
O. J. Castejón

Universidad del Zulia, Venezuela

Follow this and additional works at: https://digitalcommons.usu.edu/microscopy

Part of the Biology Commons

Recommended Citation
Available at: https://digitalcommons.usu.edu/microscopy/vol5/iss2/15

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Scanning Microscopy by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.
THREE-DIMENSIONAL MORPHOLOGICAL ANALYSIS OF NERVE CELLS
BY SCANNING ELECTRON MICROSCOPY. A REVIEW

O. J. Castejon


(Received for publication March 13, 1990, and in revised form March 1, 1991)

Abstract

The present review provides a rational and new approach to study the three-dimensional morphology of nerve cells in situ at both cellular and macromolecular levels, by means of conventional (SEM) and high resolution scanning electron microscopy (HRSEM). The slicing and ethanol-cryofracturing methods, the freeze-fracture SEM method and tissue preparation for HRSEM have been described. Nerve cell outer surface, axon hillock and initial axon segment, axonal collateral ramifications and dendritic processes were visualized either by the slicing technique or the cryofracture method displaying neuronal geometry in situ. The cleavage plane occurred at the satellite neuroglial sheath exposing somatic hidden surfaces of unfractured neurons and the outer surface synaptic morphology. "En passant" axospindendritic junctions, glomerular synapses and axosomatic contacts were examined in vertebrate cerebellar cortex. The SEM and cryofracture techniques could be applied as the Golgi light microscope technique to trace short neuronal circuits. The nerve cell inner surfaces were also studied by means of freeze-fracture SEM method and HRSEM. HRSEM provided information at both cellular and macromolecular levels. Topographic contrast of glycocalyx-like substance, synaptic junctions and myelin sheath was obtained. A comparison could be made between Au/Pd and chromium coated nerve cells. HRSEM provided SE-1 images of lipoprotein domains at the myelin sheath and globular subunits at the level of the postsynaptic membrane. This latter observation offers new potential areas for future studies on receptor morphology.

KEY WORDS: Nerve cells, synapse, myelin sheath, scanning electron microscopy, cryofracture SEM, High resolution SEM.

Address for correspondence:
Orlando J. Castejon
Instituto de Investigaciones Biológicas. Facultad de Medicina. Universidad del Zulia. Apartado 526, Maracaibo, Venezuela. Phone No. (61)515260

Introduction

More than a century has witnessed the development of several methods for neuronal visualization and nerve circuit analysis. In 1873, Camilo Golgi exposed chromate-hardened brain tissue to silver nitrate, permitting detailed observation at the light microscopic level of neuronal morphology, that had not been possible previously. The Golgi method allowed numerous neurohistologists to approach the problem of the geometrical arrangement of neurons and nerve connectivity and interrelationship, besides being the first and most comprehensive analytical tool for this type of investigation. One of the most important problems we have in the field of neurosciences is defining the total shape of an individual neuron including its dendritic and axonal branching. The description of these elements has so far been insufficient because of the irregular shape of the neuronal soma and the intricate three-dimensional expansion of dendrites and axon. Even with a very thick section, some of the dendrites and axonal branchings are obviously cut short by the plane of the section and only those branches in the plane of the soma have been fully represented (Mannen, 1978). More recently neuronal geometry has been also studied by means of injection of procion yellow or cobaltous chloride directly into a single neuron or a small region of the central nervous system (Illies and Mulloney, 1971; Stretton and Kravitz, 1973; Llinas, 1975). Photogrammetrical representation of neuronal soma and microreconstruction of expansions were introduced by Mannen (1978) for determining the real shape and the actual dimensions of neurons in Golgi's preparations.

Recent computer technology has allowed the recording and storage of information regarding neuronal form in the three dimensions of space. Glaser and Van der Loos (1965) were the first to use Golgi's preparations to demonstrate the feasibility of employing computing methods to trace axonic or dendritic arborization. Computer aided microscopy gives important data, such as hard numbers, quantitative parameters of single cell morphology, tridimensional organization of neuronal circuits and a reliable quantitative baseline to ascertain the morphological substrate of neuropathological states (Llinas and Hillman, 1975).
In the last two decades progress in the development and application of biological and physical techniques for conventional and high resolution electron microscopy has resulted in a notable increase in our knowledge of the most important surface features of nerve cell geometry. Three-dimensional observations of entire nerve cell somatic outer surface, cytoarchitectonic arrangement, plastic visualization of intracellular structures, tracing of some intracortical short nerve circuits and the morphology of synaptic contacts have been carried out in different regions of the central nervous system (Hansson, 1970; Lewis, 1971; Ohtsuki, 1972; Kristic, 1974; Phillips et al., 1974; Saetersdal and Myklebust, 1975; de Estable-Puig and Estable-Puig, 1975; Seymour and Berry, 1975; Tanaka et al., 1976; Castejon et al., 1976; Lametschwandtner et al., 1977; Antal, 1977; Bredberg, 1979; Merchant, 1979; Siew, 1979; Castejon and Valero, 1980; Castejon and Caraballo, 1980 a,b,; Castejon, 1981; Castejon and Castejon, 1981; Scheibel et al., 1981; Castejon, 1983; Castejon, 1984; Paul Castejon, 1986; Castejon and Caraballo, 1987; Castejon, 1988; Castejon and Castejon, 1988; Low, 1989; Castejon, 1990 a,b,c).

