Electron Microscopy in Anatomy

Proceedings of a Symposium held by the Anatomical Society of Great Britain on the Ultra-structure of Cells

ELECTRON MICROSCOPY IN ANATOMY

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PREFACE

A MEETING of the Anatomical Society of Great Britain and Ireland for the Session 1958–9 was entirely devoted to a Symposium on " The Ultrastructure of Cells ". The meeting was held on Thursday and Friday, 16th and 17th April, 1959, in the Department of Anatomy, University College, London, under the Chairmanship of the President of the Society, Professor F. Goldby. The present volume represents the proceedings of this Symposium and includes all but two of the papers which were read at the meeting.

At the request of the Council of the Anatomical Society the undersigned undertook the task of editing the proceedings for publication. They wish to express their thanks to Dr. J. D. Robertson and to Professor R. J. Harrison, Honorary Secretary of the Anatomical Society, for help in their task.

It should, perhaps, be added that the meeting included a demonstration of electron microscope techniques with closed circuit television (arranged by the courtesy of Messrs. Smith, Kline and French) and a panel discussion. The demonstration was conducted by Dr. J. D. Robertson, assisted by the technical staff of the University College Anatomy Department. The panel discussion was led by Professor J. Z. Young. The other panel members were Messrs. H. E. Huxley, H. Latta, E. H. Mercer and G. E. Palade. All the presentations and discussions during the meeting served to illustrate the importance of the electron microscope as a powerful weapon in biological research. The new revelations of cell structure consequent upon its use clearly indicate a vast expansion of the anatomist's horizons and leave no doubt as to his special responsibilities in this new field.

J. D. BOYD

F. R. JOHNSON

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INTRODUCTION

ELECTRON MICROSCOPY AND BIOLOGICAL PROBLEMS

By Professor J. Z. Young,
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ELECTRON microscopy is obviously valuable to Biology in numerous different ways. It may help to focus ideas about possible applications to take a definition of living processes and then see how the electron microscope can help in the study of the fundamental characteristics defined. Living systems are highly inhomogeneous aggregates of macromolecules, maintaining steady state equilibria by the operation of sets of instructions. Taking each part of this definition separately what are the advantages of the electron microscope for studying them?

(1) Highly inhomogeneous aggregates require methods giving high resolution in space. The biochemist seeks this with the centrifuge, disrupting the system. Even so he can hardly achieve the resolution possible with the electron microscope. In order to exploit our advantage we need to identify what is seen, and the development of methods that allow this is a high priority. The special advantage of the electron microscope is that we can study even very small objects in their proper positions and it is not necessarily only the more regularly arranged structures that are most worth while. Studies of muscle, myelin or collagen are clearly of great value but perhaps do not lead us as close to the understanding of biological steady states as we might reach by attention to less regularly arranged cytoplasmic or nuclear structures.

Investigation of the relations between cells and of extracellular spaces may be as important as study of their contents. This may require work at low as well as high magnification and reconstruction of the relations of a relatively large number of cells. The technical demands (including serial sections) may be as great here as at high resolution.

(2) Aggregates of macromolecules. Methods for the study of macromolecules, e.g. X-ray diffraction, become difficult when varied and inhomogeneous aggregates are involved. For the study of particles

and membranes the electron microscope is again supreme. To make use of it in this field requires a conscious approach with every possible help from biochemists and physical chemists. Every fixation is the first step in an experiment, which should end by contributing to the solution of a properly posed problem, preferably by numerical data. The electron microscope, like the light microscope, is apt to give qualitative results only, the very attraction of its " pictures " is a danger. Fixation is not " good " because it shows us pictures that are " most life-like " but when it tells us most about life.

(3) Maintaining steady states. The electron microscope here shows its greatest weakness—poor resolution in time. With electrodes we can record changes occurring in microseconds. But even the smallest electrode cannot record at smaller distances than about 1μ and is not much use for recording events say at the surface of a mitochondrion, let alone smaller objects. What can be done by suitable experiment to study with the electron microscope changes occurring over short-time stretches? It is anomalous that we have no decisive evidence of the method of formation of any of the many particles within cells. It would be useful to establish criteria by which to judge whether the apparent constrictions of tubules and vesicles indicate that movements were taking place at the time of fixation. Carefully designed experiments with suitably selected material would perhaps do more to solve fundamental questions than further " anatomical " descriptions of the appearances within cells.

