM was greatly influenced by earlier work in X-ray crystallography. In EM, the problem is somewhat more difficult as, unlike in crystallography, both the amplitude and the phase information in the data are affected by alignment procedures (which we consider distant analogues of phase-extension methods in crystallography). Therefore, resolution measures in EM reflect the self-consistency of the results; however, as the data are subject to alignment, there is a significant risk of introducing artifacts resulting from the alignment of the noise component in the data. Ultimately, these artifacts will unduly 'improve' the resolution of the map.

The resolution measures used in EM fall into two categories: measures based on averaging of Fourier transforms of individual images and measures based on comparisons of averages calculated for subsets of the data. In the first group, we have the Q-factor (van Heel & Hollenberg, 1980; Kessel *et al.*, 1985) and the spectral signal-to-noise ratio (SSNR) introduced for the 2D case by Unser and co-workers (Unser *et al.*, 1987), and for the 3D case for a class of reconstruction algorithms data are based on direct Fourier inversion by Penczek (Penczek, 2002). The second group of measures includes the differential phase residual (DPR) (Frank *et al.*, 1981) and the Fourier ring correlation (FRC) (Saxton & Baumeister, 1982). A marked advantage of these measures is that they are equally well applicable to 2D or 3D data. In the latter case, the volumes resulting from 3D reconstruction algorithms take the place of the 2D averages.

The resolution measures used in single-particle reconstruction are designed to evaluate the SSNR in the reconstruction as a function of spatial frequency (Penczek, 2002). The 'resolution' of the reconstruction is reported as a spatial frequency limit beyond which the SSNR drops below a selected level, for example below one.

The FSC is evaluated by taking advantage of the large number of single-particle images: the total data set is randomly split into halves; for each subset a 3D reconstruction is calculated (in two dimensions, a simple average); and two maps f and g are compared in Fourier space,

$$\mathrm{FSC}(f, g; u) = \frac{\sum_{|\|\mathbf{u}_n\|-r|\le\varepsilon}^{n_r} F(\mathbf{u}_n)G^*(\mathbf{u}_n)}{\left\{\left[\sum_{|\|\mathbf{u}_n\|-r|\le\varepsilon}^{n_r} |F(\mathbf{u}_n)|^2\right]\left[\sum_{|\|\mathbf{u}_n\|-r|\le\varepsilon}^{n_r} |G(\mathbf{u}_n)|^2\right]\right\}^{1/2}}. \quad (2.5.7.19)$$

In (2.5.7.19), 2ε is a preselected ring/shell thickness, the $\mathbf{u}_n$ form a uniform grid in Fourier space, $u = \|\mathbf{u}_n\|$ is the magnitude of the spatial frequency and n_r is the number of Fourier voxels in the shell corresponding to frequency u. The FSC yields a 1D curve of correlation coefficients as a function of u. Note that the FSC is insensitive to linear transformations of the densities of the objects. An FSC curve everywhere close to one reflects strong similarity between f and g; an FSC curve with values close to zero indicates the lack of similarity between f and g. Particularly convenient for the interpretation of the results in terms of 'resolution' is the relation between the FSC and the SSNR, which is easily derived by taking the expectation of (2.5.7.19) under the assumption that both f and g are sums of the same signal and different realizations of the noise, which are uncorrelated with the signal and between them (Saxton, 1978):

$$E[\mathrm{FSC}] \cong \frac{\mathrm{SSNR}}{\mathrm{SSNR}+1}. \quad (2.5.7.20)$$

By solving (2.5.7.20) for SSNR we obtain

$$\mathrm{SSNR} = \frac{\mathrm{FSC}}{1-\mathrm{FSC}}, \quad (2.5.7.21)$$

which, taking into account that the FSC was calculated from the data set split into halves, has to be modified to (Unser *et al.*, 1987)

$$\mathrm{SSNR} = 2\left(\frac{\mathrm{FSC}}{1-\mathrm{FSC}}\right). \quad (2.5.7.22)$$

In order to calculate the FSC that corresponds to a given SSNR, one inverts (2.5.7.22) to

$$\mathrm{FSC} = \frac{\mathrm{SSNR}}{\mathrm{SSNR}+2}. \quad (2.5.7.23)$$

Equations (2.5.7.21) and (2.5.7.22) serve as a basis for various 'resolution criteria' used in EM. The often-used 3σ criterion (van Heel, 1987*b*) equates resolution with the point at which the FSC is larger than zero at a 3σ level, where σ is the expected standard deviation of the FSC that has an expected value of zero, in essence finding a frequency for which the SSNR is significantly larger than zero. The 3σ criterion has a distinct disadvantage of reporting the resolution at a frequency at which there is no significant signal, while tempting the user to interpret the detail in the map at this resolution. Moreover, as the FSC approaches zero, its relative error increases, so the curve oscillates widely

around the zero level increasing the chance of selecting an incorrect resolution point. In other criteria one tries to equate the resolution with the frequency at which noise begins to dominate the signal. A good choice of the cut-off level is SSNR = 1.0, a level at which the power of the signal in the reconstruction is equal to the power of the noise. According to (2.5.7.22), this corresponds to FSC = 0.333. Another often-used cut-off level is FSC = 0.5, at which the SSNR in the reconstruction is 2.0 (Böttcher *et al.*, 1997; Conway *et al.*, 1997; Penczek, 1998).