The most important feature of nerve cells from a functional point of view is the position of their dendritic and axonal arborization in relation to the arborization of other neurons. Three-dimensional visualization of dendritic and axonal branching is therefore an essential first step in the analysis of intercellular connectivity (Mannen, 1978). Therefore the SEM analysis of nerve cells and their synaptic relationship is considered of paramount importance for understanding nerve functions.

This present review provides a rational and new approach to study the outer and inner three dimensional morphology of nerve cells in situ using conventional and high resolution scanning electron microscopy. Conventional scanning electron microscopy (SEM) was used applying slicing technique and two different freeze-fracture methods. The salient feature of outer and inner surfaces of nerve cell soma, dendrites, axons and synapses are studied. In addition, some unpublished findings on high resolution scanning electron microscopy (HRSEM) of Rhesus monkey nerve cells will be described.

Material and Methods

Slicing Technique for SEM (Castejon and Caraballo, 1980a)

Specimens of Arius spixii weighing 30-82 g, kept in aquaria at room temperature were used. Pieces of tissue were fixed: 1) by immersion in 5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4; or 2) by vascular perfusion with 4% glutaraldehyde in 0.1M phosphate buffer solution, pH 7.4; or 3) by immersion with the Karnovsky fixative. Slices of 2-3 mm thick were cut with a razor blade and fixed overnight in the same buffer fixed. After washing in buffered saline, the tissue blocks were dehydrated through graded concentrations of ethanol, dried by the critical point method with liquid CO2 as recommended by Anderson (1951), mounted on copper coated with carbon and gold-palladium. The specimens were examined in a JEOI 100B Electron Microscope with EM-ASID high resolution scanning device at 20kV.

Ethanol-cryofracturing technique (Humphreys et al., 1975, Castejon and Caraballo, 1980b)

This technique, originally designed by Humphreys et al. (1975) to scan liver and kidney tissue, was applied by us to study pathological cerebellar cortex obtained from autopsy material, 4-6 hours after death by drowning in the Maracai-bo Lake. Postmortem changes were macroscopically observed in the cerebellar cortex. The samples 3-5 mm thick were fixed for 2 to 16 h in 4% glutaraldehyde-phosphate buffer solution, pH 7.4, dehydrated in ethanol and frozen in liquid nitrogen. The fracture was made with a precooled razor blade and the fragments placed in ethanol at room temperature for thawing. The critical point drying was done with liquid CO2 followed by a coating of carbon or gold-palladium in JED-46 high vacuum evaporator. The tissue was observed with a JEDOL 100B Electron Microscope with EM-ASID high resolution scanning device at 20kV.

Freeze-fracture SEM method (Haggis and Phipps-Todd, 1977; Castejon, 1981)

This method was applied to the cerebellar cortex of two teleost fishes: Arius spixii and Salmo Trutta. After Karnovsky fixation, cerebellar slices, 2-3 mm thick, were cut with a razor blade and fixed by immersion in the same fixative for 4-5 h. After washing in buffered saline, they were postfixed in 1% osmium tetroxide in 0.1M phosphate buffer solution, pH 7.4 for 1 h. After rinsing in a similar buffer, tissue blocks were dehydrated through graded concentrations of ethanol, rapidly frozen by plunging into Freon 22, cooled by liquid nitrogen (Haggis, 1970; Haggis and Phipps-Todd, 1977) and fractured with a precooled razor blade. The fracture fragments were returned to fresh absolute ethanol for thawing. According to Haggis and Phipps-Todd, in the Fix-Dehydrate-Freeze fracture (FDF) method, the cytoplasmic and nuclear soluble proteins are washed out, presumably during the thawing step, leaving anfractuous cavities surrounding the cytomembrane and allowing visualization of the surface details of cytoplasmic and nuclear structures. The tissue was then dried by the critical point method with liquid CO2 as recommended by Anderson (1951) and coated with gold-palladium. Specimens were examined in a JEDOL 100B Electron Microscope with EM-ASID high resolution scanning device at 90 kV.


Excised rhesus monkey cerebellar cortex was minced into 2 mm3 pieces and further fixed in 2.5% E.M. grade glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, overnight in order to provide complete intracellular and extracellular proteinaceous cross-linking. Cacodylate buffer, pH 7.4 was used to completely remove the primary fixative by rinsing the tissue several times under gentle agitation. Postfixation of phospholipid moieties was accomplished by immersion into 1%
Scanning Electron Microscopy of Nerve Cells

Dried and mounted specimens were chromium coated with a continuous 2 nm film in a Denton DV-602 turbo pumped sputter deposition system operated in a vacuum of argon at 5 x 10^-3 (Apkarian and Joy, 1988).

Specimens were introduced onto the C/0 lens stages (SE-I signal mode operation) of either a 151IS DS-130 SEM equipped with LaB6 emitter or a Hitachi S-900 SEM equipped with a cold cathode field emitter. Both instruments were operated at 25-30 kV in order to produce minimal spot size and adequate instrumental noise to ratio noise at all magnifications. Micrographs were soft focus printed to reduce instrumental noise (Peters, 1985b).

Results

Nerve cell outer surface.

