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A review of resolution measures and related aspects in 3D Electron Microscopy

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ABSTRACT

Fourier Shell Correlation, Spectral Signal-to-Noise Ratio, Fourier Neighbour Correlation, and Differential Phase Residual are different measures that have been proposed over time to determine the spatial resolution achieved by a certain 3D reconstruction. Estimates of B-factors to describe the reduction in signal-to-noise ratio with increasing resolution is also a useful parameter. All these concepts are interrelated and different thresholds have been given for each one of them. However, the problem of resolution assessment in 3DEM is still far from settled and preferences are normally adopted in order to choose the "correct" threshold. In this paper we review the different concepts, their theoretical foundations and the derivation of their statistical distributions (the basis for establishing sensible thresholds). We provide theoretical justifications for some common practices in the field for which a formal justification was missing. We also analyze the relationship between SSNR and B-factors, the electron dose needed for achieving a given contrast and resolution, the number of images required, etc. Finally, we review the consequences for the number of particles needed to achieve a certain resolution and how to analyze the Signal-to-Noise Ratio for a sequence of imaging operations.

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1. Introduction

Single particle analysis of biological specimens is a useful experimental technique to acquire structural information of macromolecular complexes (Frank, 2006). The highest resolution structures are currently below 2 Å (Merk et al., 2016). However, the exact resolution of 3D reconstructions is still somehow unclear, since several views on the precise manner to calculate it still coexist. Intuitively, the resolution of a structure is the size of the smallest detail one can trust. How to translate this into a measurable function is not straightforward and several criteria have been provided in Physics. The most representative is Rayleigh criterion, by which an image of two points is diffraction limited when the minimum of the intensity of the diffraction of one of the points coincides with the maximum of the other (Born and Wolf, 1975). However, even this definition is rather arbitrary and any other distance could have been defined as resolution (Shahram and Milanfar, 2006).

Single-particle analysis by 3DEM has also devised its own definitions of resolution. All of them are based on different curves like the Fourier Shell Correlation (FSC) (Saxton, 1978; Saxton and Baumeister, 1982; Harauz and van Heel, 1986), the Spectral Signal-to-Noise Ratio (SSNR) (Unser et al., 1987; Penczek, 2002), the Differential Phase Residual (DPR) (Frank et al., 1981; Penczek et al., 1994) or B-factor (Rosenthal and Henderson, 2003; Fernández et al., 2008). Liao and Frank (2010) made a review on all these concepts. The problem is how to simplify this curve into a single number defining the resolution. A threshold is used for this and there is a strong debate about which is the "correct" threshold and on which curve. Moreover, some of these curves, like the SSNR, can be extended into anisotropic resolution measures (Unser et al., 2005) making the problem of defining the resolution even more difficult. Recently, the concept of local resolution has been introduced (Cardone et al., 2013; Kucukelbir et al., 2014). The idea is to locally measure the energy content at different frequencies using a frequency-time signal decomposition. In this way, we may identify areas in the map that have better resolution than other areas. Instead of using the map alone to estimate its resolution, we may make use of a fitting with an atomic model to help to determine the resolution of a map (we can see α-helices, β-sheets, side chains, …). However, the quantification of the resolution in this way may be involved.

In this article we review concepts like FSC, SSNR, DPR, and B-factor. We provide a solid background of their statistical distributions and in doing so we gain deep insight into their properties, theoretical limits, conditions under which they can be applied, etc. We also derive consequences in terms of number of particles needed in order to achieve a certain resolution and the way of analyzing pipelines of image processing/acquisition steps. The content of this article generalizes much of the work already performed in the field, and sets the basis for a unified vision of the Fourier concepts employed in 3DEM.

This paper is primarily concerned about the resolution for the so-called Single Particle Analysis. Resolution in Electron Tomography follows a similar, although significantly different, approach and the reader interested in this topic is referred to Penczek (2002); Cardone et al. (2005); Unser (2005); Penzcek and Frank (2007); Diebolder et al. (2015).

2. On the definition of the Fourier Shell correlation and its signal implications

The FSC is currently the most widely applied resolution measure in use in the EM field. However, the analysis of the FSC statistical distribution remains yet to be completed, especially in the context of two important applications: (1) The calculation of FSC confidence limits per shell, complementing the usual plot of its expected value, (2) The use of hypothesis testing threshold values. In the following we make an in-depth analysis of the FSC that we place in the perspective of a number of previous works that are reevaluated. The net result of this work is a simple and intuitive way to view the FSC, a clear calculation of per shell confidence limits as well as the derivation of statistical based thresholds.

The Fourier Shell Correlation is normally defined as (Saxton and Baumeister, 1982; Harauz and van Heel, 1986)

$$FSC(R,\Delta R)=\frac{\sum_{\mathbf{R}\in\Im(R,\Delta R)}F_1(\mathbf{R})F_2^*(\mathbf{R})}{\sqrt{\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left|F_1(\mathbf{R})\right|^2\right)\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left|F_2(\mathbf{R})\right|^2\right)}},\tag{1}$$

being $\mathbf{R}$ the three-dimensional frequency vector, R its module, F_i the Fourier transform of map i, and $\Im(R_0,\Delta R)$ the shell of those frequencies such that $R_0\le\|\mathbf{R}\|<R_0+\Delta R$. However, other definition has also been issued (Frank, 2006)

$$FSC(R,\Delta R)=\frac{\sum_{\mathbf{R}\in\Im(R,\Delta R)}\mathrm{Re}\{F_1(\mathbf{R})F_2^*(\mathbf{R})\}}{\sqrt{\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left|F_1(\mathbf{R})\right|^2\right)\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left|F_2(\mathbf{R})\right|^2\right)}},\tag{2}$$

where $\mathrm{Re}\{\cdot\}$ is the operator extracting the real part of a complex number. Both definitions are equivalent thanks to the Hermitian symmetry of the Fourier transform of real-valued volumes as can be easily verified. However, one may wonder what is the exact meaning of this measurement whose justification seems to be based on the Fourier correlation theorem. This theorem states that the cross-correlation function of lag $\mathbf{r}_0$ between two maps may be computed in real space or Fourier space as

$$CC_{f_1,f_2}(\mathbf{r}_0) = \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_1(\mathbf{r}+\mathbf{r}_0)f_2(\mathbf{r}) = \mathcal{F}^{-1}\{F_1(\mathbf{R})F_2^*(\mathbf{R})\}, \quad (3)$$

where the functions f_1 and f_2 are supposed to be finitely supported in the cube $\left[-\frac{N}{2},\frac{N}{2}\right]^3$, and $\mathcal{F}^{-1}$ is the inverse Fast Fourier Transform (FFT) operator. Particularizing for $\mathbf{r}_0=\mathbf{0}$, we obtain the well-known Rayleigh's theorem for real signals (Bracewell, 2006):

$$CC_{f_1,f_2}(\mathbf{0}) = \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_1(\mathbf{r})f_2(\mathbf{r}) = \sum_{\mathbf{R}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} F_1(\mathbf{R})F_2^*(\mathbf{R}), \quad (4)$$

The cross-correlation between two maps is usually normalized between −1 and 1, so that it is easier to compare correlation values. This can be done by dividing the original cross-correlation by the norm of the two input maps (Proakis and Manolakis, 2006)

$$NCC_{f_1,f_2}(\mathbf{r}_0) = \frac{CC_{f_1,f_2}(\mathbf{r}_0)}{\sqrt{CC_{f_1,f_1}(0)CC_{f_2,f_2}(\mathbf{0})}} \quad (5)$$

which, particularizing at $\mathbf{r}_0=\mathbf{0}$ and using Rayleigh's theorem, can be simplified to

$$NCC_{f_1,f_2}(0) = \frac{\sum_{\mathbf{R}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} F_1(\mathbf{R})F_2^*(\mathbf{R})}{\sqrt{\left(\sum_{\mathbf{R}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3}\left|F_1(\mathbf{R})\right|^2\right)\left(\sum_{\mathbf{R}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3}\left|F_2(\mathbf{R})\right|^2\right)}} \quad (6)$$

that is formally quite similar to the definition of the FSC except for the range of the frequencies being summed and the fact that we are summing in the numerator $F_1(\mathbf{R})F_2^*(\mathbf{R})$ instead of $\mathrm{Re}\{F_1(\mathbf{R})F_2^*(\mathbf{R})\}$. The difference in the numerators are easily overcome if we take into account that $NCC_{f1,f2}(\mathbf{0})$ is a real value and, therefore, $NCC_{f_1,f_2}(0)=\mathrm{Re}\{NCC_{f_1,f_2}(0)\}$ (in fact, the sum $\sum F_1(\mathbf{R})F_2^*(\mathbf{R})$ should be real-valued, but we may take its real part as a computational trick to protect against numerical inaccuracies). The different sum ranges (Eq. (6) is summed over the whole frequency cube, while Eq. (2) is summed within a shell of Fourier coefficients) indicate that the FSC has pre-filtered the two maps so that it is measuring the normalized cross-correlation between the two input maps after applying a hard band-pass filter whose frequency response is the indicator function of the shell $\Im(R,\Delta R)$ (i.e., within the shell the filter response is 1, and outside the shell the filter response is 0). To the best of our knowledge this intuitive definition of the FSC is lacking in the field of Electron Microscopy.

Finally we would like to highlight that, as already pointed out by Unser et al. (2005), the FSC is invariant to isotropic filtering, i.e., filtering the map by an isotropic, non-vanishing filter in Fourier space (e.g. a Gaussian filter) gives the same FSC curve. This problem is also shared by the Differential Phase Residual curve (introduced below). This is clearly a drawback of the two methods. van Heel (1987) discusses some variants of these two methods, although they are not so widespread.

3. On the distribution of the SSNR and its meaning

In Unser et al. (1987) the statistical distribution of the SSNR is discussed. Although the conclusions derived by Unser et al. (1987) are essentially the same as in the following analysis, the distributions and variables involved in their analysis and this one are not exactly the same.

Let us consider K different observations of the same signal corrupted by noise. For each observation $k\in\{1,2,\ldots,K\}$, the image observation model in Fourier space is

$$F_k(\mathbf{R}) = X(\mathbf{R}) + N_k(\mathbf{R}), \quad (7)$$

where $F_k(\mathbf{R})$ is the observed Fourier coefficient at frequency $\mathbf{R}$, $X(\mathbf{R})$ is the underlying, deterministic signal, and $N_k(\mathbf{R})$ is a realization of a random noise.

The underlying signal is estimated by averaging each Fourier coefficient

$$\widehat{X}(\mathbf{R}) = \frac{1}{K}\sum_{k=1}^{K} F_k(\mathbf{R}) = X(\mathbf{R}) + \frac{1}{K}\sum_{k=1}^{K} N_k(\mathbf{R}) \quad (8)$$

We estimate the signal power within a Fourier Ring, $\Im(R,\Delta R)$, as

$$\widehat{S}(R,\Delta R) = \frac{1}{|\Im(R,\Delta R)|}\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left|\widehat{X}(\mathbf{R})\right|^2, \quad (9)$$

where $|A|$ represents the number of elements of set A.

Similarly, the noise power is estimated by

$$\widehat{N}(R,\Delta R) = \frac{1}{|\Im(R,\Delta R)|}\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left(\frac{1}{K-1}\sum_{k=1}^{K}\left|F_k(\mathbf{R})-\widehat{X}(\mathbf{R})\right|^2\right). \quad (10)$$

Let us assume that noise is a complex random variable with Gaussian distribution, independent (from the signal and other noise variables) and identically distributed for a fixed R (note that different Fourier shells may have different noise variances). The real and imaginary parts of a Gaussian complex variable are also Gaussian. We will assume that they are distributed with zero-mean and variance $\frac{1}{2}\sigma_R^2$, with no covariance between them. For simplicity, we will consider a complex random variable as a bivariate random variable (the two components being the real and imaginary part). In this way, a complex random variable describing noise in Fourier space is a bivariate Gaussian variable with zero-mean and covariance matrix $\Sigma_R=\frac{1}{2}\sigma_R^2 I$.

In the absence of signal, our estimate of the signal power becomes

$$\widehat{S}(R,\Delta R) = \frac{1}{|\Im(R,\Delta R)|}\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left|\frac{1}{K}\sum_{k=1}^{K} N_k(\mathbf{R})\right|^2 \quad (11)$$

The average of K random Gaussian complex numbers is another random Gaussian complex number that we will refer to as $N^K(\mathbf{R})$. The mean of the new complex number is zero (since the averaged numbers are also zero-mean) and the variance of its real and imaginary parts is $\frac{1}{K^2}K\frac{\sigma_R^2}{2}=\frac{\sigma_R^2}{2K}$. Let us study the distribution of the variable $\chi_1^2 = \left|\Im(R,\Delta R)\right|\frac{\widehat{S}(R,\Delta R)}{\frac{\sigma_R^2}{2K}}$

$$\chi_1^2 = \sum_{\mathbf{R}\in\Im(R,\Delta R)} \frac{\left|N^K(\mathbf{R})\right|^2}{\frac{\sigma_R^2}{2K}} = \sum_{\mathbf{R}\in\Im(R,\Delta R)} \left(\frac{\mathrm{Re}\{N^K(\mathbf{R})\}}{\frac{\sigma_R}{\sqrt{2K}}}\right)^2 + \left(\frac{\mathrm{Im}\{N^K(\mathbf{R})\}}{\frac{\sigma_R}{\sqrt{2K}}}\right)^2 \tag{12}$$

For every point in $\Im(R,\Delta R)$ there are two terms in the sum, each one being a standardized Gaussian variable, therefore, χ_1^2 has a χ^2 distribution apparently with $2|\Im(R,\Delta R)|$ degrees of freedom because there are $2|\Im(R,\Delta R)|$ addends. However, we must remind that, because of the Hermitian symmetry, only half of these Fourier coefficients are truly independent and consequently the number of degrees of freedom would be $|\Im(R,\Delta R)|$ as stated in Unser et al. (1987).

Analogously we can study the noise power in the absence of signal

$$\widehat{N}(R,\Delta R) = \frac{1}{|\Im(R,\Delta R)|}\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left(\frac{1}{K-1}\sum_{k=1}^{K}|N_k(\mathbf{R}) - \frac{1}{K}\sum_{k'=1}^{K}N_{k'}(\mathbf{R})|^2\right)$$
$$= \frac{1}{|\Im(R,\Delta R)|}\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left(\frac{1}{K-1}\sum_{k=1}^{K}|\frac{K-1}{K}N_k(\mathbf{R}) - \frac{1}{K}\sum_{\substack{k'=1\\k'\neq k}}^{K}N_{k'}(\mathbf{R})|^2\right) \tag{13}$$

Let us consider a new random variable

$$\chi_2^2 = \sum_{\mathbf{R}\in\Im(R,\Delta R)}\left(\frac{1}{K-1}\sum_{k=1}^{K}\frac{\left|N^k(\mathbf{R})\right|^2}{\frac{\sigma_R^2}{2K}}\right) = \sum_{\mathbf{R}\in\Im(R,\Delta R)}\sum_{k=1}^{K}\frac{\left|N^k(\mathbf{R})\right|^2}{\frac{K-1}{K}\frac{\sigma_R^2}{2}}$$
$$= \sum_{\mathbf{R}\in\Im(R,\Delta R)}\sum_{k=1}^{K}\left(\frac{\mathrm{Re}\left\{N^k(\mathbf{R})\right\}}{\frac{\sigma_R}{\sqrt{2}}\sqrt{\frac{K-1}{K}}}\right)^2 + \left(\frac{\mathrm{Im}\left\{N^k(\mathbf{R})\right\}}{\frac{\sigma_R}{\sqrt{2}}\sqrt{\frac{K-1}{K}}}\right)^2 \tag{16}$$

χ_2^2 has a χ^2 distribution with $K|\Im(R,\Delta R)|$ degrees of freedom (in which we have already taken into account the Hermitian symmetry to count the actual number of independent random variables).

The ratio of the two χ^2 variables divided by their respective number of degrees of freedom is distributed as an F-Snedecor distribution with $|\Im(R,\Delta R)|$ and $K|\Im(R,\Delta R)|$ degrees of freedom:

$$F(R,\Delta R) = \frac{\frac{\chi_1^2}{|\Im(R,\Delta R)|}}{\frac{\chi_2^2}{K|\Im(R,\Delta R)|}} = K\frac{\chi_1^2}{\chi_2^2} = K\frac{|\Im(R,\Delta R)|\frac{\widehat{S}(R,\Delta R)}{\frac{\sigma_R^2}{2K}}}{|\Im(R,\Delta R)|\frac{\widehat{N}(R,\Delta R)}{\frac{\sigma_R^2}{2K}}} = K\frac{\widehat{S}(R,\Delta R)}{\widehat{N}(R,\Delta R)}. \tag{17}$$

In other words, in the absence of signal, the estimate of the SSNR after averaging is distributed as a F-Snedecor distribution with $d_1 = |\Im(R,\Delta R)|$ and $d_2 = K|\Im(R,\Delta R)|$ degrees of freedom whose average and variance are

$$\mathrm{E}\{F(R,\Delta R)\} = \frac{d_2}{d_2-2} = \frac{K|\Im(R,\Delta R)|}{K|\Im(R,\Delta R)|-2}$$
$$\mathrm{Var}\{F(R,\Delta R)\} = \frac{2d_2^2(d_1+d_2-2)}{d_1(d_2-2)^2(d_2-4)} = \frac{2K^2\left|\Im(R,\Delta R)\right|((K+1)|\Im(R,\Delta R)|-2)}{(K|\Im(R,\Delta R)|-2)^2(K|\Im(R,\Delta R)|-4)} \tag{18}$$

$$N^k(\mathbf{R}) = \frac{K-1}{K}N_k(\mathbf{R}) - \frac{1}{K}\sum_{\substack{k'=1\\k'\neq k}}^{K}N_{k'}(\mathbf{R}) \tag{14}$$

A linear combination of two independent, multivariate Gaussians, $Z = aX + bY$, has a mean $\mu_Z = a\mu_X + b\mu_Y$ and a covariance matrix $\Sigma_Z = a^2\Sigma_X + b^2\Sigma_Y$. In this way, the covariance of $N^k(\mathbf{R})$ is given by

$$\Sigma_{N^k(\mathbf{R})} = \left(\frac{K-1}{K}\right)^2\Sigma_R + \frac{1}{K^2}(K-1)\Sigma_R = \frac{K-1}{K}\Sigma_R = \frac{K-1}{K}\frac{\sigma_R^2}{2}I \tag{15}$$

Let us now define the variable $\chi_2^2 = |\Im(R,\Delta R)|\frac{\widehat{N}(R,\Delta R)}{\frac{\sigma_R^2}{2K}}$ and study its distribution (note that this variable was incorrectly standardized by Unser et al. (1987) as $\chi_2^2 = |\Im(R,\Delta R)|\frac{\widehat{N}(R,\Delta R)}{\frac{\sigma_R^2}{2(K-1)}}$, though this mistake does not invalidate their main results)

For large degrees of freedom (usually larger than 30, and in the EM application the number of degrees of freedom is in the order of thousands and consequently $d_2-4 \approx d_2-2 \approx d_2$) can be safely approximated by a Gaussian with parameters

$$\begin{aligned}\mathrm{E}\{F(R,\Delta R)\} &\approx 1\\ \mathrm{Var}\{F(R,\Delta R)\} &\approx 2\frac{K+1}{K}\frac{1}{|\Im(R,\Delta R)|}\end{aligned} \tag{19}$$

In the presence of signal the distribution of χ_2^2 does not change, but the one of χ_1^2 does:

$$\chi_1^2 = \sum_{\mathbf{R}\in\Im(R,\Delta R)}\frac{\left|X(\mathbf{R})+N^K(\mathbf{R})\right|^2}{\frac{\sigma_R^2}{2K}} = \sum_{\mathbf{R}\in\Im(R,\Delta R)}\left(\frac{\mathrm{Re}\{X(\mathbf{R})+N^K(\mathbf{R})\}}{\frac{\sigma_R}{\sqrt{2K}}}\right)^2 + \left(\frac{\mathrm{Im}\{X(\mathbf{R})+N^K(\mathbf{R})\}}{\frac{\sigma_R}{\sqrt{2K}}}\right)^2 \tag{20}$$

Now, each standardized random variable is centered at a

different mean. Thus, χ_1^2 follows a non-central χ^2 distribution with the same degrees of freedom as before and non-centrality parameter

$$\lambda = \sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)} \left(\frac{\mathrm{Re}\{X(\mathbf{R})\}}{\frac{\sigma_R}{\sqrt{2K}}}\right)^2 + \left(\frac{\mathrm{Im}\{X(\mathbf{R})\}}{\frac{\sigma_R}{\sqrt{2K}}}\right)^2 = \sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)} \frac{\left|X(\mathbf{R})\right|^2}{\frac{\sigma_R^2}{2K}} \tag{21}$$

Considering that $\mathrm{E}\{\left|N_k(\mathbf{R})\right|^2\} = \frac{\sigma_R^2}{2}$ (the ½ factor appears because of the Hermitian symmetry), and defining the average power of the signal coefficients in the shell as

$$\overline{\left|X(\mathbf{R})\right|^2} = \frac{1}{|\mathfrak{S}(R,\Delta R)|}\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)} |X(\mathbf{R})|^2, \tag{22}$$

then we can reexpress the non-centrality parameter as

$$\lambda = \frac{\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)} \left|X(\mathbf{R})\right|^2}{\frac{\sigma_R^2}{2K}} = \frac{\left|\mathfrak{S}(R,\Delta R)\right|\overline{\left|X(\mathbf{R})\right|^2}}{\frac{\mathrm{E}\{\left|N_k(\mathbf{R})\right|^2\}}{K}} = K|\mathfrak{S}(R,\Delta R)|\cdot SSNR(R,\Delta R). \tag{23}$$

In this equation $SSNR(R,\Delta R)$ should be understood as the average SNR between individual Fourier coefficients within the shell $\mathfrak{S}(R,\Delta R)$.

