Biomedical Research Applications of Scanning Electron Microscopy

Volume 1

edited by
Gisele M. Hodges
and Richard C. Hallowes

Academic Press
London New York San Francisco
A Subsidiary of Harcourt Brace Jovanovich, Publishers

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Imperial Cancer Research Fund, London, UK

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ACADEMIC PRESS INC. (LONDON) LTD.
24/28 Oval Road,
London NW1

United States Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue
New York, New York 10003

Library of Congress Catalog Card Number 78–72550
ISBN: 0–12–351001–5

Printed in Great Britain by
Latimer Trend & Company Ltd, Plymouth

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Preface

The remarkable development of scanning electron microscopy (SEM) over the last few years has resulted in increasingly wider use of this technique as a research and teaching tool throughout biomedical science. Although a number of volumes summarizing current knowledge of the physical concepts and applications of SEM are now available (see p. viii), there are as yet no comprehensive publications dedicated to a discussion of advancing biomedical applications. It was with this raison d'être that the present new series was conceived both as a forum for discussion and as a reference source for those progressing into this rapidly expanding field.

Since its commercial inception in 1966, the SEM has been shown to be a most versatile instrument for examination and analysis of the microstructural characteristics of solid objects. Its principal advantages reside in the large areas of bulk objects that can be monitored at continuously variable levels of resolution, and in the various parameters that can be investigated in a given specimen by the integration of one or more of the many emissive interactions that occur when electrons hit a surface. Insights into structural–functional relationships can be conveniently provided thereby from the emission of electrons of various energies including primary and secondary electrons, Auger electrons, X-rays and photons.

It is widely acknowledged that cell and tissue surface features accessible in the past by time-consuming procedures such as serial sectioning and stereometric reconstructions, may now be readily studied by SEM. The particular impact of this technique has lain in its ability to provide direct overall information of the architectural organization of large areas of specimen surface resulting in a new appreciation of cell and tissue morphology both in normal, pathological and experimentally induced situations. This recent upsurge of interest in the importance of distinctive surface changes will be further stimulated by the newly developing techniques of SEM marker probes which

enable macromolecular functional and structural units to be correlated.

The primary purpose of the series is, then, to present a collective and critical survey of research activities in biomedical scanning electron microscopy of vertebrate tissues integrating insights into their general structure, composition and function into a comprehensive framework, and including other analytical techniques where appropriate. Subject matter has been chosen particularly on the basis of emphasis given to those areas in the biomedical scanning electron microscopy literature. It is intended to up-date individual parts, independently of the others, at such time as it is considered necessary and to include new titles as the application of scanning electron microscopy progresses.

We would wish to express our gratitude to our authors not only for their contributions but also for their forbearance and patience during the subsequent long period of gestation, and to our publishers for their unfailing consideration and co-operation.

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Contents

CHAPTER 1

Embryonic and Foetal Tissues of Vertebrates

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INTRODUCTION AND GENERAL COMMENTS REGARDING TECHNIQUE

A sizeable literature regarding scanning electron microscope (SEM) observations of embryonic and foetal tissues has evolved [320] since the publication of studies stressing the value of critical point- or freeze-drying methods for preserving delicate surface features [2, 3, 18, 123, 318] and scanning electron micrographs increasingly appear in the popular press and as textbook illustrations. The vast majority of these investigations involve the use of the SEM in the secondary electron mode, and only a few studies involving the X-ray or backscattered electron modes have been reported [80, 313]. This chapter reviews the contributions SEM techniques have made to our current understanding of gametes and embryonic tissues. Such a survey will hopefully provide insights into the variety of specimens and problems examined to date, and by their absence, focus on certain topics still to be explored.

