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One number does not fit all: mapping local variations in resolution in cryo-EM reconstructions

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Abstract

The resolution of density maps from single particle analysis is usually measured in terms of the highest spatial frequency to which consistent information has been obtained. This calculation represents an average over the entire reconstructed volume. In practice, however, substantial local variations in resolution may occur, either from intrinsic properties of the specimen or for technical reasons such as a non-isotropic distribution of viewing orientations. To address this issue, we propose the use of a space-frequency representation, the short-space Fourier transform, to assess the quality of a density map, voxel-by-voxel, i.e. by local resolution mapping. In this approach, the experimental volume is divided into small subvolumes and the resolution determined for each of them. It is illustrated in applications both to model data and to experimental density maps. Regions with lower-than-average resolution may be mobile components or ones with incomplete occupancy or result from multiple conformational states. To improve the interpretability of reconstructions, we propose an adaptive filtering approach that reconciles the resolution to which individual features are calculated with the results of the local resolution map.

Keywords

resolution; cryo-electron microscopy; three-dimensional reconstruction; image heterogeneity; masking; short-space Fourier transform

1. Introduction

In single particle analysis (SPA) reconstructions of cryo-electron micrographs, the final density map represents the end product of a complex sequence of processing steps, which include filtering, alignment and orientation determination, deconvolution to correct for the contrast transfer effects, and reconstruction. Its quality reflects the accuracy of the algorithms employed for these steps, as well as the quality and number of input particles. To interpret the map, it is important to know the resolution to which reliable structural details extend. This is commonly done by using Fourier space techniques applied to the transform of the reconstruction. Several methods for assessing resolution have been proposed (Liao and Frank, 2010). Chronologically, they include Differential Phase Residual (Frank et al., 1981), Q-factor (Kessel et al., 1985), Fourier Shell Phase Residual (van Heel, 1987), Fourier

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Shell Correlation (FSC) (Harauz and van Heel, 1986), Spectral Signal-to-Noise Ratio 3D (SSNR3D) (Penczek, 2002; Unser et al., 2005), and R measure (Sousa and Grigorieff, 2007).

In SPA it is assumed that the sample is homogeneous, so that all the images can be treated as projections of the same particle. However, in practice, some complexes are quite heterogeneous: for example, they may have components that are intrinsically flexible (Leschziner and Nogales, 2007) (Ishikawa et al., 2004) or present at less than full occupancy (Belnap et al., 2003). In other cases, the complexes may exist in different conformations in solution. Specimen heterogeneity translates into reconstructions in which distinct regions show different levels of detail, and they need to be interpreted accordingly. Some approaches to this issue have been already made. In studies of spherical viruses, resolution has been calculated as a function of radius (Harris et al., 2006; Huiskonen et al., 2004). More generally, substructures of interest can be selected from the map by masking out everything else, thus restricting the resolution estimation to the remaining sub-volume (Gao et al., 2004; Marles-Wright et al., 2008; Menetret et al., 2007). Here, we study the extent to which this approach can be localized. Based on the results of this analysis, we propose an adaptation of the approach for the integral analysis of resolution in a voxel-wise manner. Within the framework of our localization formalism, resolution is specified in terms of the familiar Fourier Shell Correlation coefficient (Harauz and van Heel, 1986), but it can be readily extended to other criteria. The approach is illustrated in terms of some applications to both synthetic and experimental data. We also present the implementation of two tools for performing the analysis. Finally, we propose an adaptive filtering approach, which is based on the results of the localized resolution analysis, to improve interpretability of maps that are affected by marked inhomogeneities in resolution.

2. Theory

All resolution measures commonly used in SPA are based on data represented in the Fourier domain. In many cases, two reconstructions are obtained from half data sets and their Fourier transforms are compared. The FSC yields a curve that conveys correlation as a function of spatial frequency (Harauz and van Heel, 1986). The point at which this curve crosses a given threshold defines the resolution. By definition, the Fourier transform gives a global representation of the spectral features of the reconstruction. In order to assess sub-regions in the same way, the spectral information must be localized. The simplest approach would be to divide the experimental volume into small cubes and to calculate the Fourier transform separately for each cube. If the cubes were small enough, the resolution estimate would be representative of the quality of the voxel at the center of the cube. However, this approach creates discontinuities at the cube edges that generate high frequency components, an effect that is sometimes called “ringing”, or “spectral leakage” (Harris, 1978). As these high frequency components are similarly present in both cubes compared, their correlation would exaggerate the apparent resolution as they relate to the boxing more than to the densities within the box. A way to overcome this problem is to analyze the map in a localized manner, while preserving its spectral properties.

2.1. Short-space Fourier Transform

A space-frequency representation of a signal provides a simultaneous representation of it in real space and in the Fourier domain. It is related to the better-known time-frequency representation (Oppenheim and Schaffer, 1989), which has long been used in signal processing for analyzing one-dimensional signals containing time-varying spectral information. In this context, time-frequency analysis is a generalization of Fourier analysis in which frequencies are assumed to be constant, or slowly changing, over time. The major distinction between space-frequency and time-frequency analyses is that space-frequency

usually operates on three-dimensional data, while time-frequency operates on one-dimensional signals. A space-frequency representation is commonly obtained by means of the short-space Fourier transform (SSFT) (Hinman et al., 1984). Basically, the SSFT localizes the signal by modulating it with a window function, before performing the Fourier transform. For convenience, the formulation given below is for one-dimensional signals. Its extension to three-dimensional data is straightforward and is discussed later.

