Interpretation of Electron Micrographs

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Abstract

Courses in electron microscopical techniques should include training in the active reading of electron micrographs. The student should be made aware of the fact that every micrograph contains a wealth of information, evident and hidden, and that a careful inspection is required to retrieve the information. More time should normally be spent in scrutinizing the micrograph than in its manufacture. Active reading of the micrograph is aided by a curiosity in the functional significance of the various details of the picture; there has to be a dialogue between the mind and the eye concerning the structural elements and their significance. The investigator also has to be critical with respect to the possibility of technical flaws and should further be on guard against "seeing" such patterns that others may have seen and have described but which actually do not exist in the micrograph. Among examples given for an analysis in this paper are flaws in the metal shadowing technique and in ultrathin sections that have undergone deformation.

Keywords: Electron micrograph interpretation, Artefacts in electron microscope techniques, Non-random orders, Polystyrene latex particles, Ciliary ultrastructure, Metal evaporation technique, Plastic deformation of ultrathin sections.

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Introduction

A training course in electron microscopical techniques has come halfway only when the student has learnt to prepare a usable specimen and has succeeded to make a well focused electron micrograph of it. One of the most demanding steps in ultrastructure research remains, namely reading the electron micrographs. An active viewing of the picture on the fluorescent screen of the microscope or of the electron micrograph is to be performed and a maximum of information to be retrieved. The student has to be taught that equally much - or more - time should be spent in scrutinizing the electron micrographs than in the preparatory procedures. There are exceptions to this statement, for instance when a morphometric analysis of a known component is to be performed; then a machine rather than a trained person can be put to work to calculate the relative volume of a certain component. In this kind of work it may actually be advantageous not to examine the electron micrographs other than to detect flaws in the preparation and in the sampling of areas to be automatically extracted for information.

Exercise 1

During various training courses in electron microscopical techniques I have made use of one or two rather simple electron micrographs as a test object, which the students have to read. An interesting micrograph for this purpose is the one reproduced here as Fig 1 and which was kindly provided by Drs C and X Mattei. It is from a paper that was published in 1972 (6). I will use it here to illustrate how a training in the reading of an electron micrograph can be performed. This exercise is meant for a small group (5-25) of biology students, who already have some elementary knowledge in electron microscopy as well as in cell biology.

From the outset I inform the students that the electron micrograph comes from the gill lamellae of a mussel and that the magnification is appr. 50,000 times. Then I request that the students do two things:
1. They have to describe as accurately as possible the contents of the micrograph
2. They have to make an interpretation of the various details that they see in the electron micrograph

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The reader is encouraged to do this exercise for himself or herself. Study the micrograph carefully and try to understand its peculiarities before reading this text further. In the appendix to this paper some of the information contained in the figure is given.

The micrograph—being what it is—at first sight appears to present no problem to a student with some knowledge in biology; it shows a number of cross-cut cilia. However, the simplicity is deceptive; the figure contains more information than first meets the eye. On the other hand, it is much simpler than would any region of the internal cytoplasm of the cell be with all its organelles, granules, fibers, vesicles, crystals, etc. The ground cytoplasm would be more difficult to analyze within a limited time span.

The students in this test tend to start by claiming that they see a pattern of 9 + 2 ring-like structures which they interpret as an array of tubules or microtubules and that they recognize the arrays as cross-cut cilia. They are then satisfied with themselves and think that they have seen and said all that is worth while seeing in and saying about the micrograph. They expect praise and the freedom to walk away to do something else. In fact, so much remains to be detected in the micrograph that the exercise usually lasts for another two hours and when we finally stop we all feel exhausted but we have the impression that we have not exhausted the information in the micrograph.

**Reading the electron micrograph**

During the two hours we also gradually discover some of the mechanisms behind an active reading of an electron micrograph (or of any other photograph of the reality). The key secret is that the student has to be curious about the functions of the cilia and of the various details in it and has constantly to ask questions. The mind constantly has to formulate theories of these functions and the eyes have to check whether there is any evidence for the truth of these theories. The mind may for instance make the hypothesis that the beat direction of the cilia bears a relation of the machinery of the nine-plus-two microtubules (analogous to the fact that our own legs can be bent in one plane only relative to femur, fibula and tibia). If this is the case, the orientation of the cilia within a narrow field should be roughly the same throughout the viewing field. With the further assumption that all cilia are co-ordinated in that they beat in the same direction, the orientation of the nine-plus-two units can be checked. What do you see?

