Structural studies by cryo-electron microscopy: Achievements, Prospects, and Challenges

Elena Orlova
Institute of Structural and Molecular Biology, Birkbeck College,
Malet Street, London, WC1E 7HX, UK
e.orlova@mail.cryst.bbk.ac.uk

The last decade has witnessed an extremely impressive rise in the role of cryo-electron microscopy (cryo-EM) for structural studies of biocomplexes. For many years, EM was like the Cinderella of structural methods that was mainly used for visualisation of shapes of large biocomplexes or organelles in cells. However, the decades of rigorous efforts of many scientists to fulfil theoretical promises of using electrons for imaging of biological molecules at the level of atomic resolution were not in vain and the best representatives of the EM community were recognised in 2017 by the Nobel Prize awarded to J. Dubochet, J. Frank, and R. Henderson.

The last five years in EM were manifested by crucial technical advances in micro technology, improving the electron sources and systems for digital registration of images. It is important to acknowledge the role of improvements in sample preparation where they allowed the retrieval of high resolution structural information from two-dimensional images. The progress in technology was accompanied by the development of mathematical approaches describing image formation in microscopes, algorithms for the fast and efficient processing of recorded images and automation of processing, where subsequent analysis facilitated the determination of structures at near-atomic resolution. These years were distinguished by a large number of structures resolved at a resolution better than 4Å, where one can recognise the interface of protein-protein interactions, reveal different conformations of complexes and understand their function through their dynamic properties.

Now we need to fully automate the data collection on electron microscopes, increase the dimensions of digital detectors (whilst reducing at the same time the sizes of individual sensors) and to get powerful computers that will allow us to do statistical analysis of data to distinguish variations in structures. The next important step would be to resolve functional changes of complexes in time.

The single particle cryo-EM workflow for structural studies of biological macromolecules

Nadav Elad
Electron Microscopy Unit, Departments of Chemical Research Support, Weizmann Institute of Science
234 Herzl Street, Rehovot, Israel
nadav.elad@weizmann.ac.il

Single particle cryo-electron microscopy (cryo-EM) involves the 3D visualization of biological macromolecules in solution. Substantial improvements in the technique now enable to determine macromolecular structures to atomic resolution, attracting great interest in the structural biology field. These breakthroughs have awarded the 2017 Nobel Prize in Chemistry to Jacques Dubochet, Joachim Frank and Richard Henderson, who played a major role in the development of the method over the past 5 decades. Notably, although the improvement in map resolution has been an important technological driving force, single particle cryo-EM experiments entail additional elaborate information about the investigated biomolecule, such as the existence of multiple populations in the solution, oligomeric states and conformational heterogeneity.

A single particle experiment starts with purification of the target macromolecular complex from its cellular environment. Purification and imaging parameters are optimized using negative stain EM, and, once ready, the sample is vitrified in a thin layer of buffer. Samples are then imaged in the transmission electron microscope (TEM) at cryogenic temperatures. A typical data set contains tens to hundreds of thousands of 2D images, each containing a single complex. These images are processed using specialized software in order to calculate 2D class averages, followed by reconstruction of a 3D map, or multiple maps to account for sample heterogeneity.

I will discuss different aspects of single particle cryo-EM, concentrating on practical aspects in the workflow. What are the requirements for a successful experiment, and what information can one expect to obtain. I will also discuss our local workflow in the WIS EM Unit and the new state-of-the-art instruments, which will be installed in 2018.

