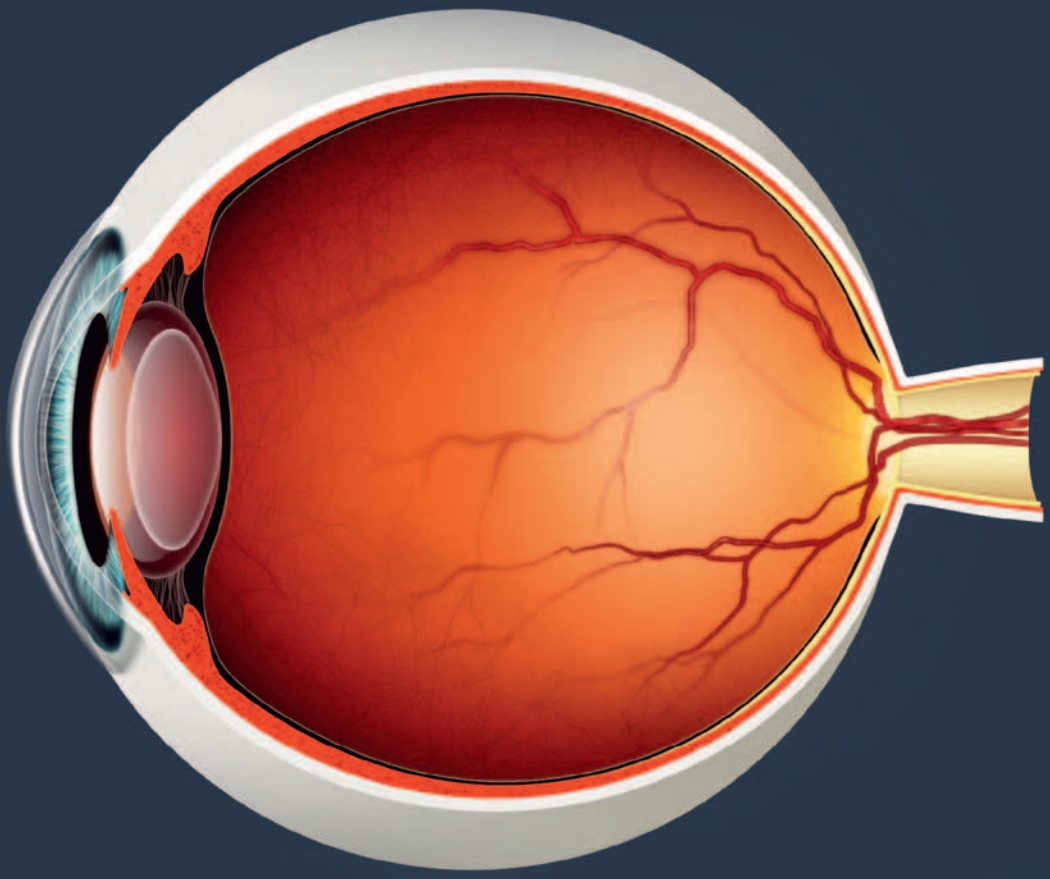


Proteomics, Multi-Omics and Systems Biology in Optic Nerve Regeneration

Edited by
Sanjoy K. Bhattacharya

PROTEOMICS, MULTI-OMICS AND SYSTEMS BIOLOGY IN OPTIC NERVE REGENERATION

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Edited by

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Dedication

Dedicated to the scientific legacy of Dr. Karl H. Pfenninger (1944–2015).

Dr. Pfenninger served as a Professor of pediatrics at the University of Colorado School of Medicine and Director of the Colorado Intellectual and Developmental Disabilities Research Center (IDDRC). He left a lasting and impactful legacy to understand growth cone composition and as well as the application of a systems biology approach to understand axon regeneration. He ignited curiosity toward understanding plasma membrane expansion, its importance in axon growth, and the mechanisms of reinnervation.

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CHAPTER

21

The use of stereological and electron microscopic techniques in the assessment of peripheral nerve regeneration success

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1. Introduction: Basic principles of stereology

The origin of stereological thinking is derived from the German scientist Hans Elias (1907–85), who lived in the early 20th century (Hildebrandt, 2012). The scientist, who pioneered the establishment of the "International Society for Stereology" in the following years, emphasized the obstacles of the three-dimensional interpretation of microscopic sections by revealing stereograms showing the real architecture of some organs (Elias, 1967, 1971). Since those years, many studies have been carried out by obtaining 3D data from the 2D views of the structures by the use of stereological techniques (Bolender, 1981; Brown, 2017; Glaser & Glaser, 2000; Schmitz & Hof, 2005). The volume, area, length, and point assessments of structures can be performed via convenient and simple test systems (e.g., sets of points, lines, or planes) for 3D analysis (Gundersen et al., 1988). The test system should be preferred according to the size of the parameter of interest (e.g., volume or surface area, or length). The use of point test systems (0-dimensional) for volumetric estimations, line for area measurement (one-dimensional), 2D plane for surface area estimation, and finally, 3D test systems for particle counting will be appropriate (Fig. 21.1).