(4) Living processes are controlled by a code of instructions. Here the electron microscope may again seem to be a weak tool because the control is dynamic. But the code substances (genes and their products) are present in such small amounts that the electron microscope may be the only means for study of the units that make the set of them, even though transforming nucleoprotein fractions can be chemically isolated in particular cases. A particularly important problem is obviously to find means for identifying the specific deoxyribose- and ribose-nucleoproteins as they move about in the cells that they control.

There are so many exciting things to do with the electron microscope that it may seem unnecessary to pause to choose between them. Yet if we are not careful it may appear in the future that the problems we are attacking are parochial, even that they are merely adjuncts to already well-developed types of anatomical and physiological investigation. Can we match the resolution of the instrument with an equal efficiency in the design of problems for it?

CONTENTS

CHAPTER 1

PRESENT AND FUTURE POSSIBILITIES OF ELECTRON MICROSCOPY

By A. W. Agar, Aeon Laboratories

IN presenting, for the benefit of anatomists, a survey which will discuss the present and future possibilities of electron microscopes, it is necessary to review the physical factors involved. This will be done in a simplified way, leaving those who wish to follow the arguments in greater detail to consult the reference books on the subject (Zworykin, Morton, Ramberg, Hillier and Vance, 1945 ; Cosslett, 1951 ; Haine, 1954 ; Hall, 1953).

The limit of resolution of an optical microscope is expressed in a formula first presented by Abbe as :

$$d = \frac{0.61\lambda}{n \sin \alpha} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where d is the resolution limit, λ the wavelength of the light used to illuminate the object O, and α is the semi angular aperture of the lens (Text-fig. 1.1), and n is the refractive index of the material between

TEXT-FIG. 1.1. The Semi-Angular Aperture of a Lens

the lens and the object. As is well known, a powerful lens on an optical microscope may be very close to the object, so that α may

approach 90°, and with oil immersion lenses, the term $n \sin \alpha$ may exceed 1. Thus, the resolution limit can be about half the wavelength of the illumination used, and d may approach $\frac{1}{4}$ micron for visible light.

It should be noted that eqn. 1 makes no reference at all to any property of the objective lens of the microscope, only to the acceptance angle of light from the object.

Defects in the lens would impair the resolution attainable, but lens design is now so advanced that the residual errors are almost negligible. A resolving power approaching the theoretical limit is therefore obtainable.

The electron microscope.

Since the optical microscope is limited in performance by the illumination which is used, an improved resolution is dependent on the use of illumination of a much smaller wavelength.

De Broglie showed that a beam of electrons could be regarded as a wave motion, and that the effective wavelength was given by :

$$\lambda = \sqrt{\frac{150}{V}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where V is the voltage accelerating the electron beam, and λ is the wavelength in Å. Thus, with an accelerating voltage of 60,000v, an effective wavelength of 0.05Å is obtained. Used as illumination for a microscope, this would offer a theoretical improvement in resolving power of a factor of 100,000.

It was shown by Busch in 1926 that a suitably shaped magnetic field would operate as an electron lens, and converge a beam of electrons. An electron microscope then became a practical possibility and the first model was built in 1932 by Knoll and Ruska.

Lens defects.

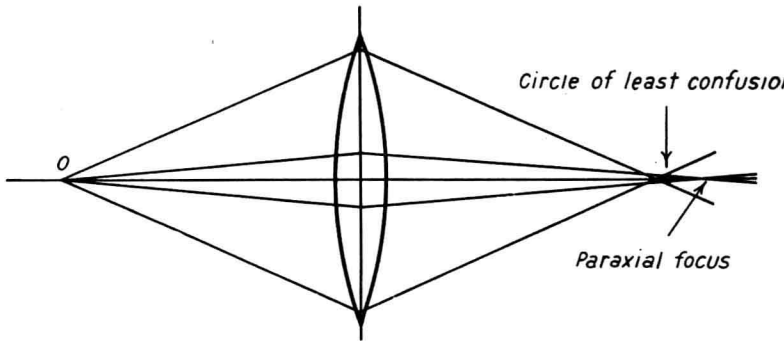
Electron lenses suffer from defects comparable to those of glass lenses for light optics, besides possessing a few additional aberrations. Unfortunately, although great improvements have been made in the development of electron lenses, it has not so far proved possible to correct these lenses for certain quite serious aberrations. It is these aberrations which set the limit to the performance of an electron microscope, and they are briefly discussed below.