Given a signal sampled in real space $f_j, j=1 \dots N$, where N is the total number of samples, its short-space Fourier Transform component at location m and frequency k , $F_{m,k}$, is given by

$$F_{m,k} = \sum_{j=1}^N f_j w_{m-j} \Pi_{m-j}^{\Delta} e^{-2\pi i \frac{kj}{N}}, \quad (2.1)$$

where Π_i^{Δ} is the rectangular function with width Δ , and w is a window function. The rectangular function is defined as

$$\Pi_i^{\Delta} = \begin{cases} 1, & |i| \leq \Delta/2 \\ 0, & \text{otherwise} \end{cases}, \quad (2.2)$$

representing a region, or kernel, around each point where spectral information is acquired. w is a symmetric function that tapers the signal at both ends of the selected region.

When using the SSFT, two choices must be made: the window function w , and the width of the rectangular function Δ , or kernel size. Various window functions have been proposed (Harris, 1978), and each provides a different trade-off between reduction of spectral leakage and separation of adjacent frequency components (resolvability). In the present context, we chose a raised cosine function, specifically the Hann window (Harris, 1978), defined as

$$w_n = 0.5 \left(1 + \cos \left(\frac{2\pi n}{\Delta} \right) \right). \quad (2.3)$$

Notice that, when n is in the range $[-\Delta/2, \Delta/2]$, the rectangular function in Eq. (2.1) becomes redundant. An immediate advantage of this window function is that it smoothly reduces the signal to zero at the edges, without requiring the setting of any additional parameter other than the width. Its spectral properties are also well characterized (Oppenheim and Schaffer, 1989), and the width of the main lobe is twice that of the rectangular function. In the following, we define this bandwidth as $\Delta = 2$. In the present context, its representation in the Fourier domain is equivalent to halving the size of the rectangular window.

Choice of the width of the function also involves a trade-off: a wide window gives better sampling of frequency components (frequency resolution) but less localized information (spatial resolution), while the opposite is true with a narrow window. Our practice is to calibrate the width according to the statistics of the resolution criterion adopted, as shown below.

Extension of the above formulation to the three-dimensional case is readily obtained by modulating the data with three one-dimensional windowing functions.

2.2. Fourier Shell Correlation statistics

The Fourier Shell Correlation, originally derived (Harauz and van Heel, 1986) as a three-dimensional extension of an earlier two-dimensional formulation (Saxton and Baumeister, 1982), requires partitioning the data so as to generate two nominally independent maps. The FSC between the two map transforms, F and G , is given by

$$FSC(k) = \frac{\sum_{m,n,p \in R(k)} \text{Re} \{ F_{m,n,p} G_{m,n,p}^* \}}{\sqrt{\left(\sum_{m,n,p \in R(k)} |F_{m,n,p}|^2 \right) \left(\sum_{m,n,p \in R(k)} |G_{m,n,p}|^2 \right)}}, \quad (2.4)$$

as a function of the radial frequency coordinate k . The Fourier components are indexed with (m,n,p) ; the asterisk denotes the complex conjugate; and $\text{Re}\{\}$ indicates that only the real part is retained. Finally, $R(k)$ refers to a shell in Fourier space of mean radius k , and its thickness is usually given by the reciprocal of the side-length of the volume under analysis. This measure has a complex dependence on the power of the signal present in the data, and it is related to the spectral signal-to-noise ratio of the reconstruction (Frank and Al-Ali, 1975; Penczek, 2002; Unser et al., 1987). The resolution is given by a single number, the reciprocal of the radial frequency at the point at which the FSC falls to a given threshold, or cutoff.

Eq. (2.4) provides an estimate of the correlation coefficient for each radial frequency coordinate, and its variance is given by (Saxton, 1978) (Appendix A)

$$\text{var}[FSC(k)] = \frac{1}{N_k} \frac{(1 + 6FSC(k) + FSC^2(k))(1 - FSC(k))^2}{(1 + FSC(k))^4}, \quad (2.5)$$

where N_k is the number of frequency components in the shell $R(k)$. The formula shows that the variance of the correlation decreases with increasing values of both N_k and $FSC(k)$. This means that the uncertainty associated with the resolution estimate depends on the choice of the threshold value and the size of the experimental volume.

2.3. Localized Fourier Shell Correlation

Whereas the FSC gives a single number as a global measure of resolution, this information can be localized in a space-frequency representation-based approach. Given two maps calculated from half data sets, a cube can be extracted from each map at the same location. Using the two cubes, the FSC is then calculated according to Eq. (2.4), using the frequency components obtained by SSFT as from Eq. (2.1), with the Hann window as tapering function. The approach is shown schematically in Figure 1. The resolution estimate is obtained from the resulting FSC curve. To better detect the point at which the threshold is crossed, the cubes can be zero-padded before calculating the Fourier transforms. Finally, the resolution estimate is assigned to the voxel located at the center of the cube. After repeating this procedure for each voxel, all the estimates are assembled into a “resolution map”. With this approach, only two parameters need to be defined: the threshold for the FSC curve, and the kernel size, i.e. the side-length of the sampling cube. Both parameters affect the resolution assessment, as described in Section 2.2.