Cryo-STEM analysis of protein-bound metals

Michael Elbaum
Dept of Chemical and Biological Physics
Weizmann Institute of Science
Rehovot, Israel
michael.elbaum@weizmann.ac.il

Metal ions play essential roles in biological chemistry. Oxygen transport by iron or copper, calcium in muscle contraction, and zinc in enzyme catalysis are all common examples. Detection of protein-bound metals is a difficult problem in structural biology, however. Cryo-EM by defocus phase contrast is not well suited to distinguish metals. Scanning transmission electron microscopy (STEM), on the other hand, is inherently sensitive to atomic number *Z* according to the scattering cross-sections. This approach is ideally suited to distinguish heavier metals on the background of the light elements hydrogen, carbon, nitrogen, and oxygen that dominate the composition of proteins and other biomacromolecules. Nanometric granules of amorphous calcium phosphate, for example, appear prominently in the matrix of mitochondria[1]. With *Z*=20, calcium is hardly a heavy metal. An obvious question is how far the sensitivity extends; can it reach the level of single atoms? To address this question, human heavy chain ferritin was labeled with Zn or Fe at very low stoichiometry, and subjected to a single particle cryo-STEM analysis[2]. Fe^{2+} normally enters the ferritin shell, undergoes oxidation at specific ferroxidase motifs, and then nucleates to solid mineral form as Fe^{3+} at nucleation sites. Zn binds the ferroxidase motifs tightly and blocks further Fe uptake. 3D reconstructions show essentially identical protein forms, but striking differences in the metal distributions. These observations show vast potential for cryo-STEM to complement traditional single particle reconstructions, as well as for macromolecular labeling in cryo-tomography.

1. Wolf, S. G. *et al.* 3D visualization of mitochondrial solid-phase calcium stores in whole cells. *eLife* **6,** e29929 (2017).
2. Elad, N., Bellapadrona, G., Houben, L., Sagi, I. & Elbaum, M. Detection of isolated protein-bound metal ions by single-particle cryo-STEM. *Proc. Natl. Acad. Sci. U. S. A.* **114,** 11139–11144 (2017).

EBSD analysis of beam sensitive bio materials; Calcite and Aragonite in Mollusc Shell

Keith Dicks[1], Peter Chung[2]
[1]*Oxford Instruments NanoAnalysis,* Halifax Road, High Wycombe, Bucks, UK, HP12 3SE keith.dicks@oxinst.com [2]*School of Geographical and Earth Sciences, University of Glasgow,* Glasgow, G12 8QQ peter.chung@glasgow.ac.uk

EBSD can be applied to crystalline biological materials, notably calcite and aragonite found in mollusc shell, and other instances of biomineralisation where crystalline forms occur. However, such phases in biological systems are likely to be very beam sensitive, warranting reduced beam current, and/or accelerating voltage (kV). Reducing the beam current reduces the total flux, i.e. the total number of electrons available for diffraction, and so diminishes the number of electrons arriving at the phophor screen, with a corresponding reduction in intensity. Similarly, reducing the kV reduces the striking energy at the phopshor, which also diminshes the light yield. Further, reducing the kV changes the wavelength of the illumination and causes broadening of the Kikuchi bands projected onto the screen, which may compromise accurate band detection. Thus the EBSD detector sensitivity is a critical factor, as is the ability of the software to correctly identify the Kikuchi band positions accurately, when band broadening is present at low kV's.

Effect of Oceanic Acidification on Mollusc Shell

Increasing atmospheric CO_2 leads to Oceanic Acidification (OA) as the CO_2 concentration of seawater rises. EBSD has been successfully applied to study the affect of OA on mollusc shells, grown under controlled conditions in the lab over a three year period, both for present day seawater and elevated CO_2 concentration. OA places stress on many marine organisms, most notably affecting the manner in which biomineralisation progresses, and the nature of the resultant structures.

EBSD has shown that mollusc shell grown in acidified seawater is subject to modified growth characteristics, to the detriment of the integrity and strength of the shell, caused by diminshed crystallographic control of the growth mechanism.

These findings have been greatly facilitated by the use of a new generation of EBSD detector (Aztec Symmetry), which uses a Complimentary Metal Oxide Semiconductor (CMOS) imaging device, rather than the conventional Charge Coupled Device (CCD), with the benefit of greatly enhaced sensitivity and speed.