The nerve cell somatic outer surface and the proximal parts of the axon and dendritic processes could be appreciated with the conventional scanning microscope equipped with tungsten filament, using either the slicing technique or the cryofracture methods. The cleavage plane occurred at the neuroglial sheath covering the nerve cell soma, which occupies an intercellular position, thus exposing the outer somatic cell surface. Remnants of satellite neuroglial cells were observed attached to the neuronal outer surfaces, which exhibited an irregular and rough appearance. Teleost fish cerebellar nerve cells, coated with 10-20 nm thick gold-palladium to increase electrical conductivity and surface contrast, exhibited SE-II and SE-III signal components characterized by a strong microroughness contrast (Fig. 1). At low magnification, large specimen features were scanned which allowed us to appreciate the neuronal shape and proximal part of nerve processes. The large depth of focus of SEM permitted the visualization of other neighboring neurons and the analysis of the complex interrelationship of nerve cells in situ. The cryofracture process exposed in addition, the hidden surfaces of nerve processes, which makes it possible to explore regions such as the axon hillock, the initial axonal segment and the dendritic trunk or to trace short neuronal circuits.

The microneurons, 6-10 μm in diameter, such as the cerebellar granule cells (Fig. 2) or stellate cells, exhibited round or ovoid shape cell bodies, two to four branched dendritic profiles and a filiform axon.

The macroneurons, 20-30 μm in diameter, as the cerebellar Purkinje cells (Fig. 3), displayed a primary dendritic trunk and the axon hillock region, from which the initial axon segment is given off.

Axon hillock and initial axon segment.

As illustrated in Figs. 2 and 3 the axon hillock outer surface of microneurons and macroneurons could be observed with the scanning electron microscope. It appeared as a cone or triangular-shape elevation from which the axon originates. As the axon hillock narrows down into the initial axon segment, there is a gradual diminution of the axonal caliber. The axon beyond the initial segment exhibited a brilliant surface, filiform shape and a straight or wavy course. Unmyelinated axons, as for example, longitudinally orientated granule cell axons or parallel fibers (Fig. 4) form compact bundles surrounded by neuroglial cells in the cerebellar molecular layer.

Axonal collateral ramifications.

One of the greatest contributions of scanning electron microscopy has been to trace axonal collaterals from the parent fibers. According to the cleavage plane of the tissue fracture and taking advantage of the large depth of focus of SEM, it was possible to follow the wavy course of axonal ramifications directed toward the dendrites, penetrating between neighboring nerve cells or going from one layer to another. Fig. 5 displays the crossing-over pattern type of ramification of climbing fiber collaterals in the human cerebellar molecular layer, as seen by the ethanol-cryo fracturing technique.

Dendritic processes.

Although there is a great variation in the form of the dendritic tree of different microneurons or macroneurons, they are essentially multiple extensions of the perikaryon (Fig. 6), with irregular contours, collateral protrusions and different branching patterns according to the...
Fig. 1. Teleost fish round or ovoid cerebellar granule cells showing the emergence of dendritic processes (arrowheads). Slicing technique. Gold-palladium coating. JEOL 100B EM-ASID scanning attachment at 20 kV.

Fig. 2. Teleost fish cerebellar granule cell (GC) showing the axon hillock region (arrowhead) and the filiform axon (arrows). Slicing technique. Gold-palladium coating. JEOL 100B EM-ASID scanning attachment at 20 kV.

Fig. 3. Teleost fish cerebellar Purkinje cell (Pc). The slicing technique has disclosed the axon hillock region and the initial axon segment (arrow). At the upper part of the figure the site of emergence of the dendritic trunk is appreciated (asterisk). The somatic surface exhibits the coarse granularity of gold-palladium coating. JEOL 100B EM-ASID scanning device at 20 kV.

Fig. 4. Teleost fish cerebellar molecular layer. Bundles of granule cell axons (GA) or parallel fibers partially surrounded by neuroglial cell cytoplasm (NC). Slicing technique. Gold-palladium coating. Hitachi 2500 at 15 kV.

Fig. 5. Human cerebellar molecular layer. A parent axon, apparently of a climbing fiber (CF), shows axon collaterals spreading between Purkinje dendrites (asterisks). Ethanol-cryofracturing technique. Gold-palladium coating. JEOL 100B EM-ASID scanning device at 80 kV.

Fig. 6. Teleost fish cerebellar stellate neuron (SN). The dendritic processes (arrows) appear extending from the cell body. They exhibit irregular contours and collateral protrusions. Freeze-fracture SEM method. Gold-palladium coating. Hitachi 2500 at 15 kV.

Fig. 7. Teleost fish cerebellar Purkinje cell (Pc) showing the emergence of the primary dendritic trunk (arrowhead) and its bifurcation into secondary branches in the molecular layer (arrow). SEM freeze-fracture method. Gold-palladium coating. Hitachi 2500 at 15 kV.
neuronal type. For example, Purkinje cell of the cerebellum exhibited a primary trunk (Fig. 7), secondary branches and tertiary branchlets (Fig. 8), which arborize profusely in the cerebellar molecular layer forming an extensive and elaborate dendritic tree. The soma and the dendritic processes are provided with specialized collateral protrusions, termed thorns or spines, which are postsynaptic structures. The dendritic spines (Fig. 9) exhibited a narrow neck joined to the dendritic stem and an ovoid or round body in
The outer surface synaptic morphology.

