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Since I moved into the field of cryo Electron Microscopy (EM), from X-ray crystallography in mid 2006, it has changed significantly. At the time, I was becoming interested in systems that were highly challenging by crystallographic means, such as large protein complexes and membrane proteins. However, the resolution afforded by cryo-EM, especially for non-symmetric systems, was still routinely above 1nm, meaning secondary structural elements could not be resolved. Although >1nm reconstructions could still provide valuable information about a protein or protein complex, especially with regards to subunit stoichiometry and organisation they lacked information on the organisation of the secondary structure elements. All that changed a few years ago with the introduction of direct electron detectors, new processing algorithms and more stable microscopes moving the field of cryo-EM from modest (~1nm) resolution reconstructions to ~3-6Å, even for non-symmetric targets. Along with these developments has come a range of new processing algorithms, sample preparation techniques and data analysis tools. It is difficult to detail all of these advancements within the field in one special edition, given that the field is undergoing such a steep developmental curve. Therefore, to try and capture the current EM landscape this issue has collected a range of *Methods* papers that provide an overview of the EM pipeline, from sample preparation to data processing and analysis of the resulting maps.

The recent developments in the cryo-EM field has resulted in significant interest from groups whose core activities are more focussed in other fields, for example X-ray crystallography. So, if one were to embark on an EM experiment, what are the options, how much protein do I need and what are the processes? The first article [1] aims to address these points and introduce the reader to the basics of EM sample preparation. This starts with an introduction to the use of negative-stain EM, which although limited in resolution does provide a powerful means by which you can quickly assess the quality of a sample and gain important information on stoichiometry and subunit arrangement. This then moves towards a more detailed look at the production of grids for single particle cryo-EM and their subsequent imaging. Data processing in EM has always been unavoidably computationally expensive due to the requirement for the averaging of a large number of molecular views and this is discussed, especially with the onset of direct detectors producing typically 5Tb per dataset.

A significant advantage of EM over other structural techniques such as NMR and X-ray crystallography is the requirement for lower quantities of protein sample. However, even the amount of protein required for EM studies is dependent on some degree of protein production, be it overexpression or purification from native sources, which still occludes many targets from study. The second paper by the Jiang group [2] addresses this problem through a new affinity cryo-EM approach called cryo-SPIEM (Solid Phase Immune Electron Microscopy). This method directly purifies a sample onto a grid surface, removing the need for high levels of protein, providing the ability to purify directly from crude cell lysates. This provides new opportunities for the study of unstable, short lived or low abundant targets. By working with antibodies to the target protein, rather than to a specific tag, the need for target modification is

removed. This technology could greatly increase the scope of systems that can be studied through EM approaches.

Getting good quality EM data is only the start of the process, to get a reliable reconstruction one must carry out a number of distinct stages including particle picking, classification, structure refinement and validation. There are a range of software packages that are available, some of which carry out specific parts of the data processing pipeline and others that can go from particle picking through to structure generation and validation in one multi-component package. One of the leading software packages is the EMAN suite developed by the Ludtke group, which has now been cited over 2500 times. The latest release of the EMAN package (EMAN2.1) is a complete image processing suite for, amongst other things, single particle reconstructions, heterogeneity analysis and subtomogram analysis. The corresponding paper provides a detailed description of the single particle analysis pipeline along with the philosophy behind the different strategies [3]. This provides an excellent starting point for those wanting to use the EMAN2.1 suite of programs and to understand some of the general principals behind image processing.

A significant hurdle in obtaining high resolution structural information is accounting for sample movement within the microscope caused through both mechanical drift and beam-induced sample movement. The introduction of direct electron detectors, which collect a series of frames over the total exposure as opposed to one single average micrograph allows this movement to be modelled. A range of programs are available to deal with this movement and the paper by Rawson *et. al.* [4] describes a number of these approaches and provides scripts to allow for the stand-alone programs to integrate with the RELION software package. A further cause for degraded resolution is due to the inherent flexibility within a protein or protein

complex whereby domains can move relative to each other. By masking out these different domains during image processing, the same authors use the example of a vacuolar ATPase to show how a significant improvement in resolution can be achieved by accounting for this flexibility.

Despite the significant improvement in the resolution obtainable by cryo-EM, the majority of maps produced are still at a more modest resolution (5-10Å). Although the position of individual side chains cannot be determined at this resolution, it does provide the ability to accurately dock higher resolution crystal structures, which may account for all, or part of the EM structure. Two methods are described; Flex-EM [5] and MDFF [6] which both permit the flexible fitting of known crystal structures or homology models to produce pseudo-atomic models. The MDFF (Molecular dynamics flexible fitting) method is a powerful approach to flexibly fit atomic structures into density maps [6]. The paper by the Schulten group in *Methods* describes the challenges faced when carrying out flexible fitting in MDFF and provides an overview of the strategies used to overcome these to successfully perform MDFF simulations. Flex-EM provides an alternative approach and was originally developed as part of MODELLER and used primarily to fit into more modest resolution EM maps (5-15Å). However, the paper presented in this issue of *Methods*, shows that Flex-EM can now refine models into higher resolution maps (2.5-4.5Å), with GroEL and eIF6 bound to the ribosome used as examples.

A powerful feature of EM is its ability to capture not just one but many conformational states. This can be achieved through conformational sorting within programs such as EMAN2.1, whereby a number of discrete conformations can be resolved [3]. An exciting development of this is to use manifold embedding which has

the potential to observe an entire work-cycle of a molecular machine, rather than just a handful of discrete conformations [7]. By taking the ribosome as an example, the Frank group have led the way in this field and their paper shows the power of this approach as a general utility to characterise multiple states of macromolecular machines. This holds great promise in moving away from the traditional "static" view of structural biology where one or only a handful of states are observed. Applications of this new method may lead therefore to a greatly increased mechanical understanding of a range of important biological systems.

I would like to take this opportunity to thank all authors for their contributions to this edition of *Methods*, which I hope has given an overview of not just where EM is at the moment but more importantly where it is heading. The ability to do on-grid purifications, to use new computational approaches, to be able to accurately fit higher resolution structures to more modest EM maps and to be able to move away from the traditional "static" structural view provides new opportunities within EM. The last 10 years has seen an incredible shift in the capabilities of cryo-EM and with the introduction of the next generation of detectors, phase plates, new computational approaches and improved grid preparation techniques the next 10 years holds great promise.

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