The mind may speculate further and make the assumption that the effective stroke of a cilium has a certain relationship to the ciliary machinery (again by analogy of our own legs and with the assumption that the microtubules do not twist around along the length of the cilium). If this would be so, the nine peripheral microtubules (actually microtubular doublets) will have unequal tasks during ciliary beating; doublets with a lateral position in the stroke direction will have less to do or have other work to do than the doublets that are in the beat direction, as a first assumption. The inequality could be expected to be visible as differences in the individual appearance of the nine microtubular doublets. At this stage another aid in the analysis of the electron micrograph will be discovered. It will be noticed that the search for an individuality of any of the nine-plus-two microtubules will be simplified (and our discussions about them will be greatly facilitated) if the different doublets are given different designations. This is analogous to naming the stars in the sky; only after they have been named can you begin to find individual differences—there are no more an aggregation of dots. The most common (and least fanciful) system of designation is to number the microtubular doublets.

An unambiguous numbering was first published in 1959 (1) and is still the accepted one. Looking now for individual markers of the nine doublets it may be seen that two of the doublets often are joined by a bridge. Extensions from what is from now on called doublet number 6 will meet the dynein arms from doublet number 5. The mind may further ask whether or not the two units of the microtubular doublets are identical in appearance and a close examination (performed at higher magnifications) will reveal four or five differences between them (1, 4, 8, 9). Again, it is helpful to give the two subunits designations as was done by Gibbons, subtubule A and subtubule B (4). The most conspicuous difference between the A- and B-tubules is the presence of projections from one of the subtubules (the one called A-subtubule). When a student at a certain stage of this exercise detects these projections he (or she) will claim that each doublet has two projections or will claim that there are three; the third one going centrifugally and joining the doublet to the circular line that is interpreted as the cell membrane.

If the first description of the dynein arms (as they are now called) had claimed that there were three arms on each doublet, then it is likely that most investigators thereafter also would see three dynein arms on each doublet. We are guided by our training and our preconceived ideas on how the system is organized. It is much easier it is to notice that the dynein arms go clockwise in some of the cilia and counterclockwise in others. Why is this so?

It is easy enough to formulate a hypothesis. For instance: Cilia in forward stroke and in backward stroke; cilia that have to sweep material in one direction and cilia that sweep in the opposite direction; cilia that are bent in a hairpin shape and cut both across their externally directed limb and their returning limb; cilia from two cells that are opposite each other and in antiparallel directions. Without new information from other micrographs it is impossible to decide between these and other hypotheses, but a longitudinal section from this particular...
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Fig 1: Ultrathin section through cilia from the mussel *Mytilus parma*. The reader is recommended to inspect the figure closely and to describe as many details as possible. Upon close inspection it may also be seen that both this figure and the following ones are marked with a number in one of the lower corners, and a magnification mark in the other lower corner. Micrograph kindly provided by C. and X. Mattei. Bar = 0.5 µm.

Material would show that the fourth hypothesis is the correct one.

When it has become clear that we are dealing here with two populations of cilia from two cells, then new questions can be asked, for instance: Is there a difference between the "clockwise" cilia and the "counterclockwise" ones? It will be seen that the clockwise cilia tend to appear more electron dense or their doublets will appear compact rather than mostly hollow as in the counterclockwise cilia.

If the student then starts to examine whether this generalization indeed is true, he (or she) will notice that even in the counterclockwise cilia one or usually two microtubular doublets are darker than the others. If the student is familiar with the numbering system, he (or she) is able to decide that the two dense doublets are those called numbers 3 and 4 (numbering according to reference 1). It may also be noticed that the limiting membrane outside these two doublets is more prominent and electron dense than at other places and also straighter than elsewhere. And further: Where the cell membrane is dense and straight in a counterclockwise cilium it is adjacent to a dense and straight portion of the membrane of a clockwise cilium.

At this stage of the analysis the students may come back to the question of whether the two central microtubules all have the same orientation. It will be seen that they have not, yet there is one regularity. Within each pair the two sets of central microtubules are not quite on a straight line; rather, the two lines through the two sets of central microtubules will meet at an angle of about 160°.

The question of how these cilia move now becomes a problematic one. If the direction of ciliary beat is related somehow to the arrangement of the nine-plus-two microtubules or perhaps only to the central microtubules, as would seem tempting to assume, then there is a problem in that the cilia differ in their orientation. After some discussion a student may suggest that these cilia do not move at all; they may instead have another function. The electron density of the membrane might provide a clue to another function. The cilia may act as adhesive devices and
the entire tissue be similar to a bur fastener. The density of the membrane is reminiscent then of a desmosome. This is the interpretation by Mattei and Mattei (6).

