Electron microscopy: from 2D to 3D images with special reference to muscle

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Abstract

This is a brief and necessarily very sketchy presentation of the evolution in electron microscopy (EM) imaging that was driven by the necessity of extracting 3-D views from the essentially 2-D images produced by the electron beam. The lens design of standard transmission electron microscope has not been greatly altered since its inception. However, technical advances in specimen preparation, image collection and analysis gradually induced an astounding progression over a period of about 50 years. From the early images that redefined tissues, cell and cell organelles at the sub-micron level, to the current nano-resolution reconstructions of organelles and proteins the step is very large. The review is written by an investigator who has followed the field for many years, but often from the sidelines, and with great wonder. Her interest in muscle ultrastructure colors the writing. More specific detailed reviews are presented in this issue.

Key Words: Transmission EM, High Voltage EM, Cold Stages, Single Particle Analysis

Eur J Transl Myol - Basic Appl Myol 2015; 25 (1): 5-13

Electron microscopy optics, despite the high resolution, have a depth of focus of several hundred nanometers at the relatively low accelerating voltages of 60-80 KV that were available in the early times and that are still commonly used for standard thin sections of biological specimens. Images at higher accelerating voltages often span larger depths. From the beginning, a major concern has been how to extract the three dimensional information that is contained within the "flat" images, a quest that is essentially similar to that of the early work with fluorescent microscopy. As in the case of the latter, the quest has driven both hardware (the microscopes and image collection systems) and the software to high levels.

The first step: separating top from bottom of images

Early microscopists could only collect two dimensional images, because the stages allowing specimen tilt and thus streo views were not available. The first steps in extracting in depth information from 2-D images were taken at the MRC Cambridge, a hot spot of scientists, trainees and discoveries (M Perutz, HE Huxley, A Klug, KC Homes, D De Rosier, N Unwin, R Henderson, J Murray, J Haselgrove). In the early sixties the newly devised negative staining technique using uranyl acetate[1] allowed observation of structural surface details and imaging of small objects (viruses were the preferred specimens) but it did not help in the quest for depth information, because surface details of both lower and upper surfaces facing the grid and away from it were outlined by the dried stain and thus were superimposed in the images. A recent review by D. De Rosier,[2] one of the major movers to the solutions to these problems, details the early agonizing, often controversial, efforts at separating the two superimposed images of surface viral capsids that came from the top and bottom views of a virus. These included the constructions of models with demonstrations that they could mimic the observed images, and attempts at "staining" only one side of the virus.[3] A simple shadowing would have gone a long way towards solving some of the questions, because the shadowed images show only one side of an object, but a means of drying objects without flattening due to surface tension effects was not available in those times. They became available in the early eighties.[4-6]

The first decisive step took advantage of helical structures that are found in some viruses and the realization that the optical diffraction pattern due to the scattering of a collimated monochromatic beam through an EM image recorded on a transparent film contained information on both sides of the virus.[7] An appropriate filter mask located in the diffraction plane could be used to separate and eliminate the information coming from one side of the virus from its mirror

image that came from the opposite side and an imaging lens allowed reconstruction of filtered images from the mask. Images of model and real objects (TMV virus and bacteriophage tails) filtered through a mask consisting of "opaque material containing appropriately located holes" punched by hand showed the details coming from one side of the virus. This paper is a "must" read for anybody starting on optical diffraction work.

Eureka: full helical reconstructions

Complete 3-D reconstruction, even for a helical structure, is considerably more complicated than the earlier attempts, as explained in De Rosier.[2] The seminal step towards achieving this goal was taken by De Rosier and Klug[8] who took advantage of digitized images and the use of calculated Fourier transforms that allowed the extraction of phase as well as amplitude information to obtain a 3-D map of the virus surface. Those who use optical diffraction filtering and reconstructions in modern times starting with digitized images directly from the microscope and simply calling up a program from their computer to handle the calculations, should read De Rosier[2] to understand the magnitude and difficulty of the initial steps.
Although De Rosier and Klug[8] used objects with a helical symmetry as their first examples, they correctly predicted that the basic principles could be applied to object that may not have an intrinsic symmetry