The fertilization process and early stages of embryonic development are presented largely in a chronological manner, and studies of later embryonic and foetal development are discussed by tissue or organ systems. Extra-embryonic membranes and embryonic cells *in vitro* are not extensively considered. Although a major emphasis of these chapters is directed toward

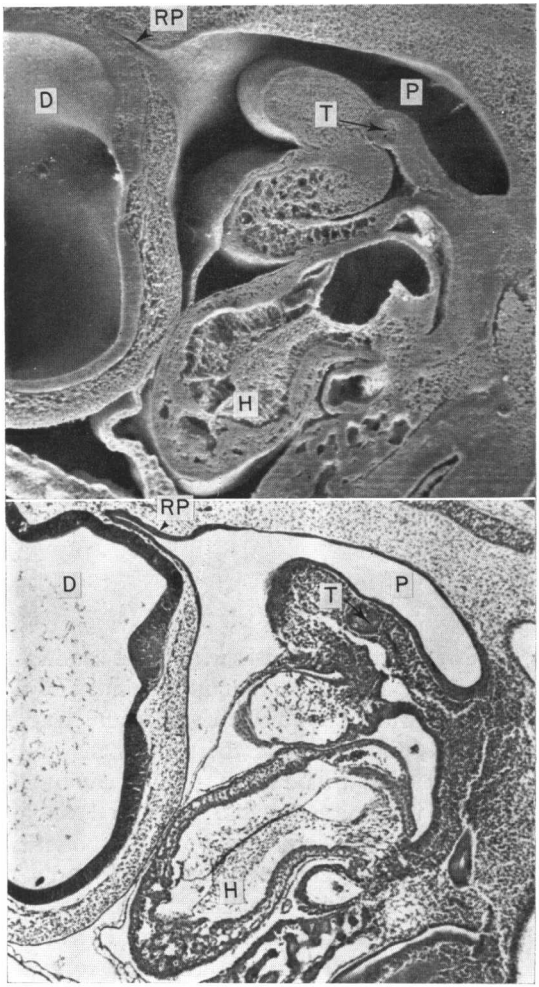
human tissues, SEM observations of human embryonic and foetal tissues are exceedingly limited, and most deal with older embryonic and foetal stages [319, 327, 329] or abnormal, aborted specimens [330]. Many general concepts of human embryogenesis are therefore derived from examination of lower forms which are accessible, and much of the information included in this chapter deals with non-mammalian species.

Gametes and embryos are subject to the same specimen preparation considerations and limitations as adult specimens, and the majority of recent studies have utilized similar protocols for specimen preparation. Samples are usually fixed initially with buffered glutaraldehyde or a combination of aldehydes, followed in most cases by additional exposure to OsO_4. After fixation, specimens are dehydrated in graded series of alcohols (ethanol or methanol) or acetone prior to either freeze drying or critical point drying from liquid CO_2 or Freon 113. Amyl acetate is occasionally used as an intermediate fluid prior to critical point drying with CO_2, although this is unnecessary. Dried specimens are generally coated with a thin layer of gold or gold–palladium alloy, occasionally preceded by deposition of a layer of carbon. Both sputter-coating or vacuum evaporation techniques are commonly utilized. In addition, a method for improving specimen conductivity by increased binding of osmium to the tissue utilizing a bivalent ligand, thiocarbohydrozide, has been developed primarily for use with amphibian embryos [178] and subsequently applied to other tissues. Air drying is utilized only in special cases due to increased awareness of the artefacts produced by the resulting surface tension forces. Because most of the observations discussed in this chapter are derived from tissues prepared by these general techniques, details of specimen preparation are specified only when they differ from the above. Each protocol has advantages and disadvantages which must be considered in interpreting the resulting observations. A comparison of more than one method of preparation is preferable, although this is seldom done.

Several procedures have been described for handling and transporting these small and delicate specimens through the phases of processing, and for affixing dried specimens to stubs for viewing. Adherence of gametes and preimplantation embryos to various substrates such as glass cover slips or millipore filters [108], or enclosure in small containers constructed of filter or lens paper [300], empty ant pupae cases [61], or of more complex mechanical design [18, 269], have all been utilized.

