ATLAS OF HUMAN REPRODUCTION

BY SCANNING ELECTRON MICROSCOPY

EDITED BY
E.S.E. HAFEZ P. KENEMANS

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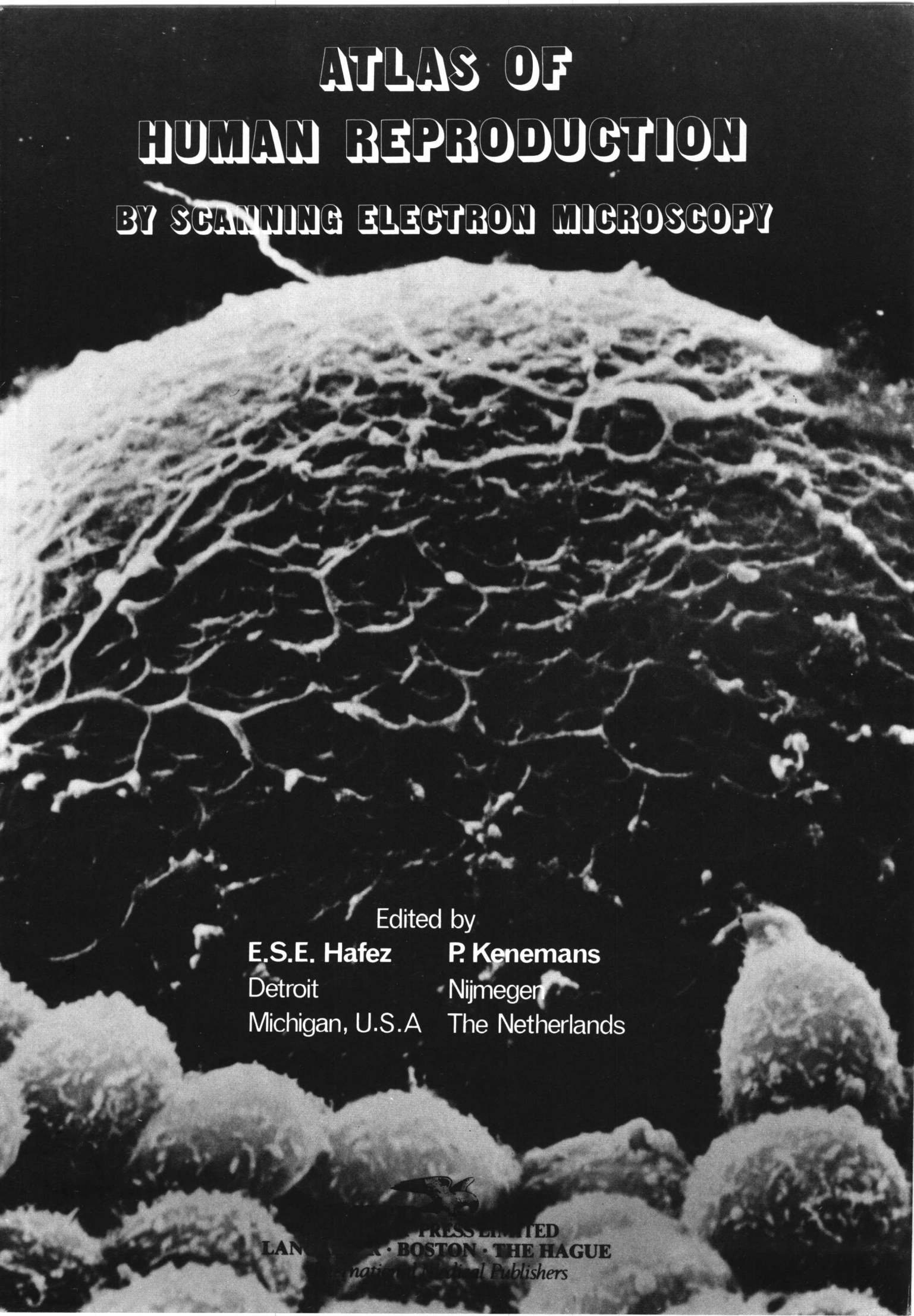

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Foreword

The suggestion of Max Knoll that an electron microscope could be developed using a fine scanning beam of electrons on a specimen surface and recording the emitted current as a function of the position of the beam was launched in 1935. Since then several investigators and clinicians have used this concept to develop techniques now known as scanning electron microscopy (SEM) and scanning transmission electron microscopy (STEM). The choice to study the female reproductive organs was a logical one because cells and tissue samples can be sampled relatively easily; furthermore, these cells and organs are influenced continuously by the cyclic production of hormones.