The ratio between the two χ^2 variables, $F(R,\Delta R)$, is still defined in the same way, although its distribution changes to a non-central F Snedecor distribution with the same degrees of freedom as before and with non-centrality parameter λ (given by the previous equation). The average and variance of $F(R,\Delta R)$ relevant for EM (large degrees of freedom, i.e., $d_2-4\approx d_2-2\approx d_2$) are now

$$\begin{aligned}\mathrm{E}\{F(R,\Delta R)\} &= \frac{d_2(d_1+\lambda)}{d_1(d_2-2)} \approx 1 + K\cdot SSNR(R,\Delta R)\\ \mathrm{Var}\{F(R,\Delta R)\} &= 2\frac{(d_1+\lambda)^2+(d_1+2\lambda)(d_2-2)}{(d_2-2)^2(d_2-4)}\frac{d_2^2}{d_1^2}\\ &\approx 2\frac{K^2SSNR^2(R,\Delta R)+2K(K+1)SSNR(R,\Delta R)+(K+1)}{K|\mathfrak{S}(R,\Delta R)|}\end{aligned} \tag{24}$$

The expected value of $F(R,\Delta R)$ is a biased estimate of the SSNR after averaging. Interestingly, the variance of the estimate decreases with K (the number of observations) and the size of the Fourier shell.

This bias suggests the unbiased estimate of the SSNR after averaging the K coefficients is

$$\widehat{SSNR}(R,\Delta R) = \begin{cases} F(R,\Delta R)-1 & F(R,\Delta R)\geq 1 \\ 0 & F(R,\Delta R)<1 \end{cases} \tag{25}$$

For all practical purposes, the standard deviation of $F(R,\Delta R)$ is much smaller than its mean. This means that, in the presence of signal, the clipping induced by Eq. (25) is seldom applied, and the variance of $\widehat{SSNR}(R,\Delta R)$ is similar to that of $F(R,\Delta R)$ and, due to the large number of degrees of freedom involved in the EM analysis, the Gaussian distribution is a safe approximation for the distribution of $\widehat{SSNR}(R,\Delta R)$.

Traditionally, the threshold for the SSNR has been set to $\widehat{SSNR}(R,\Delta R)=1$, i.e., as that frequency at which there is so much noise as signal and, therefore, the interpretation of the map beyond this point could be confounded by noise. However, this threshold may be a little conservative. Alternatively, we could use the distribution of $\widehat{SSNR}(R,\Delta R)$ in the absence of signal and define the threshold as that frequency at which the null hypothesis that there is no signal cannot be rejected with a given confidence level $1-\alpha$. Considering Eq. (19), this occurs when the $\widehat{SSNR}(R,\Delta R)$ drops below $z_{1-\alpha}\sqrt{2\frac{K+1}{K|\mathfrak{S}(R,\Delta R)|}}$, where $z_{1-\alpha}$ is the $1-\alpha$ percentil of a standard normal variable.

In case of a symmetric macromolecule, part of the terms in the sums defining χ_1^2 and χ_2^2 are identical and the noise in symmetrically equivalent positions is not independent due to the symmetrization process. In this case, the sums should be performed in the asymmetric unit of the shell and the number of degrees of freedom would be reduced accordingly.

As already suggested by Unser et al. (2005), the SSNR can be successfully extended to work in 3D Fourier space. By doing this, we can explore the anisotropy of resolution, identifying those directions with better resolution than others, instead of reducing this complex reality to a single resolution number. Note that this approach leads to the concept of directional resolution, but not to a local resolution, due to the unlocalized nature of features in Fourier space.

4. On the relationship between the Fourier Shell correlation and the signal-to-noise ratio

The Signal-to-Noise Ratio (SNR) is normally defined in image processing and EM as the ratio between the power of the signal of interest (our molecule) and the power of the noise (any other signal in the image that corrupts the signal of interest). Normally the noise is considered to be a random variable while the signal in EM is normally considered to be deterministic. The power can be unambiguously defined for both kinds of signals as the autocorrelation of the signal (or noise) with itself in the absence of lag. In the case of noise, its power corresponds to its variance; in the case of the signal, it is the average value of the square of the signal values. In the field of EM, the relationship

$$SNR = \frac{NCC}{1-NCC} \tag{26}$$

has been extensively used (e.g., Frank and Al-Ali (1975)). This relationship is based on a result of Bershad and Rockmore (1974). However, let us review that paper to see the conditions under which this result holds. Let us state the problem in the same framework as in Bershad and Rockmore (1974), and then we will see if those conditions hold in EM. Let $x(t)$ and $y(t)$ be two signals generated as noisy observations of a stationary, white, Gaussian, zero-mean random process:

$$\begin{aligned} x(t) &= s(t) + n_x(t)\\ y(t) &= s(t) + n_y(t)\end{aligned} \tag{27}$$

Let us presume that the two noise processes are equally distributed, independent from each other, and independent of the underlying signal. Although this is the original problem statement

in Bershad and Rockmore (1974), such white noise is a difficult mathematical entity to work with (Unser and Tafti, 2014)[Chap. 4]. Instead, a more tractable object is a bandlimited version of such a white noise, which is no longer independent as a continuous signal. However, it has the remarkable property that its uniform samples are i.i.d., thanks to the orthogonality of the sinc functions. Let us then take N samples from both continuous signals fulfilling Nyquist criterion obtaining the values $x_1,x_2,\ldots,x_N$ and $y_1,y_2,\ldots,y_N$. Let us study under these circumstances the correlation between x_i and x_j:

$$\begin{aligned}\mathrm{E}\{x_i x_j\} &= \mathrm{E}\{(s_i+n_{xi})(s_j+n_{xj})\} = \mathrm{E}\{(s_i s_j + n_{xi}s_j + s_i n_{xj} + n_{xi}n_{xj})\} \\ &= (\sigma_s^2+\sigma_n^2)\delta_{ij} = \sigma_n^2(1+SNR)\delta_{ij},\end{aligned} \tag{28}$$

where σ_s^2 and σ_n^2 are the variances of the signal and noise respectively, and δ_{ij} is Kronecker's delta ($\delta_{ij}=1$ if $i=j$, and $\delta_{ij}=0$ otherwise). We have also defined $SNR=\frac{\sigma_s^2}{\sigma_n^2}$. For this result we need to make use of the independence between the noise and the signal, as well as the whiteness and stationarity of both. Analogously, we can compute the correlation between y_i and y_j, and x_i and y_j:

$$\begin{aligned}\mathrm{E}\{y_i y_j\} &= \sigma_n^2(1+SNR)\delta_{ij} \\ \mathrm{E}\{x_i y_j\} &= \sigma_s^2\delta_{ij} = \sigma_n^2 SNR\,\delta_{ij}\end{aligned} \tag{29}$$

We may define two new variables, σ^2 and ρ_{xy} such that

$$\begin{aligned}\mathrm{E}\{x_i x_j\} &= \mathrm{E}\{y_i y_j\} = \sigma^2\delta_{ij} \\ \mathrm{E}\{x_i y_j\} &= \rho_{xy}\sigma^2\delta_{ij}\end{aligned} \tag{30}$$

It is easy to check that with these definitions, one must have

$$\rho_{xy} = \frac{SNR}{1+SNR}. \tag{31}$$

where ρ_{xy} is the normalized correlation between the variables x and y. So, in the notation of this paper, we could have also written

$$NCC_{x,y} = \frac{SNR}{1+SNR} \tag{32}$$

or

$$SNR = \frac{NCC_{x,y}}{1-NCC_{x,y}}, \tag{33}$$

which is the formula so much used in EM.

The true correlation between x and y is unknown and Bershad and Rockmore (1974) studied how to estimate it from the samples:

$$\widehat{NCC}_{x,y} = \frac{\sum_{i=1}^N x_i y_i}{\sqrt{\left(\sum_{i=1}^N x_i^2\right)\left(\sum_{i=1}^N y_i^2\right)}} \tag{34}$$

The estimate of SNR using this sample correlation is known to be biased, and Bershad and Rockmore (1974) proposed to use instead the unbiased estimate

$$\widehat{SNR} = e^{-\frac{2}{N-3}}\left(\frac{\widehat{NCC}_{x,y}}{1-\widehat{NCC}_{x,y}} - \frac{1}{2}\left(e^{\frac{2}{N-3}}-1\right)\right) \tag{35}$$

For very large N (as is the case of EM, where maps are in the order of 10^5 and 10^6 voxels) this estimate yields

$$\widehat{SNR} \approx \frac{\widehat{NCC}_{x,y}}{1-\widehat{NCC}_{x,y}} \tag{36}$$

and the variance of this estimate (Bershad and Rockmore, 1974) is

$$\mathrm{Var}\left\{\widehat{SNR}\right\} = \left(e^{\frac{4}{N-3}}-1\right)\left(SNR+\frac{1}{2}\right)^2 \approx \frac{(1+2SNR)^2}{N-3} \tag{37}$$

These are the results so much advocated in EM. However, the conditions under which all these formulas were derived do not strictly hold in EM:

- The underlying signal is not stationary. Stationarity requires that none of the signal statistics change over space. But, an EM map of size N^3 has different local mean, local variance and local autocorrelation depending on whether we consider a point inside the macromolecule, in its border, or outside it.
- The underlying signal is not white. If we consider points inside the macromolecule there exist a significant cross-correlation between neighbouring points due to the macromolecular structure and to the presence of the Contrast Transfer Function. If the data collection geometry misses some region in Fourier space, this masking results into a convolution in real-space which further increases local correlation.
- If the images are normalized so that the background noise is zero-mean and it has unit variance, then the reconstructed macromolecule has positive values violating also the zero-mean condition on $s(t)$. If the images are normalized to be zero-mean and have unit variance, then we are violating the condition that the two noises $n_x(t)$ and $n_y(t)$ are equally distributed.

Note that the whole derivation of Bershad and Rockmore (1974) is based on real-space signals and uses parameters (SNR, $NCC_{x,y}$) computed over the whole map. Sometimes, it has been argued without any proof that the same relationship holds for Fourier coefficients

$$SNR(\mathbf{R}) = \frac{NCC_{x,y}(\mathbf{R})}{1-NCC_{x,y}(\mathbf{R})}. \tag{38}$$

or Fourier shells

$$SNR(R) = \frac{NCC_{x,y}(R)}{1-NCC_{x,y}(R)}. \tag{39}$$

We show in the following section that these relationships (Eqs. (38) and (39)) are still valid in Fourier space although for different reasons as those argued by Bershad and Rockmore (1974) as long as the filter applied is even.

5. On the distribution of the FSC and its thresholds

Several thresholds have been proposed for the definition of the resolution using the FSC. In this section we review most of them. We start with the $K\sigma$ family, then we continue with the constant thresholds and, finally, the i-bits.

The $K\sigma$ family, proposed by Saxton and Baumeister (1982), uses the distribution of the FSC in the absence of signal to establish a relevant threshold. The study of this distribution

dates back to Saxton (1978). In fact, Saxton's derivation was not intended for the FSC, but for the distribution of the cross correlation coefficient between two noisy images. Let us review Saxton's derivation and its applicability to the FSC. Saxton's reasoning was performed in real-space and for the general case of the correlation of two independent, noisy images under an arbitrary shift. We will particularize it here to the case of identical underlying images with no shift (as we already saw, this is the most relevant case for the FSC since it can be understood as the cross-correlation coefficient between two band-pass filtered versions of noisy observations of the same map). Let $f_1(\mathbf{r}) = x(\mathbf{r}) + n_1(\mathbf{r})$ and $f_2(\mathbf{r}) = x(\mathbf{r}) + n_2(\mathbf{r})$ be two independent, noisy observations of the same map (assumed to be of zero-mean). The cross-correlation coefficient between the two maps is

$$NCC_{f_1,f_2} = \frac{\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_1(\mathbf{r})f_2(\mathbf{r})}{\sqrt{\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_1^2(\mathbf{r})}\sqrt{\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_2^2(\mathbf{r})}} = \frac{c}{\sqrt{d}}, \tag{40}$$

where c and d are convenient variables that will be used to shorten the notation.

Three well-known statistical properties of the mean and variance are (Mood et al., 1974)

$$\begin{aligned}
&\mathrm{E}\{XY\} = \mathrm{E}\{X\}\mathrm{E}\{Y\} + \mathrm{Cov}\{X,Y\}\\
&\mathrm{Var}\{XY\} = \mathrm{Var}\{X\}\mathrm{E}^2\{Y\} + \mathrm{E}^2\{X\}\mathrm{Var}\{Y\} + \mathrm{Var}\{X\}\mathrm{Var}\{Y\}\\
&\qquad + 2\mathrm{E}\{X\}\mathrm{E}\{Y\}\mathrm{Cov}\{X,Y\} - \mathrm{Cov}^2\{X,Y\}\\
&\qquad + \mathrm{E}\left\{(X-\mathrm{E}\{X\})^2(Y-\mathrm{E}\{Y\})^2\right\}\\
&\qquad + 2\mathrm{E}\{Y\}\mathrm{E}\left\{(X-\mathrm{E}\{X\})^2(Y-\mathrm{E}\{Y\})\right\}\\
&\qquad + 2\mathrm{E}\{X\}\mathrm{E}\left\{(X-\mathrm{E}\{X\})(Y-\mathrm{E}\{Y\})^2\right\}\\
&\mathrm{Var}\{X+Y\} = \mathrm{Var}\{X\} + \mathrm{Var}\{Y\} + 2\mathrm{Cov}\{X,Y\}
\end{aligned} \tag{41}$$

where Cov{X,Y} is the covariance between two random variables. In the case of two independent variables these simplify to

$$\begin{aligned}
&\mathrm{E}\{XY\} = \mathrm{E}\{X\}\mathrm{E}\{Y\}\\
&\mathrm{Var}\{XY\} = \mathrm{Var}\{X\}\mathrm{E}^2\{Y\} + \mathrm{E}^2\{X\}\mathrm{Var}\{Y\} + \mathrm{Var}\{X\}\mathrm{Var}\{Y\}\\
&\mathrm{Var}\{X+Y\} = \mathrm{Var}\{X\} + \mathrm{Var}\{Y\}
\end{aligned} \tag{42}$$

Assuming that the two noise observations are independent and that the noise at each voxel is identically distributed with zero mean and variance σ^2, then, using the previous two properties, it is easy to check that

$$\begin{aligned}
\mathrm{E}\{c\} &= \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \mathrm{E}\{f_1(\mathbf{r})f_2(\mathbf{r})\}\\
&= \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \Big(x^2(\mathbf{r}) + \mathrm{E}\{x(\mathbf{r})n_2(\mathbf{r})\} + \mathrm{E}\{n_1(\mathbf{r})x(\mathbf{r})\}\\
&\quad + \mathrm{E}\{n_1(\mathbf{r})n_2(\mathbf{r})\}\Big)\\
&= \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} x^2(\mathbf{r}) = (N+1)^3\sigma_s^2,
\end{aligned} \tag{43}$$

where σ_s^2 is the signal power defined as $\sigma_s^2 = \frac{1}{(N+1)^3}\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} x^2(\mathbf{r})$.

We may also compute its variance

$$\begin{aligned}
\mathrm{Var}\{c\} &= \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \mathrm{Var}\{f_1(\mathbf{r})f_2(\mathbf{r})\}\\
&= \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \Big(\mathrm{Var}\{f_1(\mathbf{r})\}\mathrm{E}^2\{f_2(\mathbf{r})\} + \mathrm{E}^2\{f_1(\mathbf{r})\}\mathrm{Var}\{f_2(\mathbf{r})\}\\
&\quad + \mathrm{Var}\{f_1(\mathbf{r})\}\mathrm{Var}\{f_2(\mathbf{r})\}\Big)\\
&= \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \left(\sigma^2x^2(\mathbf{r}) + x^2(\mathbf{r})\sigma^2 + \sigma^2\sigma^2\right)\\
&= (N+1)^3\sigma^2\left(2\sigma_s^2 + \sigma^2\right) = (N+1)^3\sigma^4(2SNR+1)
\end{aligned} \tag{44}$$

In the computation of Var{c} we have made use of the fact that

$$\mathrm{Cov}\left\{f_1(\mathbf{r})f_2(\mathbf{r}), f_1\left(\mathbf{r}'\right)f_2\left(\mathbf{r}'\right)\right\} = 0. \tag{45}$$

This is true for independent noise voxels, but as we discuss below, this is not the case of the FSC due to the bandpass filters introduced by the Fourier shells.

Let us study the mean and variance of $f_i^2(\mathbf{r})$ before addressing the mean and variance of the denominator of the cross-correlation coefficient

$$\mathrm{E}\left\{f_i^2(\mathbf{r})\right\} = \mathrm{E}\left\{(x(\mathbf{r}) + n_i(\mathbf{r}))^2\right\} = x^2(\mathbf{r}) + \sigma^2 \tag{46}$$

$$\begin{aligned}
\mathrm{Var}\left\{f_i^2(\mathbf{r})\right\} &= \mathrm{E}\left\{\left((x(\mathbf{r}) + n_i(\mathbf{r}))^2 - \mathrm{E}\left\{(x(\mathbf{r}) + n_i(\mathbf{r}))^2\right\}\right)^2\right\} = \mathrm{E}\left\{\left(\left(n_i^2(\mathbf{r}) - \sigma^2\right) + 2x(\mathbf{r})n_i(\mathbf{r})\right)^2\right\}\\
&= \mathrm{E}\left\{\left(\left(n_i^2(\mathbf{r}) - \sigma^2\right)\right)^2\right\} + \mathrm{E}\left\{(2x(\mathbf{r})n_i(\mathbf{r}))^2\right\} + 2\mathrm{E}\left\{2x(\mathbf{r})n_i(\mathbf{r})\left(n_i^2(\mathbf{r}) - \sigma^2\right)\right\} = 2\sigma^4 + 4x^2(\mathbf{r})\sigma^2,
\end{aligned} \tag{47}$$

where we have assumed Gaussian noise, as is the case in EM (Sorzano et al., 2004a), and made use of the fact that for Gaussian noise $\mathrm{Var}\{n_i^2(\mathbf{r})\} = 2\sigma^4$.

Now we proceed to the calculation of the mean and variance of the sums in the denominator of the cross-correlation coefficient

$$\mathrm{E}\left\{\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_i^2(\mathbf{r})\right\} = \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \mathrm{E}\left\{f_i^2(\mathbf{r})\right\} = \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \left(x^2(\mathbf{r})+\sigma^2\right)$$
$$= (N+1)^3\left(\sigma_s^2+\sigma^2\right) = (N+1)^3\sigma^2(SNR+1), \tag{48}$$

where we have defined $SNR = \frac{\sigma_s^2}{\sigma^2}$. For the variance we obtain

$$\mathrm{Var}\left\{\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_i^2(\mathbf{r})\right\} = \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \mathrm{Var}\left\{f_i^2(\mathbf{r})\right\} = \sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} \left(2\sigma^4+4x^2(\mathbf{r})\sigma^2\right)$$
$$= (N+1)^3\left(2\sigma^4+4\sigma_s^2\sigma^2\right) = 2(N+1)^3\sigma^4(1+2SNR), \tag{49}$$

where we have assumed that

$$\mathrm{Cov}\left\{f_i^2(\mathbf{r}), f_i^2\left(\mathbf{r}'\right)\right\} = 0. \tag{50}$$

Again, as we discuss later, this assumption is not correct in the case of the FSC because of the bandpass filter introduced by the Fourier shells.

We may use the statistical properties of the expectation and variance of the product to compute the mean and variance of d, we have

$$\mathrm{E}\{d\} = (N+1)^6\sigma^4(1+SNR)^2$$
$$\mathrm{Var}\{d\} = 2\mathrm{E}^2\left\{\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_i^2(\mathbf{r})\right\}\mathrm{Var}\left\{\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_i^2(\mathbf{r})\right\} + \mathrm{Var}^2\left\{\sum_{\mathbf{r}\in\left[-\frac{N}{2},\frac{N}{2}\right]^3} f_i^2(\mathbf{r})\right\}$$
$$= 2(N+1)^3\sigma^4(1+2SNR)\left(2(N+1)^6\sigma^4(1+SNR)^2 + 2(N+1)^3\sigma^4(1+2SNR)\right)$$
$$\approx 4(N+1)^9\sigma^8(1+2SNR)(1+SNR)^2 \tag{51}$$

Now, we note that c and d are narrowly distributed about their means and making a Taylor expansion of the function $\frac{c}{\sqrt{d}}$ about the means of c and d (there is a mistake in the Taylor expansion performed by Saxton affecting the variance of $NCC_{f1,f2}$, although it does not affect the result without underlying signal):

$$NCC_{f_1,f_2} = \frac{c}{\sqrt{d}} \approx \frac{\mathrm{E}\{c\}}{\sqrt{\mathrm{E}\{d\}}} + \frac{1}{\sqrt{\mathrm{E}\{d\}}}\Delta c - \frac{\mathrm{E}\{c\}}{2(\mathrm{E}\{d\})^{\frac{3}{2}}}\Delta d \tag{52}$$

Note that this expansion is linear in Δc and Δd. We can approximate the variance of the correlation coefficient considering that $\mathrm{E}\{\Delta c\} = \mathrm{E}\{\Delta d\} = 0$, $\mathrm{Var}\{\Delta c\} = \mathrm{Var}\{c\}$ and $\mathrm{Var}\{\Delta d\} = \mathrm{Var}\{d\}$

$$\mathrm{E}\left\{NCC_{f_1,f_2}\right\} \approx \frac{\mathrm{E}\{c\}}{\sqrt{\mathrm{E}\{d\}}} = \frac{(N+1)^3\sigma_s^2}{\sqrt{(N+1)^6\sigma^4(1+SNR)^2}} = \frac{SNR}{1+SNR}$$
$$\mathrm{Var}\left\{NCC_{f_1,f_2}\right\} \approx \left(\frac{1}{\sqrt{\mathrm{E}\{d\}}}\right)^2\mathrm{Var}\{\Delta c\} + \left(\frac{\mathrm{E}\{c\}}{2(\mathrm{E}\{d\})^{\frac{3}{2}}}\right)^2\mathrm{Var}\{\Delta d\} = \frac{(N+1)^3\sigma^4(2SNR+1)}{(N+1)^6\sigma^4(1+SNR)^2} + \left(\frac{(N+1)^3\sigma_s^2}{2\left((N+1)^6\sigma^4(1+SNR)^2\right)^{\frac{3}{2}}}\right)^2 \tag{53}$$
$$4(N+1)^9\sigma^8(1+2SNR)(1+SNR)^2$$
$$= \frac{1+2SNR}{(N+1)^3(1+SNR)^2}\left(1+\frac{SNR^2}{(1+SNR)^2}\right)$$

For high *SNR* values we have

$$\mathrm{Var}\left\{NCC_{f_1,f_2}\right\} \approx \frac{2}{(N+1)^3 SNR} \tag{54}$$

while for low *SNR* values or in the case of absence of signal we have

$$\mathrm{Var}\left\{NCC_{f_1,f_2}\right\} \approx \frac{1}{(N+1)^3} \tag{55}$$

Note that these results were obtained for the normalized cross-correlation in real-space between two independent, noisy observations of a zero-mean map. The map is supposed to be of size $(N+1)^3$ voxels, and it is assumed that neighbouring voxels in the map are uncorrelated ($\mathrm{Cov}\{f_1(\mathbf{r})f_2(\mathbf{r}), f_1(\mathbf{r}')f_2(\mathbf{r}')\} = \mathrm{Cov}\{f_i^2(\mathbf{r}), f_i^2(\mathbf{r}')\} = 0$). The SNR used in this case is the SNR of the map in real space.