Techniques for examination of structures not normally exposed to view in intact specimens include simply cutting or fracturing specimens before or after drying [42, 225]. Better control over the plane of section may be obtained by supporting young embryos on solidified albumin prior to cutting with a razor blade [312], or by sectioning paraffin-embedded embryos until the desired level is reached, after which the unsectioned portion of the specimen

is de-paraffinized with xylene or toluene prior to critical point drying [2, 3] (Figs 1 and 2). Images obtained from such preparations are valuable for overall orientation of internal structure, but cells at the surface cut by razor blade or



Figs 1 and 2 Figure 1 (top) is a scanning electron micrograph of de-paraffinized, partially sectioned chick embryo (stage 20; 3·5 days incubation). Rathke's pouch (RP); diencephalon (D); thyroid primordia (T); heart with endocardial cushions (H); pharynx (P). ×50. (Courtesy of G. C. Schoenwolf.) Figure 2 (bottom): photomicrograph of nearly adjacent serial paraffin section cut from specimen shown in Fig. 1. Haematoxylin. Labelling as in Fig. 1. ×50. (Courtesy of G. C. Schoenwolf.)

microtome blade usually show some degree of distortion which may interfere with detailed examination of these regions. A smoother surface results from ethanolic cryofracture as described by Humphreys *et al.* [162], or fracture of specimens impregnated with unpolymerized resin [41]. The plane of fracture is unpredictable, however, and tends to pass through cells, making visualization of their external surfaces difficult. Simple cracking or tearing of dried specimens also produces somewhat unpredictable results, but the plane of fracture tends to pass between cells, thereby exposing their lateral surfaces and cytoplasmic extensions to view. Adhesive tape may be used for removal of individual cells or layers of cells, and complementary surfaces may be obtained by placing a specimen between two pieces of tape and pulling them apart [114].

GAMETES

Studies of gamete development and transport in non-mammalian species are scarce [7, 8, 36, 93], and only aspects of the larger literature pertaining to mammalian species are considered here.

MALE GAMETES

Seminiferous Tubules

Cells at various stages of spermatogenesis within the seminiferous epithelium may be readily viewed in cut or cryofractured specimens of the testis [57, 74, 76, 203, 243]. Charging of the specimen due to insufficient metal coating of the mat of sperm tails in the lumina of the tubules may be greatly reduced by treating the specimen with a modified O-T-O technique [74, 203]. Back-scattered electron (BSE) imaging has been applied to developing spermatozoa by Vogel *et al.* [313] and offers a technique of great potential usefulness for obtaining information at a range between that provided by the light microscope after histochemical staining and ultrastructure as observed in thin sections with the transmission electron microscope (TEM). In seminiferous tubules stained with Gomori's silver methenamine reagent prior to cryofracturing in ethanol, the nuclear chromatin, outer dense fibres of the sperm flagella, and the basement membrane of the tubule are selectively stained and the silver deposits appear bright in the BSE mode. Images from a depth of several micrometers can be obtained at moderate magnification from specimens which are critical point dried, coated with carbon and viewed at an accelerating voltage of 25 kV in an electron microscope equipped with a field emission