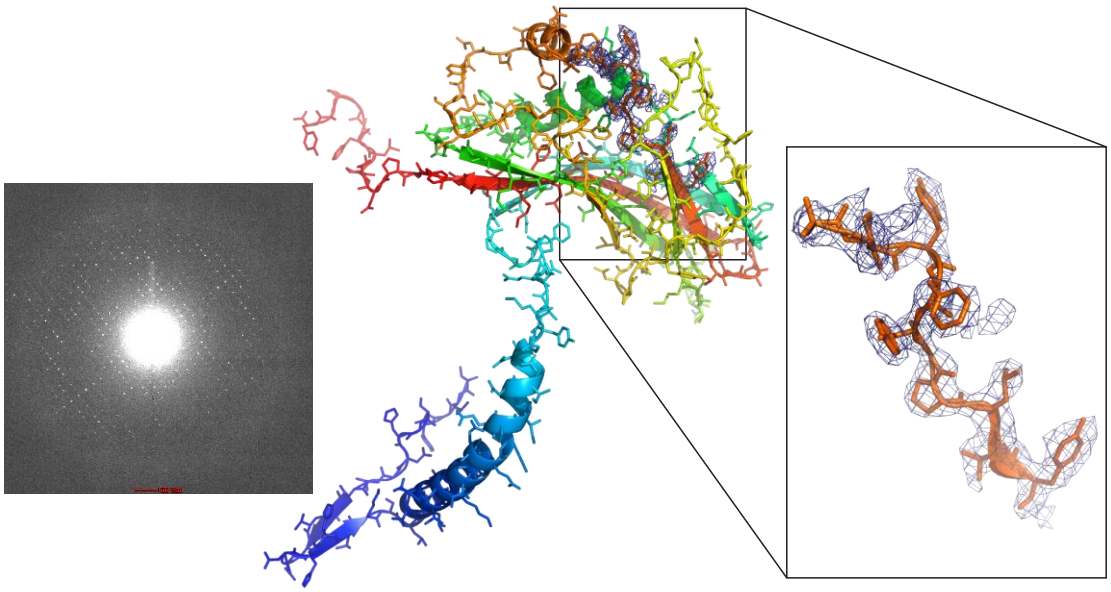
Micro-Electron Diffraction – A Complementary Structural Biology Technique

Bart Buijsse[1], Lingbo Yu[1], Richard Bunker[2]
[1]*Thermo Fisher Scientific*, Eindhoven, The Netherlands
[2]*Friedrich Miescher Institute for Biomedical Research*, Basel, Switzerland
bart.buijsse@thermofisher.com

Micro-Electron Diffraction (micro-ED) is targeting single 3D crystals, or crystal regions, which are too small to be analyzed by conventional X-ray diffraction techniques. Many proteins may form such nanocrystals, which often remain undetected in the crystallization drops. Currently only X-ray free-electron laser (XFEL) based crystallography is able to analyze these nanocrystals, if the crystals are available in large quantities. In this study, we have used micro-ED to analyze the smallest protein crystals to yield a structure by X-ray crystallography, the protective coat of naturally occurring granulovirus particles, which is formed by a 248-amino acid polypeptide and contain on average 9000 unit cells [1]. Using only a few crystals we obtained a molecular structure at high resolution.

The study was done to show that micro-ED is another interesting member in the family of cryo-EM techniques for structural biology. MED has the potential to unravel structures from nanocrystals of proteins and other biological molecules down to small pharmaceutical molecules. This can be done with samples of small size and at a resolution that may be out or reach by imaging techniques (like Single Particle Analysis, SPA) due to limitations of the optics, camera, and radiation damage.

In this presentation we will highlight the hardware requirements for micro-ED and the optimal workflow for data collection. Furthermore, the granulovirus results will presented and the critical steps in data processing.



(left) Characteristic diffraction pattern of Granulovirus. (right) Details of the protein structure, showing well-resolved side chains.