For the purpose of localizing resolution assessment, the kernel size is the most critical parameter. If it is chosen too small, the number of frequency components derived from the

extracted cubes is insufficient to give a reliable estimate; if it is chosen too large, we diminish the concept of locality. This implies a trade-off between reliability and locality, in a statistical sense. In order to relate the kernel size to the precision of the resolution measure, we express the FSC variance in Eq. (2.5) as a function of a new parameter, called the kernel-to-resolution ratio, or krr . The krr is defined as the ratio between the side-length of the cube containing the selected volume and the resolution as measured. In effect, it specifies how large the box size is, compared to the resolution to be estimated, and it is proportional to the number N_k of Fourier terms in the shell $R(k)$ corresponding to the measured resolution (Appendix B). The relative error of the FSC, defined as $\sqrt{\text{var}[FSC]}/FSC$, is then

$$\text{Err}[FSC_t, krr] = \frac{1}{krr \sqrt{2\pi\beta}} \frac{(1-FSC_t)}{FSC_t(1+FSC_t)^2} \sqrt{1+6FSC_t+FSC_t^2}, \quad (2.6)$$

where FSC_t is the threshold value. In Figure 2 we plotted Eq. (2.6) as a function of krr , for three different values of FSC_t , viz. 0.5, 0.3 and 0.143, assuming $\beta=2$ (Hann window). The relative error decreases with increasing FSC_t and krr , as expected. In order to achieve a relative error of less than 5% for the correlation estimate, the krr needs to be greater than 5, 13 and 35, for thresholds of 0.5, 0.3 and 0.143, respectively. Remarkably but not surprisingly, this implies that assessing the resolution at lower FSC threshold values, while keeping the same accuracy, requires larger amounts of data, i.e. larger volumes. This is in agreement with the FSC being directly related to the signal-to-noise ratio (SNR) of the reconstruction, since lower values of FSC correspond to higher levels of noise. These curves also apply to the global FSC measure: in this case the krr is very high, because the size of the kernel is equal to the size of the map. Nevertheless, the curves also make the point that estimating the resolution of reconstructions of small complexes is intrinsically less accurate than for larger ones. It is noteworthy that the error curve relates to the FSC, and only indirectly to the resolution. Thus a 5% error in estimating the FSC does not necessarily correspond to a 5% error in the estimate of resolution, which instead is proportional to the slope of the FSC curve (i.e. higher the slope, the lower the resolution error). Furthermore, a smaller error in the variance of the FSC will provide closer estimates between cubes extracted around voxels with the same nominal resolution. The best choice for the cutoff in the FSC curve is therefore 0.5, localizing the resolution estimate with a relatively small kernel.

2.4. Influence of background noise on resolution estimates

All methods currently used to assess resolution relate, less or more directly, to the signal-to-noise ratio of the map (Frank and Al-Ali, 1975; Penczek, 2002; Unser et al., 1987). However, in a reconstructed volume some voxels contain density information about the complex of interest, albeit contaminated by noise, while the rest just contain background noise. Therefore, a resolution measure is influenced by the ratio between these two different populations of voxels, and it inevitably underestimates the resolution of the reconstruction, in an unpredictable way (LeBarron et al., 2008). This behavior is exacerbated when estimating the resolution in a localized manner as presented here. For each voxel analyzed, the surrounding kernel encloses a variable amount of background-only voxels, which necessarily biases the resolution estimate. In practice, this suggests that different values of resolution may be assigned to different parts of a structure that are actually reconstructed with the same quality. To address this issue, we minimize the contributions from background noise by assessing the local resolution only after applying a soft-mask around the structure.

2.5. Localized filtering

The information provided by a localized resolution analysis helps to determine the level of reliable detail in the various components of a reconstructed macromolecular complex. If the quality of the reconstruction is homogeneous, it is sufficient to apply a low-pass filter to the nominal resolution estimated, in order to obtain a map ready for interpretation. In the event that regions are visualized at different resolutions, additional processing steps are required. A conservative approach is to band-limit the final map to the lowest resolution of any component. However, this discards the more detailed information associated with the better resolved regions. A better approach is to mask out all the different subregions, filter them to their estimated resolution, and then recombine them in a single density map.

The localized resolution affords an alternative approach, which does not require separating the reconstruction into multiple subvolumes to be processed separately. In fact, it is possible to follow a similar procedure to the localized resolution analysis, but tailored to filtering. Given the final reconstruction, each voxel can be rendered at the resolution given by the local resolution analysis, by applying the filter to a cube extracted around that voxel, and replacing its value with the one centered on the filtered cube. In this way it is possible to obtain an adaptively filtered reconstruction. Of note, this operation does not require the choice of any additional parameter, since the size of the box (i.e. kernel) can be conveniently set to the same value used for the resolution estimate.