This atlas demonstrates the state of the art in 1983. Having such predecessors as *Mammalian Reproduction* and *The Human Female Reproductive Tract* one can judge the progress made in techniques and their application. The basis for this research was laid during an international SEM Symposium, 'Human Reproduction in Three Dimensions', held in Nijmegen, The Netherlands, in September 1981. As one of the organizers, and especially not a morphologist, I was fascinated by the numerous SEM photographs, the wealth of information and the enthusiasm of the researchers covering a variety of disciplines. All aspects of the female and male genital tract have been covered, culminating in the prizewinning award showing the *in vitro* fertilized human egg.

In clinical diagnostics SEM also proved to be a valuable complementary technique, shedding new light on oncology, the pathogenesis of tubal disease and the maturation process of the placenta. Future research has still to be accomplished; e.g. quantification of SEM photographs for meaningful and sound biological, scientific and statistical evaluation in diagnostic gynecology, obstetrics, andrology and oncology.

If this atlas does encourage the investigators in the field and their offspring to take up this challenge, the numerous people who used their 'electrons' to make the Nijmegen SEM Symposium so successful, and this atlas possible, will be completely satisfied.

Nijmegen, October 1982 TOM ESKES

Preface

Within only two decades of its commercial innovation, SEM has become an indispensable morphological tool, not only in basic research but also in clinical application in gynecology, andrology, oncology and pathology. This atlas represents an international symposium on 'Human Reproduction in Three Dimensions' held in Nijmegen, the Netherlands, 13–16 September 1981, under the presidency of Prof. T. K. A. B. Eskes and Prof. G. P. Vooys. A major objective of this symposium was to describe the state of the art of scanning electron microscopy in the field of human reproduction. Several investigators in the field of human reproduction who work with SEM (alone or in combination with other techniques) were brought together on this occasion for the first time. The clinical significance of scanning electron microscopy was evaluated, and advanced techniques and future developments were discussed.

The symposium was co-sponsored by the C. S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, Detroit, Michigan, USA; Department of Obstetrics/ Gynecology, Faculty of Medicine, Catholic University, Nijmegen, The Netherlands; Cilag Chemie, Philips Nederland B.V., Organon Nederland B.V., Upjohn International and the Sint Radboud Hospital, University of Nijmegen, The Netherlands.

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Note:

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The Proceedings of the Annual Scanning Electron Microscope Symposium sponsored by IIT Research Institute, Chicago between 1968 and 1977, are abbreviated to SEM/IITRI: the subsequent Proceedings, from 1978 onwards, are abbreviated to SEM Symposium. Each annual number is considered as a volume number. This applies in the References throughout this volume.

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Specimen preparation techniques

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Scanning electron microscopy (SEM) provides a highly appropriate and effective method for studying the shape and surface morphology of cells and tissues. Objects studied with SEM must be non-volatile, mechanically stable, and conductive. However, seldom do biological specimens fulfill any of these demands naturally. Therefore, various methods must be used to transform the material into a proper state for examination by SEM. Paradoxically, however, while the primary objective of such methods is to preserve the structure of the surfaces of the specimens with minimal change in spatial arrangement these obligatory techniques can, and often will, modify the cell morphology considerably (Table 1). Since spatial changes, or artifacts, do occur in specimen preparation, a thorough knowledge of their location and extent is a prerequisite for an adequate interpretation of the findings. Various preparation techniques, and problems related to these methods, are hereby described. Special attention is given to fundamental aspects of fixation which were studied using *in vitro* cultivated cells as a model system.