[1] C.Gati *et al.*, Atomic structure of granulin determined from native nanocrystalline granulovirus using an X-ray free-electron laser, PNAS **114**, 2247-2252 (2017)

3D precession diffraction tomography at nm scale in TEM: Solving structures of pharmaceutical compounds with new generation direct detection pixelated detectors

Stavros Nicolopoulos [1]
[1]*NanoMEGAS SPRL Blvd Edmond Machtens 79 B-1080* Blvd Edmond Machtens 79 B-1080 Brussels Belgium info@nanomegas.com

The renewal of interest in electron crystallography the last 20 years [1,2] has been strongly influenced by the use of Precession Electron Diffraction (PED) [2,3] which allows structure solution of nanostructures using TEM PED intensities where dynamical interactions are greatly diminished. Over the last 10 years PED-TEM studies allowed structure solution of various materials such as metals /alloys, minerals , semiconductors and even organic materials.

One relatively recent development in electron crystallography in data collection , has been the use of ADT 3D (3D automatic diffraction tomography combined with precession diffraction) which allows 3D reciprocal space reconstruction from tilted ED series (usually every 1 deg) from nanocrystals as small as 20 nm. This way unit cell, symmetry & structure determination can be obtained by measuring ED intensities from unknown structures [4] where even H atomic positions can be located with picometer level precision via dynamical refinements [5].

The advent of direct detection /gew generation pixelate detcctors enables new possibilities for structure determination of beam sensitive organic and pharmaceutical samples , as we can work with very low effective doses of 0.01 e/A2 s at room. Our team has solved several pharmaceutical structures using ADT tomography and working with low dose at room temperatures [6].

[1] D.Dorset Structural Electron Crystallography Plenum 1995
[2] New Fronteers in Electron Crystallography Ultramicroscopy 2007, Vol.107 (issues 6-7) Guest Eds S.Nicolopoulos, T.Weirich
[3] R.Vincent & P.Midgley (1994) Ultramicroscopy 53, 271-282
[4] U.Kolb, T. Gorelik , C.Kubel , M.Otten, D. Hubert (2007) Ultramicroscopy , 107, 507-513
[5] Palatinus et al., Science 355, 166-169 (2017)
[6] v. Genderen et al Acta Cryst A72 (2016) 236-242

Analysis of crystalline biological materials using EBSD in the SEM

Aimo Winkelmann[1], Gert Nolze[2]
[1]*Bruker Nano GmbH*
Berlin, Germany
aimo.winkelmann@bruker.com
[2]*Federal Institute for Materials Research and Testing (BAM)*
Berlin, Germany

In this contribution, we demonstrate some alternative approaches to analyze electron backscatter diffraction (EBSD) data with applications to the imaging of biological materials in the SEM. For example, image processing techniques provide powerful tools which enable us to extract essential crystallographic orientation information by the offline analysis of saved Kikuchi pattern data (Fig.1). In addition, the comparison of experimentally measured Kikuchi patterns with dynamical electron diffraction simulations provides a way to improve the indexing success for sensitive biological materials in the presence of noise and reduced signal-to-background ratios.