3. Material and methods

3.1. Implementation

The proposed approach to calculating localized resolution has been implemented in C language, and the program is available in Bsoft (Heymann and Belnap, 2007) with the name of *blocres*. It accepts as input two maps calculated from half data-sets. The maps should not be filtered in frequency, or at most low-pass filtered at very high resolution, and background noise should have been suppressed by applying a soft mask to the structure of interest. The user-defined parameters are the cutoff for the FSC curve and the kernel size. The output is a volume that gives the estimated resolution for each voxel. The computational cost depends on the size of the maps and the kernel width, but it can be reduced by restricting the analysis to only those voxels with non-zero densities. A mask specifying these voxels can be provided to the program as an option. The output volume can be used to color the surface rendering of the final reconstruction (resolution-colored reconstruction), to provide a visual representation of its heterogeneity. For this purpose we used the tool *Surface Color* in Chimera (Pettersen et al., 2004). The output volume of *blocres*, i.e. the resolution volume, can be used to filter the final reconstruction in an adaptive manner using *blocfilt*, also available in Bsoft. Also in this case the kernel size is given as input parameter, and is used to determine the size of the bounding box around each voxel. The subvolume is then transformed in the Fourier domain, low-pass filtered to the resolution read in the resolution volume for that voxel, and transformed back in the real domain. All the values are discarded but the central voxel, which is assigned to the output volume. The final result is a reconstruction that is adaptively filtered according to the localized resolution estimate.

Furthermore, we developed a plug-in for the visualization program Chimera (Pettersen et al., 2004), *LocalFSC*, which allows determining on-the-fly the local resolution around selected voxels. The user can select regions of a map by placing markers at their center with the tool *Volume Tracer*, and then estimate their resolutions. The kernel size can be set manually, otherwise it is adaptively chosen by the program in order for the kernel-to-resolution ratio to be close to 7. An FSC plot is shown when the resolution is estimated around a single marker.

3.2. Synthetic dataset

A synthetic dataset was used to test the approach in a defined framework. Initial density maps were simulated from the crystal structure of the elongation complex of the *Thermus thermophilus* 70S ribosome with three tRNAs and mRNA (Jenner et al., 2010). To generate the 70S ribosome, the atomic coordinates of the 50S (PDB: 3I8F) and 30S (PDB: 3I8G) subunits were combined and converted to density using the EMAN program *pdb2mrc* (Ludtke et al., 1999). The map had a size of $200 \times 200 \times 200$ voxels, sampled at $3 \text{ \AA}/\text{voxel}$, and it was band-limited to $(7 \text{ \AA})^{-1}$. A map representing only the 50S subunit was generated using the same approach and conditions. Sets of projection images were obtained from these maps by simulating the same imaging conditions: specifically, identical orientations, noise levels, and contrast transfer function (CTF) values were applied. Each data set had 15000 projection images with randomly distributed orientations. Noise was added at two steps (Baxter et al., 2009). After projection, zero-mean random Gaussian noise was added to the images in order to simulate structural noise with a SNR of 1. Then the phases were flipped according to the CTF of a microscope working at 300kV ($C_s=2.0 \text{ mm}$), each time randomly picking a defocus from the values of 1.5, 2 and $2.5 \text{ \mu m}$. Finally, zero-mean random Gaussian noise with SNR of 0.1 was added to the images to simulate the effects of shot noise and digitization noise. Thus the final SNR was ~ 0.05 .

In one experiment, only the 70S data were used. In the second experiment, two data sets respectively from the 70S and the 50S maps were combined to simulate variable occupancy of the 30S subunit. Specifically, 6000/9000 and 12000/3000 images were selected from the 70S/50S sets to simulate datasets with the 30S subunit present in 40% and 80% of the particles, respectively.

In all experiments two half-set maps were reconstructed, and resolution analysis was performed using a kernel size of $105 \text{ \AA}$ (~ 35 pixels). Masks used to exclude background contributions were generated in two steps. First the reconstruction was visualized in Chimera, the contour level for surface rendering being chosen to enclose 100% of the expected mass, and the *mask* command used to generate a new map containing only the densities enclosed within this surface. Then the new map was transformed into a binary mask by converting all the positive density values to one and the rest to zero. Finally the binary mask was dilated two times and its edges softened by convolution with a $3 \times 3 \times 3$ averaging kernel. The last two operations were performed with *bmask* and *bfilter* in Bsoft (Heymann and Belnap, 2007).

3.3. Experimental datasets

The localized resolution approach was tested on three experimental data sets: icosahedral ($T=1$) particles of the capsid (CA) protein of Rous Sarcoma Virus (RSV) (Cardone et al., 2009); the mature DNA-filled capsid (C-capsid) of herpes simplex virus type 1 (HSV-1) (Trus et al., 2007); and the clathrin D6 basket (Heymann et al., 2005). The resolution of each map was estimated after applying a spherical mask with soft edges (see above) to both half-set reconstructions (LeBarron et al., 2008). We refer later to this value as global resolution. All parameters used in these analyses are listed in Table 1. Furthermore, the zero-padding factor was set equal to 2, and the kernel-to-resolution ratio equal to 7, unless specified otherwise. All the maps were analyzed by localized FSC, using a threshold of 0.5 (localized $FSC_{0.5}$).