Muscle participates in the structural harvest of helical reconstructions

Muscle has been a major beneficiary of the helical analysis approaches developed by De Rosier and Klug[8] and DeRosier and Moore[9] because muscle filaments have helical symmetry. Uranyl acetate specifically protects macromolecules and macromolecular assemblies from flattening during the drying process[10] so this relatively simple approach has been extensively used and later supplemented by frozen hydrated and freeze-dried specimens as the latter technique became available. Actin filaments were studied under a variety of states and conditions and over many years.[11,-13] The basic native helix is present in all cases but interestingly subtle variations in twist and conformation of the helix indicate a fair degree of flexibility.[14-16] The actin filament fully decorated by myosin heads in the absence of ATP, first imaged by HE Huxley,[17] imposes a helical symmetry on the myosin heads that are attached to each actin monomer. Thus helical analysis can be applied to reveal the structure of the actin-associated myosin in a configuration presumably corresponding to the end of the power stroke in full detail.[18-21] Study of native actin filaments associated with tropomyosin and troponins[22] have been very fruitful once image quality improved to allow direct visualization of the long tropomyosin helices located within the actin groove. The proposed steric model of tropomyosin blocking acto-myosin interaction by its position of in the actin helix groove was directly confirmed by imaging of the native actin filaments in the presence and absence of calcium.[23,24]
For some not well understood reasons, the dispositions of myosin heads on the surface of relaxed myosin filaments, that had been well established by x-ray diffraction could not be preserved for EM by chemical fixation. Negative staining and rapid freezing finally achieved the appropriate preservation.[10,25,26]
The sarcoplasmic reticulum (SR) calcium pump (SERCA) is inserted at high density in the SR membrane but mostly in a random distribution. However, when driven into a single step of the pump cycle, SERCA assembles into 2-D crystals and in so doing it imposes a helical symmetry which could be imaged in frozen hydrated vesicles. Detailed images of SERCA showing the trans-membrane component, the cytoplasmic head piece domains and the stalk connecting them were clearly visualized in 3-D based on helical reconstructions of EM images.[27-29] These lower resolution images were later supplanted by X ray diffraction, revealing the conformational states of the pumping cycle, the first views of a pump in action.[30]

Computational fitting of atomic models

Once a high resolution 3-D EM image is available, the next logical step is to fit the atomic structure (determined by X-ray crystallography) into the EM reconstruction. A wonderful example of how far this can lead is given by the revolution in models of muscle contraction that derived from the combination of EM and x-crystallograhy data between 1990 and 1993. The first construction of actin filament at the atomic level was obtaining by fitting the X-ray diffraction model of the actin monomer[31] into the helical EM structure.[32] When the first atomic resolution structure of muscle myosin's motor domain was triumphantly revealed in 1993,[33] the actomyosin complex could be modeled, with unexpected predictions.[34] The crystal structure of myosin's motor domain in the pre power stroke configuration[35] finally permitted a formal acceptance of the revolutionary concept that the powers stroke consists of a large swing of the lever arm connecting the heads' catalitic domain to S2, with little changes at the acto-myosin interface. The swing of the lever arm has been directly confirmed in EM images of insect flight muscle rapidly frozen during activity by fitting the crystal structure of the myosin head into the EM cross bridge profiles.[36]
Fitting the crystal structure of selected ryanodine recptor (RyR) fragments (particularly those containing clusters of disease related hot spots) into the relatively low resolution 3-D image of the channel, has been extremely effective not only in the further resolution of the channel structure but also in providing a direct link to function,[37-39] (see also Baker *et al.* review in this issue for questions and problems)[97].

The evolution of EMs, preservation techniques and analysis software

Starting in the late sixties and continuing to present times a steady, tightly interconnected progression of improvements in microscopes, preservation techniques and software for image analysis have lead to increasing resolution and 3-D understanding of ultrastructure at all levels, from cells to organelles to macromolecules. Examples of specific advances due to the introduction of high accelerating voltages, tilt stages, cold stages, low dose microscopy, new image recording hardware, various freezing techniques and image analysis software are presented below.