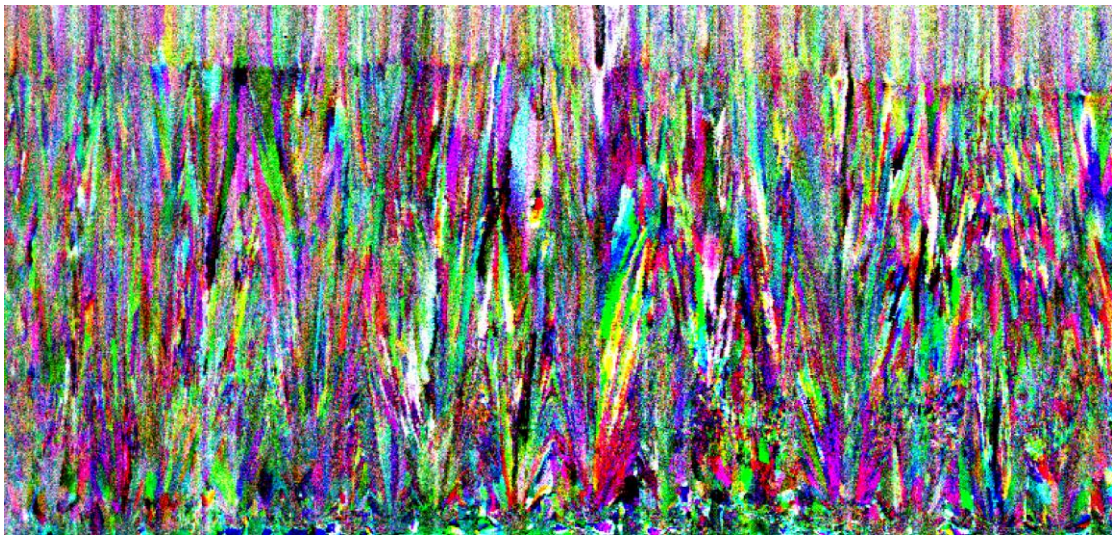


Fig.1 Microstructure of an ostrich egg shell cross section emphasized by differential Kikuchi pattern signals (top: outside edge, bottom: inside, thickness ~ 2mm)

Dynamic self-assembly of biomolecules

Uri Raviv
Hebrew University of Jerusalem,
Institute of Chemistry
Jerusalem, Israel
uri.raviv@mail.huji.ac.il

Modern synchrotron time-resolved X-ray scattering experimnts from solution of proteins that dyanmiclly self-assembly to form large biomolecular complexes can be performed at temporal resolution of msec. In this talk, we will focus on the early nucleation events of tubulin to form microtubule, an importnat filamentous protein polymer that is part of the cytoskeleton, and on the assembly of protein capids (Hepatitis B and SV40).

In both cases time-resolved X-ray scattering reveal unique information about the early stage of the nucleation events that are too tranisent for TEM and too small for optical microscopy.

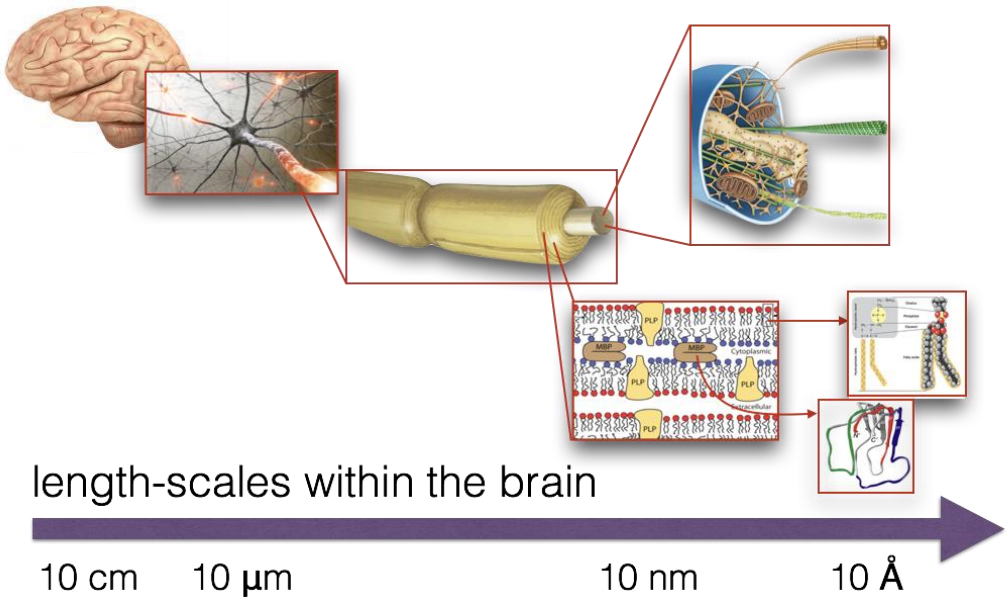
Our lab is developing experimental protocols to perform time reolved experiments and is establishing analysis methodoly for resolving the structural information in the data, using maximum entropy optimization methods.