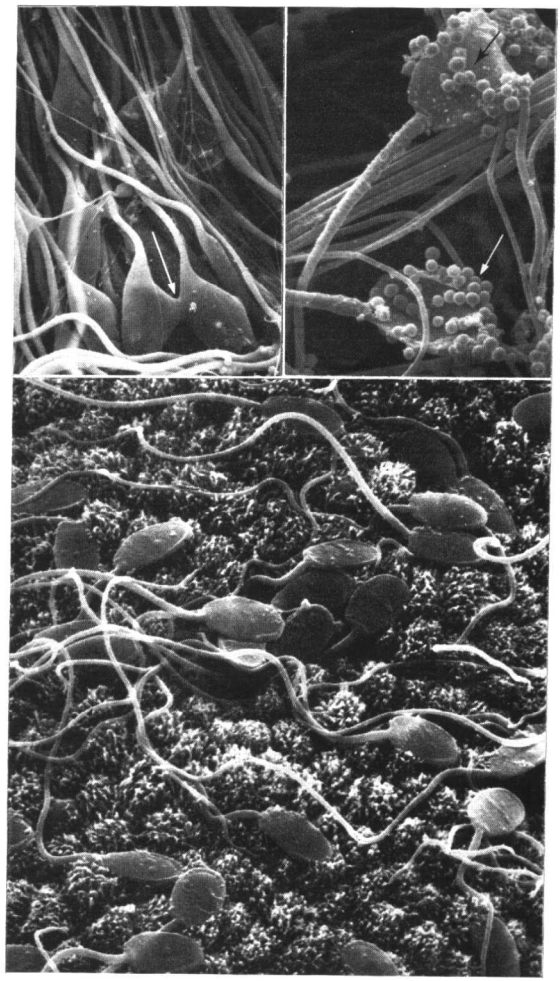
source. This allows visualization of nuclear ultrastructure such as the synaptonemal complex which can be studied particularly well in stereopairs of micrographs. Staining of non-chromatin structures is suppressed in specimens in which disulphide bonds are reduced with benzyl mercaptan followed by acetylation of the resulting sulphydral bonds with iodoacetate.

Prior to their detachment from the seminiferous epithelium (spermiation), the elongated heads of several spermatids lie within recesses of the Sertoli cells, with a portion of cytoplasm located near the neck of each spermatid attached to other such residual lobules by intercellular bridges resulting from incomplete cytokinesis of spermatogonia during spermatogenesis (Fig. 3). These portions of excess cytoplasm remain stationary near the apices of the Sertoli cells while the other components of the spermatid are gradually displaced towards the lumen. In this manner, the connection between the neck of the spermatid and the excess cytoplasm becomes reduced to a slender stalk which breaks during final separation of the spermatozoa. The small portion of the stalk attached to the spermatozoon retracts to form a "cytoplasmic droplet", the larger portion of the discarded cytoplasm remaining as the "residual body" at the apical end of the Sertoli cell [106].

Excurrent Duct System

Spermatozoa may appear in SEM preparations of the male excurrent duct system if not completely washed out or otherwise removed during specimen preparation [57, 243, 266, 340]. Spermatozoa leave the seminiferous tubules of the testis following spermiation by passing through the rete testis and ductuli efferenti which lead into the highly coiled, single ductus epididymidis which together with connective tissue and blood vessels forms the epididymis. Of particular interest to reproductive biologists are the behavioural, physiological, biochemical and morphological changes undergone by the spermatozoa during their stay within various regions of the duct system (Fig. 4). These changes collectively referred to as "maturation" of the sperm are essential for producing spermatozoa exhibiting the normal motility and other characteristics of ejaculated sperm. It has long been recognized that spermatozoa removed from the proximal portions of the ductus epididymidis (i.e. "head" or "caput epididymis") are less capable of successfully fertilizing an ovum than sperm removed from the distal portion ("tail" or "cauda epididymidis") or vas deferens. Morphological changes have also been described at the light microscopical and TEM level primarily in the head and cytoplasmic droplet (for review see Hamilton, 146).

Since the initial study of air-dried spermatozoa of the bull and rabbit by Dott [85], the surface morphology of normal and abnormal spermatozoa recovered from the cauda epididymidis, vas deferens or fresh ejaculates of



Figs 3–5 Figure 3 (top left) shows developing rat spermatozoa in seminiferous tubule. Cytoplasmic droplets connected by a cytoplasmic bridge (arrow) are seen around the sperm tails within the lumen of the tubule. ×1350. Figure 4 (top right): heat-killed *Staphylococcus aureus* (arrows) coupled to rabbit antibodies against guinea-pig testis have been used to localize testicular cell–sperm differentiation autoantigen (TSDA) on the heads of epididymal guinea-pig spermatozoa. ×3150. (Courtesy of E. P. Evan.) Figure 5 (bottom): rabbit spermatozoa on the surface of the vagina 24 h after mating. ×3000. (From D. M. Phillips and S. Mahler, 1977. Phagocytosis of Spermatozoa by the Rabbit Vagina, *Anat. Rec. 189*, 61–72, by permission of Wistar Institute Press.)