GENERAL ASPECTS OF SPECIMEN PREPARATION

All preparative techniques of vertebrate tissues for SEM (de Harven *et al.*, 1975; Bell and Revel, 1980; Boyde, 1976) have in common that cells stop living during the procedures. It is here that SEM loses the temporal dimension of organization. It is essential that cell death is instantaneous, otherwise the process of cell dying is

Table 1 Specimen preparation

Technique	*Main goal*	*Main problems*
Preparation	observation with high magnification; preservation of *in vivo* cell shape and surface	deformation; miniaturization (shrinkage); loss of time aspect
Sampling	attainment of the tissue, or population of cells wanted	selectivity; contamination
Cleaning	removal of extracellular material, such as mucus, lymph, serum	obscuration of cell surface; osmotic or biochemical effects
Mounting	attachment of specimen to solid support	contamination; selective cell loss; crowding of cells
Fixation	stabilization of cell morphology	osmotic artifacts
Dehydration and drying	removal of cell water; removal of cell fluid leaving the solids in their original location	lipid extraction; shrinkage; distructions
Coating	attainment of electrical conductivity	charging; heating; melting

depicted rather than processes of cell life. Cells, either alive or having been just killed, must be treated with utmost care until all structural elements (relevant for SEM) are suitably stabilized. This stabilization should be able to sustain crude conditions such as vacuum, irradiation, heating and charging. As cells from various organ systems are different in nature, there cannot be a single procedure which is optimal for all cell populations. Little is known about extracellular environment and intercellular fluid conditions. Thus, individual experimentation and experience is necessary to discover the optimal procedures to be used. In order to determine which procedure will be best suited, an overall strategy for preparing samples routinely should include the use of at least two different procedures.

METHODS OF SPECIMEN COLLECTION, CLEANING AND MOUNTING

Small blocks of specimen tissue obtained by surgery and pinned into a cork plate with the surface of interest upwards are suitable for processing. Isolated cells are generally best collected as suspensions. The suspensions are either natural (e.g. ascites cells; amniotic cells) or artificial in nature (e.g. cervical cells suspended in buffer or buffered fixative) obtained after aspiration, or by gentle scraping from the tissue of origin.

Extracellular material, such as mucus, lymph, or serum proteins, is removed before fixation, as it might obscure the surface architecture when fixed. This is mostly done by rinsing with an iso-osmotic warm buffer solution. Mucolytic enzymes and agents can also be used. As the specimens must be cleaned prior to structural stabilization by fixation, the maintenance of the cell surface integrity has to be explicitly guaranteed during this phase. Cells in suspensions can be attached to a support prior to or after fixation, which also results in some differences.

Living cells, when not yet fixed, show a reactive alteration in their surface features when placed onto the surface of a substratum (de Harven *et al.*, 1975; Westbrook *et al.*, 1975).

Cells can either be centrifuged onto glass slides (Thornthwaite *et al.*, 1975) or allowed to sediment onto coverslips, which are preferably coated with a polycationic substance e.g. poly-L-lysine (Mazia *et al.*, 1974). Cells can also be collected by gentle filtration onto commercial filter discs of 1–3 μm pore size, such as the millipore and nucleopore filter (Westbrook *et al.*, 1975) or the Flotronic silver membrane (de Harven *et al.*, 1975). Metal substrates are more suitable for freeze-drying.

Nucleopore filters, which may be made transparent, and glass supports (with a location grid), allow the relocation and re-examination of the same cells in SEM, once they have been classified by light microscopy (LM). In many studies it is essential to have correlative LM/SEM observations performed consecutively on the same material. This allows proper identification by conventional cytological criteria as a basis for SEM characterization (Wetzel *et al.*, 1973; Noonan and Riddle, 1977; Kenemans *et al.*, 1981).

Cells adhering to a substratum may be lost during further processing. Cell loss need not be at random, but instead may be selective.