By means of the freeze-fracture technique for SEM the surface of intracellular structures of fractured nerve cells were visualized. The cytoplasmic and nuclear soluble proteins were washed out disclosing mainly the surface of endoplasmic reticulum, Golgi complex, cytoskeletal and nuclear chromatin (Fig. 16). The outer surface of endoplasmic reticulum was seen as an interconnected network of vesicles, tubules and cisterns extended between the plasma membrane inner surface and the nuclear envelope outer surface (Figs. 17 and 18). In addition, the spatial distribution of cytoskeletal structures was seen as a fine filamentous network supporting cell organelles (Fig. 19).

High resolution scanning electron microscopy

Nerve cells processed according to delicate tissue handling for high resolution electron microscopy, coated with chromium, 1-2 nm in size and studied with an analytical scanning electron microscope equipped with LaB6 emitter showed a smooth surface, revealing presumably the glycoprotein macromolecular arrangement of outer cell coat or glycosylated layer (Fig. 20).

In fractured nerve cells, the topographic relief contrast of plasma membranes and endoplasmic reticulum profiles was appreciated (Fig. 21). The myelin sheath (Fig. 22) was observed as a continuous band with high topographic contrast. The major dense lines were vaguely discerned and the myelin periodicity was practically distinguished in certain areas. The surface of the myelin sheath exhibited a mixed population of particles 10-30 nm in diameter, which corresponded to protein and phospholipid microdomains. In addition, the axolemma and the outer oligodendroglial cytoplasm were seen.

Besides, high resolution SEM (SE-I mode) allowed us to distinguish in details, the inner structures of fractured presynaptic endings. In addition to the 50 nm spheroidal synaptic vesicles, a columnar structure 100 nm in length, attached to the presynaptic membrane (Fig. 23) by a 40 nm base was visualized. The presynaptic dense projection and the spheroidal synaptic vesicles appeared surrounded by an amorphous homogeneous extravesicular material. These presynaptic structures resembled the presynaptic grid proposed by Akert and coworkers (1972). The postsynaptic membrane and the associated postsynaptic density showed a discontinuous surface formed by round subunits, about 25-35 nm in diameter, which apparently correspond to neurotransmitter receptors.

Discussion

Conventional and High Resolution Scanning Electron Microscopy.

The present review provides a rational and new approach to study the three-dimensional morphology of nerve cells in situ at both cellular and macromolecular levels by means of conventional SEM and HRSEM. The slicing technique and the cryofracture methods: the ethanol cryofracturing technique and the freeze-fracture method for SEM produced the cleavage plane at the level of neuroglial cells, which ensheathe the nerve cells and are therefore easily removed, thus exposing the hidden neuronal outer surfaces. Successful and optimal preparation procedure depends on glutaraldehyde fixation either by immersion or vascular perfusion. This step is also essential especially for visualization of...
Scanning Electron Microscopy of Nerve Cells

Fig. 8. Human cerebellar molecular layer. Secondary and tertiary dendritic branches of Purkinje dendrites (asterisks) appear surrounded by afferent axons (arrowheads). Ethanol-cryofracturing technique. Gold-palladium coating. JEOL 100B EM-ASID at 30 kV.

Fig. 9. Teleost fish cerebellar molecular layer. "En face" view of Purkinje dendritic spines showing the neck (arrowheads) and the round body (asterisks). SEM freeze-fracture method. Gold-palladium coating. JEOL 100B. EM-ASID scanning device at 30 kV.

Fig. 10. Teleost fish cerebellar granular layer. A granule dendrite (arrowhead) arising from the granule cell soma (GC) is observed establishing axodendritic contact (asterisks) by means of a terminal enlargement or dendritic claw with a fine beaded Golgi axonal ramification (Ga). SEM freeze-fracture method. Gold-palladium coating. JEOL 100B. EM-ASID scanning device at 30 kV.

Fig. 11. Human cerebellar molecular layer. A granule cell axon (arrowheads) appears making multiple "en passant" contacts with Purkinje dendritic branchlets (asterisks). Ethanol-cryofracturing technique. Gold-palladium coating. JEOL 100B EM-ASID scanning device at 30 kV.
Fig. 12. Teleost fish cerebellar granular layer. Mossy glomerular synapses. A central mossy axonal rosette (AR) appears surrounded by numerous granule cell dendrites circumferentially arranged (arrowheads). Numerous granule cell bodies (GC) contribute to the glomerular synapse. Freeze-fracture SEM method. Gold-palladium coating. JEOL 100B. EM-ASI0 scanning device at 30 kV.

Fig. 13. Teleost fish cerebellar granular layer. Fractured glomerular synapse. The fracture process has exposed the central axonal rosette (asterisks) which appears surrounded by the granule cell (GC) dendritic digits (arrowheads). Freeze-fracture SEM method. Gold-palladium coating. JEOL 100B. EM-ASI0 scanning device at 30 kV.

Fig. 14. Human cerebellar Purkinje cell layer. Tangential view of an axosomatic synapse. The basket cell axonal endings (arrows) appear intimately applied to the Purkinje cell (Pc) body. Ethanol-cryofracturing technique. Gold-palladium coating. JEOL 100B. EM-ASI0 scanning device at 30 kV.