The symmetry effects discussed by Orlova et al. (1997) are easily introduced in this analysis. Symmetry relationships remove variance from the map since symmetrical voxels have exactly the same value, and we may get a better estimate by averaging the symmetric coefficients. For this reason, the number of independent variables drops from $(N+1)^3$ (the total number of voxels) to $\frac{(N+1)^3}{S}$ where S is the number of repetitions of the asymmetrical unit (e.g. for C5 symmetry $S=5$, for D5 symmetry $S=10$). In particular, under these circumstances the variance of the cross correlation between two symmetrical, noisy maps in the absence of signal is

$$\mathrm{Var}\left\{NCC_{n_1,n_2}\right\} \approx \frac{S}{(N+1)^3}. \tag{56}$$

The variance of the correlation between two random maps has been used as a threshold for the FSC substituting the number of voxels $(N+1)^3$ by the number of coefficients in the Fourier shell $|\Im(R,\Delta R)|$. Then, the resolution is defined as that frequency at which the FSC drops below a certain multiple of the standard deviation of the correlation without signal. This multiple depends on the user and common values are 2 (Saxton and Baumeister, 1982; van Heel and Stoffler-Meilicke, 1985), 3 (Orlova et al., 1997) and 5 (Radermacher, 1988). Choosing such a multiple could have been avoided by using inferential statistics and the resolution could have been defined as the frequency at which the hypothesis that the observed correlation is not null or negative (one-sided hypothesis) could not be rejected. Thanks to the large number of voxels involved, Fisher's transformation of the correlation coefficient (Fisher, 1915) is rather accurate and this hypothesis is rejected for $NCC_{f_1,f_2} > NCC_{n_1,n_2,\alpha}$ being

$$NCC_{n_1,n_2,\alpha} = \tanh\left(\frac{z_{1-\alpha}}{\sqrt{(N+1)^3-3}}\right) \approx \frac{z_{1-\alpha}}{(N+1)^{\frac{3}{2}}}, \tag{57}$$

where tanh is the hyperbolic tangent, $1-\alpha$ is a confidence level (typically 95% or 99%), and $z_{1-\alpha}$ is the $1-\alpha$ percentil of a standardized Gaussian distribution. The final result is similar to the current practice of choosing a multiple of the σ curve, and still the user has to choose a free parameter (the confidence level), but she is within a most established framework. The 2σ criterion proposed by Saxton and Baumeister (1982) corresponds to a confidence level of 97.7%, the 3σ to 99.9%, and the 5σ to 99.99997%.

However, there are more fundamental objections to the use of $K\sigma$ family of thresholds for the FSC. First, this result applies to statistics with real numbers and not complex numbers; therefore, the variance is using the number of voxels in real space, and not the number of Fourier coefficients in a Fourier Shell. Second, the bandpass filter interpretation of the FSC discussed in this paper introduces a significant correlation between adjacent voxels in real-space which invalidates all the results.

In the following paragraphs, let us derive a similar result for the case of the FSC explicitly considering the filter introduced by the Fourier shell. Let the two maps in real space be written in vector form as $\mathbf{F}_1 = \mathbf{X} + \mathbf{N}_1$ and $\mathbf{F}_2 = \mathbf{X} + \mathbf{N}_2$. These two maps are the reconstructed maps before applying the filter imposed by the Fourier shell. $\mathbf{F}_1$ and $\mathbf{F}_2$ are assumed to be distributed as a multivariate normal whose mean is $\mathbf{X}$ and whose covariance matrix is $\Sigma = \sigma^2 I$, being I an identity matrix whose rank is the number of voxels in the map. Let be C the filter matrix bandpass transforming the input map $\mathbf{F}_i$, the output of the filter is therefore $\tilde{\mathbf{F}}_i = C\mathbf{F}_i$. C is the convolution matrix of an even filter (due to the Fourier shell symmetry), therefore, $C^t = C$ (Ohm, 2004). The Frobenius norm of a matrix is defined as $\|A\|^2 = \mathrm{Tr}\{A^tA\}$ where $\mathrm{Tr}\{A\}$ is the trace of matrix A and can be understood as a quantity related to the energy of filter A. $\tilde{\mathbf{F}}_i$ is distributed as a multivariate Gaussian whose mean is $C\mathbf{X}$ and whose covariance matrix is $\sigma^2 CC^t = \sigma^2 C^2$. In this notation $c = \tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_2$ and $d = \tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_1\tilde{\mathbf{F}}_2^t\tilde{\mathbf{F}}_2$ (these represent similar quantities as the c and d analyzed in Eq. (40)). Let us rederive the expected values and variances of c and d making use of the following properties for independent random vectors $\mathbf{A}$ and $\mathbf{B}$ (Brown and Rutemiller, 1977)

$$\begin{aligned}&\mathrm{E}\{\mathbf{A}^t\mathbf{B}\} = \boldsymbol{\mu}_A^t\boldsymbol{\mu}_B\\ &\mathrm{Var}\{\mathbf{A}^t\mathbf{B}\} = \boldsymbol{\mu}_A^t\Sigma_B\boldsymbol{\mu}_A + \boldsymbol{\mu}_B^t\Sigma_A\boldsymbol{\mu}_B + \mathrm{Tr}\{\Sigma_A\Sigma_B\}\end{aligned} \tag{58}$$

Applying these properties we have

$$\begin{aligned}\mathrm{E}\{c\} &= \mathrm{E}\left\{\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_2\right\} = \mathbf{X}^tC^2\mathbf{X} = \|C\mathbf{X}\|^2\\ \mathrm{Var}\{c\} &= \mathrm{Var}\left\{\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_2\right\} = 2\sigma^2\mathbf{X}^tC^2\mathbf{X} + \sigma^4\mathrm{Tr}\left\{C^4\right\}\\ &= 2\sigma^2\|C\mathbf{X}\|^2 + \sigma^4\left\|\left|C^2\right|\right\|^2\end{aligned} \tag{59}$$

Since the Fourier Shell is a binary mask in Fourier space, we can apply the same filter several times without changing the result, i.e., $C^2 = C$. If we define the SNR after applying the bandpass filter as

$$SNR_C = \frac{\|C\mathbf{X}\|^2}{\sigma^2\|C\|^2}, \tag{60}$$

then we can rewrite

$$\mathrm{Var}\{c\} = \sigma^4\|C\|^2(2SNR_C + 1). \tag{61}$$

Note that if no filter is applied $C = I$, these expressions simplify to the ones we already derived in the case of independent noise. Note that at this moment we are not saying that $SNR_C = SSNR(R,\Delta R)$, we will study this relationship in the next paragraphs.

For the mean and variance of d we need first the mean and variance of the terms $\tilde{\mathbf{F}}_i^t\tilde{\mathbf{F}}_i$. Since $\tilde{\mathbf{F}}_i$ are normally distributed, then $\tilde{\mathbf{F}}_i^t\tilde{\mathbf{F}}_i$ follows a non-central, $(N+1)^3$-dimensional Wishart distribution with non-centrality parameter $\Gamma = \mathbf{X}^t\mathbf{X}(\sigma^2C)^{-1}$, 1 degree of freedom and covariance matrix σ^2C (Timm, 2002). Its mean and variance are (Brown and Rutemiller, 1977)

$$\begin{aligned}\mathrm{E}\left\{\tilde{\mathbf{F}}_i^t\tilde{\mathbf{F}}_i\right\} &= \mathbf{X}^tC^tC\mathbf{X} + \sigma^2\mathrm{Tr}\{CC^t\} = \|C\mathbf{X}\|^2 + \sigma^2\|C\|^2\\ &= \sigma^2\|C\|^2(1+SNR_C)\\ \mathrm{Var}\left\{\tilde{\mathbf{F}}_i^t\tilde{\mathbf{F}}_i\right\} &= 4\sigma^2\mathbf{X}^tC^tCC^tC\mathbf{X} + 2\sigma^4\mathrm{Tr}\{CC^tCC^t\}\\ &= 4\sigma^2\|C\mathbf{X}\|^2 + 2\sigma^4\|C\|^2 = 2\sigma^4\|C\|^2(1+2SNR_C)\end{aligned} \tag{62}$$

Now, we can compute the mean and variance of d applying the standard rules of the variance of the product of two random variables

$$\begin{aligned}\mathrm{E}\{d\} &= \sigma^4||C||^4(1+SNR_C)^2\\ \mathrm{Var}\{d\} &= 4\sigma^8||C||^6(1+SNR_C)^2(1+2SNR_C)\end{aligned} \tag{63}$$

Let us analyze the mean of the FSC

$$\begin{aligned}\mathrm{E}\{FSC\} &= \mathrm{E}\left\{NCC_{f_1,f_2}\right\} = \mathrm{E}\left\{\frac{c}{\sqrt{d}}\right\} \approx \frac{\mathrm{E}\{c\}}{\sqrt{\mathrm{E}\{d\}}} \quad (\text{Eq.}(53))\\ &= \frac{\|C\mathbf{X}\|^2}{\sqrt{\sigma^4\|C\|^4(1+SNR_C)^2}} = \frac{||C\mathbf{X}||^2}{\sigma^2\|C\|^2(1+SNR_C)}\\ &= \frac{SNR_C}{1+SNR_C} \quad (\text{Eq.}(60))\end{aligned} \tag{64}$$

where we have made use of the results from Eqs. (53) and (60). If we now solve for SNR_C is this latter equation we have

$$SNR_C \approx \frac{\mathrm{E}\{FSC\}}{1-\mathrm{E}\{FSC\}} \tag{65}$$

We may also analyze the variance of the FSC

$$\begin{aligned}\mathrm{Var}\{FSC\} &= \mathrm{Var}\left\{NCC_{f_1,f_2}\right\} = \mathrm{Var}\left\{\frac{c}{\sqrt{d}}\right\} \approx \left(\frac{1}{\sqrt{\mathrm{E}\{d\}}}\right)^2\mathrm{Var}\{\Delta c\} + \left(\frac{\mathrm{E}\{c\}}{2(\mathrm{E}\{d\})^{\frac{3}{2}}}\right)^2\mathrm{Var}\{\Delta d\} \quad (\text{Eq.}(53))\\ &= \frac{1}{\sigma^4||C||^4(1+SNR_C)^2}\left(\sigma^4\|C\|^2(1+2SNR_C)\right) + \frac{\left(\|C\mathbf{X}\|^2\right)^2}{4\left(\sigma^4\|C\|^4(1+SNR_C)^2\right)^3}\left(4\sigma^8\|C\|^6(1+SNR_C)^2(1+2SNR_C)\right)\\ &= \frac{(1+2SNR_C)}{\|C\|^2(1+SNR_C)^2} + \frac{\|C\mathbf{X}\|^4(1+2SNR_C)}{\sigma^4||C||^6(1+SNR_C)^4} = \frac{(1+2SNR_C)}{||C||^2(1+SNR_C)^2}\left(1+\frac{\|C\mathbf{X}\|^4}{\sigma^4\|C\|^4(1+SNR_C)^2}\right)\\ &= \frac{(1+2SNR_C)}{\|C\|^2(1+SNR_C)^2}\left(1+\frac{SNR_C^2}{(1+SNR_C)^2}\right)\end{aligned} \tag{66}$$

If we now substitute SNR_C by its value as a function of E{FSC} (Eq. (65))

$$\mathrm{Var}\{FSC\} \approx \frac{1+2\frac{\mathrm{E}\{FSC\}}{1-\mathrm{E}\{FSC\}}}{\|C\|^2\left(1+\frac{\mathrm{E}\{FSC\}}{1-\mathrm{E}\{FSC\}}\right)^2}\left(1+\frac{\left(\frac{\mathrm{E}\{FSC\}}{1-\mathrm{E}\{FSC\}}\right)^2}{\left(1+\frac{\mathrm{E}\{FSC\}}{1-\mathrm{E}\{FSC\}}\right)^2}\right) = \frac{1-\mathrm{E}^4\{FSC\}}{\|C\|^2} \tag{67}$$

that is significantly different from the variance given in Penczek (2002), which was based directly on the variance of the cross-correlation coefficient between two images derived by Saxton (1978)[p. 209]; however, as we have already shown along this article real space relationships cannot be transferred to Fourier space in a straightforward way. In our derivation we have made used of a Taylor expansion of order 0, meaning that the so much advocated relationship between the FSC and the SSNR in EM holds only after an approximation of order 0 and the assumption of two independent reconstructions. Under these conditions, as we show, the variance of the FSC is rather different from the results currently used in the field.

In the previous paragraphs we have proved that Eqs. (38) and (39) extend to Fourier space as long as C is an even filter (this is true for a pointwise filter, Eq. (38), and for the Fourier shell, Eq. (39)). However, the SNR_C, Eq. (60) in the formula must be understood as the SNR of the map after filtering both, signal and noise, with the filter C. Note that the SNR_C is defined as the ratio of energy of the signal over the energy of noise. Energies are dependent on the number of voxels of the corresponding signal. This dependence is pretty obvious in the denominator ($\sigma^2 \| C\|^2$). In the case that there is no filtration, then $C = I$ and $\|C\|^2 = (N+1)^3$. If there is a filtration, then $||C||^2$ is the energy of the filter. Thanks to the orthogonality of the Fast Fourier Transform, the energy of the filter in real-space is the same as in Fourier's space (Bracewell, 1986), therefore, if the filter is binary in Fourier space (as is the case of the Fourier shell or a pointwise filter), then $\|C\|^2$ corresponds to the number of voxels within the filter.

Note that our assumptions are that the filter is binary in Fourier space and that the noise added to the map observations is Gaussian, zero-mean, independent and identically distributed over voxels. We do not assume stationarity, whiteness or zero-mean of the underlying map.

One of the most interesting features of this expression is that the variance of the cross-correlation of filtered noisy maps depend on the filter, i.e., on the exact shape of the Fourier shells. In this way, resolution thresholds based on the expected variance of noise correlations can be altered arbitrarily simply by choosing different widths of the Fourier shells. Let us analyze now the thresholds most widely used in the EM field.

One of the most widely used thresholds for the FSC is 0.5 (Harauz and van Heel, 1986; Böttcher et al., 1997) which is based on Eq. (32) and the threshold $SNR = 1$ (as much noise as signal). However, as we have discussed in this section this really corresponds to $SNR_C = 1$. Another argument for the 0.5 FSC threshold has been provided based directly on the FSC formula (Eq. (2)) (Rosenthal and Henderson, 2003). If we assume that $F_1(\mathbf{R}) = X(\mathbf{R}) + N_1(\mathbf{R})$ and $F_2(\mathbf{R}) = X(\mathbf{R}) + N_2(\mathbf{R})$. Assuming independent, zero-mean noise realizations, then it was argued (Rosenthal and Henderson, 2003) that

$$\mathrm{E}\{FSC(R,\Delta R)\} = \mathrm{E}\left\{\frac{\sum_{\mathbf{R}\in\Im(R,\Delta R)}(X(\mathbf{R})+N_1(\mathbf{R}))(X(\mathbf{R})+N_2(\mathbf{R}))^*}{\sqrt{\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left|X(\mathbf{R})+N_1(\mathbf{R})\right|^2\right)\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)}\left|X(\mathbf{R})+N_2(\mathbf{R})\right|^2\right)}}\right\} = \frac{\sum_{\mathbf{R}\in\Im(R,\Delta R)}\mathrm{E}\left\{\left|X(\mathbf{R})\right|^2\right\}}{\sum_{\mathbf{R}\in\Im(R,\Delta R)}\mathrm{E}\left\{\left|X^2(\mathbf{R})+N^2(\mathbf{R})\right|\right\}} \tag{68}$$

and that when $\mathrm{E}\{\left|X(\mathbf{R})\right|^2\} = \mathrm{E}\{\left|N(\mathbf{R})\right|^2\}$, then $FSC = 0.5$. Although the mathematical derivation of this conclusion is significantly simplified, the conclusion is correct. A more precise derivation follows:

$$\mathrm{E}\{FSC(R,\Delta R)\} \approx \frac{\mathrm{E}\{c\}}{\mathrm{E}\left\{\sqrt{d}\right\}} = \frac{\|C\mathbf{X}\|^2}{\sqrt{\sigma^4\|C\|^4(1+SNR_C)^2}} = \frac{\|C\mathbf{X}\|^2}{\sigma^2\|C\|^2(1+SNR_C)} = \frac{\|C\mathbf{X}\|^2}{\|C\mathbf{X}\|^2+\sigma^2\|C\|^2} \tag{69}$$

Assuming a white spectrum for $\mathbf{X}$ and thanks to Plancherel's theorem, we can write $\|C\mathbf{X}\|^2 = \|C\|^2\sigma_s^2$, and therefore

$$\mathrm{E}\{FSC(R,\Delta R)\} = \frac{\sigma_s^2}{\sigma_s^2+\sigma^2} \tag{70}$$

For $\sigma_s^2 = \sigma^2$, we have $\mathrm{E}\{FSC(R,\Delta R)\} = 0.5$. The most important consequence of this digression is that the 0.5 threshold used in the field is assuming that the power spectrum of the macromolecule is flat, which is clearly not the case in reality.

Rosenthal and Henderson (2003) also proposed a threshold of 1/3 to account for the fact that the FSC is normally computed from two maps, each one computed with half of the data, therefore the SNR is divided by 2 and the expected value of the FSC would be

$$\mathrm{E}\{FSC(R,\Delta R)\} \approx \frac{SNR_C^{(half)}}{1+SNR_C^{(half)}} = \frac{\frac{SNR_C^{(full)}}{2}}{1+\frac{SNR_C^{(full)}}{2}} = \frac{SNR_C^{(full)}}{2+SNR_C^{(full)}} \tag{71}$$

for $SNR_C^{(full)} = 1$ we obtain a threshold of 1/3. If we want to relate the observed FSC with the FSC that would be observed by using the full dataset, then we may use the relationship

$$\mathrm{E}\left\{FSC^{(full)}(R,\Delta R)\right\} \approx \frac{SNR_C^{(full)}}{1+SNR_C^{(full)}} \tag{72}$$

yielding

$$\mathrm{E}\{FSC(R,\Delta R)\} \approx \frac{\mathrm{E}\left\{FSC^{(full)}(R,\Delta R)\right\}}{2-\mathrm{E}\left\{FSC^{(full)}(R,\Delta R)\right\}}. \tag{73}$$

or equivalently as stated in Rosenthal and Henderson (2003).

$$\mathrm{E}\left\{FSC^{(full)}(R,\Delta R)\right\} \approx \frac{2\mathrm{E}\{FSC(R,\Delta R)\}}{1+\mathrm{E}\{FSC(R,\Delta R)\}} \tag{74}$$

An important difference between our derivation and that in Rosenthal and Henderson (2003) is that in our derivation it is clear that this relationship is only approximate (it appears as a consequence of a zeroth-order Taylor expansion), while in Rosenthal and Henderson (2003) it is presented as an accurate relationship.

The 0.143 threshold proposed by Rosenthal and Henderson (2003) is based on the correlation between the perfect (unknown) map $x(\mathbf{r})$ and the map computed from the full dataset. Again, their derivation can be made more rigorous. Assuming $f_1(\mathbf{r}) = x(\mathbf{r}) + n_1(\mathbf{r})$ and $f_2(\mathbf{r}) = x(\mathbf{r})$ and repeating our analysis for this particular case, we have

$$\mathrm{E}\{c\} = \|C\mathbf{X}\|^2$$

$$\mathrm{E}\left\{\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_1\right\} = \|C\mathbf{X}\|^2 + \frac{\sigma^2}{2}||C||^2$$

$$\mathrm{E}\left\{\tilde{\mathbf{F}}_2^t\tilde{\mathbf{F}}_2\right\} = ||C\mathbf{X}||^2$$

$$\mathrm{E}\{d\} = \left(\|C\mathbf{X}\|^2 + \frac{\sigma^2}{2}||C||^2\right)\|C\mathbf{X}\|^2$$

$$\mathrm{E}\left\{FSC_{ref}(R,\Delta R)\right\} \approx \frac{\mathrm{E}\{c\}}{\sqrt{\mathrm{E}\{d\}}} = \frac{\|C\mathbf{X}\|^2}{\sqrt{\left(\|C\mathbf{X}\|^2 + \frac{\sigma^2}{2}||C||^2\right)||C\mathbf{X}||^2}}$$

$$= \frac{1}{\sqrt{1+\frac{1}{2SNR_C}}} = \sqrt{\frac{2SNR_C}{2SNR_C+1}} \tag{75}$$

Substituting SNR_C by its relationship to the FSC between two halves (Eq. (65)), we have

$$\mathrm{E}\left\{FSC_{ref}(R,\Delta R)\right\} \approx \sqrt{\frac{2\mathrm{E}\{FSC\}}{1+\mathrm{E}\{FSC\}}} \tag{76}$$

as was also stated in Rosenthal and Henderson (2003), although in our derivation we have highlighted the approximate nature of this relationship. The FSC value between two halves that makes $\mathrm{E}\{FSC_{ref}(R,\Delta R)\} = \frac{1}{2}$ is $\mathrm{E}\{FSC\} = \frac{1}{7} \approx 0.143$.