Spherical aberration. This is the property of a lens whereby it focuses the beam more strongly from its outer zones than from the region near the axis (Text-fig. 1.2). The effect of this aberration is that a point

object can only be imaged as a disc, whose minimum diameter d_s at the circle of least confusion is given by :

$$d_s = \tfrac{1}{2} C_s \alpha^3$$

where C_s is a constant depending on lens properties, and α the semi-aperture angle as defined in Text-fig. 1.1. It will be seen that the blurring of the image increases very rapidly with increasing lens aperture. Since no practicable corrector lens has yet been devised which could counteract this error, the only solution is to stop down the lens until the loss of resolution from this aberration becomes tolerable. This process must not be taken too far, as the limitation imposed by eqn. 1, where α appears in the denominator, then becomes important.



TEXT-FIG. 1.2 Spherical Abberration in an Objective Lens

A compromise between these opposing conditions is best reached at a value for α of about 10^{-2} radians—only about 36′ of angle. This places a practical limit on the resolution of the electron microscope of 2–3Å.

The limit is further worsened by other factors, for example :

Chromatic aberration. By analogy with light optics, this expresses the inability of a lens to focus illumination of different wavelengths at the same point. This again is a defect not readily corrected in an electron lens, but it is possible to obtain a reasonably monochromatic beam of electrons, and so largely avoid the difficulty. The degree of stabilisation required is 1 or 2 volts in 100,000 volts, which is a severe condition, but quite attainable. Such a variation would of itself limit the resolution to 2—4 Å.

Astigmatism. Apart from the two above errors, the only lens defect which seriously limits the resolution of an electron microscope objective is astigmatism. This arises from inaccuracies in machining the pole pieces of the magnetic circuit, and from magnetic inhomogeneities in the iron itself. The mechanical tolerances for making a lens sufficiently

free of astigmatism for a resolution of 3 Å, or even of 10 Å, are beyond attainable limits. Fortunately however, a corrector lens for astigmatic defect can be easily made, and such a corrector is an essential feature of a high resolution instrument. A limit of 5 Å due to astigmatism can be achieved without difficulty. Mechanical vibration of the column may impose a similar limit.

Desirable features of a high resolution instrument.

(*a*) The accelerating voltage available should be variable. This enables the penetrating power of the electron beam to be changed to suit the specimen being examined, and it also has an effect on the contrast of the picture. Thus, thin biological objects are easily penetrable, but are low in contrast, and are better examined with a low voltage beam (say 50 kV). A thicker object of high contrast may require a beam voltage of 100 kV. Increased accelerating voltage might appear to offer a means of improving the resolution, but in practice the effect is too slight to be important, over the range of voltages normally available (40—100 kV).

(*b*) The area of the object illuminated by the electron beam should be restricted as far as possible, both to reduce the amount of heat dissipated in it, and to prevent radiation damage to unexamined parts of the object. A single condenser lens projecting an image of the electron source on to the object will illuminate an area of at least 50 microns in diameter. If a powerful demagnifying lens is placed between the electron source and the normal condenser lens however, the area illuminated can be reduced to about 2 microns diameter. This feature is most important in a high resolution instrument.

(*c*) The arrangements for scanning the specimen must be mechanically excellent. A stage must be constructed which enables the speciment to be scanned controllably even when very high magnifications (say 100,000) are being used. When the movement is stopped, the stage must not drift by an amount comparable with the resolution of the instrument while a photograph is being recorded (a few atom diameters in 5 seconds).

Likewise, various apertures in the microscope, perhaps only 50 μ in diameter must be accurately centred round the beam, and these too require micrometer controls.

(*d*) For operational convenience, it must be possible to record a number of photographs without reloading the camera. A multiplate camera or film recording is therefore essential.

(*e*) Specimen changes must be quick and easy. A specimen airlock is therefore required.

(*f*) If the instrument is to be employed to best effect over a wide

range of operating conditions, the instrument must be provided with adequate lens alignments.

There are a number of other operational requirements which need not be discussed here. The high resolution instruments available commercially adhere to the principles enumerated above, and are briefly reviewed below.

High resolution electron microscopes.

Siemens Elmiskop I. This was the first of the instruments which could truly be called a high resolution microscope, and it appeared in 1954. It is of very solid construction, as is necessary for the mechanical stability required, and to ensure trouble-free operation over long periods of concentrated work. There is a specimen airlock and a camera with a capacity of 12 plates or 40 exposures on 35 mm. film. The astigmatism of the objective lens is corrected by a magnetic stigmator controlled mechanically. The various alignment controls can be seen protruding from the column (Fig. 1).

Japan Electron Optics JEM.5. This was the next high resolution instrument to appear, and it resembles the Elmiskop I in bulkiness of column. It differs in having the lens current stabilisers and high tension supply behind the microscope column instead of in a separate cabinet, as in the Siemens microscope, and all other high resolution instruments. The microscope has been designed to be as universal as possible, and has a very large specimen chamber which can accommodate heating and cooling stages. It has facilities for reflexion electron microscopy (a feature not of great interest to biologists).