Local scattering conditions on Au/Pd decorated nerve cell surface produced micro-roughness contrast (SE-II signal component) and did not reveal topographic information. In order to improve the substructural surface information the cerebellar samples were coated with fine-grained chromium films. Therefore this improved the signal to noise ratio at high magnification. In the present study, images of chromium coated nerve cells enriched in SE-I type contrast, provided excellent topographic contrast of glycoalyx-like substance and revealed a distinct granular substructure, presumably corresponding to glycoproteins or proteoglycans (Castejón, 1970). Detailed recognition was further enhanced by soft focusing SE-I micrographs during enlargement as recommend-
Scanning Electron Microscopy of Nerve Cells

Fig. 15. Human cerebellar Purkinje cell layer. "En face" view of an axosomatic synapse of basket cell axonal endings (arrows) upon the Purkinje cell soma (Pc). Ethanol-cryofracturing technique. Gold-palladium coating. JEOL 100B. EM-ASID scanning device at 30 kV.

Fig. 16. Fractured teleost fish cerebellar nerve cell showing the nuclear anastomotic heterochromatin bands (N) and cytoplasmic strands (arrows) extended between the nuclear envelope and the plasma membrane. The surface of vesicles, tubules and cisterns of endoplasmic reticulum (ER) are also distinguished. SEM Freeze-fracture method. Gold-palladium coating. JEOL 100B. EM-ASID scanning attachment at 80 kV.

Fig. 17. Fractured teleost fish nerve cell processed by the Freeze-fracture SEM method. Endoplasmic reticulum profiles (ER) are observed extended between the nuclear surface and the plasma membrane and exposed due to the washing out of cytoplasmic soluble fraction. Gold-palladium coating. JEOL 100B. EM-ASID scanning attachment at 80 kV.

Fig. 18. Fractured teleost fish cerebellar stellate cell processed by the SEM freeze-fracture method. The surface details of nuclear chromatin (N), endoplasmic reticulum (ER) and Golgi complex (GC) are appreciated. Note the extension of ER profiles toward the dendritic process (D). Gold-palladium coating. JEOL 100B. EM-ASID scanning attachment at 80 kV.
Fig. 19. Fractured teleost fish cerebellar nerve cell. Elements of cytoskeleton are observed forming an intra-cytoplasmic network extended between the nucleus (N) and the plasma membrane (arrowheads). Freeze-fracture SEM method. Gold-palladium coating. JEOL 100B. EM-ASID scanning attachment at 80 kV.

Fig. 20. Rhesus monkey cerebellar Golgi cell outer surface exhibiting the fine texture of glyocalyx associated with the somatic outer surface (circles). Chromium coating. Freon freeze-fracture SEM technique. ISI DS-130 SEM at 30 kV equipped with LaB₆ gun.
Scanning Electron Microscopy of Nerve Cells

Fig. 21. Rhesus monkey fractured cerebellar nerve cell showing high resolution SE-I image of the limiting plasma cell membrane (arrows) and the outer surface of endoplasmic reticulum profiles (ER). Freon freeze-fracture SEM technique. Chromium coating. ISI-DS-130 SEM at 30 kV equipped with a LaB6 gun.

Fig. 22. Rhesus monkey fractured cerebellar myelinated axon (Ax). High resolution SE-I edge contrast of globular structures are visualized (asterisks) at the fractured surface of myelin sheath, revealing proteo-lipid macromolecular arrangement. The axolemma (arrowheads) appearing limiting the axoplasm. Thick arrows indicate myelin vacuoles. The oligodendroglial cytoplasm (OL) appears attached to the myelin sheath outer surface. Freon freeze-fracture SEM technique. Chromium coating. Hitachi S-900 at 30 kV equipped with field emission emitter.

Fig. 23. Rhesus monkey cerebellar cortex. High resolution SE-I contrast of parallel fiber-Purkinje spine synapse. The presynaptic ending shows spheroidal synaptic vesicles (SV) and a presynaptic dense projection (arrow). The pre- and postsynaptic membranes (arrowheads) appear separated by the synaptic cleft (small asterisks). Note the attachment of pre-synaptic dense projection to the presynaptic membrane. Round or oval subunits are appreciated in the postsynaptic membrane (large asterisks). Freon freeze-fracture technique. Chromium coating. Hitachi S-900 at 30 kV equipped with field emission emitter.