Other thresholds have been proposed for the FSC based on information theory (van Heel and Schatz, 2005). The idea is to define the resolution as that frequency at which the Fourier coefficients carry 1 or 0.5 bits of information as defined by Shannon. Shannon's theorem on channel capacity relates the maximum channel capacity in bits to the SNR through

$$C = \log_2(1+SNR) \tag{77}$$

A channel capacity of 1 bit corresponds to $SNR = 1$ and a channel capacity of 0.5 bits corresponds to $SNR = 0.4142$. Again the user is left to the choice of an appropriate threshold. The choice $SNR = 1$ (1 bit) seems to be more natural than $SNR = 0.4142$, and there is no theoretical justification to this latter choice. Additionally, it should be noted that the analysis (van Heel and Schatz, 2005) is performed using real-valued statistics instead of complex statistics which invalidates all the mathematical derivations for the FSC in Fourier space. Finally, one of the keystones of their reasoning is

$$\mathrm{E}\{n_1(\mathbf{R})n_2(\mathbf{R})\} \approx \frac{\sigma^2}{\sqrt{|\mathfrak{S}(R,\Delta R)|}} \tag{78}$$

which is incorrect for independent random, zero-mean signals.

6. On the relationship of the FSC and the SSNR

Let us study now the relationship between the FSC and the SSNR. From Eq. (23) we see that

$$\frac{\left|\mathfrak{S}(R,\Delta R)\right|\overline{\left|X(\mathbf{R})\right|^2}}{\frac{\mathrm{E}\left\{\left|N_k(\mathbf{R})\right|^2\right\}}{K}} = K\left|\mathfrak{S}(R,\Delta R)\right|SSNR(R,\Delta R) \tag{79}$$

From where solving for $SSNR(R,\Delta R)$ we have

$$SSNR(R,\Delta R) = \frac{\overline{\left|X(\mathbf{R})\right|^2}}{\mathrm{E}\left\{\left|N_k(\mathbf{R})\right|^2\right\}} = \frac{\frac{1}{\left|\mathfrak{S}(R,\Delta R)\right|}\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)}\left|X(\mathbf{R})\right|^2}{\mathrm{E}\left\{\left|N_k(\mathbf{R})\right|^2\right\}}$$

$$= \frac{\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)}\left|X(\mathbf{R})\right|^2}{\left|\mathfrak{S}(R,\Delta R)\right|\sigma^2} = \frac{\|C\mathbf{X}\|^2}{\sigma^2||C||^2} = SNR_C^{(full)} \tag{80}$$

This result states that the $SSNR(R,\Delta R)$ can be calculated as the SNR within the binary filter C when all the data available has been used (remind that this binary filter C is the result of summing over shells as we discussed in Section 2). If two independent reconstructions are performed using two halves of the data, then the $SSNR(R,\Delta R)$ is double than the SNR of each one of the two halves

$$SSNR(R,\Delta R) = 2SNR_C \tag{81}$$

Note that this SSNR is the average SSNR along a Fourier shell before the averaging due to the 3D reconstruction takes place. When we do this averaging and K coefficients from the images are combined into a single 3D Fourier coefficient, then, the SSNR after averaging is given by $\widehat{SSNR}(R,\Delta R)$ (Eq. (25)). The expected value of the SSNR after 3D reconstruction is (see Eqs. (24) and (25))

$$\mathrm{E}\left\{\widehat{SSNR}(R,\Delta R)\right\} = \mathrm{E}\{F(R,\Delta R)-1\} = K\cdot SSNR(R,\Delta R) = 2K\cdot SNR_C \tag{82}$$

From where

$$SNR_C = \frac{\mathrm{E}\left\{\widehat{SSNR}(R,\Delta R)\right\}}{2K} \tag{83}$$

We saw in Eq. (64) that

$$\mathrm{E}\{FSC(R,\Delta R)\} \approx \frac{SNR_C}{1+SNR_C} = \frac{\mathrm{E}\left\{\widehat{SSNR}(R,\Delta R)\right\}}{2K+\mathrm{E}\left\{\widehat{SSNR}(R,\Delta R)\right\}} \tag{84}$$

In Penczek (2002) it is argued that

$$\mathrm{E}\{FSC(R,\Delta R)\} = \frac{\mathrm{E}\left\{\widehat{SSNR}(R,\Delta R)\right\}}{1+\mathrm{E}\left\{\widehat{SSNR}(R,\Delta R)\right\}}. \tag{85}$$

Note that as defined in Penczek (2002) and Unser et al. (2005), the number of coefficients averaged at each Fourier location is variable. On average, for an even angular distribution of projections, all the coefficients within a Fourier shell should get a similar amount of coefficients to be averaged and we might talk of $K(R,\Delta R)$ instead of a single K for all coefficients. However, for uneven angular distributions, this relationship is not so straightforward since each Fourier location has its own K. The problem with the equation above (Eq. (9) of Penczek (2002)) is that when the relationship between FSC and SSNR is carried over to 3D, the number of 2D Fourier coefficients from the projections affecting a single 3D Fourier coefficient is not the total number of images divided by 2, but a number that depends on each coefficient, or in a simplification, of its shell.

7. On the Fourier Neighbour Correlation

Sousa and Grigorieff (2007) introduced the interesting concept

of the Fourier Neighbour Correlation (FNC). The idea is to analyze the local correlation of neighbouring Fourier coefficients. Although similar in definition to the FSC, the FNC is different in the sense that it does not need to split the dataset in two, instead the resolution is estimated through the local correlation of Fourier coefficients. This correlation is caused by a mask in the real-space domain. Let us analyze this method here.

Let us consider a map whose Fast Fourier transform is $F(\mathbf{R})$. If we mask this map in real space, its Fourier components are circularly convolved (represented by the $\circledast$ operator) with the Fourier transform of the mask to give

$$F_M(\mathbf{R}) = F(\mathbf{R}) \circledast M(\mathbf{R}) \tag{86}$$

The FNC is defined as

$$FNC(R,\Delta R) = \frac{\sum_{\mathbf{R}\in\Im(R,\Delta R)} \sum_{\mathbf{R}'\in N(\mathbf{R})} F_M(\mathbf{R}) F_M^*(\mathbf{R}')}{\sqrt{\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)} \sum_{\mathbf{R}'\in N(\mathbf{R})} \left|F_M(\mathbf{R})\right|^2\right)\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)} \sum_{\mathbf{R}'\in N(\mathbf{R})} \left|F_M(\mathbf{R}')\right|^2\right)}}, \tag{87}$$

where $N(\mathbf{R})$ are the Fourier locations that are neighbour to a given location $\mathbf{R}$. Assuming that each Fourier location has the same number of Fourier neighbours (this number is set to 6 in Sousa and Grigorieff (2007)), we can rewrite the FNC as

$$FNC(R,\Delta R) = \frac{\sum_{\mathbf{R}\in\Im(R,\Delta R)} F_M(\mathbf{R}) \left(\sum_{\mathbf{R}'\in N(\mathbf{R})} F_M^*(\mathbf{R}')\right)}{\sqrt{|N(R)|}\sqrt{\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)} \left|F_M(\mathbf{R})\right|^2\right)\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)} \sum_{\mathbf{R}'\in N(\mathbf{R})} \left|F_M(\mathbf{R}')\right|^2\right)}}. \tag{88}$$

This is pretty similar to the FSC between the maps $F_1(\mathbf{R}) = F_M(\mathbf{R})$ and $F_2(\mathbf{R}) = \sum_{\mathbf{R}'\in N(\mathbf{R})} F_M(\mathbf{R}')$. However, the FNC is not an FSC since the denominator uses $\sum_{\mathbf{R}\in\Im(R,\Delta R)} \sum_{\mathbf{R}'\in N(\mathbf{R})} \left|F_M(\mathbf{R}')\right|^2$ which is not $\sum_{\mathbf{R}\in\Im(R,\Delta R)} \left|\sum_{\mathbf{R}'\in N(\mathbf{R})} F_M(\mathbf{R}')\right|^2$ that is what is needed for the FSC. Assuming that within a thin shell $\left|F_M(\mathbf{R}')\right|^2 \approx \left|F_M(\mathbf{R})\right|^2$, we have that

$$\sum_{\mathbf{R}\in\Im(R,\Delta R)} \sum_{\mathbf{R}'\in N(\mathbf{R})} \left|F_M\left(\mathbf{R}'\right)\right|^2 \approx \left|N(R)\right| \sum_{\mathbf{R}\in\Im(R,\Delta R)} \left|F_M(\mathbf{R})\right|^2. \tag{89}$$

Then, we can rewrite again the FNC as

$$FNC(R,\Delta R) \approx \frac{\sum_{\mathbf{R}\in\Im(R,\Delta R)} F_M(\mathbf{R}) \left(\frac{1}{|N(R)|}\sum_{\mathbf{R}'\in N(\mathbf{R})} F_M^*(\mathbf{R}')\right)}{\sqrt{\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)} \left|F_M(\mathbf{R})\right|^2\right)\left(\sum_{\mathbf{R}\in\Im(R,\Delta R)} \left|F_M(\mathbf{R})\right|^2\right)}} \tag{90}$$

that is approximately the FSC between $F_M(\mathbf{R})$ and a map where each Fourier coefficient has been substituted by a local average of its neighbours.

We can now perform an analysis similar to the one we performed in the case of the FSC by expressing the maps involved as vectors. Let $\mathbf{F}$ be the vector of voxel values in the original observed map in real space. Let $\mathbf{F}_M$ the masked map. Note that we can obtain $\mathbf{F}_M$ from $\mathbf{F}$ by a simple multiplication by a diagonal matrix M representing the mask $\mathbf{F}_M = M\mathbf{F}$ (the elements of this matrix are either 0 or 1 depending on whether a given voxel is masked out or not). The first map involved in the FSC is simply $\mathbf{F}_1 = \mathbf{F}_M$. We observe that the second map is a local average in Fourier space, which can be achieved by the convolution with an averaging kernel. Convolving in Fourier space is equivalent to multiplying by a mask in real-space. Therefore, $\mathbf{F}_2 = A\mathbf{F}_M$ where A is a diagonal matrix representing the mask in real-space.

If we now assume that $\mathbf{F}$ is normally distributed with mean $\mathbf{X}$ and covariance matrix $\sigma^2 I$, then $\mathbf{F}_1$ is normally distributed with mean $M\mathbf{X}$ and covariance matrix $\sigma^2 MM^t = \sigma^2 M$. Similarly, $\mathbf{F}_2$ is normally distributed with mean $AM\mathbf{X}$ and covariance matrix $\sigma^2 AMM^t A^t = \sigma^2 A^2 M$. After filtering through the Fourier shells, we have that $\tilde{\mathbf{F}}_1 = C\mathbf{F}_1$ is normally distributed with mean equal to $M\mathbf{X}$ and covariance matrix $\sigma^2 CMC^t = \sigma^2 CMC$, while $\tilde{\mathbf{F}}_2 = C\mathbf{F}_2$ is normally distributed with mean equal to $CAM\mathbf{X}$ and covariance matrix $\sigma^2 CA^2MC^t = \sigma^2 CA^2MC$.

The expected value of the numerator $\tilde{\mathbf{F}}_1^t \tilde{\mathbf{F}}_2$ is

$$\begin{aligned}\mathrm{E}\left\{\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_2\right\} &= \mathrm{E}\left\{(CM(\mathbf{X}+\mathbf{N}))^t(CAM(\mathbf{X}+\mathbf{N}))\right\}\\ &= \mathbf{X}^tM^tC^tCAM\mathbf{X} + \mathrm{E}\{\mathbf{N}^tM^tC^tCAM\mathbf{N}\}\\ &= \mathbf{X}^tMCAM\mathbf{X} + \mathrm{E}\{\mathbf{N}^tMCAM\mathbf{N}\}\\ &= ||\mathbf{X}||^2_{MCAM} + \sigma^2\mathrm{Tr}\{MCAM\},\end{aligned} \tag{91}$$

where $||\mathbf{X}||^2_W = \mathbf{X}^tW\mathbf{X}$ denotes the Euclidean-weighted norm with weight matrix W.

For the mean of the denominator of the FNC we need the mean of $\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_1$. Since $\tilde{\mathbf{F}}_1$ is normally distributed, then $\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_1$ follows a non-central, $(N+1)^3$-dimensional Wishart distribution with non-centrality parameter $\Gamma = \mathbf{X}^tM\mathbf{X}(\sigma^2CMC)^{-1}$, 1 degree of freedom and covariance matrix σ^2CMC (Timm, 2002). Its mean is (Brown and Rutemiller, 1977)

$$\mathrm{E}\left\{\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_1\right\} = \mathbf{X}^tM^tC^tCM\mathbf{X} + \sigma^2\mathrm{Tr}\{CMC\} = ||\mathbf{X}||^2_{MCM} + \sigma^2||CM||^2 \tag{92}$$

Finally, the expected value of the FNC is

$$\mathrm{E}\{FNC(R,\Delta R)\} = \frac{\mathrm{E}\left\{\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_2\right\}}{\mathrm{E}\left\{\tilde{\mathbf{F}}_1^t\tilde{\mathbf{F}}_1\right\}} = \frac{||\mathbf{X}||^2_{MCAM} + \sigma^2\mathrm{Tr}\{MCAM\}}{||\mathbf{X}||^2_{MCM} + \sigma^2\mathrm{Tr}\{MCM\}} \tag{93}$$

As we can see, it is difficult to relate this quantity to a SNR since the measure functions used in the numerator and denominator are different due to the matrix A (the local averaging operator in Fourier space).

Sousa and Grigorieff (2007) define FNC_F as the FNC when there is no noise and FNC_N as the FNC when there is no signal. By simply, substituting we get the expected values at each frequency

$$\begin{aligned}FNC_F(R,\Delta R) &= \frac{||\mathbf{X}||^2_{MCAM}}{||\mathbf{X}||^2_{MCM}}\\ FNC_N(R,\Delta R) &= \frac{\mathrm{Tr}\{MCAM\}}{\mathrm{Tr}\{MCM\}}.\end{aligned} \tag{94}$$

As expected, they depend on the averaging operator, the mask and the shape of the Fourier shell. We have been unable to justify the relationship given in Sousa and Grigorieff (2007).

$$FNC(R,\Delta R) \approx FNC_F(R,\Delta R)\sqrt{\frac{SNR(R,\Delta R)SNR'(R,\Delta R) + FNC_N(R,\Delta R)}{(SNR(R,\Delta R)+1)(SNR'(R,\Delta R)+1)}} \tag{95}$$

being $SNR'(R,\Delta R)$ the SNR of a Fourier shell as estimated by the neighbours of the coefficients in that shell.

8. On the distribution of the DPR

The differential phase residual (DPR) has also been introduced in the field (Frank et al., 1981; Penczek et al., 1994) as a measure of resolution. The resolution is defined as the frequency at which the differential phase residual goes above 45°. In this section we will explore the meaning and distribution of the DPR.

Let us consider two independent, noisy observations of the same map in Fourier space: $F_1(\mathbf{R}) = X(\mathbf{R}) + N_1(\mathbf{R})$ and $F_2(\mathbf{R}) = X(\mathbf{R}) + N_2(\mathbf{R})$. The DPR is defined as

$$\begin{aligned}DPR(R,\Delta R) &= \sqrt{\frac{\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)}(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\measuredangle\{F_1(\mathbf{R}),F_2(\mathbf{R})\})^2}{\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)}(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)}}\\ &= \sqrt{\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)} w(\mathbf{R})(\measuredangle\{F_1(\mathbf{R}),F_2(\mathbf{R})\})^2},\end{aligned} \tag{96}$$

where $\measuredangle\{A,B\}$ is the angle between the complex numbers A and B and $w(\mathbf{R}) = \frac{|F_1(\mathbf{R})|+|F_2(\mathbf{R})|}{\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)}(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)}$ is a weight defined for each location. Note that $\sum_{\mathbf{R}\in\mathfrak{S}(R,\Delta R)} w(\mathbf{R}) = 1$, so the DPR is a squared weighted average of the angle difference between $F_1(\mathbf{R})$ and $F_2(\mathbf{R})$ at each Fourier coefficient within a shell. In the absence of noise, the phase difference between these two complex numbers is 0°. In the absence of signal, assuming that $N_1(\mathbf{R})$ and $N_2(\mathbf{R})$ are Gaussian, randomly distributed, then the phase difference is uniformly distributed within −180° and 180°.

Let us study the mean and variance of $\measuredangle\{F_1(\mathbf{R}),F_2(\mathbf{R})\} = \measuredangle\{F_1(\mathbf{R})\} - \measuredangle\{F_2(\mathbf{R})\}$ (for simplifying the notation let us rewrite this phase difference as $\Delta\theta_{12}(\mathbf{R}) = \theta_1(\mathbf{R}) - \theta_2(\mathbf{R})$). For this we need the distribution of

$$\theta_i(\mathbf{R}) = \tan^{-1}\frac{\mathrm{Im}\{X(\mathbf{R})\}+\mathrm{Im}\{N_i(\mathbf{R})\}}{\mathrm{Re}\{X(\mathbf{R})\}+\mathrm{Re}\{N_i(\mathbf{R})\}}. \tag{97}$$

This problem is known in communications as the one of a sinusoidal corrupted by narrow-band gaussian noise (Lathi, 1998) and its solution gives a probability density function for the phase difference between $F_i(\mathbf{R})$ and $X(\mathbf{R})$, $\Delta\theta_i(\mathbf{R}) = \measuredangle\{F_i(\mathbf{R})\} - \measuredangle\{X(\mathbf{R})\}$,

$$p_{\Delta\theta_i(\mathbf{R})}(\Delta\theta_i(\mathbf{R})) = \frac{1}{2\pi}e^{-\frac{|X(\mathbf{R})|^2}{\sigma^2}}\left(1 + 2\frac{|X(\mathbf{R})|}{\sigma}\sqrt{\pi}\cos(\Delta\theta_i(\mathbf{R}))e^{\frac{|X(\mathbf{R})|^2\cos^2(\Delta\theta_i(\mathbf{R}))}{\sigma^2}}Q\left(\frac{\sqrt{2}|X(\mathbf{R})|\cos(\Delta\theta_i(\mathbf{R}))}{\sigma}\right)\right) \tag{98}$$

being $\Delta\theta_i(\mathbf{R})\in[-\pi,\pi]$ and $Q(x) = \frac{1}{\sqrt{2\pi}}\int_{-\infty}^{x}e^{-\frac{\tau^2}{2}}d\tau$. The statistical moments of this distribution do not yield an easy analytical formula (although can be numerically evaluated), and in this paper we will study its distribution in two limiting cases: no signal and high SNR.

When there is no signal $|X(\mathbf{R})| = 0$, this distribution simplifies to

$$p_{\Delta\theta_i(\mathbf{R})}(\Delta\theta_i(\mathbf{R})) = \frac{1}{2\pi} \tag{99}$$

i.e., a continuous uniform distribution whose mean and variance are

$$\begin{aligned}&\mathrm{E}\{\theta_i(\mathbf{R})\}=\mathrm{E}\{\Delta\theta_i(\mathbf{R})\}=0\\&\mathrm{Var}\{\theta_i(\mathbf{R})\}=\mathrm{Var}\{\Delta\theta_i(\mathbf{R})\}=\frac{\pi^2}{3}.\end{aligned}\tag{100}$$

When the SNR is high $|X(\mathbf{R})|\gg\sigma$, the distribution simplifies to

$$p_{\Delta\theta_i(\mathbf{R})}(\Delta\theta_i(\mathbf{R}))=\frac{1}{\sqrt{\pi}}\frac{|X(\mathbf{R})|}{\sigma}\cos(\Delta\theta_i(\mathbf{R}))e^{-\frac{|X(\mathbf{R})|^2}{\sigma^2}\left(1-\cos^2(\Delta\theta_i(\mathbf{R}))\right)}.\tag{101}$$

Taking into account that $\Delta\theta_i(\mathbf{R})$ is small (since the signal is much larger than the noise), then

$$\begin{aligned}p_{\Delta\theta_i(\mathbf{R})}(\Delta\theta_i(\mathbf{R}))&\approx\frac{1}{\sqrt{\pi}}\frac{|X(\mathbf{R})|}{\sigma}e^{-\frac{|X(\mathbf{R})|^2}{\sigma^2}(\Delta\theta_i(\mathbf{R}))^2}\\&=\frac{1}{\sqrt{2\pi}}\frac{\sigma}{\sqrt{2}|X(\mathbf{R})|}e^{-\frac{1}{2}\left(\frac{\Delta\theta_i(\mathbf{R})}{\frac{\sigma}{\sqrt{2}|X(\mathbf{R})|}}\right)^2},\end{aligned}\tag{102}$$

i.e., the phase noise is approximately normally distributed. Its mean and variance are

$$\begin{aligned}&\mathrm{E}\{\Delta\theta_i(\mathbf{R})\}=0\\&\mathrm{Var}\{\Delta\theta_i(\mathbf{R})\}=\frac{\sigma^2}{2|X(\mathbf{R})|^2}=\frac{1}{2SNR(\mathbf{R})}.\end{aligned}\tag{103}$$

and those of $\theta_i(\mathbf{R})$

$$\begin{aligned}&\mathrm{E}\{\theta_i(\mathbf{R})\}=\mathrm{E}\{\measuredangle\{X(\mathbf{R})\}+\Delta\theta_i(\mathbf{R})\}=\measuredangle\{X(\mathbf{R})\}\\&\mathrm{Var}\{\theta_i(\mathbf{R})\}=\mathrm{Var}\{\Delta\theta_i(\mathbf{R})\}=\frac{1}{2SNR(\mathbf{R})}.\end{aligned}\tag{104}$$

We can now proceed with the distribution of $\Delta\theta_{12}(\mathbf{R})$. In the case of no signal, this difference is again uniformly distributed between $-\pi$ and π and its mean and variance are already computed in Eq. (100). In the case of large SNR and assuming independent noise observations, it gives a normal distribution with parameters

$$\begin{aligned}&\mathrm{E}\{\Delta\theta_{12}(\mathbf{R})\}=0\\&\mathrm{Var}\{\Delta\theta_{12}(\mathbf{R})\}=\frac{1}{SNR(\mathbf{R})}.\end{aligned}\tag{105}$$

Finally, the distribution of $(\Delta\theta_{12}(\mathbf{R}))^2$ in the case of no signal is the distribution of the squared of a uniform variable. $(\Delta\theta_{12}(\mathbf{R}))^2$ is distributed between 0 and π^2 and its probability density function is

$$p_{(\Delta\theta_{12}(\mathbf{R}))^2}(x)=\frac{1}{2\pi}\frac{1}{\sqrt{x}}.\tag{106}$$

Its mean and variance are

$$\begin{aligned}&\mathrm{E}\left\{(\Delta\theta_{12}(\mathbf{R}))^2\right\}=\frac{\pi^2}{3}\\&\mathrm{Var}\left\{(\Delta\theta_{12}(\mathbf{R}))^2\right\}=\frac{4\pi^4}{45}.\end{aligned}\tag{107}$$

In the case of large SNR, $SNR(\mathbf{R})(\Delta\theta_{12}(\mathbf{R}))^2$ is distributed as χ^2 with one degree of freedom (whose mean and variance are 1 and 2, respectively), therefore

$$\begin{aligned}&\mathrm{E}\left\{(\Delta\theta_{12}(\mathbf{R}))^2\right\}=\frac{1}{SNR(\mathbf{R})}\\&\mathrm{Var}\left\{(\Delta\theta_{12}(\mathbf{R}))^2\right\}=\frac{2}{SNR^2(\mathbf{R})}.\end{aligned}\tag{108}$$

We next need the distribution of the terms $|F_i(\mathbf{R})|$. This distribution is well known in Statistics and is called Rice distribution (Lathi, 1998). In our case its parameters are $|X(\mathbf{R})|$ and $\sigma^2/2$. The mean and variance of $|F_i(\mathbf{R})|$ are

$$\begin{aligned}&\mathrm{E}\{|F_i(\mathbf{R})|\}=\frac{\sigma\sqrt{\pi}}{2}L_{\frac{1}{2}}(-SNR(\mathbf{R}))\\&\mathrm{Var}\{|F_i(\mathbf{R})|\}=\sigma^2\left(1+SNR(\mathbf{R})-\frac{\pi}{4}L_{\frac{1}{2}}^2(-SNR(\mathbf{R}))\right),\end{aligned}\tag{109}$$

where $L_{\frac{1}{2}}(x)$ is the Laguerre polynomial of degree ½. In case of no signal $SNR(\mathbf{R})=0$ and $L_{\frac{1}{2}}(0)=1$, and the Rice distribution is called Rayleigh distribution.