The next step in the examination of the figure is now obvious. The investigator becomes curious to know whether each cilium from one cell has made contact with one (and only one) cilium from the other cell. This will be found; there is indeed a remarkable effective mating and the students will now discuss the mechanisms by which such an effective mating can take place. We are however without a good answer to the one-to-one correspondence. (Maybe the reader of this paper will be able to provide a clue?)

It is not quite correct, however, that all cilia have a mate. There is one - but only one - non-mated cilium in this figure. And there is also one cilium that differs from the others by having a $1 + 9 + 2$ formula rather than a $9 + 2$ formula. It takes some time to find these two deviant cilia, and as soon as a student has found them the question comes: Why is this cilium abnormal? I object to this way of looking at things, however, and insist that the peculiarity is that so many cilia have a normal morphology (or are mated). The problem to be explained is the regularity rather than the few deviations from the regularity, even though these can be interesting enough.

During analysis some students will have enquired why a few cilia appear diffuse rather than sharply outlined. This gives an opportunity to discuss the difference in appearance of a structure that has been transversely and oblique-ly sectioned. The effect of chromatic aberration might also be noticeable in this rather thick section, which does not appear as sharp in the periphery as in the center. Alternatively this effect was due to an inadequate enlarger.

The exercise of reading an electron micrograph has one characteristic that perhaps can be said to be unsatisfactory. When analysis is completed (because of the exhaustion of students and teacher), there are more unsolved questions than when the analysis started. It can be said that our ignorance is greater after analysis than before, but hopefully the ignorance is at a higher level.

A non-biological test specimen

In the analysis of the section shown in Fig 1, emphasis was on the biological component of the section, on the cilia. The purpose was to demonstrate that even "simple" structures may turn out to have great complexity and that it may pay off well to look closely on each electron micrograph of the biological specimen.

The technical aspects of the preparatory procedures preceding the electron microscopical inspection of the section was intentionally excluded from the analysis. This was done in order to simplify analysis and must not be taken as a sign that these procedures would be unimportant or poorly understood. A discussion of artefacts in fixation, dehydration, and sectioning would be the topic of another long paper.

In order to demonstrate some of the comple-
Fig 2: Shadow cast preparation of polystyrene latex particles with an average diameter of 880 Å. The micrograph has reversed contrast as an intermediate diapositive has been printed. Can you give a description of the micrograph? Bar = 0.2 µm.

Fig 3: Polystyrene latex particles that have arranged themselves in a hexagonal array. Bar = 0.2 µm.

Fig 4: Polystyrene latex particles in a square packing array. Bar = 0.2 µm.
3) each particle has six closest neighbors, in the second case (Fig 4) four closest neighbors. Fig 5 shows a third preferential pattern, namely a linear arrangement.

To me it is a mystery that non-biological latex particles can arrange themselves in one, another, or a third packing pattern or that they may lie with no particular order and that they do this within different fields of the same preparation. Which long-distance forces have been at play to introduce an orderly arrangement of chemically uniform latex particles over large areas comprising hundreds or thousands of particles? When an answer will be found we may also get an answer to the problem of why for instance insect spermatozoa in the sperm ducts arrange themselves in regular arrays (Fig 6). Other examples of regularly arranged insect spermatozoa may be seen in the paper by Phillips (7).

**Latex particles in sections**

It is also instructive to embed and section latex particles. When this is done, it is found that all latex particles are deformed in the same direction. They are no more round but elliptic with the long axis parallel to the knife edge. The deformation is greater the thinner the section has been made. What is perplexing here is that the section itself is not deformed or only minimally deformed. An ultrathin section may thus be compressed in the sectioning direction with less than 2% whereas the latex particles it contains have been compressed by 30% or more. When measuring the dimensions of the sectioned latex particles it is found that the particle can be expanded in a direction parallel to the knife edge whereas it is compressed in the direction perpendicular to it, e.g., the direction of the cutting movement. One way of showing this is to deposit latex particles from the same batch as the embedded ones on top of the section and to compare the long axis of the sectioned particles with the diameter of the deposited particles (Fig 7). The size difference between deposited and sectioned latex particles is too great to be explained by a shrinkage in the electron beam of the deposited particles (3). An expansion of the latex particles was not noted in similar experiments performed by Jésoir (5).

It hence appears that the Epon section with its latex particles behaves as a soft compressible material, not unlike a rubber film. An epon section of a biological section may also exhibit a similar plastic deformation, particularly around holes in the section which expand in the electron beam (Fig 8). This condition is particularly often seen when the epon section has been put on a naked copper grid, thus with no supporting film. When instructive changes of this order are found it becomes obvious that measurements of dimensions should not be trusted too much. There is thus no sense in giving a dimension with three decimals.