However it should be considered that smallest detectable details may not be true surface features but subsurface structures (Peters, 1980).
Neuronal Geometry and Scanning Electron Microscopy

Conventional SEM using the slicing and ethanol-cryofracturing techniques has provided surface morphology of unfractured nerve cells displaying neuronal geometry in situ. It is felt, therefore, that these methods might be of interest to neuropathologists and neuroscientists for studying the fine three-dimensional details of central nervous system. They should be considered as complementary methods for transmission electron microscope studies using ultrathin sections or for studying membrane morphology by means of freeze-etching technique (Castejón and Castaño, 1987; 1988; Castejón, 1990 a,b). SEM offers in addition some potential values in the studies of interneuronic relationships, especially in short neuronal connections, as those established by short-axon neurons. Surface morphology of dendrites and axons ensures accurate identification of nerve cell processes, especially at the proximal parts. The sectioning and the fracture procedures, the large size of nerve processes and their tortuous course, as well as the complex organization of the neuropil impose some limitations to trace, for some distance, the wavy course or the complete trajectory of nerve processes. The fact that the cleavage plane occurred through neoglial cells and that large areas were exposed by the fracture process, allowed the almost complete visualization of the intracortical trajectory of an afferent fiber. In addition, the large depth of focus of SEM permitted us to estimate with a more reliable degree of certainty the amount of branching or lateral collateralization of an axonal process. In the study of short neuronal connections, conventional SEM and cryofracturing techniques offer some advantages for tracing neuronal circuits in comparison with Golgi-light microscope technique, which has been classically used for more than a century since Ramón y Cajal’s pioneering studies (Ramón y Cajal, 1955).

Besides, SEM offers the advantages of following tortuous course and extreme tenuity of delicate collaterals, overcoming the inherent difficulties and time consuming work derived from the interpretation of TEM fine serial section studies (Castejón, 1983). In certain regions, the ethanol-cryofracturing technique allows the observation of complete nerve processes. Apparently the nerve processes are kept intact due to the freezing of ethanol impregnated tissue, which is transformed into an amorphous solid (Humphrey et al. 1975), which in turn acts as a solid matrix supporting the delicate neuronal processes.

The results obtained with the ethanol-cryofracturing technique and the freeze-fracture method for SEM provided a sophisticated methodology for the study of synaptic junction geometrical arrangement and new vistas of the synaptic contact outer surface. Such a novel contribution has not been obtained until now for other ultrastructural studies.

The most challenging aspect of SEM studies of nerve cell is related to the identification criteria of a given cellular type or an afferent or efferent fiber. Such identification criteria are obtained from previous light or transmission electron microscopic studies. Therefore the SEM methods should be considered as supplementary techniques. SEM contributes to confer and extend previous light microscopical studies, to bring out new facts and facilitate three-dimensional studies of neuronal organization. When the low resolution power of conventional SEM limits the observation, the freeze-etching technique for transmission electron microscopy or the high resolution scanning electron microscopy should be complementarily used (Castejón, 1988, Castejón and Apkarian. In preparation, 1990).

SEM Freeze-Fracture Method.

The SEM freeze-fracture method allows direct access to the interior and inner surface cytoplasmic details of teleost fish nerve cells. According to Haggis and Phipps-Todd (1977), in the FDFF method, the cytoplasmic and nuclear soluble proteins are washed out presumably during the thawing step, disclosing the surface of cytomembranes, cell organelles, cytoskeletal structures and nuclear chromatin. An interesting contribution of the SEM freeze-fracture method is the three-dimensional visualization of the spatial arrangement and organization of endoplasmic reticulum surface. High resolution electron microscopy of chromium coated Rhesus monkey cerebellar cells showed the surface characteristics of plasma membrane, endoplasmic reticulum and synaptic membranes. Again, as above mentioned, different surface membrane features were observed between gold-palladium decorated intracytoplasmic surfaces and chromium coated cytomembranes.

Synaptic Junction types.

Conventional scanning electron microscopy using the slicing technique or the ethanol-cryofracturing technique exposed the outer surface of axodendritic, axosomatic and gliomerular synapses. The images obtained give a new and better insight into the topographical relationship between pre- and postsynaptic structures than that obtained from transmission electron microscopic studies either by thin sectioning or freeze-etching replicas. The cruciform or crossing-over type of synaptic relationship between parallel fibers and Purkinje dendrites postulated by Hamori and Szentagothai (1964), by thin-section electron microscopy was confirmed by means of ethanol cryofracturing technique applied to human cerebellum (Castejón and Valero, 1980) and by the SEM method freeze-fracture in teleost fishes (Castejón, 1981, 1988).

The radial arrangement of granule cell dendrites in relation to a mossy fiber rosette in a gliomerular synapse was firstly demonstrated by Castejón and Caraballo (1980a) and Castejón (1981). The images of mossy gliomerular synapses obtained by earlier transmission electron microscopic studies (Gray, 1961; Castejón and Castejón, 1972; Palay and Chan-Palay, 1974) only showed the cross sections of dendritic digits surrounding the central mossy rosette fiber, but not the simultaneous topographic relationship with neighbouring granule cells. The radial arrangement demonstrated only by SEM gives an accurate idea of the degree of nerve impulse
Scanning Electron Microscopy of Nerve Cells

divergence from a mossy fiber toward as many as 18 different granule cells (Castejon and Carabal­

lo, 1980a).

In relation to the axosomatic contacts, SEM and ethanol-cryofracturing techniques displayed the pericellular nest formed by basket cell axon ending around the Purkinje cell body, supporting Ramon y Cajal’s (1901) classical studies by means of Golgi-light microscope technique. In addition the “en face” view of such axosomatic contacts firstly obtained by SEM (Castejon and Valero, 1980) gave an idea about the surface area of the postsynaptic somatic surface occupied by the presynaptic endings.

Synaptic Morphology.