The distribution of $|F_1(\mathbf{R})|+|F_2(\mathbf{R})|$ has no specific name and its probability density function is the convolution of two Rice distributions. We may compute its mean and variance using the properties in Eq. (41) and assuming independence between the two noisy observations

$$\begin{aligned}&\mathrm{E}\{|F_1(\mathbf{R})|+|F_2(\mathbf{R})|\}=\sigma\sqrt{\pi}L_{\frac{1}{2}}(-SNR(\mathbf{R}))\\&\mathrm{Var}\{|F_1(\mathbf{R})|+|F_2(\mathbf{R})|\}=2\sigma^2\left(1+SNR(\mathbf{R})-\frac{\pi}{4}L_{\frac{1}{2}}^2(-SNR(\mathbf{R}))\right).\end{aligned}\tag{110}$$

Let us now compute the mean of the terms $(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2$. For this we make use of the fact that $|F_1(\mathbf{R})|+|F_2(\mathbf{R})|$ and $(\Delta\theta_{12}(\mathbf{R}))^2$ are independent of each other

$$\begin{aligned}&\mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\}\\&=\mathrm{E}\{|F_1(\mathbf{R})|+|F_2(\mathbf{R})|\}\mathrm{E}\left\{(\Delta\theta_1(\mathbf{R}))^2\right\}\end{aligned}\tag{111}$$

In the case of no signal this gives

$$\mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\}=2\frac{\sigma\sqrt{\pi}}{2}\frac{\pi^2}{3}=\frac{\sigma\pi^2\sqrt{\pi}}{3}\tag{112}$$

While in case of large SNR

$$\mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\}=\sigma\sqrt{\pi}L_{\frac{1}{2}}(-SNR(\mathbf{R}))\frac{1}{SNR(\mathbf{R})}\tag{113}$$

Let us now compute the variance of the terms $(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2$.

$$\begin{aligned}&\mathrm{Var}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\}\\&=\mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)^2(\Delta\theta_{12}(\mathbf{R}))^4\right\}-\mathrm{E}^2\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|\right.\\&\left.\times)(\Delta\theta_{12}(\mathbf{R}))^2\right\}\end{aligned}\tag{114}$$

The module of $F_i(\mathbf{R})$ and its phase are independent, therefore, in the case there is no signal present we get

$$\mathrm{Var}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\} = \mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)^2\right\}\mathrm{E}\left\{(\Delta\theta_{12}(\mathbf{R}))^4\right\} - \left(\frac{\sigma\pi^2\sqrt{\pi}}{3}\right)^2 \tag{115}$$

On one side we have

$$\mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)^2\right\} = \mathrm{E}^2\{|F_1(\mathbf{R})|+|F_2(\mathbf{R})|\} + \mathrm{Var}\{|F_1(\mathbf{R})|+|F_2(\mathbf{R})|\} = (\sigma\sqrt{\pi})^2 + 2\sigma^2\left(1-\frac{\pi}{4}\right) = \sigma^2\left(2+\frac{\pi}{2}\right) \tag{116}$$

On the other side, $\Delta\theta_{12}(\mathbf{R})$ is uniformly distributed between $-\pi$ and π, thus, $\mathrm{E}\{(\Delta\theta_{12}(\mathbf{R}))^4\}$ is the fourth moment of the uniform distribution

$$\mathrm{E}\left\{(\Delta\theta_{12}(\mathbf{R}))^4\right\} = \frac{\pi^4}{5} \tag{117}$$

Summarizing

$$\mathrm{Var}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\} = \sigma^2\left(2+\frac{\pi}{2}\right)\frac{\pi^4}{5} - \left(\frac{\sigma\pi^2\sqrt{\pi}}{3}\right)^2 = \sigma^2\pi^4\left(\frac{2}{5}-\frac{\pi}{90}\right) \tag{118}$$

Repeating the same analysis with large SNR

$$\mathrm{Var}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\} = \mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)^2\right\}\mathrm{E}\left\{(\Delta\theta_{12}(\mathbf{R}))^4\right\} - \left(\sigma\sqrt{\pi}L_{\frac{1}{2}}(-SNR(\mathbf{R}))\frac{1}{SNR(\mathbf{R})}\right) \tag{119}$$

On one side we have

$$\mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)^2\right\} = \mathrm{E}^2\{|F_1(\mathbf{R})|+|F_2(\mathbf{R})|\} + \mathrm{Var}\{|F_1(\mathbf{R})|+|F_2(\mathbf{R})|\} = \left(\sigma\sqrt{\pi}L_{\frac{1}{2}}(-SNR(\mathbf{R}))\right)^2 + 2\sigma^2\left(1+SNR(\mathbf{R})-\frac{\pi}{4}L^2_{\frac{1}{2}}(-SNR(\mathbf{R}))\right) = \sigma^2\left(2+2SNR(\mathbf{R})+\frac{3\pi}{4}L^2_{\frac{1}{2}}(-SNR(\mathbf{R}))\right) \tag{120}$$

On the other side,

$$\mathrm{E}\left\{(\Delta\theta_{12}(\mathbf{R}))^4\right\} = \mathrm{E}^2\left\{(\Delta\theta_{12}(\mathbf{R}))^2\right\} + \mathrm{Var}\left\{(\Delta\theta_{12}(\mathbf{R}))^2\right\} = \frac{1}{SNR^2(\mathbf{R})} + \frac{2}{SNR^2(\mathbf{R})} = \frac{3}{SNR^2(\mathbf{R})} \tag{121}$$

Summarizing

$$\mathrm{Var}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\} = \sigma^2\left(2+2SNR(\mathbf{R})+\frac{3\pi}{4}L^2_{\frac{1}{2}}(-SNR(\mathbf{R}))\right)\frac{3}{SNR^2(\mathbf{R})} - \left(\sigma\sqrt{\pi}L_{\frac{1}{2}}(-SNR(\mathbf{R}))\frac{1}{SNR(\mathbf{R})}\right)^2 = \frac{\sigma^2}{SNR^2(\mathbf{R})}\left(6+6SNR(\mathbf{R})-\frac{5\pi}{4}L^2_{\frac{1}{2}}(-SNR(\mathbf{R}))\right) \tag{122}$$

We now turn our attention to the numerator of the DPR (for notation simplification, let us call it c)

$$c(R,\Delta R) = \sum_{\mathbf{R}\in\circledS(R,\Delta R)}(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\measuredangle\{F_1(\mathbf{R}),F_2(\mathbf{R})\})^2. \tag{123}$$

It consists of the summation of $|\circledS(R,\Delta R)|$ random variables whose mean and variance have been derived above. We will assume a constant SNR within each shell. Then, for a sufficiently large number of variables the central limit theorem is applicable (in EM the number of Fourier coefficients in each shell is large enough for this purpose). This theorem states that c is distributed as a normal variable whose mean and variance are

$$\begin{aligned}\mathrm{E}\{c(R,\Delta R)\} &= \left|\circledS(R,\Delta R)\right|\mathrm{E}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\}\\ \mathrm{Var}\{c(R,\Delta R)\} &= \left|\circledS(R,\Delta R)\right|\mathrm{Var}\left\{(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|)(\Delta\theta_{12}(\mathbf{R}))^2\right\}\end{aligned} \tag{124}$$

That is

$$\begin{aligned}\mathrm{E}\{c(R,\Delta R)\} &= \left|\circledS(R,\Delta R)\right|\frac{\sigma\pi^2\sqrt{\pi}}{3}\\ \mathrm{Var}\{c(R,\Delta R)\} &= \left|\circledS(R,\Delta R)\right|\sigma^2\pi^4\left(\frac{2}{5}-\frac{\pi}{90}\right)\end{aligned} \tag{125}$$

for the case without signal and

$$\begin{aligned}\mathrm{E}\{c(R,\Delta R)\}&=\left|\Im(R,\Delta R)\right|\frac{\sigma\sqrt{\pi}}{SNR(R)}L_{\frac{1}{2}}(-SNR(R))\\ \mathrm{Var}\{c(R,\Delta R)\}&=\left|\Im(R,\Delta R)\right|\frac{\sigma^2}{SNR^2(R)}\left(6+6SNR(R)-\frac{5\pi}{4}L_{\frac{1}{2}}^2(-SNR(R))\right)\end{aligned} \tag{126}$$

for the case with large SNR.

The denominator of the DPR is

$$d(R,\Delta R)=\sum_{\mathbf{R}\in\Im(R,\Delta R)}(|F_1(\mathbf{R})|+|F_2(\mathbf{R})|), \tag{127}$$

and the central limit theorem can also be applied yielding

$$\begin{aligned}\mathrm{E}\{d(R,\Delta R)\}&=|\Im(R,\Delta R)|\sigma\sqrt{\pi}\\ \mathrm{Var}\{d(R,\Delta R)\}&=\left|\Im(R,\Delta R)\right|2\sigma^2\left(1-\frac{\pi}{4}\right)\end{aligned} \tag{128}$$

for the case without signal and

$$\begin{aligned}\mathrm{E}\{d(R,\Delta R)\}&=\left|\Im(R,\Delta R)\right|\sigma\sqrt{\pi}L_{\frac{1}{2}}(-SNR(R))\\ \mathrm{Var}\{d(R,\Delta R)\}&=\left|\Im(R,\Delta R)\right|2\sigma^2\left(1+SNR(R)-\frac{\pi}{4}L_{\frac{1}{2}}^2(-SNR(R))\right)\end{aligned} \tag{129}$$

for the case with large SNR.

To determine the mean and variance of the DPR we expand it in Taylor series of first order around the means of c and d

$$\begin{aligned}DPR(R,\Delta R)&=\sqrt{\frac{c(R,\Delta R)}{d(R,\Delta R)}}\\ &=\sqrt{\frac{\mathrm{E}\{c(R,\Delta R)\}}{\mathrm{E}\{d(R,\Delta R)\}}}\\ &\quad+\frac{1}{2}\frac{1}{\sqrt{\mathrm{E}\{c(R,\Delta R)\}\mathrm{E}\{d(R,\Delta R)\}}}\Delta c(R,\Delta R)\\ &\quad-\frac{1}{2}\sqrt{\frac{\mathrm{E}\{c(R,\Delta R)\}}{\mathrm{E}\{d(R,\Delta R)\}}}\frac{1}{\mathrm{E}\{d(R,\Delta R)\}}\Delta d(R,\Delta R).\end{aligned} \tag{130}$$

From this we can easily determine that

$$\begin{aligned}\mathrm{E}\{DPR(R,\Delta R)\}&=\sqrt{\frac{\mathrm{E}\{c(R,\Delta R)\}}{\mathrm{E}\{d(R,\Delta R)\}}}\\ \mathrm{Var}\{DPR(R,\Delta R)\}&=\frac{1}{4}\frac{1}{\mathrm{E}\{c(R,\Delta R)\}\mathrm{E}\{d(R,\Delta R)\}}\mathrm{Var}\{c(R,\Delta R)\}+\frac{1}{4}\frac{\mathrm{E}\{c(R,\Delta R)\}}{\mathrm{E}^3\{d(R,\Delta R)\}}\mathrm{Var}\{d(R,\Delta R)\}.\end{aligned} \tag{131}$$

In the case of no signal we get

$$\mathrm{E}\{DPR(R,\Delta R)\}=\sqrt{\frac{\left|\Im(R,\Delta R)\right|\frac{\sigma\pi^2\sqrt{\pi}}{3}}{|\Im(R,\Delta R)|\sigma\sqrt{\pi}}}=\frac{\pi}{\sqrt{3}}$$

$$\begin{aligned}\mathrm{Var}\{DPR(R,\Delta R)\}&=\frac{1}{4}\frac{1}{\left|\Im(R,\Delta R)\right|\frac{\sigma\pi^2\sqrt{\pi}}{3}\left|\Im(R,\Delta R)\right|\sigma\sqrt{\pi}}\left|\Im(R,\Delta R)\right|\sigma^2\pi^4\left(\frac{2}{5}-\frac{\pi}{90}\right)+\frac{1}{4}\frac{\left|\Im(R,\Delta R)\right|\frac{\sigma\pi^2\sqrt{\pi}}{3}}{\left(|\Im(R,\Delta R)|\sigma\sqrt{\pi}\right)^3}\left|\Im(R,\Delta R)\right|2\sigma^2\left(1-\frac{\pi}{4}\right)\\ &=\frac{\pi}{20|\Im(R,\Delta R)|}\left(\frac{28}{3}-\pi\right).\end{aligned} \tag{132}$$

Interestingly the mean expected value in the absence of signal is 103.9° and not 90° as argued in Unser et al. (1987). Experimentally this mean of 103.9° and the variance decreasing with the shell radius were observed in de la Fraga et al. (1995).

In case of large SNR

$$\mathrm{E}\{DPR(R,\Delta R)\} = \sqrt{\frac{\left|\mathfrak{S}(R,\Delta R)\right|\frac{\sigma\sqrt{\pi}}{SNR(R)}L_{\frac{1}{2}}(-SNR(R))}{\left|\mathfrak{S}(R,\Delta R)\right|\sigma\sqrt{\pi}L_{\frac{1}{2}}(-SNR(R))}} = \frac{1}{\sqrt{SNR(R)}}$$

$$\begin{aligned}\mathrm{Var}\{DPR(R,\Delta R)\} &= \frac{1}{4}\frac{\left|\mathfrak{S}(R,\Delta R)\right|\frac{\sigma^2}{SNR^2(R)}\left(6+6SNR(R)-\frac{5\pi}{4}L_{\frac{1}{2}}^2(-SNR(R))\right)}{\left|\mathfrak{S}(R,\Delta R)\right|\frac{\sigma\sqrt{\pi}}{SNR(R)}L_{\frac{1}{2}}(-SNR(R))\left|\mathfrak{S}(R,\Delta R)\right|\sigma\sqrt{\pi}L_{\frac{1}{2}}(-SNR(R))} \\ &+\frac{1}{4}\frac{\left|\mathfrak{S}(R,\Delta R)\right|\frac{\sigma\sqrt{\pi}}{SNR(R)}L_{\frac{1}{2}}(-SNR(R))\left|\mathfrak{S}(R,\Delta R)\right|2\sigma^2\left(1+SNR(R)-\frac{\pi}{4}L_{\frac{1}{2}}^2(-SNR(R))\right)}{\left(\left|\mathfrak{S}(R,\Delta R)\right|\sigma\sqrt{\pi}\,L_{\frac{1}{2}}(-SNR(R))\right)^3} \\ &= \frac{8+8SNR(R)-\frac{3\pi}{4}L_{\frac{1}{2}}^2(-SNR(R))}{4\pi\left|\mathfrak{S}(R,\Delta R)\right|SNR(R)L_{\frac{1}{2}}^2(-SNR(R))}\end{aligned} \tag{133}$$

For setting the DPR threshold one is tempted of setting it for $SNR(R)=1$ as $\mathrm{E}\{DPR(R,\Delta R)\}=\frac{1}{\sqrt{SNR(R)}}=1(\mathrm{rad})=57.2°$. Interestingly, this value is not so far from the generally accepted threshold at 45° (Frank, 2006) although this latter threshold was intuitively defined upon the behaviour of two sine waves. However, the expectation of the DPR in the presence of signal was derived under the assumption of large SNR, and $SNR(R)=1$ does not meet this condition. Alternatively, we could set the threshold based on hypothesis testing and the distribution of the DPR in the absence of signal. The distribution of the DPR in this case is unknown, although its first two moments (mean and variance) have been derived. We may use Chebyshev's inequality which is valid for any distribution

$$\Pr\left\{\left|DPR(R,\Delta R)-\mathrm{E}\{DPR(R,\Delta R)\}\right| \geq \sqrt{\frac{\mathrm{Var}\{DPR(R,\Delta R)\}}{\alpha}}\right\} \leq \alpha \tag{134}$$

Therefore, we would reject the hypothesis that there is no signal if

$$DPR(R,\Delta R) \geq \frac{\pi}{\sqrt{3}} - \sqrt{\frac{\pi}{20|\mathfrak{S}(R,\Delta R)|\alpha}\left(\frac{28}{3}-\pi\right)}, \tag{135}$$

that is the threshold depends on the number of Fourier coefficients in the shell and the confidence level, and it is expected to be lower than 103.9°. Interestingly, the DPR equivalent of the 2σ threshold for the FSC (we already saw that this threshold is very much related to hypothesis testing on the FSC) is about 85° (Radermacher, 1988; de la Fraga et al., 1995).

9. On the effects of alignment on resolution measures

Grigorieff (2000) pointed out an interesting effect on the resolution measures when maps (or images) are aligned before measuring the resolution. Let us assume that we observe two noisy maps without any signal inside. We already saw (Eq. (55)) that the correlation coefficient is distributed as a zero-mean Gaussian with variance

$$\mathrm{Var}\left\{NCC_{f_1,f_2}\right\} \approx \frac{1}{(N+1)^3} \tag{136}$$

The alignment explores a list of S possible shifts looking for the best correlation coefficient. For simplicity let us consider the case of cyclic shifts (whatever comes out on one side due to the shift comes in from the other side; this is normally done for avoiding border effects and does not modify the statistics of the correlation coefficient since the number of voxels being compared remains the same). The problem is that, even if the cross correlation coefficient is centered around 0, drawing S samples of this distribution and taking its maximum may yield a relatively large value giving the false impression of high-correlation. This is known in Statistics as the distribution of the extreme values.

