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# Biophysical methods: bigger, finer, faster and dynamic

## Editorial overview

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Current Opinion in Structural Biology 2005,  
15:535–537

0959-440X/\$ – see front matter

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DOI 10.1016/j.sbi.2005.09.003

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For many of us, biology drives the nature of the questions that can be posed, but biophysical and computational methods successfully yield answers to these questions. Generating pointed scientific questions is the easy part. Deciding exactly what experimental techniques can best be deployed, or must be developed from scratch, is often the hard part. The methods described in this collection of short reviews represent significant advances in existing successful techniques, such as X-ray crystallography, cryo-EM, NMR and computational structural biology, and also introduce quite new techniques, such as cellular X-ray tomography and the deployment of microfluidics in applications to macromolecular crystallization. These biophysical tools address the structures of objects whose length scales vary from that of cells to large macromolecular assemblies and to single protein molecules. The reviews describe enhancements in instrument resolving power, sample preparation, detector technology, computer-driven data collection, and sophisticated algorithms for data analysis and interpreting structure in relation to functional mechanisms.

Results derived from macromolecular crystallography provide the structural underpinnings of much of modern biology; however, crystal structures, elegant though they are, are static in nature. The time domain is limiting. How then to proceed from the limitations of static crystallography to explore mechanism, whereby an array of short-lived structures forms and decays as all reactions proceed? Bourgeois and Royant identify the four main classes of experimental strategies that constitute ‘kinetic crystallography’, which they denote equilibrium/steady state, trigger freeze, freeze trigger and time resolved. They cogently summarize the advantages and limitations of each, which differ in their inherent time resolution, and quasi-static or explicitly dynamic nature, and in the demands placed on the experiment itself during data acquisition and on subsequent data analysis. Experiments in kinetic crystallography representing each of these four classes have to confront the question of structural heterogeneity. Are all molecules in the crystal in the same structural state or is heterogeneity introduced, by design or inadvertently, by reaction triggering or freezing? Can heterogeneity be confidently detected and resolved into homogeneous structural components? Can those components be confidently assigned to particular intermediates? Answers to these questions are bolstered by combining the X-ray-based approaches with spectroscopic and computational techniques.

The space domain is also limiting. Two further reviews address this in quite different ways: the development of microfluidics-based techniques for exploring crystallization space and growing small crystals (Zheng, Gerdts and Ismagilov); and the design and implementation at the European

Synchrotron Radiation Facility (ESRF) of an X-ray beamline in which the intense X-ray beam is  $\sim 5 \mu\text{m}$  in diameter and thus suitable for probing tiny crystalline or fiber samples (Riekkel, Burghammer and Schertler).

Crystallization robots increasingly take the tedium out of initial crystallization experiments, in which a wide range of solution conditions must be explored, seeking just those that yield promising microcrystals and not the all-too-common amorphous precipitate. The robots typically employ an array of fine tubing and valves as plumbing. What if these could be replaced by channels in a chip and valves dispensed with altogether, by clever exploitation of fluid dynamic properties on a micro scale? In essence, this is the heart of the approach reviewed by Ismagilov and colleagues, still very much a work in progress. However, growing crystals in micro plugs of liquid is only the first part of the problem, because the object is to examine them by X-ray techniques. These tiny crystals are fragile and, although they may be swept out of the plugs on to a cryo loop or into a conventional external capillary, it would be more convenient by far and potentially less damaging to examine them *in situ*. This would require both that a microcapillary constitute part of the chip — entirely feasible — and that a suitable intense, micro X-ray beam be available.