High resolution SEM (SE-I mode) provided a new contribution to the study of synaptic mor­

phology, facilitating the visualization of pre­
synaptic grid, postulated by Akert and coworkers (1972) by means of ultracytochemical studies. According to Akert et al. the presynaptic dense projection would be the “locus” of interaction between the synaptic vesicles and the presynaptic membrane. Round subunits, 25-35 nm in diameter, were seen in the postsynaptic membrane and asso­
ciated postsynaptic density. In this context, high quality imaging of macromolecular synaptic membrane structures offers new potential areas for future studies on receptor morphology.

The Myelin Sheath.

The resolution obtainable in an in-lens field emission scanning electron microscope is close to transmission electron microscope studies and image reconstructions of the same specimen (Hermann et al., 1988). The images obtained with the field emission S-900 Hitachi allowed us (Castejon and Apkarian, in preparation, 1990) to visualize at the myelin sheath globular structures at the level of myelin period, between the major dense lines, which apparently correspond according to X-ray diffraction data (Finean, 1961) to lipoprotein domains.

Acknowledgement

This investigation was partially supported by the National Research Council of Venezuela (CONICIT), Scientific and Humanity Council of Zulia University (CONDES) and Biological Research Institute. Faculty of Medicine, LUZ. Venezuela. The author thanks Dr. D.C. Joy of Analytical E. M. Facility. University of Tennessee and Hitachi Scientific Instruments for use of the S-900 FE SEM. High resolution SEM study was supported in part by NIH grant RR-00165 from the Division of Research Resources to Yerkes Regional Primate Research Center, Emory University, Atlanta. The collaboration of Frederick King and Robert Apkarian is deeply appreciated. Thanks are also due to Prof. Geoffrey Haggis, Canada Department of Agriculture, Ottawa for his invaluable help with the freeze-fracture method for SEM.

References

Akert K, Pfenninger C, Sandri C, Moor H. (1972) Freeze-etching and cytochemistry of vesi-


Anderson TF (1951) Techniques for the pres­
servation of three dimensional structures in preparing specimens for the electron microscope. Trans NY Acad Sci 13, 130-134.


Bredberg G (1979) Scanning electron micros­


Castejon, HV (1970) Histochemical demon­

stration of sulphated polysaccharides at the surface coat in the mouse central nervous system. Acta Histochem 43, 253-263.


cros. 1980; IV, 197-207.


Castejon OJ (1983) Scanning electron micro­

Castejon OJ (1984) Low resolution scanning electron microscopy of cerebellar neurons and neuroglial cells of the granular layer. Scanning

Microscopy Study of Cerebellar Synaptic Junctions. Scanning Microsc 21, 2181-2193.


Gogli C (1873) Sulla struttura della sostanza grigia del cerebello (On the structure of cerebellar gray matter). Gazl Med Ital 21, 244-246.


Peters KR (1985b) Noise reduction in high magnification micrographs by soft focus printing and digital image processing. Scanning 7, 205-


Discussion With Reviewers

F. N. Low: All figures show blebs of various sizes, cellular fragments and congealed materials? These for the most part, have no counterparts in TEM. I am particularly concerned about the nearly ubiquitous blebs. A study in my laboratory with extremely delicate early chick embryos (Litke and Low, Amer. J. Anat., 148(1):121-127, 1977) showed that the toxicity of fluid substrated had a marked effect on plasmalemmal integrity. And a separate study showed that physiological holding solutions caused formation of blebs and microvilli in otherwise smooth surfaces after only 3 to 5 minutes immersion (Peine and Low, Amer. J. Anat. 142(2):137-58, 1975). The extreme sensitivity and rapid instability of plasmalemmal contours are now well accepted in SEM. This circumstance coupled with the failure of numerous blebs to appear in TEM raises serious questions about the fidelity to living structure in Dr. Castejon's preparations.

D.E. Hillman: The difficulty that this review has with the approach is that there are too many distracting artifacts to effectively analyze organization of the neurons and glia or to study cell surfaces.

Author: The artifactual formation of blebs due to glutaraldehyde fixation has been also reported in SEM studies (Shelton, E. and Mowczko, W.E. (1977): Membrane blebs: A fixation artifact. J. Cell Biol., 72:206a.; Shelton E. and Mowczko, W.E. (1978): Membrane blisters: A fixation artifact. A study in fixation for scanning electron microscopy, Scanning, 1:166-173). In the present review we have shown normal experimental animal material (fish and monkey) and pathological human samples. Fresh teleost fish cerebellar nerve cells fixed either by vascular perfusion or by immersion with 5% glutaraldehyde in phosphate buffer solution, pH 7.4 and osmolarity 300-320 mOs/liter did not show bleb or cyst formations. Control teleost fish nerve tissue processed in our laboratory for TEM studies following the same fixation protocol has not shown blebs. In previous papers we have published corresponding SEM and TEM preparations (Castejon, 1981; 1984; Castejon and Castejon,1987; 1988) which do not exhibit blebs. Until now we have found formation of blebs in mouse central nerves myelin sheath processed according to the freeze-etching technique. In such replicas, the outer surface and the inner fractured surfaces could be simultaneously observed and the formation of blebs can be positively demonstrated (Fig. 24).

Fig. 24. Mouse myelinated nerve fiber. Freeze-etching technique. Artifactual bleb formations (B) are observed induced by glutaraldehyde fixation. The blistering process produce enlargement of myelin periods (arrows).