Metropolitan-Vickers EM.6. The latest model to appear on the market, it shows its more recent development in a very smooth external appearance. This has been achieved by operating the gun alignments, and astigmatism correction, by electrical means rather than by mechanical movement, which also makes for convenience in operation. The deflector system permits of reflexion operation with oblique incidence of the electron beam on to the object. It has a large specimen chamber and a six-plate camera. It has a single large viewing window for the final screen in preference to the three smaller ones of the other instruments.

Philips Superscope. This microscope was shown in prototype form at the International Conference on Electron Microscopy at Berlin in 1958. It is not yet in production. It shows similarities in appearance to the EM.6 and has gone even further in substituting electronic controls for mechnical ones. Although it appears to have fewer controls on the column, it has in fact more than any other microscope so far produced, since every lens is capable of individual alignment.

RCA EM.3. This instrument has a very sleek appearance and is more fully equipped for push button operation than any of the other designs. However, it does not have the same magnification range as the other instruments and is not fitted with a double condenser as standard. It is, however, claimed to have a comparable performance.

There is thus available a range of commercial instruments capable of giving a resolution of 10 Å or better on suitable specimens. The actual test resolutions obtained are difficult to assess, especially since the definition of resolution has not been internationally agreed. It is however, certain that they are all capable of giving excellent results on the best biological preparations yet made. Before considering what further improvements could be expected or required in instrumental development, it is profitable to consider what other limitations to resolution arise.

Limitation due to the specimen.

It has been plausibly argued that when a thin section is examined in the electron microscope, the size of the finest detail which will be resolvable will be of the order of one tenth of the specimen thickness. Thus, a section of 500 Å thick will not permit a better resolution than 50 Å, and a section thinner than 100 Å will be needed before the available resolution of the microscope can be employed. Of course, the matter is not as simple as this, and dense objects in a section can be resolved considerably below these limits. Nevertheless, the specimen preparation technique for high resolution working is clearly very demanding. When thin sections are prepared thin enough for high resolution photography, the contrast of the detail may become so low that focusing is very difficult.

If, however, a specimen can be obtained which permits of photography at high magnification, it must then be asked if the fine structure which can be seen can be believed. Fig. 2 shows an area from the pericardial organ of a prawn, fixed in potassium permanganate ; the area shown is a section through one of the secretory nerves. The more or less circular bodies within the nerve fibre can be clearly recognised, as can the membrane round these bodies. This membrane measures about 25 Å across. However, individual black dots appear in these membranes, and it seems very unlikely that they are in fact particulate in this fashion. It is most probable that the dots represent accretions of fixative, aggregated by the effect of an intense electron beam. In such a case therefore, the microscope is resolving detail smaller than the limit which can at present be explained biologically. It may be that there is some mechanism in the fixation process causing the segregation, and that it bears some significant relationship to the

initial structure of the material. For the moment however, the preparation procedure is often not equal to the potentialities of the microscope.

Damage to the Specimen.

(*a*) *Heating.* The electron beam illuminating the object must be extremely intense—for example, at a magnification on the final screen of 40,000x the electrons are spread out over an area 1,600,000,000x as large as that at the object. The electron beam may therefore cause severe damage to the specimen, both by heating and by radiation, unless suitable precautions are taken. In particular, a small area of illumination is most useful in restricting the damage to the specimens, and the photograph must be recorded with the minimum intensity required for obtaining an accurate focus.

(*b*) *Contamination.* When an electron beam strikes a surface on which organic molecules are resting, these can be decomposed to form carbon. In an electron microscope, there is a copious supply of organic molecules—from the oil of the diffusion pump, vacuum grease, and even from fingerprints (Ennos, 1953). These molecules moving at random, reach the specimen and the various apertures in the microscope. Since these surfaces are exposed to the electron beam, a layer of carbon contamination forms on them. This may grow at an alarming rate—the highest recorded is about 8 Å/sec., but even at the very common rate of 1 Å/sec., it does not take long to build up a sufficient thickness of amorphous material to hide much of the fine detail in the specimen. A more dangerous effect from the point of view of interpretation, is that the contamination has the effect of increasing the apparent size of fine detail.

Errors of interpretation due to mal-operation of the microscope.