Provision of the latter is the subject of the review by Riekkel *et al.* They emphasize that careful mechanical design of the diffractometer and centering devices must be incorporated into a beamline that both confers micro-focusing and, critically, affords low noise. As there may be as few as  $10^9$  unit cells illuminated by a  $5 \mu\text{m}$  X-ray beam, the diffraction signal is very low and can only be measured if exquisite attention is paid to minimizing all sources of noise (i.e. background). Part of the background derives from any liquid surrounding the crystal. This poses perhaps the biggest challenge to examining crystals *in situ* in a microfluidics-based crystallization chip — how will the liquid surrounding the microcrystal be removed, leaving the crystal or crystallites in place? For more conventionally grown microcrystals and fibers, Riekkel *et al.* show that excellent data can be obtained using their ESRF beamline, provided due attention is paid to the factor that ultimately limits data quality — radiation damage. Providing another example of links between seemingly disparate methods, the scaling of individual diffraction images, perhaps only one per microcrystal, is mathematically related to the problem encountered in single-particle cryo-EM, whereby the relative orientation of individual particles in an image must be established before their complete, three-dimensional continuous transform can be computed.

NMR is a high-resolution technique that is amenable to providing molecular dynamics information experimentally. Bax and Grishaev describe the use of residual

internuclear dipolar couplings in solution NMR. Combined improvements in measurement sensitivity and computational analysis begin to demonstrate the prospect of revealing the internal motion of proteins on a timescale slower than rotational diffusion. Although the systems under study are at equilibrium, perhaps transient intermediates might ultimately be accessible.

Cryo-EM of single particles with high or low inherent symmetry reaches subnanometer resolution (0.6–1.0 nm), at which long  $\alpha$  helices and large  $\beta$  sheets of the protein components of a large macromolecular assembly can be discerned (Jiang and Ludtke). Only a few thousand to tens of thousands of structurally homogeneous particle images are sufficient to yield a subnanometer-resolution structure. This enhanced capability is primarily attributable to improvements in algorithms and software for structure refinement and structural feature recognition. Visualization of secondary structure elements provides a new level of confidence in the reliability of this methodology, which has been used to provide only low-resolution ‘blobs’. As structures derived from cryo-EM can represent structures derived in solution, they are complementary to crystal structures, which may be influenced by crystal packing forces. This imaging modality has become a mainstream biophysical tool as the trend in structural biology moves towards studying larger and more complex assemblies in the structural proteomics era.

Homology modeling of a protein subunit based on high sequence similarity and the crystal structure of a homologous protein is now routine. However, a novel hybrid that combines homology modeling with restraints arising from a low- or intermediate-resolution cryo-EM density map has opened up the possibility of building pseudo-atomic models of molecular components or domains within a large macromolecular assembly (over one million daltons) with high reliability (Topf and Sali). There are sufficient numbers of experimental structures for which both high- and low-resolution structures exist and can be used to validate this approach. This computational structural biology method is particularly useful for generating reliable models of a system for which it is inherently difficult to obtain a high-resolution structure directly using experimental approaches.

Because biological molecules are not static objects, a quantitative description of the dynamics of molecules is necessary for understanding their functional mechanisms. Traditionally, molecular dynamics are computed using atomic crystal or NMR structures as input for the simulations. Bahar and Rader review progress in the field of computational biophysics, whereby coarse-grained normal mode analysis has been deployed to predict protein domain motions, to make a flexible fit of the crystal structure into cryo-EM maps, and to generate a template

for model-based structure refinement of low- and intermediate-resolution cryo-EM density maps. Normal mode analysis and molecular dynamics calculations also play a significant role in interpreting kinetic crystallography, as noted by [Bourgeois and Royant](#).

Moving up in scale, structural biology of a whole cell is the ultimate dream of cell biologists. X-ray tomography of a whole cell becomes feasible using a dedicated synchrotron beamline and a cryogenic sample stage (see the review by [Le Gros, McDermott and Larabell](#)).

Preliminary tomographic images of a rapidly frozen budding yeast cell at 50 nm are encouraging, but further instrumentation development will be necessary to improve the resolution of this methodology to the point at which it can directly visualize subcellular features in a living cell.

The reviews were all solicited and prepared independently; however, as the above discussion indicates, there are links between them. Perhaps other linkages will occur to readers — “only connect”.