X-ray Scattering – resolving order and disorder in nano-scale biological complexes

Roy Beck
School of Physics and astronomy, Tel Aviv University
Tel Aviv, Israel
roy@tauex.tau.ac.il

During the 20th century, the protein sequence-structure-function paradigm was uniformly accepted as a key concept in molecular cell-biology. The central dogma is that the biological function of proteins is inherently encoded in their folded 3D structures. This idea, introduced in 1894 by Emil Fischer and known as the "lock-and-key" model, explained the high specificity of enzyme-substrate recognition and was validated over and over to create the basis of modern proteomics. Protein folding occurs mainly due to short-ranged specific interactions between amino-acids encoded in its sequence. This is the core reason why point mutations have a dramatic effect on protein conformation which in-turn significantly distorts protein-protein recognition.

Utilizing synchrotron and lab-based small-angle X-ray scattering we are now able to explore dynamic and flexible biological materials that lack 3D order. In this talk I will present some of our recent experimental results aiming to address the fundamental relation between order and disorder in functional biological complexes with key emphasize on nano-scale objects originating from the neuronal system.



Small Angle X-ray diffraction revealing Structural adaptations for seed crawling

Rivka Elbaum[1], Yael Abraham[2]
[1]*The RH Smith Institute of Plant Sciences and Genetics in Agriculture, Hebrew University of Jerusalem*
Faculty of Agriculture, Rehovot, Israel
[2]*Hanoter St. 6/14,* Rehovot, Israel
rivka.elbaum@mail.huji.ac.il

Seed dispersal enables plants to scatter their progeny into new growing locations. Many times hygroscopic tissues are involved in dispersal by contracting in specific directions as they mature and dry. The hygroscopic structure may react to the diurnal humidity cycle and facilitate the release of seeds from the mother plant (e.g. in pinecones (*1*)) and their propulsion along the ground (e.g. in wheat (*2*)). One example for such mechanism exists in the coiling awns of stork's bills (*Erodium gruinum*): Cells that build the hygroscopically active tissue coil as they dry (*3*). Using small angle X-ray diffraction, we detected cellulose microfibrils arranged in an unusual tilted-helix, which forces the cells to adopt a coiled configuration when they dry. The modulation of the movement is done through both arrangement of the cellulose microfibrils and the hygroscopic character of the polymeric matrix in which the microfibrils are embedded. Correlating X-ray diffraction with polarized light microscopy, we mapped the tilt angle of the cellulose microfibrils within single cells (*4*). Following similar approaches, we identified tilted cellulose microfibrils also the grasses family (Poaceae). These dispersal units move in elaborate ways, adjusted to varied dispersal mechanisms, such as shooting off the mother plant, floating in the air, crawling along the soil, and borrowing into the soil. Our studies illuminate the structural principles that govern each of the hygroscopic movements, and opens ways to translate them into technical "smart" materials.

1. C. Dawson, J. F. V. Vincent, A.-M. Rocca, How pine cones open. *Nature*. **390**, 668 (1997).

2. R. Elbaum, L. Zaltzman, I. Burgert, P. Fratzl, The role of wheat awns in the seed dispersal unit. *Science*. **316**, 884–886 (2007).

3. Y. Abraham *et al.*, Tilted cellulose arrangement as a novel mechanism for hygroscopic coiling in the stork's bill awn. *J. R. Soc. Interface*. **9**, 640–647 (2012).

4. Y. Abraham, R. Elbaum, Quantification of microfibril angle in secondary cell walls at subcellular resolution by means of polarized light microscopy. *New Phytol.* **197**, 1012–1019 (2013).

