photons in each 40 femtosecond pulse, and are capable of reading out perhaps 120 of the resulting diffraction-pattern 'snapshots' every second. Such a beam, focused to micron dimensions, vaporizes the sample, but it has been discovered that a useful pattern is obtained before radiation damage commences (due to the photoelectron cascade). The method has given 2 Å resolution data from micron-sized protein nanocrystals, and, if sufficiently brief pulses are used, allows about 100 times greater dose to be delivered than the Henderson 'safe dose' (see Chapter 10.3). The snapshot data consist of partial reflections. As a consequence of the fully coherent nature of the radiation, for the smallest submicron nanocrystals the data show interference fringes between the Bragg reflections that facilitate iterative phasing (Spence *et al.*, 2011). Sample delivery has been based on a continuously flowing liquid jet of micron or submicron dimensions, freely flowing in vacuum, with gas focusing at a nozzle to prevent clogging. Merging of millions of nanocrystal snapshots to obtain full reflections has created new challenges for data analysis, as has the development of MAD phasing for the time-resolved absorption involved.

Diffraction patterns may also be obtained from single particles such as a virus or whole cell, commonly injected from a nebulizer in a gas-focused stream into vacuum. Each snapshot gives one projection of the particle in a random orientation, so that three-dimensional reconstruction requires a solution to the difficult problems of orientation determination and phasing of single-particle diffraction patterns. The available X-ray fluence per shot, together with the minimum amount of scattering needed for orientation determination, has so far limited resolution to about 30 nm. However, more powerful X-ray lasers, smaller focused spots and improvements in 'hit rate' are bound to improve resolution to the predicted 1 nm resolution limit for particles too thick for study by cryogenic electron microscopy (cryo-EM). Scattering in the water window, around 500 eV, gives greatly increased protein/water contrast, but wavelength-limited resolution. Conformational variability imposes similar limitations to those encountered in cryo-EM, and the merging of multiple projections from similar cells remains an important challenge for the future, perhaps based on topological constraints. The XFEL also offers unprecedented opportunities for time-resolved imaging, spanning the range from the femtosecond timescale important for electron-transfer reactions in biochemistry, to the slower microsecond processes of protein activity. In favourable cases, a fast optical trigger exists for pump–probe studies, while in others chemical reactions (such as the enzyme cycle) might be followed in mixed and flowing solutions. For this purpose, the correlated fluctuations in 'snapshot' small-angle X-ray (SAX) patterns may prove useful, since they offer a hit rate of 100%. Since these patterns are two-dimensional for particles frozen in space or time, they contain more information than conventional one-dimensional SAX patterns. The ability to reconstruct an image of one particle using the scattering from many randomly oriented particles frozen in space (without modelling) has recently been demonstrated (Saldin *et al.*, 2011). In summary, the XFEL has opened up many new exciting possibilities for structural and dynamic biology, based on entirely new experimental arrangements (now far from optimized) and offering great scope for developments in this highly interdisciplinary field, which spans laser, detector and particle injector physics, diffraction physics, and structural biology. We anticipate rapid progress in methods for the growth of suitable protein microcrystals, especially for membrane proteins. The XFEL is then certain to provide a wealth of new information on molecular mechanisms in biology, as techniques are refined and more powerful X-ray lasers are constructed.

1.4.5. Electron microscopy's impact on structural biology (S. Sun)

Just like crystallography half a century ago, cryo-electron microscopy (cryo-EM) has been developing rapidly and has become one of the dominant techniques in structural biology. Ever since an electron microscope was first constructed, there has been a desire to examine biological samples. There were two major obstacles to this task, one being how to obtain a sample thin enough for the electron beam to go through, the other being the preservation of the sample in the high vacuum of the microscope. Early EM studies of biological specimens combined fixation, dehydration, embedding and sectioning with application of heavy metals to provide contrast in tissue samples. These methods cannot preserve the biological samples in their native state, but merely produce an outline rather than provide intricate three-dimensional structural details. In the 1980s, Dr Jacques Dubochet and his co-workers at the EMBL developed a method of producing vitrified biological samples that made it possible to study biological specimens in their native state (Dubochet *et al.*, 1981, 1988). Together with the invention of high-stability cold stages and transfer mechanisms for electron microscopes, there has been an explosion of studies using cryo-EM for biological samples (Frank, 1989; Ruiz *et al.*, 1994; Frank, 2009; Wade & Hewat, 1994; Ben-Harush *et al.*, 2010).

