International Tables for Crystallography (2012). Vol. F, Chapter 1.4, pp. 39–44.

Chapter 1.4. Perspectives for the future

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1.4.1. Gazing into the crystal ball (E. Arnold)

We live in an era when there are many wonderful opportunities for reaching new vistas of human experience. Some of the dreams that we hold may be achieved through scientific progress. Among the things we have learned in scientific research is to expect the unexpected – the crystal ball holds many surprises for us in the future. Alchemists of ancient times laboured to turn ordinary materials into precious metals. These days, scientists can create substances far more precious than gold by discovering new medicines and materials for high-technology industries. Crystallography has played an important role in helping to advance science and the human endeavour in the twentieth century. I expect to see great contributions from crystallography also in the twenty-first century.

Science of the twentieth century has yielded a great deal of insight into the workings of the natural world. Systematic advances are permitting dissection of the molecular anatomy of living systems. This has propelled us into a world where these insights can be brought to bear on problems of design. The impact in such fields as health and medicine, materials science, and microelectronics will be continually greater.

1.4.1.1. What can we expect to see in the future of science and technology in general?

Just as few were successful in predicting the ubiquitous impact of the Internet, it is difficult to predict which specific technologies will accomplish the transition into the culture of the future. It is possible to envision instantaneous telecommunication and videoconferencing with colleagues and friends throughout the world – anytime, anywhere – using small, portable devices. Access to computer-based information *via* media such as the Internet will become continually more facile and powerful. This will permit access to the storehouse of human knowledge in unprecedented ways, catalysing more rapid development of new ideas.

Experimental tests of new ideas will continue to play a crucial role in the guidance of scientific knowledge and reasoning. However, more powerful computing resources may change paradigms in which ever more powerful simulation techniques can bootstrap from primitive ideas to full-blown theories. I still expect that experiment will be necessary for the foreseeable future, since nearly every well designed experiment yields unexpected results, often at a number of levels.

In the realm of biology, greater understanding of the structure and mechanism of living processes will permit unprecedented advances in health and medicine. Even those scientists most sceptical of molecular-design possibilities would be likely to admit that revolutionary advances have been achieved. In the area of drug design, for example, structure-based approaches have yielded some of the most important new molecules currently being introduced worldwide for the treatment of human diseases ranging from AIDS and influenza to cancer and heart disease. This is a relatively young and very rapidly changing area, and it is reasonable to expect that we have witnessed only the tip of the iceberg. Dream drugs to control growth and form, ageing, intelligence, and other physiologically linked aspects of health and well-being may be developed in our lifetime. As greater understanding of the structural basis of immunogenicity emerges, we should also expect to benefit from structure-based approaches to vaccine design.

Other areas where molecular design will play revolutionary roles include the broad field of material sciences. Traditionally, 'materials science' referred to the development of materials with desired physical properties – strength, flexibility, and resistance to damage by physical and chemical agents. Now, materials science includes key foci in development of new biomaterials and in the burgeoning field of nanotechnology. The acrobatics of new smart materials could include computation at speeds that may be much faster than can be accomplished with silicon-based materials.

1.4.1.2. How will crystallography change in the future?

Potential future advances in the fields of crystallography, structural chemistry and biology are tantalizing. Owing to the limitations of current physical theories and experimental possibilities, large numbers of molecules have generally been required for detailed investigations of molecular phenomena. Given the complexity of large biological molecules, only techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) have been suitable for describing the detailed atomic structures of these systems. New technologies, unforseen and currently under development, will make the successful imaging of single specimens and single molecules at high resolution far more commonplace than is now achievable. Even now, we are beginning to see X-ray and electron microscopy (EM) methods harnessed to visualize single particles at high resolution. Methods such as coherent X-ray diffraction microscopy (CXDM), discussed in Chapter 9.3 of this volume, and the X-ray (free-electron) laser (XFEL) are challenging the limits of our ability to image single particles directly at high resolution (see Section 1.4.4, below). Merging of information from multiple specimens, as is currently done in electron microscopy, may be very powerful in X-ray microscopy as well.

X-ray sources will continue to evolve. High-intensity synchrotron sources have allowed the development of dramatically faster and higher-quality diffraction data measurements. Complete multiwavelength X-ray diffraction data sets have been measured from frozen protein crystals containing selenomethionine (SeMet) in less than one hour, leading to nearly automatic structure solution by the multiwavelength anomalous diffraction (MAD) technique. At present, such synchrotron facilities are enormous national or multinational facilities that sap the electric power of an entire region. Perhaps portable 'table-top' X-ray sources will be developed that can be used to create synchrotron-like intensity in the laboratory. If such sources could have a tunable energy or wavelength, then experiments such as MAD would be routinely accessible within the laboratory. Better time resolution of molecular motion and of chemical reactions will be achieved with higher-intensity sources.