In Grigorieff (2000) it is argued that the asymptotic mean of the maximum cross-correlation is

$$\mathrm{E}\left\{\max_{\mathbf{s}\in\mathcal{S}}\left\{NCC_{f_1(\mathbf{r}),f_2(\mathbf{r}+\mathbf{s})}\right\}\right\} \approx \sqrt{\mathrm{Var}\left\{NCC_{f_1,f_2}\right\}}\sqrt{2\log(|\mathcal{S}|)}, \tag{137}$$

where $\mathbf{r}\in\mathbb{R}^3$ is the spatial location within the map, $\mathbf{s}\in\mathbb{R}^3$ is the spatial shift, $\mathcal{S}$ is the set of possible shifts, and $|\mathcal{S}|$ is the number of shifts in that set. The correct asymptotic mean is (Johnson et al., 1994)

$$\mathrm{E}\left\{\max_{\mathbf{s}\in\mathcal{S}}\left\{NCC_{f_1(\mathbf{r}),f_2(\mathbf{r}+\mathbf{s})}\right\}\right\} \approx \sqrt{\mathrm{Var}\left\{NCC_{f_1,f_2}\right\}} \left(\sqrt{2\log(|\mathcal{S}|)} - \frac{\log(\log(|\mathcal{S}|)) + \log(4\pi) - 1.1544}{2\sqrt{2\log(|\mathcal{S}|)}}\right), \tag{138}$$

and its variance

$$\mathrm{Var}\left\{\max_{\mathbf{s}\in\mathcal{S}}\left\{NCC_{f_1(\mathbf{r}),f_2(\mathbf{r}+\mathbf{s})}\right\}\right\} \approx \mathrm{Var}\left\{NCC_{f_1,f_2}\right\}\frac{1.64493}{2\log(|\mathcal{S}|)}. \tag{139}$$

The asymptotic mean can be bias corrected to account for the fact that the number of shifts explored is finite (Petzold, 2000)

$$\mathrm{E}\left\{\max_{\mathbf{s}\in\mathcal{S}}\left\{NCC_{f_1(\mathbf{r}),f_2(\mathbf{r}+\mathbf{s})}\right\}\right\} \approx \sqrt{\mathrm{Var}\left\{NCC_{f_1,f_2}\right\}}\sqrt{2\log(|\mathcal{S}|)} - \frac{\log(\log(|\mathcal{S}|)) + \log(4\pi) - 1.1544}{2\sqrt{2\log(|\mathcal{S}|)}} - 0.1727\left|\mathcal{S}\right|^{-0.2759}, \tag{140}$$

In fact, this result is extensible to shifts and rotations by simply setting $|\mathcal{S}|$ to the total number of rigid transformations explored. Let us give an example to get an idea of the order of magnitude of this expected correlation coefficient. Let us consider a map of size 101^3 voxels. In the worse case we could explore 101^3 shifts. Let us assume that we also explore for the best 3D rotation every $\Delta\theta = 0.5^\circ$. An approximate number of rotation samples can be estimated as follows (for a deeper analysis see Yershova and LaValle (2004)):

1. Let us consider 3D rotations as three Euler angles (α,β,γ) in a *ZYZ*-rotation convention (Frank, 2006)
2. Let us uniformly distribute the inclination angle, β, between 0° and 90°. We get $N_\beta = 90/\Delta\theta$ samples in the hemisphere. This is the number of circles parallel to the equator that we will explore. The i-th circle is at an inclination $\beta_i = i\Delta\theta$.
3. Within the i-th circle we take samples every $\Delta\alpha_i = \Delta\theta/\sin(\beta_i)$. This adaptive sampling produces more samples at the equator and less samples at the poles. The total number of samples per circle is $N_{\alpha i} = 360/\Delta\alpha_i = 360/\Delta\theta\sin(\beta_i)$. At the pole $\beta_i = 0$ and this formula would give zero samples, and therefore we must add two extra samples for considering the two poles.
4. The total count of samples in the sphere is twice that of the number of samples in the hemisphere plus the two extra samples corresponding to the two poles

$$N_{\alpha,\beta} = 2 + 2\sum_{i=0}^{N_\beta} N_{\alpha_i} = 2 + 2\frac{360}{\Delta\theta}\sum_{i=0}^{N_\beta}\sin(i\Delta\theta) = 2 + \frac{720}{\Delta\theta}\frac{\sin\left(\frac{N_\beta+1}{2}\Delta\theta\right)\sin\left(\frac{N_\beta}{2}\Delta\theta\right)}{\sin\left(\frac{1}{2}\Delta\theta\right)} \tag{141}$$

5. Finally, there are $N_\gamma = 360/\Delta\theta$ samples for each combination of α and β

10. The total number of rotations explored is $N_{\alpha,\beta,\gamma} = N_{\alpha,\beta}N_\gamma$

In our example with $\Delta\theta = 0.5^\circ$, we have $N_{\alpha,\beta,\gamma} \approx 1.2\cdot10^6$ combinations. The total number of shifts and rotations explored go up to $\left|\mathcal{S}\right| = N_{\alpha,\beta,\gamma}(N+1)^3 = 1.2\cdot10^{14}$. The expected maximum correlation observed in this sheer amount of correlations is

$$\mathrm{E}\left\{\max_{\mathbf{s}\in\mathcal{S}}\left\{NCC_{f_1(\mathbf{r}),f_2(\mathbf{r}+\mathbf{s})}\right\}\right\} \approx \sqrt{\frac{1}{N+1}^3}\left(\sqrt{2\log(|\mathcal{S}|)} - \frac{\log(\log(|\mathcal{S}|)) + \log(4\pi) - 1.1544}{2\sqrt{2\log(|\mathcal{S}|)}} - 0.1727\left|\mathcal{S}\right|^{-0.2759}\right) = 0.0076 \tag{142}$$

This result seems to point out that map alignment does not pose a major problem in the determination of the resolution. We were not able to reproduce the results in Grigorieff (2000) which showed much higher expected values.

A different but related problem is the following: let $f_1(\mathbf{r})$ and $f_2(\mathbf{r})$ be two images that are aligned against a common reference $f_0(\mathbf{r})$ producing the aligned images $f_1^*(\mathbf{r})$ and $f_2^*(\mathbf{r})$. What is the expected correlation coefficient between $f_1^*(\mathbf{r})$ and $f_2^*(\mathbf{r})$? Let us discuss this problem here for the case without signal which will help us to derive appropriate thresholds.

We first check that the result in Eq. (55) is valid even assuming

different noise variances. Let $f_1(\mathbf{r}) = n_1(\mathbf{r})$ and $f_2(\mathbf{r}) = n_2(\mathbf{r})$. Let the noise variables be normally distributed with zero mean, and variances σ_1^2 and σ_2^2, respectively. Let us analyze the mean and variance of c and d, the numerator and denominator of the $NCC_{f1,f2}$, as we did in the analysis that started at (40):

$$\begin{aligned}
&\mathrm{E}\{c\} = 0\\
&\mathrm{Var}\{c\} = (N+1)^3\sigma_1^2\sigma_2^2\\
&\mathrm{E}\{d\} = (N+1)^6\sigma_1^2\sigma_2^2\\
&\mathrm{Var}\{d\} \approx 4(N+1)^9\sigma_1^4\sigma_2^4\\
&\mathrm{E}\left\{NCC_{f_1,f_2}\right\} = \frac{\mathrm{E}\{c\}}{\sqrt{\mathrm{Var}\{d\}}} = 0\\
&\mathrm{Var}\left\{NCC_{f_1,f_2}\right\} = \frac{\mathrm{Var}\{c\}}{\mathrm{E}\{d\}} = \frac{1}{(N+1)^3}
\end{aligned} \tag{143}$$

For addressing the problem of the correlation of $f_1^*(\mathbf{r})$ and $f_2^*(\mathbf{r})$, note that $NCC_{f_1^*,f_2^*}$ can also be computed as $NCC_{f_1^*,f_2^*} = \frac{\langle F_1^*, F_2^*\rangle}{\|F_1^*\|\|F_2^*\|}$ (being $\langle F_1^*, F_2^*\rangle$ the inner product between two vectors corresponding to the lexicographical ordering of the voxels in real space), and let us study the mean of the variable

$$\begin{aligned}
e &= \left\|\frac{F_1^*}{\|F_1^*\|} - \frac{F_2^*}{\|F_2^*\|}\right\|^2 = \left\|\frac{F_1^*}{\|F_1^*\|}\right\|^2 + \left\|\frac{F_2^*}{\|F_2^*\|}\right\|^2 - 2\left\langle\frac{F_1^*}{\|F_1^*\|}, \frac{F_2^*}{\|F_2^*\|}\right\rangle\\
&= 2 - 2NCC_{f_1^*,f_2^*},
\end{aligned} \tag{144}$$

We can reformulate the previous variable as

$$\begin{aligned}
e &= \left\|\left(\frac{F_1^*}{\|F_1^*\|} - \frac{F_0}{\|F_0\|}\right) - \left(\frac{F_2^*}{\|F_2^*\|} - \frac{F_0}{\|F_0\|}\right)\right\|^2\\
&= \left\|\frac{F_1^*}{\|F_1^*\|} - \frac{F_0}{\|F_0\|}\right\|^2 + \left\|\frac{F_2^*}{\|F_2^*\|} - \frac{F_0}{\|F_0\|}\right\|^2 - 2\left\langle\frac{F_1^*}{\|F_1^*\|} - \frac{F_0}{\|F_0\|}, \frac{F_2^*}{\|F_2^*\|} - \frac{F_0}{\|F_0\|}\right\rangle\\
&= 4 - 2NCC_{f_1^*,f_0} - 2NCC_{f_2^*,f_0} - 2\left(\left\langle\frac{F_1^*}{\|F_1^*\|}, \frac{F_2^*}{\|F_2^*\|}\right\rangle - \left\langle\frac{F_1^*}{\|F_1^*\|}, \frac{F_0}{\|F_0\|}\right\rangle - \left\langle\frac{F_0}{\|F_0\|}, \frac{F_2^*}{\|F_2^*\|}\right\rangle + 1\right)
\end{aligned} \tag{145}$$

Taking expectations on both sides and exploiting that F_0, F_1 and F_2 are independent, we have

$$2 - 2\mathrm{E}\left\{NCC_{f_1^*,f_2^*}\right\} = 2 - 4\mathrm{E}\left\{NCC_{f_i^*,f_0}\right\} \tag{146}$$

or what is the same

$$\mathrm{E}\left\{NCC_{f_1^*,f_2^*}\right\} = 2\mathrm{E}\left\{NCC_{f_i^*,f_0}\right\}. \tag{147}$$

$NCC_{f_i^*,f_0}$ is the maximum cross-correlation between the best shifted and rotated version of $f_i(\mathbf{r})$ and $f_0(\mathbf{r})$ whose expected value is given in (140). Finally,

$$\mathrm{E}\left\{NCC_{f_1^*,f_2^*}\right\} = \frac{2}{\sqrt{(N+1)^3}}\left(\sqrt{2\log(|\mathcal{S}|)} - \frac{\log(\log(|\mathcal{S}|)) + \log(4\pi) - 1.1544}{2\sqrt{2\log(|\mathcal{S}|)}} - 0.1727\left|\mathcal{S}\right|^{-0.2759}\right) \tag{148}$$

Note that this result only depends on the size of the maps and the number of rigid transformations explored for the alignment, but it does not depend on the noise variance of $f_1(\mathbf{r})$ or $f_2(\mathbf{r})$. From this point of view it does not matter whether the images being compared are single projections or projection averages.

11. On the meaning and assumptions of *B*-factors

The *B*-factor is a coefficient that has been typically associated to the damping of the microscope Contrast Transfer Function (Saad et al., 2001; Huang et al., 2003; Frank, 2006). However, we can also find it describing the decay of the Fourier coefficients of the reconstructed maps (Rosenthal and Henderson, 2003; Fernández et al., 2008; Liao and Frank, 2010) and defining a compensatory highpass filter to improve resolution (Fernández et al., 2008). In this paper we show that all these interpretations are deeply intertwined and are difficult to separate experimentally. In the theory so far we have used $\mathbf{R}$ mainly as a frequency index in Fourier space (measured between $-N/2$ and $N/2$). In the following, we will abuse a little bit of the notation and we will use $\mathbf{R}$ as a continuous frequency measured in Å^{-1}.

11.1. B-factor as a consequence of thermal vibrations

The concept of *B*-factor is strongly related to the statistical distribution of Fourier coefficients. Let us revise the foundations of this distribution, which is known in the field as Wilson statistics (Ladd and Palmer, 2003).

Let us consider the atomic structure of a macromolecule as sum of Dirac's deltas located at different positions in real space, $\mathbf{r}_j$, and different weights, w_j

$$f(\mathbf{r}) = \sum_{j=1}^{N_{\text{atoms}}} w_j\delta(\mathbf{r} - \mathbf{r}_j). \tag{149}$$

The Fourier transform of this structure is

$$F(\mathbf{R}) = \sum_{j=1}^{N_{\text{atoms}}} w_j e^{-i2\pi\langle \mathbf{R},\mathbf{r}_j\rangle} = \sum_{j=1}^{N_{\text{atoms}}} w_j \cos(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle) - i\sum_{j=1}^{N_{\text{atoms}}} w_j \sin(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle) \quad (150)$$

Wilson statistics presume that the weight of each atom is known but its exact location in space follows a uniform distribution within some predefined box. Under these conditions, let us study the average and variance of the real part and imaginary parts of $F(\mathbf{R})$. Without loss of generality we can assume that each component of $\mathbf{r}_j$ is uniformly distributed between $-r_{max}$ and r_{max}, then the inner product

$$\langle \mathbf{R},\mathbf{r}_j\rangle = R_x\mathbf{r}_{jx} + R_y\mathbf{r}_{jy} + R_z\mathbf{r}_{jz} \quad (151)$$

is the sum of three random variables, each one with a uniform distribution between $-R_x r_{max}$ and $R_x r_{max}$, $-R_y r_{max}$ and $R_y r_{max}$, and $-R_z r_{max}$ and $R_z r_{max}$, respectively. The probability density function of the sum of three variables is equal to the convolution of the three individual probability density functions. In this case, the convolution of three box functions is a polynomial spline of degree two (de Boor, 2001), and the cosine of such a random variable does not follow any standard statistical distribution.

However, Wilson assumed that $\cos(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle)$ and $\sin(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle)$, both followed a uniform distribution between -1 and 1 for any frequency $\mathbf{R}$ (Shmueli, 2007). At the sight of the previous digression, it is clearly seen that this cannot be the case. Even if the distribution of the spatial locations $\mathbf{r}_j$ is not uniform, there is no spatial distribution such that all the sines and cosines are uniformly distributed for any frequency. Nevertheless, the uniform distribution is still a reasonable approximation for frequencies and atom locations such that the inner product $\langle \mathbf{R},\mathbf{r}_j\rangle$ is large. If this inner product is not large enough (which is generally the case for low frequencies), then the approximation by a uniform distribution is not valid.

Let us keep with Wilson's reasoning (Shmueli, 2007). Since the real and imaginary parts of $F(\mathbf{R})$ is the sum of many independent, random variables, then the Central Limit Theorem applies so that the real and imaginary parts are normally distributed and their mean and variance are given by

$$\begin{aligned}
\mathrm{E}\{\mathrm{Re}\{F(\mathbf{R})\}\} &= \mathrm{E}\left\{\sum_{j=1}^{N_{\text{atoms}}} w_j \cos(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle)\right\} \\
&= \sum_{j=1}^{N_{\text{atoms}}} w_j \mathrm{E}\{\cos(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle)\} = 0 \\
\mathrm{Var}\{\mathrm{Re}\{F(\mathbf{R})\}\} &= \mathrm{E}\left\{\left(\sum_{j=1}^{N_{\text{atoms}}} w_j \cos(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle)\right)^2\right\} \\
&= \sum_{j=1}^{N_{\text{atoms}}} w_j^2 \mathrm{E}\left\{\cos^2(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle)\right\} \\
&\quad + \sum_{j,j'=1 j\neq j'}^{N_{\text{atoms}}} w_j w_{j'} \mathrm{E}\{\cos(2\pi\langle \mathbf{R},\mathbf{r}_j\rangle)\cos(2\pi\langle \mathbf{R},\mathbf{r}_{j'}\rangle)\} \\
&= \sum_{j=1}^{N_{\text{atoms}}} w_j^2 \mathrm{E}\left\{\frac{1}{2} + \cos(4\pi\langle \mathbf{R},\mathbf{r}_j\rangle)\right\} = \frac{1}{2}\sum_{j=1}^{N_{\text{atoms}}} w_j^2,
\end{aligned} \quad (152)$$

similarly for the imaginary part. For convenience we will define a new variable σ_{atoms} such that $\sigma_{\text{atoms}}^2 = \sum_{j=1}^{N_{\text{atoms}}} w_j^2$. Assuming that the real and imaginary parts of $F(\mathbf{R})$ are independent, the probability density function of $F(\mathbf{R})$ is simply the joint distribution of the real and imaginary parts, and thanks to their independence, equal to the product of two normal probability density functions:

$$\begin{aligned}
p_{F(\mathbf{R})}(F(\mathbf{R})) &= \frac{1}{\sqrt{2\pi\frac{1}{2}\sigma_{\text{atoms}}^2}} e^{-\frac{1}{2}\frac{\mathrm{Re}^2\{F(\mathbf{R})\}}{\frac{1}{2}\sigma_{\text{atoms}}^2}} \frac{1}{\sqrt{2\pi\frac{1}{2}\sigma_{\text{atoms}}^2}} e^{-\frac{1}{2}\frac{\mathrm{Im}^2\{F(\mathbf{R})\}}{\frac{1}{2}\sigma_{\text{atoms}}^2}} \\
&= \frac{1}{\pi\sigma_{\text{atoms}}^2} e^{-\frac{|F(\mathbf{R})|^2}{\sigma_{\text{atoms}}^2}}
\end{aligned} \quad (153)$$

$\frac{|F(\mathbf{R})|^2}{\frac{1}{2}\sigma_{\text{atoms}}^2}$ is distributed as a χ^2 with 2 degrees of freedom and, therefore, the mean of $|F(\mathbf{R})|^2$ is

$$\mathrm{E}\left\{|F(\mathbf{R})|^2\right\} = 2\frac{1}{2}\sigma_{\text{atoms}}^2 = \sum_{j=1}^{N_{\text{atoms}}} w_j^2 \quad (154)$$

and its variance is

$$\mathrm{Var}\left\{|F(\mathbf{R})|^2\right\} = 2\cdot 2\left(\frac{\sigma_{\text{atoms}}^2}{2}\right)^2 = \sigma_{\text{atoms}}^4. \quad (155)$$

The classical approach to the analysis of the effect of thermal vibrations on the atoms takes a different perspective (Ladd and Palmer, 2003). Let us assume that the position of the atoms is randomly distributed around their central position $\mathbf{r}_j$ by adding a random variable $\Delta\mathbf{r}_j$ with a tridimensional normal distribution with zero mean and covariance matrix $\Sigma_j = \sigma_{\Delta_x}^2 I$. Then, the module of the Fourier coefficients is given by

$$\begin{aligned}
F_{\text{thermal}}(\mathbf{R}) &= \sum_{j=1}^{N_{\text{atoms}}} w_j e^{-i2\pi\langle \mathbf{R},\mathbf{r}_j+\Delta\mathbf{r}_j\rangle} \\
&= \sum_{j=1}^{N_{\text{atoms}}} w_j e^{-i2\pi\langle \mathbf{R},\mathbf{r}_j\rangle} e^{-i2\pi\langle \mathbf{R},\Delta\mathbf{r}_j\rangle}
\end{aligned} \quad (156)$$

For small vibrations we can approximate the exponential by a Taylor series of degree 2

$$\begin{aligned}
F_{\text{thermal}}(\mathbf{R}) &\approx \sum_{j=1}^{N_{\text{atoms}}} w_j e^{-i2\pi\langle \mathbf{R},\mathbf{r}_j\rangle}\left(1 - i2\pi\left\langle \mathbf{R},\Delta\mathbf{r}_j\right\rangle + \frac{(-i2\pi\langle \mathbf{R},\Delta\mathbf{r}_j\rangle)^2}{2}\right) \\
&= \sum_{j=1}^{N_{\text{atoms}}} w_j e^{-i2\pi\langle \mathbf{R},\mathbf{r}_j\rangle}\left(1 - i2\pi\left\langle \mathbf{R},\Delta\mathbf{r}_j\right\rangle - 2\pi^2\langle \mathbf{R},\Delta\mathbf{r}_j\rangle^2\right)
\end{aligned} \quad (157)$$

If we now take the expectation with respect to the distribution of the displacements $\Delta\mathbf{r}_j$ and considering that the vibrations have zero mean, we obtain

$$\mathrm{E}_{\Delta\mathbf{r}_j}\{F_{\text{thermal}}(\mathbf{R})\} = \sum_{j=1}^{N_{\text{atoms}}} w_j e^{-i2\pi\langle \mathbf{R},\mathbf{r}_j\rangle}\left(1 - 2\pi^2\mathrm{E}_{\Delta\mathbf{r}_j}\left\{\langle \mathbf{R},\Delta\mathbf{r}_j\rangle^2\right\}\right) \quad (158)$$

The expected value needed in the previous formula is

$$\begin{aligned}\mathrm{E}_{\Delta\mathbf{r}_j}\left\{\langle\mathbf{R},\Delta\mathbf{r}_j\rangle^2\right\} &= \mathrm{E}_{\Delta\mathbf{r}_j}\left\{\left(R_x\Delta\mathbf{r}_{jx}+R_y\Delta\mathbf{r}_{jy}+R_z\Delta\mathbf{r}_{jz}\right)^2\right\}\\ &= R_x^2\mathrm{E}_{\Delta\mathbf{r}_j}\left\{\Delta\mathbf{r}_{jx}^2\right\}+R_y^2\mathrm{E}_{\Delta\mathbf{r}_j}\left\{\Delta\mathbf{r}_{jy}^2\right\}+R_z^2\mathrm{E}_{\Delta\mathbf{r}_j}\left\{\Delta\mathbf{r}_{jz}^2\right\}\\ &= \sigma_{\Delta_x}^2\left|\mathbf{R}\right|^2,\end{aligned} \tag{159}$$

where we have made use of the mutual independence of the different components of the vibration displacement vector.

Finally we get

$$\begin{aligned}\mathrm{E}_{\Delta\mathbf{r}_j}\{F_{\text{thermal}}(\mathbf{R})\} &= \sum_{j=1}^{N_{\text{atoms}}} w_j e^{-i2\pi\langle\mathbf{R},\mathbf{r}_j\rangle}\left(1-2\pi^2\sigma_{\Delta_x}^2\left|\mathbf{R}\right|^2\right)\\ &\approx \sum_{j=1}^{N_{\text{atoms}}} w_j e^{-i2\pi\langle\mathbf{R},\mathbf{r}_j\rangle}e^{-2\pi^2\sigma_{\Delta_x}^2\left|\mathbf{R}\right|^2}\\ &= F(\mathbf{R})e^{-2\pi^2\sigma_{\Delta_x}^2\left|\mathbf{R}\right|^2} = F(\mathbf{R})e^{-B\left|\mathbf{R}\right|^2}.\end{aligned} \tag{160}$$

In the last equation we see that $B=2\pi^2\sigma_{\Delta_x}^2$ has units of Å^2 and is proportional to the displacements due to thermal vibration.

11.2. B-factor as a consequence of Gaussian atoms

In the derivation of the *B*-factor equation (Eq. (160)), we have assumed that the original structure is composed of infinitely narrow atoms (their description is given by Dirac's deltas) and we had to make a couple of Taylor expansions for two exponential functions, which limit the validity of the statistical derivation of the *B*-factor to relatively small frequencies.

However, we could have also derived the same equation (Eq. (160)) without any approximation if we follow a deterministic reasoning instead of a statistical one. If we now assume that our atoms are not represented by Dirac's deltas but by Gaussian functions ($\psi(\mathbf{r})$), then we obtain

$$f_{\text{Gaussian}}(\mathbf{r}) = f(\mathbf{r})\bigstar\psi(\mathbf{r}), \tag{161}$$

where $\bigstar$ represents the convolution operator. The Fourier transform of the new atomic representation is then

$$F_{\text{Gaussian}}(\mathbf{R}) = F(\mathbf{R})\Psi(\mathbf{R}). \tag{162}$$

Knowing that the Fourier transform of the real-space Gaussian $e^{-\pi\left|\mathbf{r}\right|^2}$ is $e^{-\pi\left|\mathbf{R}\right|^2}$ and making use of the scale property of the Fourier transform (the Fourier transform of $f(a\mathbf{r})$ is $\left|a\right|^{-1}F(a^{-1}\mathbf{R})$, it is easy to show that the atomic representation yielding the same equation as in Eq. (160) is

$$\psi(\mathbf{r}) = \sqrt{\frac{\pi}{B}}e^{-\pi\left(\sqrt{\frac{\pi}{B}}\left|\mathbf{r}\right|\right)^2} = \sqrt{\frac{\pi}{B}}e^{-\frac{1}{2}\left(\frac{|\mathbf{r}|}{\sqrt{\frac{B}{2\pi^2}}}\right)^2} \tag{163}$$

In this way we have shown that the approximations giving raise to the *B*-factor formulation of the thermal vibration is equivalent to representing atoms by Gaussians whose standard deviation is $B/2\pi^2$. However, note that atoms in the electron microscope are better modeled with a sum of Gaussians representing the Electron Atomic Scattering Factors as has been shown in Sorzano et al. (2015). This representation makes the atoms to be more concentrated in Fourier space than the standard Gaussian for the frequencies of interest in Electron Microscopy (whose target resolution is normally between 4-2 Å).