several species has been described and compared with the ultrastructural information obtained with the light microscope and TEM. In one of the most extensive SEM studies of spermatozoan maturation to date [110], spermatozoa were recovered from the seminiferous tubules, ductuli efferenti, corpus- and cauda epididymidis, and vas deferens of the bonnet monkey. Samples were either air dried or critical point dried. While critical point drying seems to preserve structure to a greater degree than air drying, collapse of the plasmalemma during air drying makes visualization of underlying structure (e.g. mitochondria) more apparent. Cells recovered from the testis include spermatids still attached to portions of Sertoli cells [110]. These are often in clusters, with the cytoplasmic droplet surrounding the neck of each spermatid exhibiting a lateral projection which is interconnected with similar projections from other spermatids in the cluster and with the cytoplasm of presumed Sertoli cells. Free spermatozoa recovered from the testis following spermiation have a smooth-surfaced cytoplasmic droplet at the neck. The cytoplasmic droplet moves posteriorly as the sperm progresses through the epididymidis, becoming smaller and assuming a location near the posterior end of the middle piece when present on spermatozoa in the cauda epididymidis. A constriction marks the junction between the middle piece and end piece of spermatozoa recovered from the testis, but is less apparent in epididymal sperm [243]. Similar observations of the posterior migration of the cytoplasmic droplets have been made in the rhesus monkey [57]. Some apparent ice crystal damage was seen after freeze drying in this study, however.

Ejaculated Spermatozoa

The majority of SEM studies of spermatozoa deal with ejaculated spermatozoa, and have been concerned primarily with descriptive and comparative morphology, clinical problems or animal husbandry, and forensic medicine. Descriptive studies of untreated, ejaculated spermatozoon morphology have utilized the human [54, 115, 119, 120, 125, 127, 129, 140, 142, 143, 199, 208, 209, 215, 277, 341, 342], non-human primates [71, 111, 125, 127, 143, 208, 209, 212], rabbit [33, 56, 85, 107, 109, 127, 129, 143, 199, 215, 341], horse [286], ram [55, 127, 280], bull [55, 85, 127], boar [55], guinea-pig [127], and a variety of less commonly studied vertebrate species [79, 210, 211, 213, 214]. Presumably "mature" spermatozoa from the cauda epididymidis or vas deferens have also been examined in a number of species including the hamster [129, 215, 337, 341], rabbit [108, 199], bull, dog, guinea-pig, rat, mouse and gerbil [215, 266]. Variations of sperm morphology are commonly seen in routine ejaculates of several species, particularly man, and the abnormal morphology of spermatozoa from sterile mice carrying different lethal *T/t* locus genes has been described in a combined TEM and SEM study by

Dooher and Bennett [84]. These descriptive studies reveal a close correlation between the appearance of spermatozoa viewed in the SEM, light microscope and TEM, although some differences in appearance have been described between various methods of preparation.

Specimens are usually washed several times by centrifugation in a physiological solution, fixed in glutaraldehyde and dried. Dilution of freshly ejaculated specimens in a non-coagulant fixative such as glutaraldehyde may preclude the necessity for extensive washing of spermatozoa prior to fixation [71, 119, 143], and surfaces of unwashed spermatozoa appear smoother than those washed in saline prior to fixation [143]. Air drying is commonly used for preparing spermatozoa for viewing in the SEM [55, 57, 85, 111, 119, 138, 140, 143, 209, 266, 280, 286, 337]. In most cases, drops of fixed spermatozoa suspended in either acetone, ethanol or water are dried on to a glass slide. Specimens have also been freeze dried [71, 129, 341] or critical point dried with either CO_2 or Freon [54, 55, 108, 111, 123, 208, 209, 266]. Studies comparing the results obtained by use of these three drying methods indicate that each has advantages and disadvantages regarding either the technical details of the process or the resulting morphology of the dried spermatozoa. The dimensions of air-dried spermatozoa are approximately 10% smaller than those noted in the dimensions of specific regions of spermatozoa [111]. Flattening of the surface membrane during air drying allows better visualization of underlying structure [55, 57, 127, 209], however, and it is very rapid, a feature which may be important in surveying large numbers of spermatozoa in a clinical or practical setting. Little pretreatment of spermatozoa may be necessary [71, 143], and specimens may be air dried from distilled water or from ethanol following glutaraldehyde and osmium tetroxide fixation. Post-fixation with osmium may make the spermatozoa brittle, resulting in separation between the heads and the tails [71].