(1) *Focusing.* Fig. 4 shows a through-focus series of photographs of a hole in a carbon film. The underfocused (objective lens too weak) photograph can be distinguished by the bright fringe outlining the hole, and the overfocused picture by the dark fringe around the hole. The focused picture shows that the carbon film is almost structureless, but in the out-of-focus pictures it appears to have a coarse pebbly structure, in one case with white dots, and in the other black dots. The reversed contrast in the overfocused picture would often be detected as false structure, but the underfocusing could much more easily be misinterpreted. It should be particularly noted that the additional contrast at the film edge in the underfocused picture makes it appear sharper when observed in the microscope than the properly focused edge.

(2) *Astigmatism.* It is assumed that in a high resolution instrument, the astigmatism of the objective lens will have been corrected before work begins. However, after a period of operation, the objective lens aperture will become contaminated, and will begin to introduce fresh astigmatism. This may not always be noticed at the time, and micrographs may be recorded which show the defect. Fig. 3 shows a thin film of carbon with an evaporated layer of thallium chloride which has aggregated into small spheres:

(*a*) shows a correctly focused picture,

(*b*) is overfocused, and each particle has now developed a fringe around it (this could look like a biological membrane),

(*c*) shows the effect of introducing astigmatism in the objective lens,

(*d*) shows the direction of the astigmatism changed.

The pictures (*c*) and (*d*) show that astigmatism may give rise to a striated effect in the background, whose direction corresponds to that of the astigmatism.

If striations appear on a photograph it is particularly important to be sure that the effect is not due to astigmatism. If the effect is believed to be real, but no Fresnel fringes are visible to give certain information, the safest precaution is to find material of the same kind running at right angles to the first, and also exhibiting striations.

(3) *Electron noise.* It is necessary to record a micrograph at an adequately high electron optical magnification if fine detail is to be meaningful. It is possible to observe a graininess in an enlarged photograph, which is due, not to photographic grain, but to the random arrival of electrons at the photographic plate. It is clearly necessary to ensure that the contrast due to this statistical variation of blackening should be small compared with the image contrast in areas approaching the resolving limit of the instrument. The need for this was independently stressed by Haine (1951), Boersch (1951) and Hillier (1951). A minimum magnification of nearly 30,000x is required for a resolution of 10 Å. Successive plates of the same area of the object will show a completely different arrangement of the electron noise at any given point in the image. Photographs recorded to show structure near the limit of resolution of the instrument should therefore be duplicated, so as to demonstrate that any fine detail is in fact true structure.

Shape discrimination.

In view of all the difficulties in the way of employing the full potentialities of a high resolution microscope, it might perhaps be wondered if it is worth having such an instrument. Quite apart from other considerations, it is advisable to have a microscope superior in performance to the potentialities of the preparation

techniques and the operator, since the defects of these latter can then be distinguished and steps taken to improve on them. A further most important consideration is that of distinguishing the shape of small objects. The resolution limit quoted for a microscope only expresses its capabilities in distinguishing as separate, two small objects separated by their diameter, and all such objects at the limit of resolution appear spherical. If it is desired to resolve a square object as such, the smallest that can be identified with certainty may be about three times larger than the smallest object detected (v. Borries & Kausche, 1940 ; Seeliger, 1948). For objects with a greater number of sides, the size increases roughly proportionally to the number of sides.

Future developments of electron microscopes.

(1) *Image intensification.* A study of the difficulties in operation, mentioned above, reveals that several of them (lack of contrast, overheating of the specimen, contamination, difficulty in focusing the image and in correcting the astigmatism) arise chiefly through lack of visibility of the image—the tolerable electron intensity on the object leaving insufficient intensity for viewing the image. These difficulties may be overcome by intensification of the image. A system has been devised by Haine, Ennos and Einstein (1958) which consists of a photoconductor, excited by the electron beam, which backs a charged plate. An intense illumination causes charge to leak away from the plate. The plate is scanned by an electron probe and the response is fed to a cathode ray tube display. With such an arrangement, the contrast of the picture may be increased electronically, and the brightness can be considerably increased. Although the authors have shown that no increase in resolution will be obtainable by photographing the cathode ray tube instead of using direct recording, a considerable improvement in results may be expected from the use of such a device. The increased intensity available should reduce errors due to bad focusing of the picture and to incorrect astigmatism correction. The increase in viewing intensity may be employed to reduce the electron beam intensity on to the object, which will be very important for some objects in avoiding damage or change. Although further development will be required before this system can be applied to commercial instruments, its advent will be of great importance.

(2) *Improved ultimate resolution.* It should be possible even now to design and build a microscope which would have a resolving power of 2 Å. The lens design is not unduly difficult, but the improved stabilisation of lens current and high voltage for the gun would involve considerable complication in the electronics. The shielding of the instrument against stray varying magnetic fields (e.g. from heavy