11.3. B-factor as a description of the CTF envelope

B-factors are also found in the formulations of the envelope of the CTF (Saad et al., 2001; Huang et al., 2003; Frank, 2006). Other authors prefer a complete description of the physical factors determining the damping envelope (Zhou et al., 1996; Velázquez-Muriel et al., 2003; Frank, 2006; Sorzano et al., 2007). Such envelope described by physical factors is given by the multiplication of three separate envelopes:

$$E(\mathbf{R}) = E_{spread}(\mathbf{R})E_{coherence}(\mathbf{R})E_{drift}(\mathbf{R}). \tag{164}$$

The beam energy spread envelope is computed as

$$E_{spread}(\mathbf{R}) = \exp\left(-\frac{\left(\left(\frac{\pi}{4}C_a\lambda\right)\left(\frac{\Delta V}{V}+2\frac{\Delta I}{I}\right)\right)^2}{\log(2)}\left|\mathbf{R}\right|^4\right). \tag{165}$$

where C_a is the chromatic aberration coefficient, λ is the electron wavelength which is computed as $\lambda=\frac{1.23\cdot10^{-9}}{\sqrt{V+10^{-6}V^2}}$, V is the acceleration voltage of the microscope, $\Delta V/V$ is the energy spread of the emitted electrons represented as a fraction of the nominal acceleration voltage, and $\Delta I/I$ is the lens current instability expressed as a fraction of the nominal current.

The beam coherence envelope is given by (Frank, 2006)

$$E_{coherence}(\mathbf{R}) = \exp\left(-\pi^2\alpha^2\left(C_s\lambda^2\left|\mathbf{R}\right|^3+\left|\Delta f(\mathbf{R})\right|\left|\mathbf{R}\right|\right)^2\right), \tag{166}$$

where α is the semi-angle of aperture, C_s represents the spherical aberration coefficient, and $|\Delta f(\mathbf{R})|$ is the defocus in the direction of $\mathbf{R}$.

Finally, assuming the mechanical displacement perpendicular to the focal plane ΔF and the displacement in the focal plane (drift) ΔR, the envelope due to sample shift is modeled as

$$E_{drift}(\mathbf{R}) = J_0\left(\pi\Delta F\lambda\left|\mathbf{R}\right|^2\right)\mathrm{sinc}(|\mathbf{R}|\Delta R), \tag{167}$$

being J_0 the Bessel function of first kind and order 0.

The envelope model can be well approximated by a Gaussian if $\Delta F=\Delta R=\Delta V/V=\Delta I/I=0$ and $C_s\lambda^2\left|\mathbf{R}\right|^3 \ll \Delta f(\mathbf{R})\left|\mathbf{R}\right|$. In this case, the envelope is given by

$$E(\mathbf{R}) \approx e^{-\pi^2\alpha^2\left|\Delta f(\mathbf{R})\right|^2\left|\mathbf{R}\right|^2}. \tag{168}$$

It is interesting to see that in this situation the *B*-factor becomes direction dependent and, consequently, it is not well defined (it is not a single constant) for astigmatic images.

If we now think of the Fourier coefficients reconstructed from micrographs affected by this CTF envelope, what we reconstruct is

$$F_{\text{reconstructed}}(\mathbf{R}) = F_{\text{Gaussian}}(\mathbf{R})E(\mathbf{R}) = F(\mathbf{R})e^{-\left(B+\pi^2\alpha^2\left|\Delta f(\mathbf{R})\right|^2\right)\left|\mathbf{R}\right|^2}. \tag{169}$$

We now see that the thermal effects on the position of the atoms and the microscope aberrations become inseparable in the reconstructed map and all we can see is the combined effect of both (*B*-factors have been reported to be 110 Å^2 (Miyazawa et al., 2003), 400 Å^2 (Conway and Steven, 1999), 500 Å^2 (Böttcher et al., 1997), 1200 Å^2 (Gabashvili et al., 2000), and even 2100 Å^2 (Thuman-Commike et al., 1999)). For this reason, it makes sense the common procedure in the field of correcting for the CTF envelope during the reconstruction process using the estimates of the CTF

parameters obtained from the micrographs (Frank and Penczek, 1995; Sorzano et al., 2004b), and once it is finished correcting for an additional *B*-factor estimated from the shape of the expected spectrum (Fernández et al., 2008).

11.4. B-factor as a consequence of electron scattering

B-factor correction has become a standard practice in Electron Microscopy. Specially as one of the last image processing steps in order to boost high frequencies. The strategy normally performed is to fit a straight line in the Guinier plot as a way to estimate the *B* factor, and then apply a highpass filter with the *B* factor estimated as a way to compensate for the damping allegedly introduced by the image acquisition and processing. Let us review here the rationale behind this strategy.

Guinier plots are 1D plots in which the logarithm of the modulus of the Fourier coefficients (averaged along the radial direction, $\log(\overline{|F(R)|^2})$) are plotted against the squared resolution R^2. It has been stated that in a certain frequency region, this plot has to be approximately flat. Let us see the assumptions behind this statement.

Assuming that the Born approximation holds, that is, the scattered wave from a single scatterer is not affected by the presence of other scatterers (this is true if the scattering of the electrons in the microscope is weak), then the 2D intensity scattered by a molecule whose shape is $f(\mathbf{r})$ can be described in frequency by (Brumberger, 2013)[Chap. 1]

$$I(\mathbf{R}) = |F(\mathbf{R})|^2 \tag{170}$$

where $F(\mathbf{R})$ is the Fourier transform of the molecule. For a continuous function, $f(\mathbf{r})$, the intensity can be calculated as (Brumberger, 2013)[Chap. 1]

$$|F(\mathbf{R})|^2 = \iint f(\mathbf{r}_1) f(\mathbf{r}_2) \cos(\langle \mathbf{R}, \mathbf{r}_1 - \mathbf{r}_2 \rangle) d\mathbf{r}_1 d\mathbf{r}_2 \tag{171}$$

If we think of a macromolecule as a set of N_{atoms} atoms, then the expression above is simplified to

$$|F(\mathbf{R})|^2 = \sum_{i=1}^{N_{\text{atoms}}} \sum_{j=1}^{N_{\text{atoms}}} F_i(\mathbf{R}) F_j(\mathbf{R}) \cos\left(\langle \mathbf{R}, \mathbf{r}_i - \mathbf{r}_j \rangle\right) \tag{172}$$

where $\mathbf{r}_i$ and $\mathbf{r}_j$ are the locations of the i-th and j-th atoms, and $F_i(\mathbf{R})$ and $F_j(\mathbf{R})$ are the Fourier transforms of each one of the atoms evaluated at a given frequency $\mathbf{R}$.

If we now make a radial average of this intensity, this is called Debye scattering equation, then we obtain a 1D profile given by

$$\overline{|F(R)|^2} = \iint f(\mathbf{r}_1) f(\mathbf{r}_2) \frac{\sin(R\|\mathbf{r}_1 - \mathbf{r}_2\|)}{R\|\mathbf{r}_1 - \mathbf{r}_2\|} d\mathbf{r}_1 d\mathbf{r}_2 \tag{173}$$

Similarly, for a set of N_{atoms} atoms and assuming that each atom has a spherically symmetric spectrum, we would have

$$\overline{|F(R)|^2} = \sum_{i=1}^{N_{\text{atoms}}} \sum_{j=1}^{N_{\text{atoms}}} F_i(R) F_j(R) \frac{\sin(R\|\mathbf{r}_i - \mathbf{r}_j\|)}{R\|\mathbf{r}_i - \mathbf{r}_j\|} \tag{174}$$

Note that performing a radial average assumes that we have no missing regions in Fourier space, since otherwise there would be a region of zeroes that would be taken into account in the radial average.

Given a molecule of diameter D, Debye equation can be rewritten as

$$\overline{|F(R)|^2} = \int_0^D r^2 \Phi_f(r) \frac{\sin(Rr)}{Rr} dr \tag{175}$$

where $\Phi_f(r)$ is called the correlation or characteristic function calculated as

$$\Phi_f(r) = \int_0^{2\pi} \int_0^{\pi} \int f(\mathbf{r}_1) f(\mathbf{r}_1 + \mathbf{r}) \sin(\alpha) d\mathbf{r}_1 d\alpha d\beta \tag{176}$$

where α and β are the angles of the spherical coordinates of the vector $\mathbf{r}$ (whose norm is r), and which is nothing more than the radial average of the autocorrelation function of $f(\mathbf{r})$.

Guinier approximation comes from the Taylor expansion

$$\frac{\sin(Rr)}{Rr} = 1 - \frac{R^2 r^2}{6} + \frac{R^4 r^4}{100} - \dots \tag{177}$$

for which we need $R_{max} D \ll 1$, where R_{max} is the maximum frequency we pretend to reconstruct and D is the molecule diameter. Considering only the first two terms and substituting in Eq. (175), we get

$$\begin{aligned} \overline{|F(R)|^2} &\approx \int_0^D r^2 \Phi_f(r) dr - R^2 \int_0^D \frac{r^4}{6} \Phi_f(r) dr \\ &= \int_0^D r^2 \Phi_f(r) dr \left(1 - R^2 \frac{\int_0^D \frac{r^4}{6} \Phi_f(r) dr}{\int_0^D r^2 \Phi_f(r) dr} \right) \\ &= F_0^2 \left(1 - \frac{R_g^2}{3} R^2 \right) \end{aligned} \tag{178}$$

In the last equation we have defined $F_0^2 = \int_0^D r^2 \Phi_f(r) dr$ and $R_g^2 = \frac{1}{2} \frac{\int_0^D r^4 \Phi_f(r) dr}{\int_0^D r^2 \Phi_f(r) dr}$, which is called the radius of gyration. In the case of a set of infinitely small atoms F_0^2 becomes

$$F_0^2 = \overline{|F(0)|^2} = \sum_{i=1}^{N_{\text{atoms}}} \sum_{j=1}^{N_{\text{atoms}}} w_i w_j \delta(\|\mathbf{r}_i - \mathbf{r}_j\|) = \sum_{j=1}^{N_{\text{atoms}}} w_j^2 \tag{179}$$

where w_i and w_j are the molecular weights of the i-th and j-th atoms. Note that this value is the same as the one obtained in Wilson statistics and equal to N_{atoms} as stated in Rosenthal and Henderson (2003) if all the atoms are presumed to have unit weight.

Finally, we note that Eq. (178) looks like the first two terms of the Taylor expansion of an exponential, consequently, we may write

$$\overline{|F(R)|^2} \approx F_0^2 e^{-\frac{R_g^2}{3} R^2} \tag{180}$$

The radius of gyration of a point is 0, of a solid sphere of diameter D is $3D^2/20$, of a cylinder of diameter D and height H is $H^2/12 + D^2/8$ (Johnson and Gabriel, 1981)[Chap. II]. Finally, taking the logarithm of the radial average, we get

$$\log\overline{\left|F(R)\right|^2} = \log\left(F_0^2\right) - \frac{R_g^2}{3}R^2 \tag{181}$$

11.5. B-factor correction

B-factor correction is a technique commonly used in which a line is fitted to the $\log\overline{\left|F(R)\right|^2}$ plot in a squared-frequency region normally between $1/10^2$ and $1/5^2$ (10 and 5 Å, respectively) (Fernández et al., 2008)

$$\log\overline{\left|F(R)\right|^2} \approx A - B^2R^2 \tag{182}$$

and the whole map is corrected with the transformation

$$F_{corrected}(R) = F(R)e^{BR} \tag{183}$$

followed by a low pass filter with radial shape

$$C_{ref} = \sqrt{\frac{2FSC}{1+FSC}} \tag{184}$$

This correction tends to make the Guinier plot of the corrected map to be flat in the fitted squared-frequency region. The belief in EM that the Guinier plot needs to be flat comes from the fact that R_g is 0 for a single, infinitely small atom, but it does not need to be so for a set of atoms. Consequently, this correction should be applied with care since linear Guinier plots are based on two numerical approximations, and flat Guinier plots are only expected for a single scattering atom (not a macromolecular structure). Additionally, the lowpass filter applied with C_{ref} is based on a derivation that it is not actually measuring what it meant (see Eq. (73)). Still, this correction has proved to be useful for the interpretation of EM maps since it boosts high frequencies better highlighting secondary structure features.

12. On the dose needed for detecting features of a given size

To calculate the number of electrons needed to detect features of a given size we will start from the interaction of electrons in the electron microscope with matter. At a given acceleration voltage V_0 (in Volts), the elastic cross section of an atom in the microscope is given by (Langmore and Smith, 1992)[Eq. (1)]

$$\sigma_Z = \frac{1.4\cdot10^{-4}Z^{3/2}}{\beta^2}\left(1-\frac{0.26Z}{137\beta}\right)\left[\text{Å}^2\Big/\text{atom}\right] \tag{185}$$

where Z is the atomic number of the atom and

$$\beta = \sqrt{1-\left(\frac{m_ec^2}{q_eV_0+m_ec^2}\right)^2} \tag{186}$$

being m_e the electron mass in kilograms ($m_e = 9.1\cdot10^{-31}$[kg], q_e the electron charge in Coulombs ($q_e = 1.6\cdot10^{-19}$[C]) and c the speed of light in vacuum ($c = 3\cdot10^8$[m/s]). For instance, a carbon atom at 200 kV has an elastic cross section of 41.9[pm^2]. Langmore and Smith (1992) proposed that the fraction of electrons scattered within the electron microscope objective aperture reaching a resolution of R_{max} (Å^{-1}) was $\eta = R_{max}$ (e.g., as stated in Henderson (1995), the fraction of electrons scattered to a resolution of 3 Å is 1/3rd of the total number of elastically scattered electrons). Note that Langmore and Smith (1992) states that this approximation for the fraction is valid if $R_{max}\in[0.2,0.5]$Å^{-1}, and that η is an adimensional number.

Let us now calculate the average number of carbon equivalents in a protein as a way to estimate the energy observed in an imaged particle. Let us assume a cubic protein of side D [Å]. Assuming an average density for proteins, ρ_{prot} ([Da/Å^3]; normally it is taken $\rho_{\text{prot}} = 0.8$[Da/Å^3], Henderson (1995)), the molecular weight of the protein is given by

$$MW_{\text{prot}} = D^3\rho_{\text{prot}} \quad \text{[Da]} \tag{187}$$

The number of carbon equivalents in this map is given by

$$N_C = \frac{MW_{\text{prot}}[\text{Da}]}{MW_{\text{C}}[\text{Da/Catom}]} \tag{188}$$

If we consider now a square enclosing the projection of our protein, this square has an area D^2 [Å^2], and the total cross section per unit area observed in this square image is

$$\begin{aligned}\tilde{\sigma}_{\text{prot}} &= \frac{(MW_{\text{prot}}/MW_{\text{C}}[\text{C atoms}])\left(\eta\sigma_{Z=6}\left[\text{Å}^2\Big/\text{C atom}\right]\right)}{D^2\left[\text{Å}^2\right]}\\ &= D\rho_{\text{prot}}\frac{1}{MW_{\text{C}}}R_{max}\sigma_{Z=6}\end{aligned} \tag{189}$$

Note that this number is adimensional and it represents the fraction of the incident intensity, I_0, that elastically interacts with the sample resulting in an observed intensity, I_{obs}. The time average of this observed intensity fulfills

$$\tilde{\sigma}_{\text{prot}} = \frac{\langle I_{obs}\rangle}{I_0}. \tag{190}$$

Interestingly, if we consider small cubes of proteins of size $D = 1/R_{max}$, then $\tilde{\sigma}_{\text{prot}}$ is independent of the resolution at which we are interested ($\tilde{\sigma}_{\text{prot}} = \rho_{\text{prot}}\frac{1}{MW_C}\sigma_{Z=6}$).

If instead of a cube of protein we had a parallepiped of size $D\times D\times H$ made of ice, instead of protein, then we would have obtained

$$\tilde{\sigma}_{\text{ice}} = H\rho_{\text{ice}}\frac{1}{MW_{\text{O}}}R_{max}\sigma_{Z=8} \tag{191}$$

At 200 kV, the elastic cross section of oxygen is 64.2[pm^2], and an accepted density of amorphous ice is 1.17 g/cm^3 = 0.7[Da/Å^3] (Mishima et al., 1984).

Our protein is actually embedded in a layer of amorphous ice, let us say of height H [Å], the total observed cross section would be given by

$$\begin{aligned}\tilde{\sigma}_{\text{embedded.prot}} &= D\rho_{\text{prot}}\frac{1}{MW_{\text{C}}}R_{max}\sigma_{Z=6}\\ &\quad + (H-D)\rho_{\text{ice}}\frac{1}{MW_{\text{O}}}R_{max}\sigma_{Z=8}\end{aligned} \tag{192}$$

If we now compute the relative contrast between two adjacent areas of embedded protein and ice, we have

$$C = \frac{\tilde{\sigma}_{\text{ice}} - \tilde{\sigma}_{\text{embedded.prot}}}{\tilde{\sigma}_{\text{ice}}} = \frac{D}{H}\left(1-\frac{\rho_{\text{prot}}}{\rho_{\text{ice}}}\frac{MW_{\text{O}}}{MW_{\text{C}}}\frac{\sigma_{Z=6}}{\sigma_{Z=8}}\right) \tag{193}$$

At 200 kV this contrast becomes $C = 0.0047D/H$.

We now wonder how many electrons we need to illuminate this parallepiped with the embedded protein to be able to distinguish between protein details of size D from their surroundings. We will see that the results given in this paper justify and give a generalization of the work of Glaeser (1999) (on its turn based on that of Rose (1973)). When we illuminate this part of the protein with N_e electrons per Å^2, the number of scattered electrons in the protein area follows a Poisson with parameter

$$\lambda_{\text{embedded.prot}} = N_e \tilde{\sigma}_{\text{embedded.prot}} D^2 \quad \left[\text{e}^-\right] \tag{194}$$

Similarly, for an ice region of the same size, we would have a Poisson with parameter

$$\lambda_{\text{ice}} = N_e \tilde{\sigma}_{\text{ice}} D^2 \quad \left[\text{e}^-\right] \tag{195}$$

Remind that the mean and standard deviations of a Poisson distribution are λ and λ, and that for sufficiently high λ's, the Poisson is well approximated by a Gaussian. Given a number of electron counts in the allegedly protein area and a surrounding ice area, we would reject the hypothesis that the protein area is ice with a confidence level $1-\alpha$ and a statistical power $1-\beta$ if

$$\lambda_{\text{embedded.prot}} + z_{1-\alpha}\sqrt{\lambda_{\text{embedded.prot}}} < \lambda_{\text{ice}} - z_{1-\beta}\sqrt{\lambda_{\text{ice}}} \tag{196}$$

where $z_{1-\alpha}$ and $z_{1-\beta}$ are the $1-\alpha$ and $1-\beta$ percentiles of a standardized Gaussian. This expression is the most basic version of sample size calculation (Mathews, 2010). Simplifying

$$\begin{aligned} &z_{1-\alpha}\sqrt{\lambda_{\text{embedded.prot}}} + z_{1-\beta}\sqrt{\lambda_{\text{ice}}} < \lambda_{\text{ice}} - \lambda_{\text{embedded.prot}} \\ &z_{1-\alpha}\sqrt{N_e\tilde{\sigma}_{\text{embedded.prot}}D^2} + z_{1-\beta}\sqrt{N_e\tilde{\sigma}_{\text{ice}}D^2} < N_e\tilde{\sigma}_{\text{ice}}D^2 - N_e\tilde{\sigma}_{\text{embedded.prot}}D^2 \\ &\sqrt{N_e}D\left(\tilde{\sigma}_{\text{ice}} - \tilde{\sigma}_{\text{embedded.prot}}\right) > z_{1-\alpha}\sqrt{\tilde{\sigma}_{\text{embedded.prot}}} + z_{1-\beta}\sqrt{\tilde{\sigma}_{\text{ice}}} \end{aligned} \tag{197}$$

Finally

$$\begin{aligned} N_e &> \left(\frac{z_{1-\alpha}\sqrt{\tilde{\sigma}_{\text{embedded.prot}}} + z_{1-\beta}\sqrt{\tilde{\sigma}_{\text{ice}}}}{D\left(\tilde{\sigma}_{\text{ice}} - \tilde{\sigma}_{\text{embedded.prot}}\right)}\right)^2 \\ &= \frac{\left(z_{1-\alpha}\sqrt{\frac{\tilde{\sigma}_{\text{embedded.prot}}}{\tilde{\sigma}_{\text{ice}}}} + z_{1-\beta}\right)^2}{\tilde{\sigma}_{\text{ice}}}\frac{1}{D^2C^2} \end{aligned} \tag{198}$$

This expression is the same as Eq. (2) in Glaeser (1999) in which our term $\frac{\left(z_{1-\alpha}\sqrt{\frac{\tilde{\sigma}_{\text{embedded.prot}}}{\tilde{\sigma}_{\text{ice}}}} + z_{1-\beta}\right)^2}{\tilde{\sigma}_{\text{ice}}}$ has been substituted in that article by 25 (a value that without any ice and protein considerations would correspond to an approximate confidence level and statistical power of 99.4%). This value appears from a sample size design in which the separation between signal and noise is required to be 5 standard deviations. The equivalent design corresponds to the formula

$$N_e > \frac{z_{1-\alpha}^2}{D^2C^2} \tag{199}$$

with a confidence level of 99.9999%.