High accelerating voltages and tilt stages go together

In the early versions of most EMs, the accelerating voltages in the range of 60-80 K (rarely higher) were sufficient to penetrate and to image without significant chromatic aberration thin specimens such as thinly spreadout cultured cells (as in the classic first image of endoplasmic reticulum by KR Porter, 1954)[40]; standard 50-80 nm thin sections; negatively stained small objects; shadowed replicas of small objects and of freeze-fractured cells. Higher voltages permit deeper penetrance of thick specimens (up to microns) and lower chromatic aberrations, although at the expense of image contrast. With higher voltages the necessity of sorting the information from different levels in the specimen became imperative. Fortunately, side entry stages and a larger space between the objective lens pole pieces allowed the grid holder to be tilted (in two different directions in the more sophisticated holders), so that images at different angles could be obtained. Simple fusing of two images taken at appropriate angles immediately offers a view with some 3D information, but more complete reconstruction are possible by electron tomography (ET). A famous two-story high microscope capable of 800-1000 KV accelerating voltages was introduced by K.R. Porter to the Boulder Laboratory for 3-D Electron Microscopy of Cells at the University of Colorado in 1970[41]. In muscle, the first triumph of the super high voltage microscope was the discovery that T tubules network, which are mostly located in transverse planes across the fiber, also occasionally follow a helicoid disposition involving the length of several sarcomeres.[42]

In time however, intermediate voltage microscopes (mostly in the range of 300KV) have superseded the super high voltage ones and become the standard in most facilities dedicated to image analysis. This is due to the combination of factors: lower purchase and maintenance costs; the fact that imaging of a 10 microns thick specimen is in most cases an overkill; the evolution of electron sources capable of collimated beams and the introduction of energy filtering to minimize chromatic aberration resulting in excellent images of thicker specimens even at lower voltages.[43]

Transverse tubules of skeletal muscle as test objects: LM to EM and back

Transverse (T) tubules are slender invaginations of the plasmalemma that penetrate deep into the skeletal and cardiac muscle fibers forming complex grids that carry an electrical signal. Continuities of T tubule membrane with the plasmalemma and of T tubule lumen with the extracellular space offer numerous opportunities for preferential labeling, thus T tubules have been used to test a large variety of imaging approaches and their descriptions have gone full circle from light microscopy (LM) to standard, intermediate and high voltage EM, back to LM in its recent high resolution incarnation (Jayasinghe *et al.* article in this issue)[95] with much current interest in pathological variations. The first extensive descriptions of T tubule networks were based on a Golgi infiltration (performed, of course in Camillo Golgi's department at the University of Pavia,) that made the tubules well visible in the light microscope despite their small size. Veratti's incredibly detailed comparative descriptions of T tubules networks were published in 1902, rediscovered and reprinted in 1961[41] and later confirmed by the use of the same Golgi "staining' at the EM level.[44-46] Infiltration of T tubules' lumen[47] and of the T tubule membrane[48.49] with fluorescently labeled molecules provided good LM view of the T tubule, including developmental stages. EM images of Golgi stained T tubules were obtained at 800 KV from very thick (up to 10 µm) slices, but it quickly became obvious that, despite some chromatic aberrations, thinner (1/4 to 1/2 µm) could also give sufficiently clear overview of the system.[43,48-50] Among the large numbers of publications two papers by the late Hiro Takekura[51.52] show excellent stereo views of T tubules using 100 KV voltages and calcium-potassium ferrocyanide method. Completing the circle, T tubules have returned as test objects for high resolution LM (see Jayasinghe *et al.* review in this issue)[95].

Freezing of tissues and cells as an alternative procedure: advantages and limitations

The EM preservation procedures for tissues and cells that have provided, and still provide, the bulk of ultrastructural data are a combination of chemical fixation, dehydration and embedding in plastic for thin sectioning. Obvious artifacts can arise from glutaraldehyde cross linking and from shrinkages and distortions during dehydration and sectioning. Freezing has been sought and effectively used as an alternative first "fixation" step. Its main limitation is technical: heat extraction needs to be extremely fast in order to freeze without formation of distorting ice crystals ("vitrification") and without water movements between different cellular compartments. Thus freezing has to

be "rapid" and such techniques for tissues and cells have been quite difficult to implement. The first triumphant demonstration that the exocytic events of acetylcholine vesicles discharge could be trapped at an active neuromuscular junction brought rapid freezing to the attention of electron microscopists interested in tissues.[53] The Heuser "slammer", in which the object to be frozen is slammed against a highly polished copper block required liquid helium and could be modified to rapidly freeze active muscle fibers while recording tension.[36,54] However this is not within the reach of most laboratories and alternative liquid nitrogen-based slammers are commercially available. Since the glutaraldehyde fixation faithfully preserves ultrastructure of "hardy" tissues such as muscle, a hybrid approach using an initial fixation followed by freezing in the presence of a cryoprotectant is also possible.