Sample preparation for macromolecular structural studies has undergone a complete revolution thanks to the advent of

recombinant DNA methods. Early macromolecular studies were limited to materials present in large abundance. By the late 1970s and early 1980s, molecular biology made it possible to obtain desired gene products in large amounts, and new methods of chemical synthesis permitted production of large quantities of defined oligonucleotides. Initial drafts of the entire human genome have been mapped and sequenced, allowing even broader access to genes for study. The combination of structural genomics and already ongoing studies will lead to knowing the structure of the entire human proteome in a finite amount of time. Many materials are still challenging to produce in quantities sufficient for structural studies. Engineering methods (site-directed mutagenesis, combinatorial mutagenesis and directed evolution techniques) have permitted additional sampling of molecular diversity, and we can expect that even more powerful methods will be developed. Engineering of solubility and crystallizability will help make more problems tractable for study. Perhaps, as suggested before, techniques for visualization of single molecules may become adequate for *in situ* visualization of molecular interactions in living cells and organisms. However, traditional considerations of amount, purity, specific activity *etc.* will remain important, as will hard work and good luck.

The phase problem has continued to be a stumbling block for structure determination. Experimental methods, including isomorphous replacement and anomalous-scattering measurements, are currently a mainstay, and will be for the foreseeable future. New isomorphous heavy-atom reagents and preparation methods will emerge; witness the valuable engineering of derivatives *via* mutagenesis to add such heavy-atom-binding sites as cysteine residues in proteins. Anomalous-scattering measurements from macromolecular crystals containing heavy metals or SeMet replacements of methionine residues in proteins have led to tremendous acceleration of the phase-problem solution for many structures – especially in the last two decades with the availability of 'tunable' synchrotron radiation. It should become possible to take better advantage of anomalous-scattering effects from lighter atoms already present in biological molecules: sulfur, phosphorus, oxygen, nitrogen and carbon. The increasingly higher intensity of synchrotron-radiation sources may permit structure solution from microcrystals of macromolecules. Incorporation of non-standard amino acids into proteins will become more common, leading to a vast array of new substitution possibilities. Molecular-replacement phasing from similar structures or from noncrystallographically redundant data is now commonplace and is continuing to become easier. Systematic molecular replacement using all known structures from databases may prove surprisingly powerful, if we can learn how to position small molecular fragments reliably. Systematic molecular-replacement approaches should help identify what folds may be present in a crystalline protein of unknown structure. Direct computational assaults on the phase problem are also becoming more aggressive and successful, although direct-methods approaches still work best for small macromolecular structures with very high resolution data.

Crystallography and structural biology have been helping to drive advances in three-dimensional visualization technology. Versatile molecular-graphics packages have been among the most important software applications for the best three-dimensional graphics workstations. Now that personal computers are being mass-produced with similar graphics capabilities, we are beginning to see a molecular-graphics workstation at every computer, whether desktop or portable (terms that soon may become antiquated since everything will become more compact). Modes of input will include direct access to thought processes, and computer output devices will extend beyond light and sound. Universal Internet access will provide immediate access to the rapidly increasing store of molecular information. As a result, we will achieve a more thorough understanding of patterns present in macromolecular structures: common folds of proteins and nucleic acids, three-dimensional motifs, and evolutionary relationships among molecules. Simulations of complex molecular motions and interactions will be easier to display, making movies of molecules in motion commonplace. Facile 'virtual reality' representation of molecules will be a powerful research and teaching aid. Chemical reaction mechanisms will become better understood over time through interplay among theory, experiment and simulation. The ability to simulate all coupled chemical reactions in living cells and organisms will be achieved over time.

Advances in computational productivity depend on the intricate co-evolution of hardware and software. For silicon-based transistor chips, raw computational speed doubles approximately every 18 months (Moore's law). Tools and software for writing software will continue to advance rapidly. With greater modularity of software tools, it will become easier to coordinate existing programs and program suites. Enhanced automation, parallelization and development of new algorithms will also increase speed and throughput. More powerful software heuristics involving artificial intelligence, expert systems, neural nets and the like may permit unexpected advances in our understanding of the natural world.

In summary, we eagerly await what the future of science and of studies of molecular structure will bring. There is every reason to expect the unexpected. If the past is a guide, many new flowers will bloom to colour our world in bright new ways.

1.4.2. Brief comments on *Gazing into the crystal ball* (M. G. Rossmann)

Eddy Arnold and I had planned to write a joint commentary about our vision of the future of macromolecular crystallography. However, when Eddy produced the first draft of '*Perspectives for the future*', I was fascinated by his wide vision. I felt it more appropriate and far more interesting to make my own brief comments, stimulated by Eddy's observations.

When I was a graduate student in Scotland in the 1950s, physics departments were called departments of 'Natural Philosophy'. Clearly, the original hope had been that some aspects of science were all encompassing and gave insight to every aspect of observations of natural phenomena. However, in the twentieth century, with rapidly increasing technological advances, it appeared to be more and more difficult for any one person to study all of science. Disciplines were progressively subdivided. Learning became increasingly specialized. *International Tables* were created, and updated, for the use of a highly specialized and small community of crystallographic experts.

As I read Eddy's draft article, I became fascinated by the wide impact he envisioned for crystallography in the next few decades. Indeed, the lay person, reading his article, would barely be aware that this was an article anticipating the future impact of crystallography. The average reader would think that the topic was the total impact of science on our civilization. Thus, to my delight, I saw that crystallography might now be a catalyst for the reunification of fragmented science into a coherent whole. I therefore hope that these new *Tables* commissioned by the International Union of Crystallography may turn out to be a significant help to further the trend implied in Eddy's article.

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

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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