FIXATION

The plasma membrane of cells is semipermeable and as long as it retains this property, it is quite sensitive to changes in osmolality. Glutaraldehyde (as opposed to OsO_4) does not completely destroy membrane semipermeability (Jard *et al.*, 1966). Therefore, all cells are sensitive to variations in osmolality until the OsO_4 fixation (Bone and Ryan, 1972).

The glutaraldehyde molecule is readily transported across cell membranes (Arborgh *et al.*, 1976). Therefore, it is not the total osmotic pressure of the fixative solution which must equal the osmolality of the natural cell environment, but rather the osmolality of the fixative vehicle, consisting of buffer and other additives (Figure 1). Even high concentrations of glutaraldehyde add little or nothing to the effective osmotic pressure of the fixative. The effective osmotic pressure is produced by molecules which are not capable of transversing the membrane.

Morphological changes are caused by primary glutaraldehyde fixation when hypotonic vehicles are used. Comparatively slight deviations in the osmotic pressure of the fixative vehicle may even give rise to fixation artifacts. Thus, the commonly utilized fixative, 2% glutaraldehyde in 0.1 mol/l Cac (total osmotic pressure 410 mOsmol; vehicle osmotic pressure 200 mOsmol) causes detectable swelling of glial cells cultured at 200 mOsmol (Figures 2, 3). A 2% glutaraldehyde in 0.1 mol/l Na-cacodylate-HCl buffer with 0.1 mol/l sucrose (pH 7.2; total osmolality 510 mOsmol; vehicle osmolality 300 mOsmol) produces no detectable artifacts on human cells grown in commonly used complete media which have a total osmolality of around 280–290 mOsmol (Arborgh *et al.*, 1976; Collins *et al.*, 1977). This fixative is suitable for initial fixation for all morphological, and many cytochemical studies (Figure 1a). Ruffles and microvilli appear to be the surface structures most sensitive to osmotic effects (see the swollen ruffles and microvilli in Figures 2 and 3).

The changes made by fixatives with only slightly hypotonic vehicles require high resolution and magnification for detection; but are, under such conditions, conspicuous.

The primary fixative solution should have a temperature equal to that of the site of origin of the tissues or cells used. After approximately 15 min at 37 °C, the specimen is then transferred to 4 °C until further processing. A minimal glutaraldehyde fixation time of 1 h is required. All buffer washes following glutaraldehyde fixation, up to and including the secondary fixation in OsO_4, must have the same osmolality as the vehicle of the primary fixative and the natural cell environments.

When studying cells in the low and middle magnification ranges, glutaraldehyde fixation alone may be used. This fixation should, however, be greatly prolonged for several days (Boyde, 1972; Vesely and

Boyde, 1973). If high magnifications are required, or if time is limited, a secondary OsO_4 fixation is recommended. Figures 4 and 5 demonstrate the surfaces of cells fixed in glutaraldehyde for 24 h at a proper osmotic pressure with and without ensuing osmium post-fixation.

Without osmium postfixation (Figure 5), the morphology of the plasma membrane is irregular and defective. The glutaraldehyde fixation alone does not adequately stabilize the lipid-rich plasma membrane and it therefore cannot withstand the stresses of the subsequent preparative procedures which result in multiple small tears. Even a prolonged fixation in glutaraldehyde cannot compensate for osmium post-fixation (Figure 6).

Secondary fixation is performed with 1% OsO_4 in 0.15 mol/l Cac buffer at room temperature for 90 min, following a rinse with 0.15 mol/l Cac buffer only.

DRYING OF THE SPECIMEN

Dehydration (removal of cell water) and drying (removal of intracellular fluid), while the solids remain in their original location, can be performed in one of three ways, each having its advantages and disadvantages (Table 2). While adequate fixation does not cause significant dimensional change, severe preparative shrinkage with drying is to some extent unavoidable (Boyde *et al.*, 1977).

With air-drying from the liquid phase, the solvent/vapor phase boundary is crossed and surface tension is high, depending on the solvent used. Shrinkage to less than 30% of the original volume is common.

