Principles and Techniques of Electron Microscopy

BIOLOGICAL APPLICATIONS Volume 5

Edited by **M. A Hayat**

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Biological Applications

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Preface

This is the fifth volume of a planned treatise on the principles and techniques employed for studying biological specimens with the aid of an electron microscope. Since its inception in 1970, the treatise has successfully reflected the growth of electron microscopy in instrumentation as well as in methodology. There was a pressing need to keep the readers abreast of the remarkable expansion of the field in recent years and the ever growing importance of its contributions to many areas of biological and medical sciences. This treatise serves as an international authoritative source in the field, and is designed to cover important new developments systematically.

This volume has developed through the joint efforts of six distinguished author-scientists. As a result, a most extensive compilation of methods, developed and used by a large number of competent scientists, has been achieved. The book contains new viewpoints with particular regard to current problems. Areas of disagreement and potential research problems have been pointed out. It is within the scope of this volume to provide the readers with detailed instrumentation of the analytical electron microscope. Each chapter has an exhaustive list of references with complete titles, and full author and subject indices are included at the end of the book.

It is encouraging to know that the first four volumes have been favorably received. I am confident that this volume will also fulfill its purpose: to provide an understanding of the usefulness, limitations, and potential applications of special methods employed for studying the structure, composition, size, number, and location of cellular components and to provide details of current improvements in the instrumentation.

M. A. HAYAT

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1. QUANTITATIVE MAPPING WITH THE ELECTRON MICROSCOPE

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INTRODUCTION

The enormous advances in our understanding of the structure of cells and tissues in the last two decades have depended on the increased resolution achieved with the electron microscope. Now that many organelles have been described, it is of interest to know how they are distributed in particular cells or tissues, because this can be a clue to their functions. Furthermore, if maps of the distribution of organelles can be established easily and accurately in normal material, the study of variations in distribution that result from experimental manipulation becomes possible.

This chapter discusses the problems encountered in constructing accurate maps with the electron microscope. The examples used here are drawn from neuroanatomy where the lack of a simple mapping method has been felt acutely. Although overlap with other fields may be incomplete, many of the solutions developed by neuroanatomists should have wide applicability.

USES OF MAPPING

A major preoccupation in neuroanatomy is to determine the precise distribution of particular types of synapses in various parts of the brain (Nauta and Ebbesson, 1970). Such information is essential as a step to-

ward working out detailed wiring diagrams of connections between neurons. Although the general region of the brain to which a class of synapses is distributed can often be identified with light microscopic techniques, the actual identification of particular synaptic elements generally requires the electron microscope. The technical problem is how to map the distribution of these small elements (~0.5 μm diameter) when they are scattered widely, and often sparsely, throughout a thin section several million square micrometers in area.

It is easy to imagine other fields of biology in which techniques for making such maps would be useful. In the study of development, for example, the fine structural correlates of morphogenetic gradients in embryonic tissues have been little investigated. It is known that "gap-junctions" appear and disappear in various tissues at different times during development. Although there are hints that these junctions are related to developmental processes (reviewed by Bennett, 1973), the question has not been thoroughly investigated with anatomical methods. This is because each junction must be identified at high magnification, and there has been no convenient method for recording their distribution.

The difficulties in constructing quantitative maps fall into two broad categories: those of sampling and those of record-keeping. Sampling is by far the most difficult problem and is considered first, not from a theoretical but rather from a practical point of view.

TISSUE SAMPLING WITH THE ELECTRON MICROSCOPE

In reconstructing the distribution of a particular element with the electron microscope, sampling occurs at three different levels: in the choice of blocks from the tissue, in the choice of sections from the block, and in the choice of areas to be scanned from each section. It is common to regard the selection at each of these stages as "random" though, frequently, the opposite is the case. Weibel (1969 and 1973) presents a detailed review of procedures for sampling in microscopy. Even when sampling is done properly, however, it involves a loss of information and consequently a piling of layer upon layer of uncertainty (Moroney, 1963). Weibel is concerned with stereological methods that permit calculation of such features as the size, surface area, and volume of organelles entirely on the basis of geometrical and statistical reasoning. In quantitative mapping, however, much information can be preserved by reducing as far as possible the extent to which sampling is used.