Stereology is often used in nerve regeneration studies to evaluate the effectiveness of methods used to regenerate and heal injured nerves (Brown et al., 2020; Delibas et al., 2021). With the use of stereological methods, it is possible to obtain quantitative data such as the number of regenerated axons, axon diameter, myelin sheath thickness, and the ratio of axon diameter to myelin sheath thickness (Barghash et al., 2013; Brown et al., 2020; Hunter et al., 2020). In addition, it is possible to make various measurements in ganglion tissues where the cell bodies of injured nerves are located (Hausdorf et al., 2008; Muratori et al., 2015; Noorafshan et al., 2011). Evaluation of the total ganglion volume, estimating the number, diameter, and volume of sensory neurons, is possible using stereological methods. This chapter focuses on the application of stereological techniques in evaluating the success of peripheral nerve regeneration.

2. Preparation of the samples for transmission electron microscopy

Electron microscopes are powerful tools for observing biological samples at an ultrastructural level. The stereological evaluations of the nerve tissues (sciatic nerve, optic nerve, ganglia, etc.) are generally performed using resin blocks. At this point, it is essential to prepare the samples in accordance with electron microscopic imaging to obtain high quality images. In addition to choosing the right fixatives and chemicals, tissue size also affects processing success. Also, the freshness of the embedding material and working on appropriate temperatures significantly affect the success of the result. Sectioning can be considered one of the most sensitive steps in sample preparation. It is important for researchers to successfully obtain ultra-thin sections (average less than 100 nm) using an ultramicrotome and

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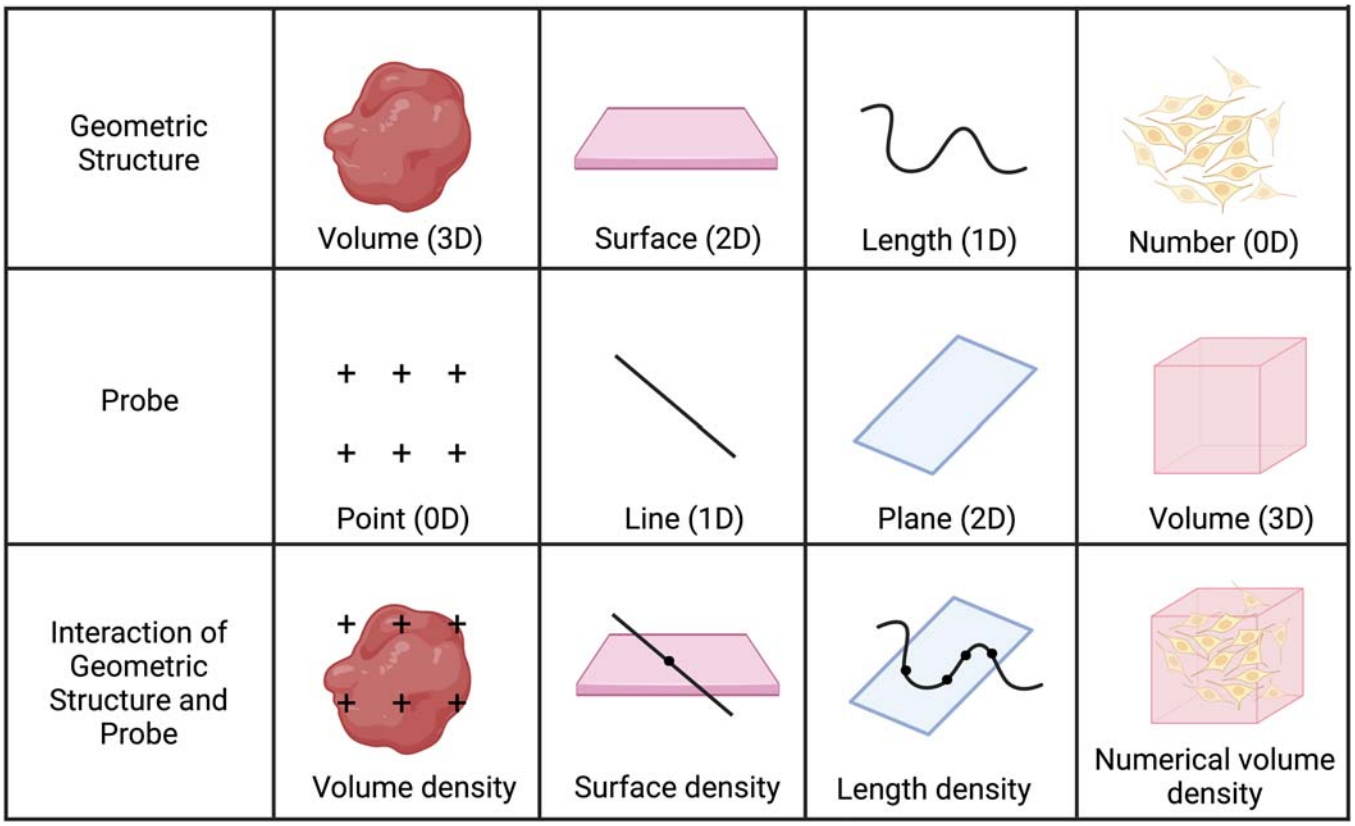