In critical point drying (CPD), the CO_2 (or Freon 13) liquid phase passes into a CO_2 (or Freon 13) gas state without changes in volume, since the transition takes place at or above the critical point (Anderson, 1951). However, shrinkage is also common with this method, even when accidental air-drying is avoided. Shrinkage (20% volume) occurring within the bomb (during substitution, heating and depressurization) adds to the shrinkage caused by dehydration in a graded ethanol (30%) volume), which is obligatory with CPD. The result is a total specimen shrinkage of some 40–50% in volume. Freeze-drying avoids the problem of the surface forces at the liquid/gas phase boundary by changing from a liquid phase to a solid state by quenching (i.e. rapid freezing; e.g. in liquefied and cooled Freon 22, or in a liquid-nitrogen slush). Subsequently, this allows the specimen to pass the solid/gas phase boundary by way of sublimation in a high vacuum system at a low temperature.

Freeze-drying of fixed material from water is fast and allows minimal shrinkage (10–15% volume) as dehydration can be omitted. However, ice crystal damage can occur if temperatures do not remain low for a prolonged period of time.

COATING

Evaporation, or more superior and more popular sputter coating using a special apparatus, allows uniform deposition of a metal (gold or gold-palladium) layer onto the cells and the substratum (Echlin, 1974; 1975). Alternatively to metal coating, metal impregnation techniques, e.g. Oto technique (Murakami, 1974), have been successfully employed for rendering specimens conductive.

CONCLUDING REMARKS

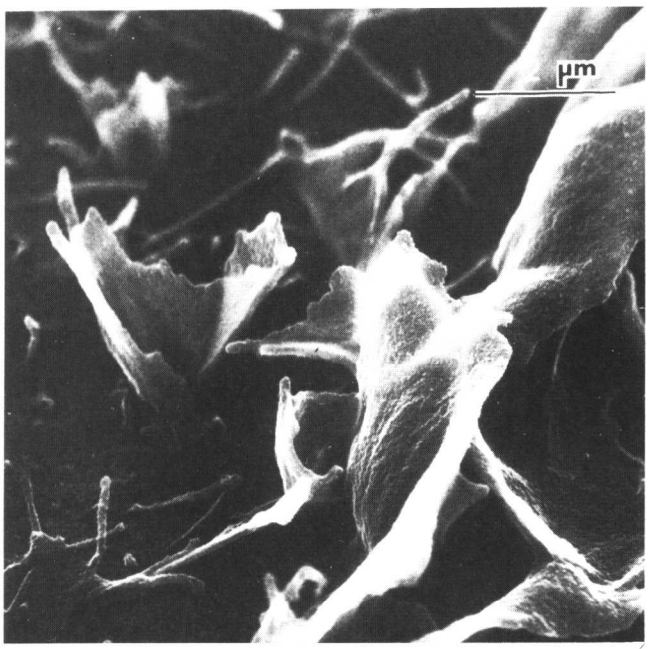
Specimen preparation methods that are necessary to obtain a technically acceptable image of the specimen studied in the SEM, at the same time unavoidably introduce specimen deformations, or artifacts. This is because all SEM preparative methods have two ideal objectives, that, in practice, are partly conflicting, viz.:

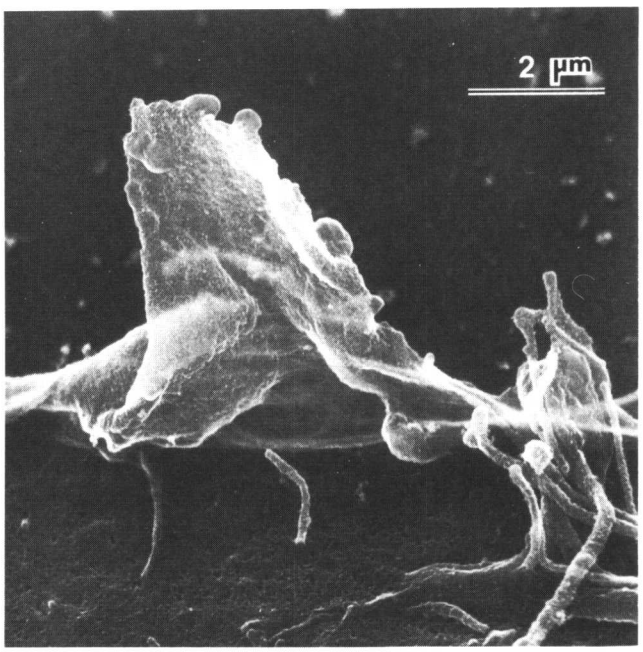
(1) attainment of a stable and conductive specimen; and
(2) preservation of surface structure of the specimen.