X-ray crystallography, 100 years old, and still a “bright” future

Haim Rozenberg

Department of Structural Biology, Weizmann Institute of Science
Rehovot 7610001, ISRAEL

haim.rozenberg@weizmann.ac.il

In 2014, the United Nations promoted the International Year of Crystallography to celebrate the centenary of the discovery of X-ray crystallography.
Indeed, Max von Laue carried out the experiment that showed that X-rays were diffracted by crystals in 1912, and a year after, the Braggs (William Henry and William Lawrence, father and son) showed that the X-rays diffractions can be used to determine the positions of atoms within a crystal and unravel their three-dimensional arrangement. The significance of these experiments was immediately realized by the scientific community; Max von Laue received the Nobel Prize in Physics in 1914, and the Braggs the year after.

Since then, X-ray crystallography has developed continuously in direct correlation with the advance of the technologies (energy, mechanics, optics, computer, programing, electronics, etc) and in close collaboration with other scientific disciplines (from fundamental physics to biology through material science and medicine).

The different steps from protein expression to structure analysis have been rationalized and systemized. In nowadays, most biological macromolecules are crystallized using commercial crystal screening kits and robots, and their X-ray crystallographic data are collected at synchrotron sources almost automatically. It took days and weeks to collect a full data set of a 200 a.a. protein crystal in the 80’s days; it takes now only a few seconds thanks to the ever brighter synchrotrons, and not much more to process the data thanks to the ever faster processers.

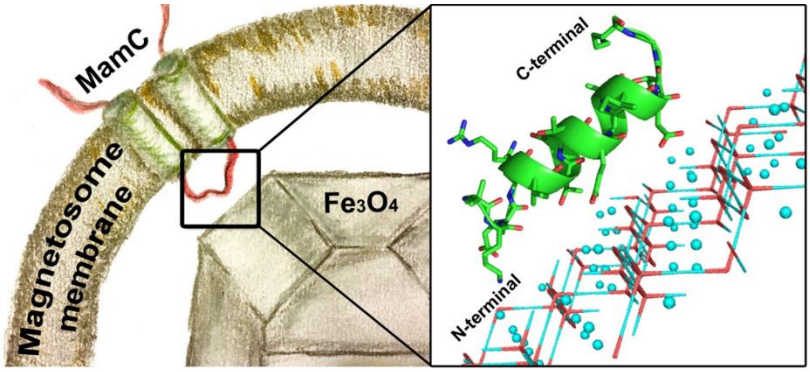
More than 121000 X-ray crystal structures of proteins, DNA or RNA oligomers, and their complexes have been solved and deposited to the open access Protein Data Bank (November 2017). This number continues to increase by the thousands annually although new techniques are now available (NMR, cryo-EM).

After a brief review of the history and the essential principles of x-ray crystallography, we will illustrate the current achievements and limitations of modern crystallography of biological macromolecules using examples from our present studies on the p53 tumor suppressor protein.

Structural studies of magnetosome-associated proteins

Raz Zarivach
Department of Life Sciences, The National Institute for Biotechnology in the Negev and The Ilse Katz Institute for Nanoscale Science & Technology, Ben-Gurion University of the Negev
P.O.B. 653, Beer-Sheva 84105, Israel
Zarivach@bgu.ac.il

Magnetic nanoparticles are key components in many technologies and biotechnologies. Yet, it is not easy to modify them and control their shape and size. Natural organisms that perform such control are magnetotactic bacteria. Magnetotactic bacteria navigate along geomagnetic fields by forming magnetosomes chains. Magnetosomes are intracellular membrane-enclosed, nanometer-sized crystals of the magnetic iron mineral magnetite (Fe_3O_4) or gregite (Fe_3S_4). Biomineralization of magnetite within these unique prokaryotic organelles involves the formation of the magnetosome, the transport of iron and the nucleation and controlled growth of magnetite via magnetosome-associated proteins. Here we present the use of X-ray crystallography to understand the structure-function relationships of magnetosome-associated proteins. Furthermore, structure based rational design is a key point for biotechnological application development.