4. Results

4.1. Influence of background noise

To assess the effect of background regions, i.e. regions void of density, on the estimate of localized resolution, we performed the following experiment. A synthetic dataset of 15000 projection images with random uniformly distributed orientations was generated, starting from the crystal structure of a 70S ribosome. The known orientations were used in calculating the density map, i.e. no alignment error was simulated. Under these conditions, the reconstruction is expected to represent all the parts of the ribosome with uniform quality. However, as shown in Figure 3a, when the surrounding background is not masked out, the analysis assigns resolution values ranging from 9.5 to 17 Å to different parts of the structure. While the resolution at the ribosome core is fairly uniform at about 10 Å, at its outer surface the resolution values are highly variable. In this zone, the cubic subvolumes contain significant and variable fractions of voxels that contain only noise. This effect is particularly evident for the protruding protein L9 of the 50S subunit. This ‘edge effect’ is strongly attenuated if the analysis is performed after the background regions are masked out (Figure 3b). In this case the resolution in the core region is almost uniform, as expected, and in most of the outer surface, albeit of somewhat lower resolution than the core, it is much more consistent (to within 10%). The global resolution estimated without excluding the background is 17.2 Å, while it is 10.3 Å when the background is masked out.

4.2. Sensitivity to occupancy

One source of heterogeneity in a reconstruction of a macromolecular complex can be the presence of a component in only a subset of particles. In this situation the component will be represented at a lower resolution than the rest of the map, because it is computed by averaging fewer particles. We simulated this situation by generating datasets of ribosome images that have differing occupancies for the 30S subunit and performing local resolution analyses on them. The results with occupancies of 40% and 80% are shown in Figures 3c,d and Figures 3e,f. In both cases the analysis detects the two subunits as distinct in quality, assigning them different values for the average resolution. When the 30S occupancy is low, its density is weak and noisy. This effect comes mostly from filtering the map to 10 Å which is too high for the 30S: while this value is appropriate for the 50S subunit. On the other hand, filtering the reconstruction to 20 Å, as the local resolution analysis would suggest for the 30S subunit, would represent 50S at a much lower resolution than is warranted. However, for 80% occupancy, the local analysis assigns average resolution values of 12 Å to the 30S subunit and 10 Å to the 50S subunit. The maps, colored according to local resolution, show that around the interface regions between two subunits, the analysis assigns intermediate resolution values. This phenomenon is similar to the ‘edge effect’ discussed above, and also in this case it is caused by the finite size of the kernel used.

4.3. Effect of kernel size

The side-length of the box used to estimate localized resolution is expected to affect its accuracy. In order to analyze this dependency, we used experimental data on RSV CA capsids (Cardone et al., 2009), HSV-1 C capsids (Trus et al., 2007), and D6 clathrin baskets (Heymann et al., 2005) as test specimens (see next Section for details on the reconstructions). The icosahedrally symmetric map obtained for the RSV CA capsid is quite uniform in terms of quality. Therefore most of its voxels should have similar resolution, close to the global value estimated as 10.4 Å by FSC_{0.5} (Cardone et al., 2009). To test the effect of kernel size on localized resolution, we designed an *ad hoc* experiment. First we generated a binary mask that selected only voxels with positive density, setting the baseline at 1.8 (= standard deviation). In all three experiments, this mask fitted tightly around the density map. Then we calculated the average of the localized resolution (localized FSC_{0.5})

estimated for the selected voxels. In Figure 4a the average resolution is shown for different kernel sizes. The kernel-to-resolution ratio (krr) is given on a secondary x-axis, and it is obtained by dividing the kernel size by the global resolution. The plot shows that the resolution becomes progressively lower (higher number) with increasing kernel size and tends asymptotically to 10 Å, close to the global value. Therefore there is apparently a systematic bias of the resolution towards lower values. For very small kernels (linear box size ~ 19 Å, $krr \sim 2$), the average resolution is given as ~ 6 Å, which is not consistent with visual assessment of the map. Thus the resolution is evidently overestimated in the first part of the curve, where it changes rapidly. As the kernel size increases, the resolution changes more slowly and, by a krr of 4, comes within 10% of the asymptotic value. We interpret this behavior as follows: too small a kernel size results in an inaccurate estimate, whereas too large a value results in an over-regularized estimate that loses the concept of localization. This behavior is in agreement with the error analysis given above, based on the FSC variance. According to Eq. (2.6), a relative error of the variance lower than 5% is achieved with a krr higher than 5, which corresponds to a resolution of 9.4 Å ($FSC_{0.5}$). This improvement over the initially estimated value (10.4 Å) is consistent with the fact that all 11 α -helices in the CA subunit are well resolved in this density map (Cardone et al., 2009). In general, a choice of krr of 5 is pragmatic and one of 7 is conservative, seeming to guarantee a reliable estimate of the localized resolution for all the voxels of practical interest. Similar results were obtained when this analysis was repeated on the HSV-1 C capsid and the clathrin basket, yielding similar plots (Figures 4b, c). For the HSV-1 C capsid, the average resolution was obtained by excluding the genome, i.e. all densities inside a radius of 50 nm. It is noteworthy that in all three examples the transition point of the curve is around a krr value of 5.