Critical point drying of spermatozoa yields an overall smooth surface and good morphology (Fig. 5), but is slightly more time consuming and may present handling problems not encountered with air drying. Collection of suspended spermatozoa on to millipore filters can be used to concentrate dilute samples, and the millipore filter then serves as a substrate on which the spermatozoa may be carried through the dehydration and critical point-drying process [54, 55]. Spermatozoa seem to adhere better if collected on the filter surface prior to fixation [108], but the irregular surface of the millipore filter may make observation of an entire individual spermatozoon difficult [55]. Critical point drying from solid CO_2 as suggested by Tanaka and Iino [301] to reduce the turbulence caused by flushing the chamber of the critical point apparatus with liquid CO_2 may decrease the loss of cells [208, 209].

Considerable attention has been given to problems related to male infertility. It is possible that T-mycoplasmas may be associated with genito-urinary

tract disease and male infertility in man. The presence of these organisms on spermatozoa of infertile human males whose semen contained T-mycoplasmas, and the absence of such structures on spermatozoa from normal fertile males, has been demonstrated in the SEM [115, 120]. T-Mycoplasmas were identified as spherical objects (160–200 nm in diameter) associated with filamentous strands whose appearance closely resembled that of cultured T-mycoplasmas similarly prepared. Many of the infected spermatozoa display coiled tails, but this may result during specimen preparation, since similar coiling is not observed in living sperm in the light microscope [115, 120].

The morphological relationships between spermatozoa and seminal coagulum following ejaculation has also been examined in the SEM relative to the role abnormal liquifaction of semen may play in human infertility [342]. Freshly ejaculated human semen initially coagulates then liquifies within 10–20 minutes. Men with semen which fails to liquify, or liquifies poorly, are often subfertile, possibly due to a restriction of sperm motility within the coagulum. Scanning electron microscope observation of fresh human ejaculates reveals a dense network of fibres with interstices which appear too small to allow passage of spermatozoa "trapped" within them. This fibrous network normally disappears as liquifaction occurs, and is replaced by globular structures. The same sequence of change is seen in semen which liquifies slowly, but takes longer. The appearance of the fibrous network in coagulated human semen is similar to that in the poorly liquified coagula of the guinea-pig and rhesus monkey, although the spaces within the coagulum of these species are larger [342].

Sperm-agglutinating antibodies in the sera are associated with infertility in the male, and several antigens have been localized over the head and tail of spermatozoa by means of standard immunological techniques such as cytotoxicity, agglutination, immobilization and immunofluorescence [245], and recently by SEM as well. The surface morphology of air-dried spermatozoa from fertile and infertile men cultured with sera containing agglutinating antibodies was examined [116, 140], and several patterns of agglutination between various regions of the spermatozoa were observed. The observations suggest that the antibodies may be directed toward antigens located at the surface of the spermatozoon. The various patterns of agglutination may also reflect biochemical differences in the composition of the plasmalemma and differences in surface charge over various portions of the spermatozoa [116, 140].

The SEM has also been used to examine possible membrane damage resulting from immunological immobilization of spermatozoa in the rabbit [270]. Spermatozoa incubated with goat or rabbit anti-rabbit semen globulin plus active complement (C′) and critical point dried reveal extensive alteration in the morphology of the acrosome region. A thickened margin of the acrosome