Even in the best circumstances slammer freezing is good to within less than 10 µm from the surface that hits the block, for various physical reasons. An alternative was offered by the observation that under high pressure vitrification could be obtained throughout relatively thick specimens.[55] High pressure freezers are now commercially available (see Wagenknecht *et al.* in this issue)[98] and although still somewhat demanding the techniques are quite extensively used.

Several choices are available after freezing. Freeze substitution, in which water is replaced without thawing and osmium acts as a fixative, leads to embedding and thin sectioning.[56] Interestingly muscle ultrastructure is not substantially different between fully chemically treated and rapidly frozen tissue, except when the requirement is to stop events, such as cycling cross bridges, in action[36] and, unexpectedly, in regard to preservation of the ordered helical arrangement of myosin head on the surface of the relaxed myosin filaments. A more direct approach is the visualization of the frozen tissue as frozen hydrated sections. This, with its difficulties and limitations is discussed in the review by Wagenknecht *et al.* in this issue.[98]

Finally, frozen tissues can be freeze-fractured, shadowed and replicated without any further intervention. Deep etching of rapidly frozen tissues followed by stereomicroscopy[57] has been extraordinarily successful in revealing cell ultrastructure, such as the disposition of thin and thick filaments and cross bridges in 3-D views of muscle[58] and the semicrystalline arrays of Ach receptors in postsynaptic membranes.[59] Interestingly chemical fixation followed by cryoprotection with a volatile solvent (methanol) and "standard" freezing also provided excellent views of cross bridges in the rigor configuration and of the M line.[60-62]

Cryoprotection and electron tomography (ET): a powerful combination for cells and tissues

ET involves the collection of images taken through a series of tilts up to high angles, which are then reconstructed into a full three dimensional view (see Wagenknecht *et al.* in this issue).[98] Teething problems with ET are mostly in the past. One of the initial difficulties was due to the instability and/or poor alignment of the stage so that alignment of the images was difficult to follow, despite the use of fiduciary markers in the sections. Modern well maintained EMs, available in one of the several facilities dedicated to image analysis, offer easy image acquisition and processing. Computer programs for parceling the information and extracting the full 3D view are accessible even to the less dedicated microscopist.[63] Averaging adds further strength to the analysis of object with inherent symmetry, such as the highly ordered insect flight muscle.[64] One problem and one limitation remain. The problem is the necessity of prolonged exposures to the electron beam with resultant downgrad of resolution. The limitation is in the thickness of specimen that can be used, mostly due to problems at high tilt angles, so that only relatively limited slices can be used. Field Emission Guns energy Filters (GIF) and the use of STEM microscopy increase the usefulness of tomography but at intermediate voltages 0.5 µm is the thickness limit for images at the molecular resolution level.[65] The dream of reconstructing an entire cell, even if small, is not achievable., unless the cell is thinly spread in culture.

The power of cryoprotection in the more accurate preservation of subcellular structures is fully revealed by electron tomography as illustrated in numerous reviews[65.66] and in published images.[67,68] However, ET is of course also applicable to chemically fixed specimens, where it has helped to reveal structural details that were less fully described in thin sections. For example, Hayashi et al.[69] have revealed that grouping of RyR2 at dyadic junctions in cardiac muscle is patchy. Although these data do not alter basic understanding of cardiac e-c coupling it, they do complete the pictures. Tethering structures between ER and mitochondria, detected in thin sections, were clearly revealed in EM tomograms by S. Boncompagni.[79]

The three-dimensional views obtained from analyzing the sequential slices of a tomogram have a limitation that is not usually recognized by the fascinated viewers of the resulting beautiful color images. The features are traced manually through each of the slices of the tomogram and are given a specific molecular identification by the operator in the "segmentation" process. This can be the source of considerable misrepresentation and the 3-D views should be looked at with some reservations.