Nano-structure of natural biominerals visualized by advanced X-ray diffraction, X-ray tomography, and electron microscopy techniques

Emil Zolotoyabko
Technion-Israel Institute of Technology
Haifa 32000, Israel
zloto@technion.ac.il

Interplay between soft and hard components in biogenic composites attracts a great deal of attention of numerous research groups worldwide, aimed at comprehensive understanding of growth mechanisms and related physical origin of improved mechanical characteristics of these natural materials, first of all, the resistance to fracture. In gross mode, this is achieved by sophisticated design of stiff and compliant materials on different length scales. In mineralized biocomposites, the stiff (hard) components are mineral particles and layers built of calcium carbonate (as in mollusk shells), biosilica (as in sponge spicules), or hydroxyapatite (as in teeth and bones). In turn, different protein and polysaccharide sub-layers and inclusions serve as soft (compliant) components. Despite intensive research, the details of atomic interactions at the organic/inorganic interfaces and their exact impact on mechanical properties of biocomposites remain unaddressed.

Nowadays, there is a tremendous progress in the development of the advanced characterization techniques, using X-rays and electrons, which provide researchers with unprecedented tools for structural investigations on the nano-scale. In our research, we apply high-resolution X-ray powder diffraction, X-ray nano-tomography and focused X-ray beam diffraction, as well as high-angle electron scattering in the STEM mode in order to visualize atomic structure and nanoscale structure of biocomposites. In this presentation, based on the obtained experimental results, we discuss the recipes used in nature toward improving mechanical properties of biocomposites, focusing on mollusk shells and sponge spicules.

Organic Nano-crystals in Organisms: Biogenic Scatterers, Mirrors, Multilayer Reflectors and Photonic Crystals

Benjamin A. Palmer[1], Anna Hirsch[2], Dvir Gur[1], Dan Oron[3], Leeor Kronik[2], Leslie Leiserowitz[2], Steve Weiner[1], Lia Addadi[1]
Depts. of Structural Biology[1], Materials and Interfaces[2], Physics of Complex Systems[3], Weizmann Institute of Science,
76100 Rehovot, Israel
Lia.addadi@weizmann.ac.il

Organisms are able to construct an array of 'devices' based on assemblies of organic crystals with optical functions including diffuse scatterers, broadband reflectors, tunable photonic crystals and mirrors. The optical properties are achieved by controlling the constituent molecules, the structure, polymorphism, size, morphology and arrangement of the organic crystals. The constitutent molecules are mostly limited to purines and pteridines. The crystal structures are characterized by dense layers of the hydrogen-bonded polycyclic aromatic molecules. This structural feature endows the crystals with unusually high refractive indexes for light impinging perpendicular to the hydrogen-bonded planes. The controlled assembly of several thin crystals creates multilayer reflectors with a variety of optical properties. Guanine crystal multilayer reflectors generate the white color of certain spiders, the metallic silvery reflectance of fish scales, the brilliant iridescent colors of some copepods, and the mirrors used for vision in several animal eyes (1). Scallops have tens of eyes, each containing a concave multi-layered mirror perfectly tiled with a mosaic of square guanine crystals, reflecting the light to form images onto the overlying retinas (2, 3). Shrimp, crayfish and lobsters possess compound eyes that also use reflective optics, and contain two sets of mirrors, composed of isoxanthopterine, a pteridine which crystal structure had not been previously determined. The two mirrors have very different ultrastructures and functions that we can rationalize in terms of the optical performance of the eye. In all these examples, the hierarchical organization is controlled from the crystal structure at the nanoscale to the complex 3D super-structure at the millimeter level.

1) D Gur, BA Palmer, S Weiner, L Addadi, *Adv Funct Mater* 2017, 1603514
2) A Hirsch, BA Palmer, N Elad, D Gur, S Weiner, L Addadi, L Kronik, L Leiserowitz, *Angew Chem* 2017, 56, 1.
3) BA Palmer, GJ Taylor, V Brumfeld, D Gur, M Shemesh, N Elad, A Osherov, D Oron, S Weiner, L Addadi, *Science*, in press.