4.4. Results on experimental data

HSV-1 capsids have T=16 icosahedral symmetry and are composed of hexamers and pentamers of the major capsid protein, UL19 (150 kDa), with six copies of the small protein UL35 (12 kDa) bound around the outer tip of each hexamer. At one of the twelve vertices, the dodecameric UL6 portal protein replaces a UL5 pentamer but this distinction is lost in the icosahedrally symmetric reconstruction. At the 320 sites of 3-fold symmetry “triplexes” (heterotrimers of UL18, 35 kDa, and UL38, 50 kDa) coordinate the adjacent capsomers. Attached to the triplexes immediately adjacent to the vertices, are heterodimers of UL25 (60 kDa) and UL17 (75 kDa), also called the C-capsid-specific component (CCSC) (Cardone et al., 2012). A cryo-EM reconstruction of the C-capsid revealed that the CCSCs exhibited only partial occupancy, around 50%, corresponding to an average complement of 30 per capsid (Trus et al., 2007). The global resolution of the reconstruction was estimated to be around 20 Å, after excluding the interior volume occupied by DNA and soft-masking around the outer surface. The map, colored according to local resolution in Figure 5a, shows that the resolution of the floor (the near-continuous innermost layer of density) and the cylindrical capsomer protrusions ranges between 17 and 20 Å, except at the outer tips of the pentamers (~ 21 Å). The CCSCs appear to be represented at slightly lower resolution, which agrees with their lower occupancy, as measured by calculating the ratio between density values at the CCSC and at other regions of the map, after background subtraction. Specifically, the resolution of the vertex-distal part of the CCSC is estimated around 19 Å, while in the vertex-proximal part it is lower than 21 Å.

Clathrin triskelions form a wide variety of polyhedral cages, an adaptability that allows them to coat vesicles of different sizes (Crowther and Pearse, 1981). One common polyhedral form of assembly is the 36-vertex “basket” (a cage without an enclosed vesicle) with D6 symmetry, featuring a lattice of hexagons and pentagons (Figure 5b). Each triskelion has a hub located at a polyhedral vertex, and three legs that bundle with leg segments from other

triskelions to form the vertex-linking spars. Each leg ends in an N-terminal α -barrel domain that is located below a polygonal face (the hook-like features inside the cage). These domains interact with the mostly unstructured adaptor protein, producing some heterogeneity in this inner layer of density. A reconstruction of this basket was estimated to have a global resolution of 28 Å (Heymann et al., 2005). However, the resolution is by no means uniform, as shown in Figure 5b. While the resolution of the outer cage is ~ 25 Å, that in the inner region occupied by the N-termini is lower, around 30 Å. Visual inspection of the density map shows lower intensity of the N-terminal domains compared to the triskelion legs, in agreement with the assessment of structural heterogeneity and flexibility.

In vitro assembly of the RSV CA protein produces small icosahedral particles (triangulation number T=1) with a diameter of ~ 17 nm (Purdy et al., 2008). Our reconstruction (Cardone et al., 2009) was estimated to have a global resolution of ~ 10 Å - see also Section 4.3. In Figure 5c is shown the rendering of this map, colored according to local resolution. The floor layer, formed by C-terminal domains of CA interacting with each other, is assigned a resolution between 9 and 10 Å. The N-terminal domains of the proteins form pentameric protrusions around the icosahedral vertices, and their resolution ranges between 10 and 13 Å. Interestingly, two regions on top of these protrusions show the lowest resolution (> 11 Å). A pseudo-atomic model obtained by fitting the subunit crystal structure into the cryo-EM density map revealed that these regions correspond to the loop between helices 4 and 5, and to the turn of the hairpin, suggesting that these motifs are relatively mobile. This result agrees with the analysis of the quality of fit of the atomic models into the corresponding density. Interestingly, the same residues are among those with the highest B-factors in the atomic model derived by X-ray crystallography (Kingston et al., 2000).

4.5. Local filtering

When regions with differing resolution levels co-exist within the same reconstructed map, a proper filtering can be critical for a correct interpretation of the density. Especially when the differences are sizeable, low-pass filtering the entire reconstruction at a single resolution value is simply not effective. On the other hand, filtering according to the highest resolution estimate will convey the regions with lowest quality embedded in a level of noise that makes difficult their interpretation, as shown in Figure 3C. We used data from this experiment to show how a local resolution analysis can be used to filter the reconstruction efficiently. By low-pass filtering each voxel according to the corresponding resolution estimated, we obtained a map that preserves the interpretable features of both subunits 30S and 50S, while minimizing the noise contributions (figure 6). An improved result could be obtained by computationally separating the two subunits, filtering each of them according to their average resolution, and then recombining them in a single volume. However, this last approach is only possible when the boundaries of all the components are exactly known.

5. Discussion and conclusions

This paper addresses the issue of localized resolution assessment in SPA. We have shown that an approach based on the short space Fourier representation of data allows a more detailed understanding of the quality of the reconstruction. All resolution measures in current use provide a single number for the whole reconstruction which relates, more or less indirectly, to the signal-to-noise ratio of the map. Residual noise can have a marked effect on resolution estimates in an unpredictable way (LeBarron et al., 2008). The situation can be stabilized by applying a soft-edged mask, which minimizes the contributions from background noise (LeBarron et al., 2008), a practice now in use. (However, a hard-edged mask risks to exaggerate the resolution, referring to the mask more than the structure imaged). A similar approach can be used to analyze the quality of a subvolume of interest

identified within a reconstruction (Gao et al., 2004; Stewart et al., 2000). The technique presented here contributes additional information on the quality of the reconstruction.