Note that this expression is valid for the detection of particle projections with respect to their background (in which D would be the diameter of the particle) and the detection of a small protein feature of size D (a small number that can be as low as 2-3 Å) outstanding from the main body of the protein. This is the total number of events (electrons) needed to detect the feature of size D. However, this number of events does not need to be given in a single shot; it can be fractionated, as is now the case with multiple frames in DDD movies or the analysis of 2D class averages. Actually, Glaeser (1999) argues that when D is a target resolution (say, $D = 2$ Å), this is the number of electrons needed to be acquired in the whole collection of images, and that it can be fractionated in the number of images that constitute our dataset. We could not establish the connection between the 2D reasoning presented in this section and the 3D arguments implied by Glaeser (1999), since in the connection between 2D and 3D we have the influence of an arbitrary angular distribution and the effect of the 3D reconstruction algorithm.

13. On the number of particles required for a given SNR

As EM is currently used, the main source of noise in the projection images comes from the ice in which the particles are embedded (this statement is true when we analyze micrographs and less so when we go down to the level of frames due to the low electron counting). In this section we will estimate the number of measurements we need of a given Fourier coefficient to have a Signal-to-Noise Ratio larger than a given threshold SNR_0. In this analysis we will disregard the effect of the CTF, since the 3D reconstruction algorithm is in charge of compensating its damping.

The cross-section of a scattering object, $f(\mathbf{r})$, is related to the Fourier transform of that object, $F(\mathbf{R})$, through the relationship (Born and Wolf, 1999)[Section 13.3]

$$\tilde{\sigma} = \int \left|F(\mathbf{R})\right|^2 d\mathbf{R} \tag{200}$$

Actually, this expression is consistent with the scattered intensity in Fourier space (Eq. (170)) and it is a consequence of energy conservation and Plancherel's theorem. Note that the integration must be performed in the Fourier plane onto which the 2D projection of the map is being taken. The exact shape of the Fourier transform of the object is unknown at the moment of estimating the number of projections. We may assume radial symmetry, make a change of variables to integrate in polar coordinates and simplify to

$$\tilde{\sigma} = \int_0^{R_{max}} \int_0^{2\pi} \left|F(R)\right|^2 R dR d\Theta = 2\pi \int_0^{R_{max}} \left|F(R)\right|^2 R dR \tag{201}$$

In Henderson (1995) it was assumed a constant amplitude, $\left|F(R)\right|^2 = F_0^2$ (Eqs. A5 and A6 of Henderson (1995) disregard the decay of the amplitude of the Fourier coefficients of a macromolecule). We propose that a more accurate estimate is given by the dependency on the radius of gyration since it suggests how the protein Fourier coefficients decay along frequency. Substituting Eq. (178) into the integral above we get

$$\tilde{\sigma} = 2\pi F_0^2 R_{max}\left(1 - \frac{R_g^2}{9}R_{max}^3\right) \tag{202}$$

from where

$$F_0^2 = \frac{\tilde{\sigma}}{2\pi R_{max}\left(1 - \frac{R_g^2}{9}R_{max}^3\right)}. \tag{203}$$

At the highest resolution we get the value

$$\left|F(R_{max})\right|^2 = F_0^2\left(1 - \frac{R_g^2}{3}R_{max}^2\right) = \frac{\tilde{\sigma}\left(1 - \frac{R_g^2}{3}R_{max}^2\right)}{2\pi R_{max}\left(1 - \frac{R_g^2}{9}R_{max}^3\right)} \tag{204}$$

If we now substitute the value of the cross section of the protein (Eq. (189)), then

$$\left|F_{\text{prot}}(R_{max})\right|^2 = \frac{D\rho_{\text{prot}}\sigma_{Z=6}\left(1 - \frac{R_{g,prot}^2}{3}R_{max}^2\right)}{2\pi MW_C\left(1 - \frac{R_{g,prot}^2}{9}R_{max}^3\right)} \tag{205}$$

Similarly, for the surrounding ice, we would get

$$\left|F_{\text{surr.ice}}(R_{max})\right|^2 = \frac{(H-D)\rho_{\text{ice}}\sigma_{Z=8}\left(1 - \frac{R_{g,ice}^2}{3}R_{max}^2\right)}{2\pi MW_O\left(1 - \frac{R_{g,ice}^2}{9}R_{max}^3\right)} \tag{206}$$

When multiple measurements, N_{meas}, of the same Fourier coefficient are taken, the signal is reinforced while the noise is decreased by a factor N_{meas}. The Signal-to-Noise Ratio becomes

$$SNR = \frac{\left|F_{\text{prot}}(R_{max})\right|^2}{\frac{1}{N_{\text{meas}}}\left|F_{\text{surr.ice}}(R_{max})\right|^2} \tag{207}$$

Consequently the number of measurements must be

$$\begin{aligned} N_{\text{meas}} &> SNR_0 \frac{\left|F_{\text{surr.ice}}(R_{max})\right|^2}{\left|F_{\text{prot}}(R_{max})\right|^2} \\ &= SNR_0\left(\frac{H-D}{D}\right)\left(\frac{\rho_{\text{prot}}MW_C\sigma_{Z=8}}{\rho_{\text{ice}}MW_O\sigma_{Z=6}}\right)\left(\frac{3 - R_{g,ice}^2R_{max}^2}{3 - R_{g,prot}^2R_{max}^2}\right)\left(\frac{9 - R_{g,prot}^2R_{max}^3}{9 - R_{g,ice}^2R_{max}^3}\right) \end{aligned} \tag{208}$$

In this expression, we see that the number of measurements increases with the target SNR and for small molecules (small D). The dependence with frequency, R_{max} is not obvious, but it depends on how the Fourier coefficients of the protein and ice fall along frequency. As an approximation we can presume that the radius of gyration of the ice will be much smaller than the radius of gyration of the macromolecule, then

$$\left(\frac{3 - R_{g,ice}^2R_{max}^2}{3 - R_{g,prot}^2R_{max}^2}\right)\left(\frac{9 - R_{g,prot}^2R_{max}^3}{9 - R_{g,ice}^2R_{max}^3}\right) \approx \frac{9 - R_{g,prot}^2R_{max}^3}{3 - R_{g,prot}^2R_{max}^2} \approx 3 + R_{g,prot}^2R_{max}^2 \tag{209}$$

Interestingly, the number of measurements needed for a given coefficient increases with the square of the radius of gyration and the square of the desired resolution, meaning that achieving high resolution for big particles requires more measurements than the same resolution for a small particle, and that the dependence is quadratic.

Bracewell showed that an object of diameter D can be recovered from samples of its Fourier transform if the sampling rate in Fourier space is smaller than $1/D$ (Bracewell, 1958). Then, he showed (Bracewell and Riddle, 1967) that in a single tilt-axis collection geometry, the 3D object could be recovered if the distance between Fourier coefficients two adjacent projections, at a given circumference of radius R_{max} was smaller that $1/D$, that is

$$\frac{\pi}{N_{imgs}}R_{max} < \frac{1}{D} \Rightarrow N_{imgs} > \pi D R_{max} \tag{210}$$

This is a result very much used in EM to suggest the number of images required to perform 3D reconstruction. However, note that this number was deduced using a very particular collection geometry (single tilt axis), and that it does not consider noise. This formula was used in Henderson (1995) (Eq. (A11)) to estimate the number of images required to achieve a given resolution. However, this formula is only valid for a single tile axis data collection. In the following, we generalize the formula to consider an even distribution of projections with randomly distributed orientations. Our reasoning is not valid for strongly biased projection distributions (Sorzano et al., 2001) or a data collection geometry with very few projections.

Based on Bracewell's reasoning of samples separated less than

$1/D$, we may deduce the number of images evenly distributed so that, on average, each Fourier coefficient receives the required number of measurements. Let us analyze a Fourier sphere of radius R. Disregarding discrete effects, within each circle of radius R, each experimental image is providing $N_{\text{samples},R}$ samples separated on both directions $1/D$ where

$$N_{\text{samples},R} = \frac{\pi R^2}{\left(\frac{1}{D}\right)^2} = \pi R^2 D^2 \tag{211}$$

If we have N_{imgs} such images, we will have a total of

$$N_{\text{total samples},R} = \pi R^2 D^2 N_{\text{imgs}} \tag{212}$$

such samples. These samples occupy a volume of

$$V_{\text{total samples},R} = \frac{4}{3}\pi R^3 \tag{213}$$

Let us analyze now the density of samples between a sphere of radius $R-\Delta$ and R. The density of samples is

$$\begin{aligned}\rho_{\text{total samples},R-\Delta,R} &= \frac{N_{\text{total samples},R} - N_{\text{total samples},R-\Delta}}{V_{\text{total samples},R} - V_{\text{total samples},R-\Delta}} \\ &= \frac{\pi D^2 N_{\text{imgs}}\left(R^2 - (R-\Delta)^2\right)}{\frac{4}{3}\pi\left(R^3 - (R-\Delta)^3\right)} \\ &= \frac{3}{4}D^2 N_{\text{imgs}}\frac{2R-\Delta}{3R^2 - 3\Delta R + \Delta^2}\end{aligned} \tag{214}$$

When Δ goes to 0, the density of samples goes to

$$\rho_{\text{total samples},R} = \frac{D^2 N_{\text{imgs}}}{2R} \tag{215}$$

This density is the superficial density of samples at a frequency R, when the samples are separated from nearby samples by $1/D$. The number of samples on the surface of the sphere of radius R is

$$N_{\text{total samples,surface},R} = \left(\frac{D^2 N_{\text{imgs}}}{2R}\right)\left(4\pi R^2\right) = 2\pi R D^2 N_{\text{imgs}} \tag{216}$$

However, there are only

$$N_{\text{Fourier coefficients, }R} = \frac{4\pi R^2}{\left(\frac{1}{D}\right)^2} \tag{217}$$

different coefficients in this surface (note that this is only an approximation, which is better fulfilled as R grows), consequently the number of measurements per different coefficient is

$$\frac{N_{\text{total samples,surface, }R}}{N_{\text{Fourier coefficients, }R}} = \frac{2\pi R D^2 N_{\text{imgs}}}{\frac{4\pi R^2}{\left(\frac{1}{D}\right)^2}} = \frac{N_{\text{imgs}}}{2R} \tag{218}$$

This number has to be larger than the number of measurements estimated at Eq. (208). For the sake of clarity let us summarize in a single (approximated) equation the number of images needed for a given resolution, assuming that these images are evenly distributed in the projection sphere

$$N_{\text{imgs}} > 2SNR_0\left(\frac{H-D}{D}\right)\left(\frac{\rho_{\text{prot}} MW_C \sigma_{Z=8}}{\rho_{\text{ice}} MW_O \sigma_{Z=6}}\right) R_{max}\left(3 + R_{g,prot}^2 R_{max}^2\right) \tag{219}$$

That is, the number of images, depend on the cube of the desired resolution (there is a quadratic dependence due to electron scattering and a linear dependence due to the data collection geometry).

It has been shown that the effect of illuminating the sample with the electron beam decreases the signal to noise ratio originally in the sample (Unwin and Henderson, 1975; Hayward and Glaeser, 1979; Stark et al., 1996; Baker et al., 2010; Grant and Grigorieff, 2015). The SNR is supposed to decay with the dose N_e as

$$SNR(N_e, R) = SNR(0, R)e^{-\frac{N_e}{N_{crit}(R)}} \tag{220}$$

where $SNR(0,R)$ is the SNR with no beam damage (0 electrons/Å^2) at a given frequency, and $N_{crit}(R)$ (electrons/Å^2) is a parameter that depends on frequency, the operating voltage and the object being imaged. For instance, Grant and Grigorieff (2015) measured

$$N_{crit}(R) = 0.245R^{-1.665} + 2.81 \tag{221}$$

at 300 kV for rotavirus VP6. Knowing this decay of SNR, the number of images above has to be multiplied by a compensatory term $e^{\frac{N_e}{N_{crit}(R_{max})}}$ to account for the SNR decay experienced by radiation damage.

Stagg et al. (2008, 2014); Heymann (2015) experimentally studied how the resolution, measured as the frequency at which the FSC drops below 0.5, depended on the number of images and a number of other variables. They showed that the resolution improves with an increasing number of particles, increased magnification and acceleration voltage. Their works seem to point to a dependency of the form

$$\begin{aligned}R_{max} &= R_0 + aM + bV + c\log_{10}(N_{\text{imgs}}) \\ &= R_0(M,V) + c\log_{10}(N_{\text{imgs}})\end{aligned} \tag{222}$$

where R_{max} is the resolution achieved (in Å^{-1}), M is the microscope magnification, V is the voltage and R_0, a, b and c are positive constants. If we solve for the number of images, we have

$$N_{\text{imgs}} = 10^{\frac{R_{max} - R_0(M,V)}{c}} \tag{223}$$

This result is compatible with the analysis performed in this section. Choosing a fixed threshold for the FSC (FSC = 0.5) is equivalent to choosing a target SNR_0. Then, it states that 1) the resolution depends on the magnification and voltage, as also do the cross-sections in Eq. (219) (higher magnification or voltage result in a smaller number of images); 2) the number of particles required to achieve a given resolution rapidly increases with R_{max}, as also does in Eq. (219). The exact form of the dependence on the target resolution differs, but in both cases the number of images rapidly grow with the target resolution, and this growth is faster than linear, as originally proposed by Henderson (1995) (which was calculated with two important simplifications as discussed along this section). In any case, it must be noted that our cubic dependence with R_{max} was achieved only after a number of simplifications trying to bring insight into the more accurate dependence calculated in Eq. (208), and the exact dependence is much more complicated. Stagg et al. (2008, 2014); Heymann (2015) take a practical approach of empirically observing how the resolution progresses with the number of particles and they confirm the more-than-linear dependence.

If the particle has some internal symmetry, obviously the number of particles above must be reduced by a factor equal to the number of Fourier replicates implied by the symmetry (for instance, in a C3 symmetric particle each experimental coefficient contributes to 3 Fourier coefficients in the map; in a D3 symmetric particle, it contributes to 6; in an icosahedral particle, it contributes to 60).

14. On the composition of systems for the calculation of the SNR

The SNR is degraded by the addition of different noise components. Baxter et al. (2009) studied the contribution of the different structural, shot and digitization noise to the final SNR. Their aim was to decompose the SNR into different SNRs corresponding to each one of the effects. It was assumed that structural noise is the first one to be added, then shot noise is added during the micrograph acquisition, and finally digitization noise is added. The following equation was used by Baxter et al. (2009) for the compound SNR for three concatenated systems

$$1+\frac{1}{SNR_{comp}}=\left(1+\frac{1}{SNR_1}\right)\left(1+\frac{1}{SNR_2}\right)\left(1+\frac{1}{SNR_3}\right) \tag{224}$$

A similar formula appeared in Frank and Al-Ali (1975).

This formula is well-known in signal processing and communication engineering but less so in the EM community. Let us develop some insight of its meaning and on our way we will discover a more complete version of it. Taking into account that $SNR=S/N$, the expression $1+1/SNR$ represents really $S+N/S$. The rationale behind this expression is to compute the ratio between the output power $(S+N)$ and the input power (S) to a given system. In this way, calling I_i the input power to the system i and O_i the output power to the system i, and considering that the output of the system i is the input of the system $i+1$, we could have derived the previous expression by considering two systems that take an input signal and add independent noises. The input to the first system I_1 is a given signal $s(\mathbf{r})$ whose power is S (in the EM context $s(\mathbf{r})$ is the original 2D projection of the underlying structure without any noise). Then, the system adds a noise whose power is N_1. The total power at the output is $O_1=S+N_1$ and the ratio between the output and input power is

$$\frac{O_1}{I_1}=\frac{S+N_1}{S}=1+\frac{1}{SNR_1} \tag{225}$$

The second system receives an input power that is the output power of the previous system $I_2=O_1$, and also adds a noise of power N_2. The final output power is $O_2=I_2+N_2$. The ratio between the input and output at the second system is

$$\frac{O_2}{I_2}=\frac{I_2+N_2}{I_2}=1+\frac{1}{SNR_2} \tag{226}$$

Another expression for this ratio could have been

$$\frac{O_2}{I_2}=\frac{S+N_1+N_2}{S+N_1} \tag{227}$$

If we now consider the ratio between the input power to the whole system and its output power, we find

$$\frac{O_2}{I_1}=\frac{S+N_1+N_2}{S}=1+\frac{N_1+N_2}{S}=1+\frac{1}{SNR_{comp}} \tag{228}$$

On the other hand

$$1+\frac{1}{SNR_{comp}}=\frac{O_2}{I_1}=\frac{O_1}{I_1}\frac{O_2}{I_2}=\left(1+\frac{1}{SNR_1}\right)\left(1+\frac{1}{SNR_2}\right) \tag{229}$$

The addition of a third system is straightforward.

In the previous derivation we have not considered the power gain or attenuation introduced by the different systems. If the two systems have gains G_1 and G_2 respectively (note that gains larger than 1 imply a power gain, while gains smaller than 1 imply a signal attenuation), then the output power of the system 1 is $O_1=G_1S+N_1$. The equivalent of Eq. (225) with gain is

$$\frac{O_1}{I_1}=\frac{G_1S+N_1}{S}=G_1+\frac{1}{SNR_1} \tag{230}$$

And the same holds for system 2. The output power of the system 2 is $O_2=G_2I_2+N_2=G_2G_1S+G_2N_1+N_2$. Thus, the overall ratio of output power to input power is

$$\begin{aligned}\frac{O_2}{I_1}&=\frac{G_2G_1S+G_2N_1+N_2}{S}=G_2G_1+\frac{G_2N_1+N_2}{S}\\&=G_2G_1+\frac{1}{SNR_{comp}}\end{aligned} \tag{231}$$

Therefore, if different gains are considered for each system, then Eq. (224) becomes

$$G_1G_2G_3+\frac{1}{SNR_{comp}}=\left(G_1+\frac{1}{SNR_1}\right)\left(G_2+\frac{1}{SNR_2}\right)\left(G_3+\frac{1}{SNR_3}\right) \tag{232}$$

The first system of Baxter et al. (2009) is the system adding structural noise. The structural noise comes from the carbon layer and ice around the particle of interest. If S is the power of the particle of interest, it is likely that this power is reduced since part of the energy coming out from the particle is absorbed by the ice and carbon coming later in the electron path. Therefore $G_1<1$.

The second system is adding shot noise due to the quantum nature of electrons. It does not seem that there is any gain or absorption of the energy coming out of the structure being imaged, and G_2 can be assumed to be 1.

The third system is adding digitization noise. This noise is added by the CCD camera or the combination film/scanner. The gain of the acquisition system will be in general a certain factor G_3 different from 1.

An important consequence of this reasoning is that the system gains have to be estimated along with the individual SNRs, otherwise the SNRs cannot be successfully estimated as pretended in Baxter et al. (2009).

This reasoning easily extends to Fourier space, Eq. (232) still holds but taking into account that the system gains and noises may be different for each frequency

$$G_1(\mathbf{R})G_2(\mathbf{R})G_3(\mathbf{R})+\frac{1}{\alpha_{comp}(\mathbf{R})}=\left(G_1(\mathbf{R})+\frac{1}{\alpha_1(\mathbf{R})}\right)\left(G_2(\mathbf{R})+\frac{1}{\alpha_2(\mathbf{R})}\right)\left(G_3(\mathbf{R})+\frac{1}{\alpha_3(\mathbf{R})}\right) \tag{233}$$

or in other words

$$\frac{S_o(\mathbf{R})}{N_o(\mathbf{R})}=\frac{G_1(\mathbf{R})G_2(\mathbf{R})G_3(\mathbf{R})S(\mathbf{R})}{G_2(\mathbf{R})G_3(\mathbf{R})N_1(\mathbf{R})+G_3(\mathbf{R})N_2(\mathbf{R})+N_3(\mathbf{R})} \tag{234}$$

This equation shows that the calculation of the Spectral SNR (SSNR) curves for each effect also needs the explicit consideration of the gain factors at each frequency which in real micrographs are

likely not to be unity over all frequencies.

The previous equation gives us the ratio between the output signal and the output noise, but not the SSNR. The SSNR at a single frequency would be

$$\frac{\left|S_o(\mathbf{R})\right|^2}{\left|N_o(\mathbf{R})\right|^2}, \tag{235}$$

and the overall SNR is

$$SNR_{comp} = \frac{\int_{\mathbb{R}^2} \left|S_o(\mathbf{R})\right|^2 d\mathbf{R}}{\int_{\mathbb{R}^2} \left|N_o(\mathbf{R})\right|^2 d\mathbf{R}} \tag{236}$$

15. Conclusions

In this paper we have reviewed and revised the theoretical foundations of the most popular resolution measures in 3DEM as well as some of their related problems. We have enhanced the original derivations of their statistical properties and have completed those results with new relationships and statistical distributions. We have seen that the FSC and the SSNR are the most tractable resolution measures and sensible thresholds have been given for them based on statistical inference. SSNR has the extra advantage of being extended to a 3D measure of resolution so that we can explore the direction-dependent resolution in Fourier space. A drawback of all methods is that the resolution curve statistics strongly depend on the actual implementation of the Fourier shells. Theoretically, thin shells should be preferred to thicker shells since they provide higher frequency resolution. How thin is thin? We have checked the implementation of the FSC in Xmipp and Relion and both calculate the distance in voxels of each Fourier coefficient to the Fourier origin and round it producing an index. The Fourier coefficient in question only contributes to the FSC at the so calculated index, implying that the corresponding shells are between 1 and 2 voxels wide depending on the specific 3D Fourier location, and probably this is the thinner implementation that does not leave any Fourier coefficient out. What is important is that to compare two FSCs calculated with two different packages, we should make sure that the shells used for the FSC calculations are the same. We have also reviewed and revised the connection between the SSNR and the electron dose and number of particles needed to achieve given contrast and resolution targets.

Despite all these theoretical advances, there is still lacking in the field a unique and measurable definition of what is resolution. The implicit definition used in this article is that the resolution is the frequency beyond which we cannot detect any signal within a given confidence level. However, the choice of the confidence level is still arbitrary and more work must be done by the community to unify criteria. More importantly, the whole article points into the direction that resolution curves may not be the more appropriate way of measuring resolution. The idea of defining the resolution as the capability of the structure to show high-resolution structures like α-helices, β-sheets and side chains is appealing. However, a quantitative method to define resolution in this way should be devised.

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