The above examples of difficulties in the interpretation of electron micrographs have all been taken from work with the transmission electron microscope, the instrument I am familiar with. Similar problems arise in work with the scanning electron microscope and hence it is advisable to practise this kind of exercise with some known sample such as the latex beads or pollen grains.

**Coda**

I end this paper with two quotations on the art of seeing and observing. Together they provide a warning that you should be critical and should not trust your eyes unconditionally.

J W Goethe is claimed to have said "Man sieht nur was man weiß" or "You see only what you know".

The other quotation of unknown origin says "When you look for something in particular you have to be very careful, or else you risk to find it".

**Appendix**

The following is an example of description and interpretation of Fig 1. A number of similar structures are seen; they are densely packed, roughly in a hexagonal array, thus with no supporting film. When instructive changes of this order are found it becomes obvious that measurements of dimensions should not be trusted too much. There is thus no sense in giving a dimension with three decimals.

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Fig 6: Cross-sectioned spermatozoa from the bug *Laccotrephes* spec. What forces make the spermatozoa align themselves in regular rows? Bar = 1 µm.

Fig 7: Sectioned latex particles with an average diameter in the non-sectioned state of 1.305 µm. Other latex particles from the same batch have been deposited on top of the sections. Sectioned particles are compressed in the sectioning direction (whereas the section was not compressed). They also have an increased diameter in the opposite direction. Bar = 1 µm.

Fig 8: Section of cilia from the nasal epthelium: In the center of the micrograph there is a hole that has widened during electron bombardment. The cilia at the edge of the hole have shortened in one direction and become stretched in the opposite direction (with up to 30%). The epon section and its biological contents hence have been plastically deformed. Bar = 0.5 µm.
interpreted as a cell membrane (it can also be called here the ciliary membrane). Its waviness is surmised to be artefactual. The cell membrane appears straighter and of a relatively higher electron density at one portion along the cell periphery. This portion lies opposite a similar portion of another cilium. In this way the cilia form pairs. The dynein arms go clockwise in one member of a pair and counterclockwise in the other. All cilia except one are paired in this fashion - the exceptional one being located 4.5 cm from the top and 5.5 cm from the left side. A line through the two central microtubules in one member of a pair meets the corresponding line from the other member to form an angle of about 160°. The region of the dense membrane is along doublets no 3 and 4 in both members, according to the numbering system in ref 1. One cilium has an extra microtubular doublet (it is located at the right side and 3 cm from the bottom). Some cross-sectioned cilia have a dense substance surrounding the microtubules; these cilia tend to have a smaller diameter. Many ciliary cross-sections are elliptical rather than circular. There is no recognizable regularity in the direction of the elliptical long axes which hence must not be interpreted as due to compression during sectioning or tissue preparation. In the upper left portion of the figure there are a few cilia that appear to be less well defined; they are interpreted as cilia that are obliquely oriented in the section. There is some material between the cilia and thus in the extracellular space; this material probably represents debris and membrane fragments.

References


and observations were abused when not fitting in these laws.

Author: I have not been able to read Goethe’s quotation in its context, but I admire his analysis of how we observe the world. My interpretation of the statement is simply either ‘new observations fit in the existing concepts’ or even ‘no new observations are made, you just confirm what other persons have already described’.

J. Murphy: Is there any possibility that the latex is reacting with the epoxy?

Author: This possibility has to my knowledge not been examined. If there is a reactivity it would be very slow as no change is seen in the particles in spite of the fact that they have a very large surface-to-volume ratio. On the other hand the particles will be dissolved by propylene oxide which thus has to be omitted during the embedding procedure. The resin used for the latex sections was Epon 812, with DDSA, NMA, and DMP-30 as described by Glauert AM: Practical methods in electron microscopy, volume 3, 1974.

J. Murphy: What is the basis of your conclusion that there is no compression of the section in Fig 7?

Author: The dimensions of the section were compared with those of the face of the block from which it was cut. These measurements can easily be made with the LKB Ultratome ultramicrotome. A similar conclusion has been reached by J.-C. Jésoir (text ref 5). In his experiment the latex particles were compressed by 29% whereas the section itself was compressed by less than 2%.

B. Forslind: Artifacts may be produced from the time of biopsy/ specimen collection or isolation to the act of microscopy and even the process of micrograph production – but which is the most important step in your mind?

Author: Probably the fixation procedure. I believe so because I feel that any artefact that is introduced at an early step in the process might be amplified in later steps. It has been comforting, however, that different preparatory protocols result in rather similar preservation, e.g., cytoplasm after conventional fixation and embedding compared with data after the new quick freeze–deep etch preparatory method.