1.4.3. Additional comments on *Gazing into the crystal ball* (D. M. Himmel)

I wish to expand briefly on Eddy's and Michael's comments to entertain the question of how macromolecular crystallography and structural biology might change the future. New detector technologies, such as shutterless detector systems that can collect data in continuous rotation mode (see, for example, Miyoshi *et al.*, 2008), are now being developed and deployed at synchrotrons. These detectors will improve the quality of data collection and integration. It is possible that the emergence of ever more rapid and more sensitive X-ray detectors, along with new X-ray technologies, will lead to multiple-wavelength rapidly pulsed X-ray beams that are synchronized by wavelength with the detector. At each crystal rotation position, the beam will rapidly run through each of the several tuned wavelengths and record a diffraction pattern for each wavelength. This sort of setup can be used to collect MAD data from a single data pass through the Ewald sphere. In cases where crystals are particularly sensitive to X-ray damage, this approach, along with the ability to detect anomalous signals from ever-smaller elements in the Periodic Table, may make MAD more competitive with molecular replacement as the method of choice for structure determination. Molecular replacement itself will become more powerful as it is combined with *ab initio* phasing and computational chemistry methods. In the future, due to other emerging technologies such as described in Section 1.4.4 below, whether or not one can crystallize a macromolecule may no longer limit one's ability to determine a structure by X-ray diffraction. Here, I will focus on two additional points: (1) how X-ray crystallography and allied fields (such as NMR and EM) have already started to transform the physical sciences, and (2) how they might transform the overall human experience as well.

In the mid-twentieth century, the physical sciences to a considerable extent were divided into separate fiefdoms that often competed with each other rather than fostered a collaborative spirit. Physics, chemistry, virology and biochemistry, to name a few, each attempted to stand on its own, each using its own independent preferred jargon and preferred explanations for scientific questions of the day. By contrast, what we see happening today is a convergence of all these once-separate fields to understand first principles right down to the molecular level and beyond. More than that, macromolecular crystallography and allied fields are playing a substantial role in catalysing this convergence. The determination and subsequent analysis of a macromolecular structure (such as a protein in complex with nucleic acid, co-factors and/or small-molecule ligands) of necessity culminates from the application of physics, mathematics and chemistry to begin with, followed by various biological sciences to understand the context of a molecular structure. But for physicists, chemists and biologists to speak to each other productively, they increasingly must share the same jargon and learn each other's disciplines. Sir Isaac Newton once wrote, 'If I have seen further it is by standing on the shoulders of giants.' The giants upon which we stand today in crystallography were the leaders of those separate fields that today find their synthesis in the determination and understanding of molecular structure. It is reasonable to assume that this unifying spirit will continue and will foster greater breakthroughs in structure determination and understanding the properties of molecules in the real world.

In addition to bringing various disciplines of science together, macromolecular crystallography and allied fields are likely to revolutionize the way people live in the future. Richard Feynman was reported to have said, 'What I cannot create, I do not understand.' Chapters 3.1, 3.2, and 4.3 of this volume describe the harnessing of gene expression and protein engineering to further the aims of the X-ray crystallographic experiment. Macromolecular engineering, however, does not stop there. Recent years have seen a rising interest in the field of nanotechnology, which, according to some definitions, aims to design machines and technologies that operate on a scale of about 100 nm or smaller (Farokhzad & Langer, 2009). These technologies generally encompass applications of our understanding of chemistry and physics. As this field matures, it will encounter many of the problems on the molecular level that have already been solved by the machines and devices that exist in biological systems, such as enzymes, molecular motors and structural proteins. Proofs of concept have recently been described in which components of biological molecules were redesigned for applications outside their usual environment (Goel & Vogel, 2008; van den Heuvel & Dekker, 2007; Lewis *et al.*, 2011), or in which principles learned from structural biology have been applied to the construction of completely artificial molecular devices (Ceroni *et al.*, 2010). The discoveries of structural biology may well light the way to the nanotechnologies of the future. These technologies, by operating at the molecular level like never before, will enable the manufacture of superior fabrics and materials, improve medical diagnostics, and revolutionize electronics and photonics in a whole host of devices, from computers and robotics to communication and the efficient harnessing of unconventional energy sources like light. Chemical catalysis will be performed in industry with such tight control that there will be far fewer unwanted side reactions than is commonplace today, so that, for example, medicines and other materials can be manufactured cheaply to an exceptionally high level of purity. Drug-delivery vehicles will be developed that precisely target a cell type, tissue type (Farokhzad & Langer, 2009) or even a pathogen. Some of these molecular vehicles will be modelled on methods used by viruses such as influenza, HIV or even bacteriophages, using a harpoon or plasma membrane fusion strategy, and will release their cargo in response to a chemical or other trigger. Others will employ active transport, in which the therapeutic agent will be guided to its target by remote control and carry diagnostics modules for the ride. The development of these nanotechnologies will both benefit from nanotechnological spin-offs of structural studies as well as require X-ray crystallography and other structural techniques to aid in the analysis of the nanomachines.

To peer at an image of individual molecules at atomic or near-atomic resolution, X-ray crystallography has served to provide the missing lens needed to focus that image. New and maturing fields described in the following sections will supply other ways to provide that missing lens, or, indeed, to obtain the initial phase information needed to determine a higher-resolution X-ray crystal structure.

1.4.4. *Gazing into the crystal ball* – the X-ray free-electron laser (J. C. H. Spence)

The recent invention of the pulsed hard X-ray (free-electron) laser (XFEL) is certain to impact structural biology, particularly in the areas of protein nanocrystal analysis (Chapman *et al.*, 2011), single-particle imaging (Siebert *et al.*, 2011), time-resolved crystallography and solution scattering (see the forthcoming reviews in *Reports on Progress in Physics* by Spence and Chapman). Current hard-X-ray machines provide about 10^{12}

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