There are two groups of artifacts: avoidable ones, that must be prevented, and unavoidable ones, that must be known in order to be recognized as such. Processing artifacts vary both with the method used and the type of specimen under study. Therefore, although some general rules for specimen processing can be given, especially as to fixation and dehydration, each student using SEM has to find and establish the optimal method for his problem.

Table 2 Drying techniques

	Advantages	*Disadvantages*
Air-drying	no special instrument; simple and fast; displays subcellular structures	enormous shrinkages; enormous distortion
Critical point drying	good morphology	special instrument; considerable shrinkages; dehydration needed; accidental air-drying; health and safety hazards
Freeze-drying	good morphology; no hydration; little shrinkage; freeze cleaving possible	special instrumentation; ice crystal damage; thermal stress damage





Figures 1 and 2 High magnification of ruffling areas of cultured human glial cells following fixation in 2% glutaraldehyde (GA) in vehicles of 300 and 200 mOsmol, respectively. The membranes are well preserved in Figure 1 but show obvious swelling artifacts in the form of small bubbles in Fig. 2. Postfixation in OsO_4, critical-point dried (CPD)

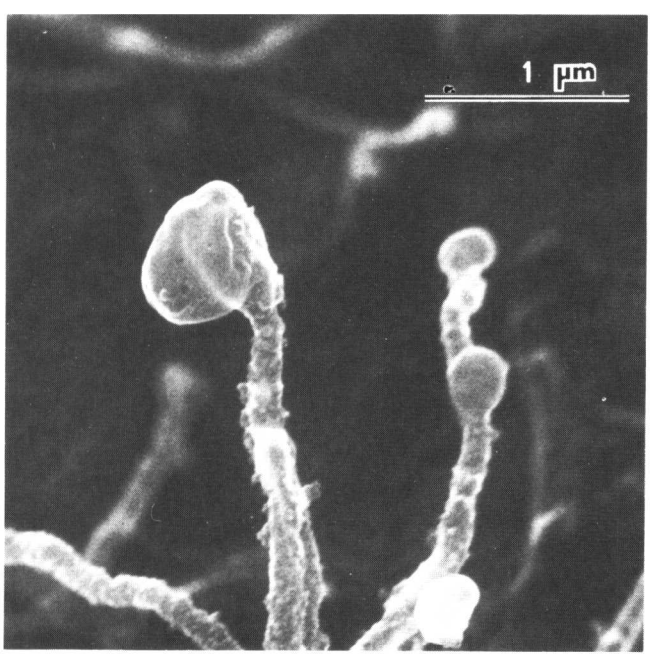
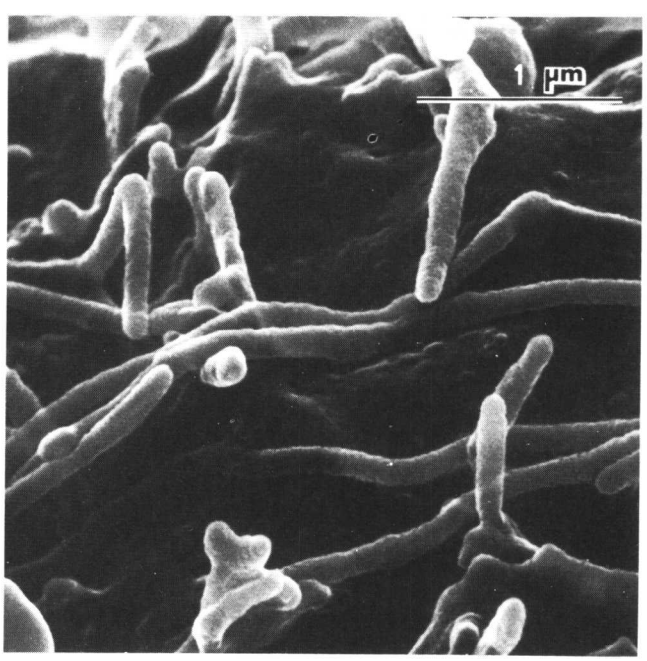