With the slicing technique we have exposed the hidden outer surface of teleost fish neurons, as illustrated in Figs. 1, 2, 3, 6, 12 and 13. These surfaces are normally ensheathed by the satellite neuroglial cells (oligodendrocytes or astrocytes). Since the fracture plane occurred at the neuroglial cells we have interpreted that cellular fragments observed in these figures are remnants of these cells.

The anoxic human cerebellum from patients who died by drowning were processed according to the ethanol cryofracturing technique. This material was removed during autopsy, 4 to 6 hours after death, and fixed only by immersion technique. The outer surface of nerve cells and their...
processes exhibited, as illustrated in Figs. 5, 8, 9, 11, 14 and 15 certain degree of shrinkage due to anoxic-ischemic or postmortem changes (Castejón and Valero, 1980; Castejón and Caraballo, 1980b). The outer surface of neurons also displayed attached vestiges of neuropilic cells removed by the cryofracture process. Therefore this is the appearance of a damaged neuron. A completely different picture was obtained from normal Rhesus monkey nerve cells. The primate cerebellar tissue was frozen by the freeze-fracture method, using Freon 22 cooled by liquid nitrogen (Fast freezing). The preservation of the outer surface of neurons was better than that obtained with slow freezing, as depicted in Fig. 20. Evidently delicate handling of the tissue as recommended by Peters (1980) and chromium coated primate cerebellar specimens are additional new efforts to improve nerve tissue preparation for SEM.

R. De Estable Puig: Was picture 6 taken from the outer molecular layer? If not, and without having an axon to follow, what criteria were used to identify this as a stellate cell (other than the fact that material is from teleost fish cerebellar cortex).

Authors: Yes, Fig. 6 was taken from the outer third molecular layer, therefore we have identified this cell as a stellate neuron. In another paper we have described in details these micro-neurons (Castejón and Castejón, 1987).

R. De Estable Puig: Were granule cells exhibiting four branched dendritic profiles observed frequently? If so, could the author provide a picture?

Authors: The number of dendritic profiles observed in a neuron at SEM level depends on the cleavage plane of the fracture process. Using the slicing technique we have observed up to four dendritic profiles, see for example the granule cell labeled GC at the bottom of Fig. 1. However, it is not a frequent observation. When special preparation procedures are used, such as the Tanaka technique (unpublished observations), the four processes can be easily and frequently observed due to the macerating procedure which digests the neighboring neuropile.

R. De Estable Puig: Neuroglial cytoplasm is identified between the bundles of parallel fibers. What criteria are used for identification? Could these fragments be of dendritic origin, from Golgi or Purkinje cells?

Authors: Neuroglial cytoplasm ensheaths the parallel fibers, so it is identified in the sections or in the fractures as remnants or vestiges of cytoplasm running along the course of the parallel fibers. On the contrary the Purkinje and Golgi dendritic profiles cross the parallel fiber bundles at a perpendicular angle ascending toward the surface of the cerebellar folia. Similarly the Bergmann glial cells follow an identical course surrounding these dendritic profiles. Therefore when the longitudinal course of parallel fibers is seen, as illustrated in Fig. 4, the surrounding cytoplasm is characterized as neuroglial cytoplasm. They are not of dendritic origin because according to the dendritic distribution of Purkinje or Golgi cells, they should cross the parallel fibers bundles at right angle.

R. De Estable Puig: Various different fixatives, buffers and protocols are referred to in this article. a) Which does the author favour to obtain mainly intracytoplasmic fractures? b) Are mitochondria preserved in the authors specimens?

Authors: Intracytoplasmic fractures were obtained only with the freeze-fracture SEM method of Haggis and Phipps-Todd (1977) and the cryofracture method for high resolution scanning electron microscopy. Freezing the samples with Freon 22 cooled by liquid nitrogen and then fracturing with a precooled razor blade by hand or using the Smith-Farquhar tissue sectioner allowed us to obtain intracytoplasmic fracture faces. The outer surface of mitochondria is illustrated in Fig. 19 and a fractured mitochondria can be observed in Fig. 22 in the axoplasm of a myelinated axon.

Reviewer IV: An objective of the study seems to be a critical comparison of previously published techniques. This a worthy objective but the paper falls short in that controls are often insufficient to permit valid comparisons and the author provides few specific conclusions.

Authors: This is a review paper where the general salient outer and inner three dimensional surface features of unfractured and fractured nerve cells have been described using conventional SEM and HRSEM. We have studied normal nerve cells (Teleost fish and Rhesus Monkey) and pathological neurons (human cerebellum). When we analyzed normal cells, the samples were prepared also for light and transmission electron microscopy (See review Castejón, 1988). More than one hundred samples were processed for these correlative techniques. The present state of knowledge obtained with these techniques does not permit more specific details or conclusions than those summarized in the present paper. There are some technical limitations in the use of scanning electron microscopy in the study of nerve such as: a) The random plane of the section or the fracture exposed by the slicing technique or the freeze-facture methods; b) The thickness of metallic coating and c) The electron microscope instrumental parameters: gun type, spot size, accelerating voltage used. In relation with the study of human material for ethical reasons it is impossible to study normal or "control" samples to compare with pathological nerve cells.