This form of localized resolution assessment has been already used successfully on experimental maps by other investigators (Lin et al., 2011). In a study on the proteasome regulatory particle (Lander et al., 2012), the information derived from the local resolution analysis helped to delineate the boundaries of the subunits in the lid complex. Furthermore, localized filtering was applied to the final reconstruction, in order to avoid over-interpretation of less-ordered portions of the map, while preserving the high-resolution elsewhere. In another study on the mammalian ribosomal 43S preinitiation complex (Hashem et al., 2013a; Hashem et al., 2013b), localized resolution assessment was used to identify components with partial occupancy. In this case, the results were compared to those obtained by three-dimensional variance analysis (Penczek et al., 2006), yielding substantial agreement. Recently, other studies have explored the use of localized resolution measures to investigate the heterogeneity of a reconstruction (Bai et al., 2012; Beck et al., 2012; Lasker et al., 2012). Although only one of them gave some information on the implementation, they all seem to be based on approaches similar to the one presented here. In this context, we have shown that the chosen kernel size is of key importance, and as such, should always be specified.

5.1. Extendability to other resolution criteria

In the approach described here, the FSC was used as resolution criterion. However, in principle, it could be adapted for any other criterion that assesses resolution by comparing two reconstructions generated from half data sets, such as DPR and FSPR. It is important to note that since each such criterion has different statistical properties, the choice of the kernel size should be chosen accordingly. Other resolution criteria require a different organization of the data. For example, the R-measure relies on just one reconstruction generated from the entire input dataset, with the only constraint that no filtering or masking is applied. Another resolution criterion, the SSNR, determines the estimate by comparing the input projections with reprojections from the final map. With both of these measures, our procedure cannot be applied in a straightforward manner, since they would require some explicit estimation of the noise statistics in the selected subvolume.

5.2. Applicability to electron tomography

This approach can be applied to any reconstruction method that is based on averaging the information from several particles. We have shown results from single particle analysis. However, we expect that this approach would provide useful also in electron tomography, to estimate localized resolution of averages of subtomograms. In fact, while the resolution of single subtomograms is estimated by *ad-hoc* methods like NLOO (Cardone et al., 2005), density maps obtained by averaging multiple subtomograms are assessed in the same way as in single particle analysis. In subtomogram averaging, the combined effect of the missing wedges associated with each particle generates a compound wedge effect (Stolken et al., 2011) that can affect the quality of the final map, especially when the particles have preferential orientations. In such cases, localized resolution analysis would identify the regions of the map that have been more severely affected.

5.3. Other applications

We have shown that localized information on the quality of a reconstruction can be exploited to low-pass filter the density map accordingly. Furthermore, the same information can be used to determine the equivalent B-factors and to sharpen the reconstruction in a localized manner, following available methods (Fernandez et al., 2008; Rosenthal and Henderson, 2003) that use resolution curves to determine optimum parameters. The

advantage of local adaptive filtering (or sharpening) goes beyond improving visual interpretability: procedures that attempt to derive a pseudo-atomic model of a macromolecular complex by fitting structures of individual components into a cryo-EM density map rely on an estimate of the map's resolution when it comes to comparing map and model. If components vary significantly in quality (resolution), filtering them according to their respective resolutions can improve the accuracy of the fitting. Filtering of this kind is even more important in the case of flexible fitting procedures. For example, approaches like MDFF (Trabuco et al., 2008) take the density in the map as an external potential field, and use local gradients in the map to steer the atoms into high-density regions. In this case also, improved filtering should increase the accuracy of the fitting, especially for those regions whose resolution would be overestimated by a global assessment.

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Appendix A: Variance of the Fourier Shell Correlation

The statistical properties of the cross-correlation between two images have been extensively studied in real space (Frank and Al-Ali, 1975; Saxton, 1978), and similar relations have been derived in the Fourier domain (Penczek, 2002). On the assumption that the two images are corrupted by uncorrelated zero-mean noise, the variance of the correlation is given by (Eq. 9.4.27 in Saxton, 1978)

$$\text{var}[\rho] = \frac{1}{N} \frac{(1+\alpha^2)^2 (1+2\alpha^2) - \rho_{ideal}^2 \alpha^4 (2\alpha^2+3)}{(1+\alpha^2)^4}, \quad (\text{A.1})$$

where N is the number of pixels in each image, α is the square root of the signal-to-noise ratio of the images, assuming that the two images have the same signal and noise levels (i.e. expected variance), and ρ_{ideal} is the noise-free cross-correlation. This equation can be used to derive the variance of the Fourier Shell Correlation FSC_k for a given shell of index k . In fact, in this case N is given by the number N_k of voxels in the shell, α^2 corresponds to the

spectral signal-to-noise ratio $SSNR_k$, and $ideal=1$, because the signal in the two volumes is assumed to be the same. It follows that the variance of FSC_k is

$$\text{var}[FSC_k] = \frac{1}{N_k} \frac{1+4SSNR_k+2SSNR_k^2}{(1+SSNR_k)^4}. \quad (\text{A.2})$$