SELECTION OF BLOCKS

There are two opposite approaches to preparing blocks. One can mince the tissue and choose small, "random" blocks for examination (Cragg, 1968). Alternatively, one can cut the tissue into larger blocks, record their precise positions and orientations and choose some fraction of these for examination ("systematic" sampling, Weibel, 1969 and 1973). For mapping, the only advantage in using the "random" blocks appears to be in the time saved during the initial trimming and embedding. There are strong disadvantages. First, by discarding information about location and orientation, the necessity for statistical treatment is introduced. This is particularly unfortunate where one wants to minimize the number of blocks to be examined. A small sample may give sensible information when the tissue is homogeneous, but the greater the heterogeneity, the larger the number of blocks will be required to reflect the truth. In the central nervous system even small pieces of tissue are often heterogeneous; for example, a vertical section of the cerebral cortex (1–2 mm deep) cuts through six distinct cell layers. When the heterogeneity is obvious, as in this example, the necessity for sampling systematically is also obvious; it would be senseless to cut such a highly oriented structure in random planes, although it has been done.

It is important to remember that many tissues may contain underlying heterogeneities that are not morphologically obvious. The cerebral cortex, for example, in addition to its dramatic horizontal layering is also organized into vertical "columns" that are demonstrable only experimentally (Hubel and Wiesel, 1972). It is known that embryonic tissues also contain local differences long before these are anatomically apparent. If heterogeneity in a tissue is suspected, but its configuration unknown, as with the cortical "columns," use of blocks whose precise position and orientation are known becomes extremely important, because it allows for the possibility that the underlying patterns might be reconstructed from the sample. With "random" sampling such a possibility may be permanently lost.

The size of the block is also of some importance. Clearly, the larger the block, the more closely it represents the actual tissue. If adequate methods are available for maintaining one's orientation in the section, it is an advantage to make the blocks as large as possible. Our blocks are routinely 1 x 2 mm. Other workers use even larger ones (10 x 10 mm) and, after examining thick sections in the light microscope, take thin sections from mesas trimmed from selected areas (McGee-Russel and Gosztonyi, 1967).

To summarize, in mapping a tissue it is an advantage to use relatively

large blocks whose position and orientation are known. The time invested in keeping such records is well spent, for then one knows precisely, rather than probably, which parts of the tissue have been examined, and no assumptions must be made about the degree of heterogeneity in the tissue or about the true randomness of the sample.

SELECTION OF SECTIONS

It is necessary to consider the extent to which a single section can be considered representative of a block. The adequacy of a particular section depends first on whether the distribution of elements is dense enough so that a pattern can be discerned (Fig. 1.1-I). Second, the adequacy of a

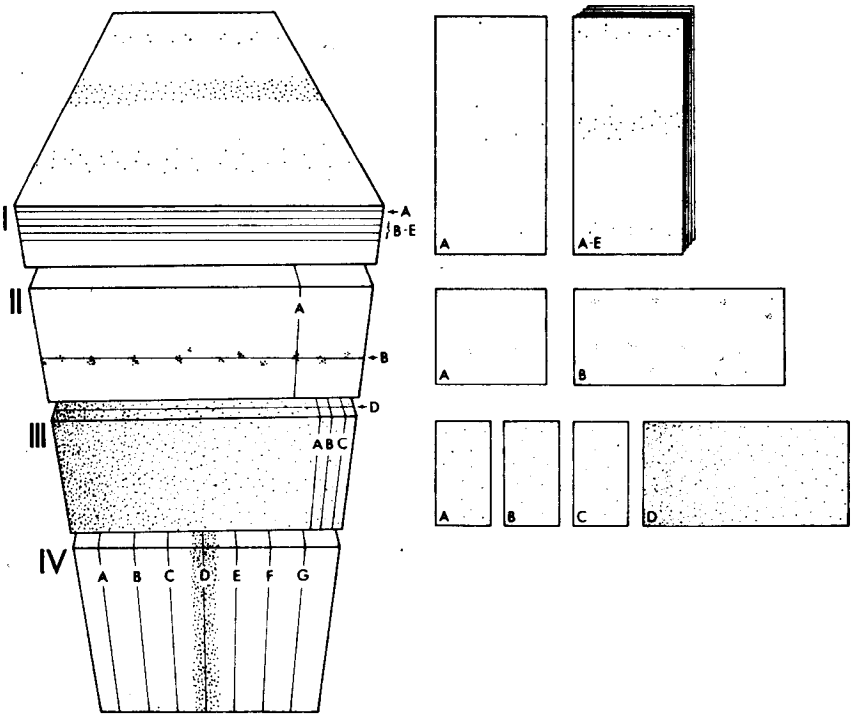

Fig. 1.1 Problems encountered in sampling blocks with ultrathin sections. I. Elements in section A are too sparse to show pattern. Layered pattern is clear when thin sections A–E are superimposed. II. Clumped distribution of elements is not apparent in section A but is clear when block is reoriented and section B is examined. III. Distribution of elements in gradient is not apparent in successive thin sections A–C but is clear when block is reoriented and section D is examined. IV. Block is searched for elements with thin sections A–G. Intervening thick sections (1–5 μm) are saved so that thick section through D can be remounted and additional thin sections from this region can be examined.