Figure 3 Details of distal, artifactually swollen ends of microvilli following initial GA fixation with a vehicle of 200 mOsmol.



Figures 4 and 5 Microvilli-covered cell surfaces after fixation in GA in a 300 mOsmol vehicle for 24 h with (Figure 4) and without (Figure 5) postfixation in OsO_4. Note pronounced artifactual alterations in the plasma membrane in the form of small holes and irregular rifts when osmium postfixation was omitted. CPD.

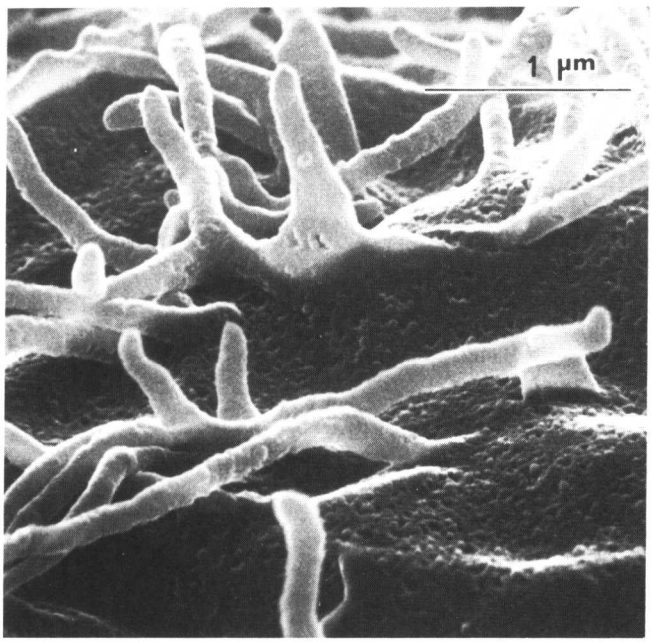


Figure 5 (see legend to Figure 4)

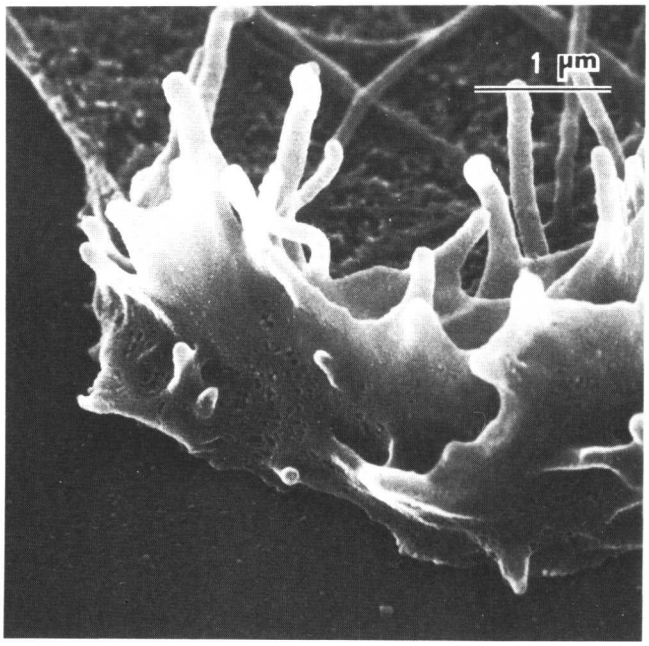


Figure 6 High magnification of a ruffling area of a cell fixed in GA in a 300 mOsmol vehicle for 14 days. No postfixation in osmium. Note same type of artifacts as are shown in Figure 5. CPD.

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