Single-particle cryo-EM and cryo-electron tomography (cryo-ET) are the most commonly used techniques for the study of biological samples. Owing to the sensitivity to radiation that causes structural damage, only small electron doses can be used to examine biological samples. As a result, the signal-to-noise ratio is low for cryo-EM and especially for cryo-ET. In single-particle cryo-EM, this can be compensated for by collecting two-dimensional (2D) projections of a large number of particles and averaging projections representing the same orientation. Viruses have been among the most popular subjects for single-particle cryo-EM reconstruction because of their high symmetry. With several hundred particles, a 20 Å or so resolution reconstruction can be obtained with relative ease. For particles with low or no symmetry, more data need to be collected to achieve the same resolution. Two of the better-studied asymmetric particles by single particle cryo-EM are the ribosome and GroEL (Frank, 2001; Roseman *et al.*, 2001).

Although the theoretically achievable resolution limit using an electron microscope should be related to the wavelength of the electron beam, in reality there are many factors that affect the resolution, some of which are microscope-related or sample-related. These factors include, but are not limited to, the quality of the microscope, the stability of the sample towards radiation damage, and sample or beam movements caused by electric or magnetic field variation, temperature changes, or mechanical vibrations. It is also essential that the individual samples are sufficiently homogeneous to allow meaningful averaging within the desired resolution. The quality of a specific cryo-EM experimental data set can be judged by the 'Thon rings', provided by the averaged radial Fourier transformation of the particle images. These rings show the averaged resolution limit of the image signal and thus show how far it might be possible to extend the resolution, provided that all relevant parameters are accurately determined. Once the best possible data have been collected on a particular sample, numerous computational steps follow to achieve a good result. These include boxing (selecting

particles within a 'box' and normalizing the density to a standard variation), contrast-transfer-function correction, 2D alignment and 3D reconstruction. Many independent groups have developed various software packages for 3D EM reconstruction such as *SPIDER* (see Chapter 19.8), *IMAGIC 4D* (Chapter 19.9), *EMAN* (see Chapter 19.10), *AUTO3DEM* and *FREALIGN* (Ludtke *et al.*, 1999; Shaikh *et al.*, 2008; Yan *et al.*, 2007; Grigorieff, 2007). Numerous aspects of the reconstruction procedure are still being developed to achieve higher resolution, such as individual-particle contrast-transfer-function correction, magnification correction, local refinement in 2D alignment, and averaging among quasi-equivalent subunits within the icosahedral asymmetric unit after the 3D reconstruction. The highest resolution structure obtained for a virus is the 3.1 Å resolution structure of the cytoplasmic polyhedrosis virus.

The definition of resolution of a single-particle cryo-EM reconstruction is different from that used for assessing a crystal structure. The resolution of an EM reconstruction is usually estimated using the 'Fourier shell correlation' method (van Heel & Schatz, 2005). This requires dividing the randomly chosen images into two separate groups and computing reconstructions assuming the previously determined orientations. The resultant maps are then Fourier transformed to permit a comparison of the Fourier amplitudes and phases. The resolution at which the correlation between the amplitudes falls below 0.5 or the average phase difference is greater than 45° is usually a conservative estimate of resolution used by many groups, although other definitions are also in use (Henderson, 2004). Just as is the case for crystallographic maps, the quality of maps at the same resolution can vary depending on the degree of accuracy of the phase determination. Therefore, a visual inspection of a 3D EM map should serve as a useful check.

In contrast to single-particle cryo-EM, cryo-ET can be used to study biological samples that lack homogeneity (Milne & Subramaniam, 2009; Li & Jensen, 2009; Hoenger & McIntosh, 2009; Subramaniam *et al.*, 2007). In this technique, images of the same sample oriented by a succession of tilt angles are taken about a common axis and are subsequently combined into a three-dimensional map. Because the same sample is repeatedly exposed to the electron beam, the allowable electron dose has to be spread over all the tilted images, making the exposure for each image much less than for single-particle cryo-EM. This significantly reduces the limit of achievable resolution. In addition, the physical limitation on the tilt angles results in a 'missing wedge' problem. Dual-axis tomography has been developed to alleviate this problem to some extent. Averaging of the homogeneous portion of the heterogeneous sample has been used when appropriate to increase the resolution of a cryo-ET experiment (Bartesaghi & Subramaniam, 2009).

Cryo-EM methods have been an important addition to the family of structural biology tools. With single-particle cryo-EM, near-atomic-resolution structures can be obtained without the requirement of crystal formation, which is still the rate-limiting step for crystallographic studies. Cryo-ET is particularly useful in studying large molecular complexes and cells that do not possess any symmetry. Further improvements, such as the use of phase plates, further development of automated data-collection software and perfection of reconstruction algorithms, make it likely that cryo-EM will overtake crystallography or NMR as the primary tool for structural biology at low to medium resolution. However, crystallography, NMR and other tools will still be essential to obtain detailed, atomic resolution information.

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