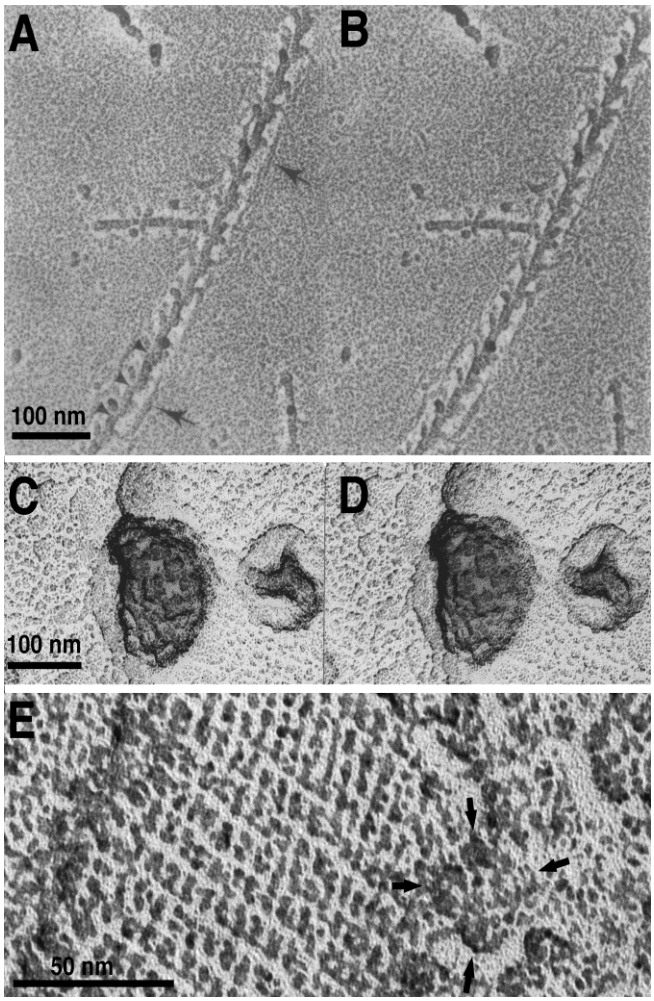

Fig 1. *Images of small objects freeze-dried and rotary shadowed illustrate the preservation of 3D information and fine structural details.* ***A*** *and* ***B*** *illustrate thin filaments isolated from a crayfish muscle that was depleted of ATP and imaged at two degrees of tilt (+/– 15°). The rigor cross bridges remain attached to the filament in the native configuration showing the periodicity of acto-myosin interactions and also reflecting the helical symmetry of the underlying actin filament.[78]* ***C*** *and* ***D*** *illustrate a stereo view of RyR1s arranged in a double row over the surface of a "heavy SR" vesicle isolated from mammalian muscle.[79]* ***E*** *High magnification detail of the rotary shadowed cytoplasmic surface of a freeze-dried SR vesicle after 2-D crystallization of SERCA.[94] The heads of individual Ca pump molecules arranged in orderly double rows are clearly resolved. Compare the size of a single RyR in this image (between arrows) with those in C and D, to get an idea of the scale. Scale bars are shown.*

Single particle analysis: shadowing techniques

Rotary shadowing with heavy metals (most commonly platinum) is a contrast enhancing technique that is very effective in outlining isolated small molecules and macromolecular assemblies, It has been particularly useful in visualizing individual myosin molecules, starting with skeletal muscle myosin II [19,71] and leading to direct views of the long myosin V lever arms,[72] of myosin V complexes[73] and of dimerized myosin VI.[74] The favorite and still currently used preservation technique for the above experiments is the glycerol spray technique of Tyler and Branton[4] that avoids surface tension effects in drying of the specimens.

Alternative approaches rely on freeze-drying, as in the "mica flakes" technique[75] which combines rapid freezing with freeze drying of molecules adhered to mica with spectacular results, e.g., see views of myosin V and of the dynactin complex.[76,77] A second simpler freeze-drying method[78] also allows preservation of 3-D dimensions and is applicable to larger objects such as isolated SR vesicles.[79] Figure 1, illustrates the retention of 3-D information in isolated filaments and SR vesicles that have been freeze dried, with images from the author's archive, see also.[5]

Single molecule/particle analysis: cold stages, low dose microscopy, sophisticated algorithms

In their 1968 paper on helical reconstructions[8] De Rosier and Klug suggested that "Because the theory underlying this method is a very general one, the method can be used for all kinds of objects. It may be necessary however to modify the various procedures…" Indeed that was the case.