The relationship between FSC_k and $SSNR_k$ is (Unser, 1987)

$$SSNR_k = 2 \frac{FSC_k}{1-FSC_k}, \quad (\text{A.3})$$

which takes into account that each of the two volumes used for calculating Fourier Shell Correlation are generated from half the dataset. By combining (A.2) and (A.3), we obtain

$$\text{var}[FSC_k] = \frac{1}{N_k} \frac{(1+6FSC_k+FSC_k^2)(1-FSC_k)^2}{(1+FSC_k)^4}. \quad (\text{A.4})$$

Appendix B: Number of samples in a Fourier shell

The number of samples in a Fourier shell can be expressed as a function of the ratio between the size of the kernel used for the Fourier analysis and the resolution corresponding to the mean radius of the shell. Given N equally spaced samples separated by δ , the result of their Fourier Transform is N Fourier components sampled every $1/N$. In the case of the FSC the correlation at a radial frequency component $f_i = i/N$ is obtained from the integration of the correlation of all the points in a shell with mean radius index i . Assuming the width of the shell to be equal to one pixel unit, the number of points in the shell can be approximated as $4\pi i^2 \delta^2$, where δ is the thickness of the shell in voxels. However, only half the points are statistically independent, because the components are Friedel symmetric. Therefore, the number of points to consider for the resolution analysis is $2\pi i^2 \delta^2$. Since the estimated resolution r is defined as the inverse of the radial frequency at the cutoff chosen $r = f_i^{-1}$, the index i can be expressed as

$$i = N\delta/r. \quad (\text{B.1})$$

Since the numerator is equal to the size of the kernel in physical units, we refer to this quantity as the kernel-to-resolution ratio krr , and the number of independent samples in a Fourier shell is then $2\pi krr^2$.

In our analysis the Fourier Transform is calculated after multiplying the volumes by a window function, the Hann window. Since in the Fourier domain the operation of multiplication corresponds to a convolution between the Fourier transforms of the volumes and the window function, this is equivalent to average the information from each voxel with the adjacent ones, according to the spectral shape of the window function. By considering only the main lobe of the Hann window in the Fourier domain, which has width of two voxels ($\Delta = 2$), the equivalent thickness of the shell is $\Delta = 2$.

Figure 1.

1D example of localized Fourier Correlation. A tapering window is applied to both the signals, and the result is transformed in the Fourier domain. The correlation is calculated between the two transformed signals to estimate the resolution, which is assigned to the center point in the window. The procedure is then repeated by moving the window successively along the data, until all the data are analyzed.

Figure 2. Accuracy of the localized Fourier Shell Correlation. The relative error of the FSC is plotted against the kernel-to-resolution ratio for three cutoff thresholds: 0.143, 0.3, 0.5.

Figure 3.

Simulated 70S ribosome map. Reconstructions are color-coded according to the localized resolution estimate, and all were low-pass filtered to 10 Å. The color bars give the resolution scale in Å. (a, b) Effect of masking out background noise on estimation accuracy. (a) unmasked (b) masked. (c–f) Sensitivity of local resolution to occupancy level.

Reconstructions from datasets with different levels of occupancy of the 30S subunit. (c) Surface rendering and (e) gray scale section for reconstruction with 40% occupancy. (d) and (f) similarly, with 80% occupancy. Scale bar, 10 nm.

Figure 4. Effect of kernel size on localized FSC estimation. The average of the localized FSC resolution estimated on a subset of voxels in the reconstruction is plotted for several values of the box size used for the estimation. (a) RSV CA icosahedral particle; (b) HSV-1 C capsid; (c) Clathrin basket. The secondary x-axis is the kernel-to-resolution ratio. Cutoff threshold: 0.5.

Figure 5.

Surface rendering of cryo-EM reconstructions, colored according to localized resolution. (a) HSV-1 C capsid. The map is filtered to 16 Å, and the global resolution calculated for the capsid shell is estimated at ~ 20 Å. Kernel size used to estimate the localized resolution: 140 Å (38 pixels). Scale bar, 20 nm. (b) Clathrin D6 basket. The map is filtered to 24 Å, and the global resolution is estimated at ~ 28 Å. Kernel size: 193 Å (35 pixels). Scale bar, 20 nm. (c) RSV CA icosahedral assembly. The map is filtered to 9 Å, and the global resolution is estimated around 10 Å. Kernel size: 71 Å (56 pixels). Scale bar, 5 nm.

Figure 6.

Example of localized filtering. The reconstruction from the synthetic data set of 70S ribosome particles, where the 30S subunit is simulated to be present in only 40% of particles, is low-pass filtered at every voxel according to the local resolution estimate. This result may be compared with Figure 3C, in which the entire reconstruction is low-pass filtered to 10 Å. The reconstruction is colored according to the localized resolution used for filtering, using the same scheme as in Figure 3c.

Resolution statistics for the experimental data sets used. The resolution is the global resolution assessed by FSC at cutoff 0.5, and the radii refer to the radial zone used for its assessment. The kernel size is the linear size of the box used for the localized resolution analysis.

Table 1

sample	map size (Å)	voxel size (Å)	resolution (Å)	min, max radius (Å)	kernel size (Å)
RSV CA icosahedral particles	324	1.27	10.4	47, 133	71
HSV-1 C capsid	1476	3.68	19.9	427, 670	140
Clathrin D6 basket	1111	5.526	28.5	144, 431	193