single section depends on whether the elements are evenly distributed in the plane perpendicular to the section. If there is a gradient in this plane, or if the elements are clumped, then a single section may be very misleading (Figs. 1.1-II and 1.1-III). In neuroanatomy, both of these problems are quite real. It is invariably a shock to proceed from thick (25 μm) light microscope sections, in which signs of a particular element are abundant and regular, to thin (0.1 μm) sections in which the distribution is sparse, and elements are often clumped, abundant in some sections and rare in others from the same block (Garey and Powell, 1971).

There are no quick solutions to these problems. It is possible with the accelerating voltages of conventional microscopes (50–125 kV) to use sections as thick as 0.3 μm (dark blue) particularly when the object is to determine a distribution and not to produce high resolution micrographs (Fig. 1.2; Varela, 1970). With 200 kV one can use 0.5–0.75 μm thick sections (Fig. 1.3; Shelton, *et al.*, 1971), and with 1 MeV, many structures can be identified in sections as thick as 3 μm (Hama and Porter, 1969). Unfortunately, the usefulness of thick sections in determining the distribution of a particular element depends on the diameter of the element relative to the section thickness, and also on its distribution. For example, it is of little use to employ a 0.3 μm section in plotting the distribution of synapses. The synapses are of this magnitude, but the distances between clumps of synapses may be of greater orders of magnitude.

When it becomes apparent that the distribution of elements varies from section to section, several strategies are possible. It is possible to determine the distribution in "random" sections taken from all levels of the block and reconstruct the distribution in the tissue using statistics. Alternatively, one might reorient the block and examine sections perpendicular to the original plane in which case the clumping, gradient, etc., might be detected either in a single section or by superimposing maps from a few adjacent ones.

Finally, one might sample thin sections at regular, but rather widely spaced intervals through the block, saving the intervening thick sections (Fig. 1.1-IV). When a thin section turns up containing a large number of the elements in question, it is a simple matter to remount the adjacent thick sections and sample them more thoroughly. See Campbell (1972) for a description of the technique for remounting thick sections and Lindsey (1974) for an ingenious illustration of its usefulness. The logical extension of this last approach is, of course, the use of serial sections. Techniques for analyzing large numbers of serial sections with the electron microscope are improving rapidly but are beyond the scope of this chapter (see Levinthal and Ware, 1973). In summary, where the direct methods (such as reorienting the block or remounting thick sections) are ap-