The main reason behind the determination of the resolution of the EM maps is the necessary step of low-pass filtration of the results before the interpretation of the map is attempted. In order to avoid mistakes, particularly the danger of overinterpretation, one has to remove from the map unreliable Fourier coefficients. Inclusion of Fourier coefficients with a low SNR will result in the creation of spurious details and artifacts in the map. Thus, the optimal filtration should be based on the SSNR distribution in the map and the solution is given by a Wiener filter:

$$W(u) = \frac{\mathrm{SSNR}(u)}{\mathrm{SSNR}(u) + 1}. \qquad (2.5.7.24)$$

Based on the relation of FSC to SSNR (2.5.7.22), we can write (2.5.7.24) as

$$W(u) = \frac{2\mathrm{FSC}(u)}{\mathrm{FSC}(u) + 1}. \qquad (2.5.7.25)$$

In practice, because of the irregular shape of typical FSC curves (particularly for small values of FSC) it is preferable to approximate the shape of the Wiener filter (2.5.7.25) by one of the standard low-pass filters, such as Butterworth (Gonzalez & Woods, 2002) or hyperbolic tangent (Basokur, 1998).

The FRC/FSC methodology can be used to compare a noise-corrupted map with a noise-free ideal version of the same object. In single-particle reconstruction this situation emerges when an X-ray crystallographic structure of either the entire EM-determined structure or of some of its domains is available (Penczek *et al.*, 1999). In this case, we assume that in (2.5.7.19) f represents a sum of the signal and additive uncorrelated noise and g represents the noise-free signal, so is straightforward to calculate the expectation of (2.5.7.19) in order to obtain the relation between the cross-resolution (CRC) and the SSNR:

$$E[\mathrm{CRC}] \cong \left(\frac{\mathrm{SSNR}}{\mathrm{SSNR} + 1}\right)^{1/2}. \qquad (2.5.7.26)$$

Thus

$$\mathrm{SSNR} = \frac{\mathrm{CRC}^2}{1 - \mathrm{CRC}^2}. \qquad (2.5.7.27)$$

Interestingly, for the same SSNR cut-off levels, corresponding values of CRC are higher than those for FSC. For example, for SSNR = 1, CRC = 0.71, while FSC = 0.33. For SSNR = 2, CRC = 0.82, while FSC = 0.5.

2.5.7.11. Analysis of 3D cryo-EM maps

The amount of structural information that can be derived from a structure of a macromolecular complex determined by cryo-EM depends on two factors: the resolution of the map and the availability of additional structural information about the system. Generally, we will refer to complexes at a resolution better than 7 Å as high-resolution structures, as at this resolution the elements of secondary structure become directly visible. Maps at resolution lower than that we will call intermediate resolution, as at this scale of detail one can only determine a general arrangement of subunits. However, it is good to realize that there is a huge difference between the amount and reliability of information derived from a map of the same complex determined at 10 Å as compared to a map determined at 30 Å resolution. Similarly, very large complexes determined at 50 Å resolution will yield more information than very small complexes determined at 15 Å resolution. On the other hand, even intermediate-resolution EM maps provide extremely valuable information if they can be placed in the context of other structural work. The single-particle structure can be also investigated within a context of a more complex system using other, lower-resolution techniques, for example electron tomography. In this case, by using docking approaches one can determine the distribution, orientation and general arrangement of smaller cryo-EM determined complexes within larger subcellular systems. On a different scale of resolution, it is quite common to have structures of some domains or even of the entire complexes determined to atomic resolution by X-ray crystallography. Again, by using docking techniques it is possible to determine whether the conformation of the EM structure differs from that determined by X-ray crystallography or to map subunits and domains of the larger complex by fitting available atomic resolution structures.

The basic mode of visualization of cryo-EM maps is surface representation. The first step involves the choice of an appropriate threshold level for the displayed surface, particularly when the scaling of the cryo-EM data is arbitrary. A good guide is provided by the total molecular mass of the complex: given a pixel size of p Å, an average protein density $d = 1.36 \times 10^{-24}$ g Å^{-3} and the total molecular mass of the complex M Da, the number of voxels N_v occupied by the complex is

$$N_\mathrm{v} = M/\left(p^3 d N_\mathrm{A}\right), \qquad (2.5.7.28)$$

where N_A is the Avogadro's number (6.02×10^{23} atoms mole^{-1}). Based on that, one can find the threshold that for a given structure encompasses the determined number of voxels N_v [appropriate functions are implemented in *SPIDER* (Agrawal *et al.*, 1996; Frank *et al.*, 1996) and *SPARX* (Hohn *et al.*, 2007)]. At a sufficiently high resolution, cryo-EM maps can be analysed in the same manner as X-ray crystallographic maps and using the same graphical/analytical packages ('backbone tracing') (Jones *et al.*, 1991) (Fig. 2.5.7.6).

The complexity of cryo-EM maps of large macromolecular assemblies combined with their limited resolution invites attempts to automate some of the steps of analysis in an attempt to make the results more robust and less dependent on the researcher's bias. A good example of semi-automated analysis is the nucleic acid–protein separation in a 11.5 Å cryo-EM map of the 70S *E. coli* ribosome (Spahn *et al.*, 2000). In the procedure, the (continuous-valued) densities were analysed making use of (i) the difference in scattering density between protein and nucleic acids; (ii) continuity constraints that the image of any nucleic acid molecule must obey and (iii) knowledge of the molecular volumes of all proteins. As a result, it was possible to reproduce boundary assignments between ribosomal RNA (rRNA) and proteins made from higher-resolution X-ray maps of the ribosomal subunits with a high degree of accuracy, and allowed plausible predictions to be made for the placements of proteins and RNA components as yet unassigned. One of the conclusions derived from this separation was that the 23S rRNA is solely responsible for the catalysis of peptide-bond formation; thus, the ribosome is a ribozyme. The same conclusion was reached independently in the studies of the X-ray crystallographic structure of the 70S ribosome (Nissen *et al.*, 2000). The method by Spahn *et al.* cannot be easily extended to other