The high resolution 3-D view of an intramembrane protein, the proton transport ATPase of Halobacterium[80] created a very strong impression because it presented the very first view of an intramembrane protein at the EM level and also because it showed the power of diffraction analysis in extracting information from micrographs. The images were obtained from unstained membranes that contain a native "crystal" of the protein. Unwin loved to show the original images (gray, noisy apparently unstructured sheets), their diffraction pattern that showed the presence of repetitive structures and the final filtered images. This type of analysis required highly ordered 2-D crystal arrays of molecules in the membranes and few samples of membrane proteins, such as the ACh receptor[80] and the Ca pump ATPase of the SR[27] have been approached by the same technique.

Four major principles were used in the work by Henderson and Unwin: frozen hydrated sample, specimen tilt, low dose microscopy so that each molecule in the array was hit by no more than one electron and reconstruction based on the combination of limited information from a very large number of equal objects.

A more widely applicable approach was initially implemented in the classic work that lead to the 3-D reconstruction of the 30S ribosomal subunit[82] immediately followed by the 50S subunit.[83] Central to the analysis was the ability of arranging a set of images according to the combined variations of several features, so that images in random orientation could be sorted to form a tilt series which was then used for reconstruction.[84] Current explosion in 3-D imaging of isolated macromolecules and organelles has been driven by EM cold stages that allow direct visualization of objects flash-frozen in a thin layer of ice and by suitable digital cameras that allow rapid collection of images. Under these conditions the objects have random orientations, so that single images contain their views at all angles and low dose restrictions can be applied since each area of the grid is hit only once by the electron beam to form the image of many molecules. Image refinement can be obtained by analyzing a large number of samples and taking advantage of the many suites of program that are available. Finally, atomic resolution samples can be fitted in the 3-D image. Although caution must be used in these fittings, these combined approaches are very powerful, as exemplified by the stunning views of chaperone proteins[85] and of the ryanodine receptor (see Baker *at al.* in this issue).[97]

Correspondence analysis can also be used in the context of thin sections but it is restricted by the necessity of a suitable, non biased reference in the image that allows identification of appropriate windows for extracting the information. For example, the structure profiles of cycling myosin cross bridges was effectively analyzed in cross sections of muscles rapidly frozen during active contraction by relying on the location of actin and myosin filament profiles (AM pairs) to define the location of small subimages windows and semiautomatically collect 10,000 images that could be used for the analysis.[54]

The race to atomic resolution is on: EM challenges X-ray crystallography

Digital cameras allowing collection of a large number of images have greatly helped in collecting the large sample size needed for 3-D reconstructions, but resolutions have stubbornly remained at levels that allow only indirect fitting of atomic resolution structures (see Baker *et al.*,[97] and Samsó[96] in this issue). Very recently however a quantum jump in detection capabilities has appeared with a digital camera that allows direct detection of electrons, increasing resolution and rapidity of image gathering[86] combined with novel ways of supporting the specimens. The first harvest has already been obtained, although at the expenses of horrendous calculations, with the near atomic level solution of a slightly truncated rat TRPV1channel in two different conformations.[87,88] Since this direcy EM imaging did not need crystallization, a step often difficult to achieve for channels,[89] the approach gives an advantage to EM against X-ray diffraction and also against NMR which works only for smaller fragments.[90] A flurry of activity is sure to follow on the part of scientists who already have purified proteins and who can afford the use of the new atomic resolution electron microscope, JEM-ARM300F, offered by JEOL. Indeed, three simultaneous publications reporting near atomic resolution images of ryanodine receptors have just appeard in Nature, from a European group, [91] from the USA[92] and from a China-UK collaboration.[93]

Computation now dominates the upper spheres of electron microscopy and the humble traditional electron microscopist takes a back seat in this rarified atmosphere. However, there is still scope for more direct, traditional descriptions of cellular structures at the EM, if scientists are willing to accept their utility.

Acknowledgements

Supported by grant from the National Institutes of Health 2P01AR052354 (PA Allen PI)

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