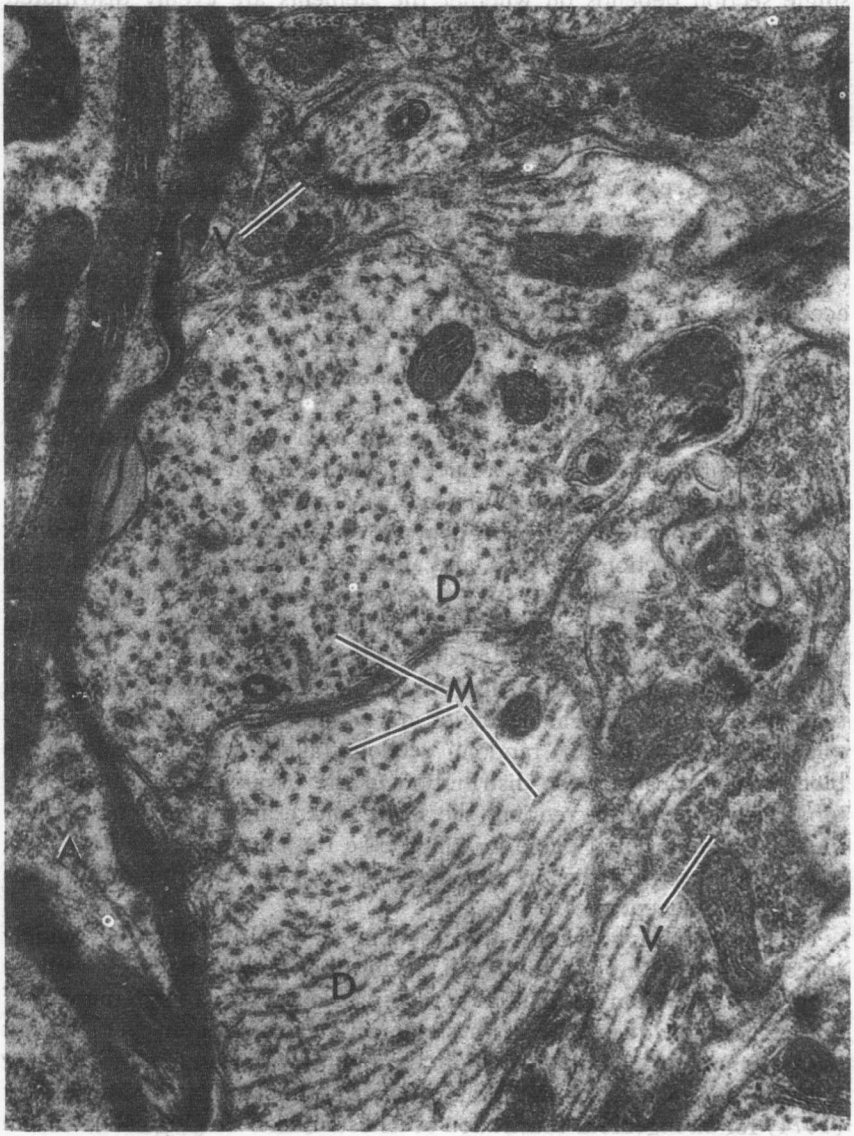

Fig. 1.2 Electron micrograph of section with blue interference color, ~0.25μm thick, taken at 50 kV. Even at this low accelerating voltage the image is adequate for mapping. Cat superior colliculus. ×25,000. A, axon; D, dendrite; M, microtubules; V, synaptic vesicles.

Fig. 1.3 Electron micrograph of section with green interference color, ~0.7 μm thick, taken at 200 kV. Even though many structures are overlapping, synapses can be identified (single arrows). Double arrow marks mitochondrion folded on itself. Cat superior colliculus. ×25,000.

propriate, they are likely to provide more information for roughly equivalent effort, than simply examining large numbers of widely spaced sections.

SAMPLING OF SECTIONS

It is common practice to mount sections on bare grids of fine mesh. Such grids are convenient in that both the step of preparing films and the possibility of their rupture in the electron beam are avoided. Furthermore, when the mesh is fine enough, it is possible to use the grid to keep rough track of the position of the elements within the section (Raisman and Matthews, 1972). Unfortunately, mesh grids, by obscuring a large fraction of each section, introduce a third, and entirely unnecessary layer of uncertainty. This is particularly disturbing when there are relatively few elements in each section.

Sampling of sections should be avoided by mounting them on grids with a single, large, Formvar-covered hole. In this way, the entire section can be scanned with none of the landmarks or possibly critical areas obscured. We can make sturdy films by dipping a glass slide into a 0.5% Formvar solution and removing it fairly rapidly (Hayat, 1970) or by redipping the slide to make a film with several layers (suggested by Margaret Wong-Riley). Such films do not rupture easily even with repeated insertion into the microscope, and carbon coating has been unnecessary. Micrographs taken through these films, though not as crisp as with a bare mesh, are adequate particularly with accelerating voltages of 80 kV and above.

RECORDING THE DISTRIBUTION OF ELEMENTS WITHIN THE SECTION

Several methods have been devised for establishing the distribution of particular elements in sections with the electron microscope. These differ in the degree of precision achieved and in their convenience. A simple method, already noted above, is to mount a section on a fine mesh grid and to make a record of the number of elements in each grid square. The distribution can then be plotted on a low power micrograph of the thin section or adjacent thick section. The accuracy with which this method can localize particular elements is limited by the size of the grid square. The smallest commercially available grid squares are ~30 μm on a side and, of course, the finer the mesh, the less tissue is available in each sample.

A second method, suggested by Molliver and Van der Loos (1970) and used by Lund and Lund (1972) produces a one-dimensional distribution very conveniently. A section is examined in a series of parallel scans, and