FIGURE 21.1 The illustration demonstrates the stereological test probes. Estimation of volume densities requires point (0D) test systems. Taking sections from 3D structures leads to dimensional loss (sections provide 2D images). In volume estimations, the usage of (OD) point systems (e.g., point grids) allows researchers to estimate the mean volume of the tissue (described in the further headings). For surface area estimations, the intersection of the surface (2D) with the line probe (1D) provides information about the surface density (e.g., surface area estimation of the alveolar ducts). Length density (1D) estimations require plane test systems (2D) (e.g., estimation of myelin sheath thickness, described in the further headings). The same rules apply to the estimation of the number of particles. To increase the dimension of the structure (note that the final dimension of the test systems and structures always sum up to 3), a virtual volume is created between two consecutive sections (e.g., physical disector) or in a single thick section (e.g., optic disector).

to subject them to appropriate staining/contrasting processes (uranyl acetate/lead citrate) at the point of obtaining the desired images. The primary fixation, secondary fixation, dehydration, and resin infiltration steps should be followed consequitvely in preparing tissue obtained from living subjects for conventional electron microscopic analysis. The details and features of the stages can be listed as follows.

2.1 Primary fixation

The most commonly used fixatives in electron microscopic processing are oxidizing agents such as osmium tetraoxide and potassium permanganate, and aldehydes such as glutaraldehyde and paraformaldehyde (Vollmer, 1983). In our laboratory, we use 4% glutaraldehyde fixation solely or in combination with 4% paraformaldehyde (4% glutaraldehyde + 4% paraformaldehyde). Approximately 24–48 h of fixation at 4°C is generally preferred.

2.2 Secondary fixation

Osmium tetraoxide is generally preferred as a secondary fixative agent. Osmium tetraoxide reacts with lipids and stabilizes the phospholipids that form the membranes of the membrane-bound organelles and other lipid-rich structures. A black insoluble precipitate forms on the membranes during fixation, leading to creating contrast with the neighboring cytoplasm. Osmification is one of the most critical steps of the EM processing, and it needs to be performed in the dark with freshly prepared solutions.

2.3 Dehydration step

Tissue can be dehydrated with the help of alcohol or acetone. By gradually increasing the concentration of the solvent used, the water in the tissue is gradually removed.

2.4 Infiltration of the resin

Following the dehydration step, the tissue is cleared using propylene oxide and prepared for epoxy resin. Epoxy resin is well soluble in propylene oxide and will be infiltrated into the tissue. After the tissues are placed in the pure resin, the temperature is increased gradually, and the resin hardens. Finally, the cured tissue is removed from the silicon embedding cups, and ultra-thin sections are taken with an ultramicrotome and a diamond knife. At this stage, it is important to have an experienced researcher or technician who has worked with an ultramicrotome and diamond knife before. Tissues taken on a copper grid are contrasted with lead citrate and uranyl acetate. These heavy metals bind to cell components and scatter the incident beam electrons. The areas of the section which scatter electrons more are recorded as darker pixels, which stand out against the brighter background.

3. Total volume estimation of the ganglion tissue (Cavalieri's principle)

In studies carried out on biological samples, data on total volume and volume ratios appear as an important requirement. Estimating the volume of a particular tissue, cell, or cellular organelle can be performed unbiasedly using stereological methods. Previous studies showed that the volumes of various organs (kidney, brain, dorsal root ganglion, nerve fascicle, uterus, spleen) could be calculated unbiasedly by using Cavalieri's principle (Chen et al., 2020; Modabberi et al., 2021; Noorafshan et al., 2011; Sadeghinezhad & Nyengaard, 2019). Also, in peripheral nerve regeneration studies, the volume of the peripheral nerve and the volume of the dorsal root ganglion tissue where the cell bodies of the sensory fibers are located can be calculated using this principle (Brown et al., 2020; Degn et al., 1999; Delibas et al., 2021; Tandrup, 2004; Vestergaard et al., 1997). Most of the cell bodies of the sensory fibers belonging to the sciatic nerve are located in the L4, L5 level dorsal root ganglia Fig. 21.2. Ganglions at this level are frequently used in neuropathy studies and spinal disc herniation studies, as well as illuminating cellular events that occur after mechanical injury to the sciatic nerve (Boada et al., 2019; Moon et al., 2010; Tomita et al., 2020).

The Cavalieri's principle, which was first introduced by the Italian mathematician Bonaventura Cavalieri in the 17th century, is known as the most used volume calculation method among stereological methods (Gundersen & Jensen, 1987; Sandau, 1999). In the application of the Cavalieri principle, the structure of interest is divided into equally spaced parallel sections. Pictures in small magnification (typically at ×4 to ×10 objectives) should be obtained from the entire tissue at regular intervals to calculate the total volume of the dorsal root ganglion tissue. Then, the point count should be done by placing the point grid on the image Fig. 21.3. The surface area of interest

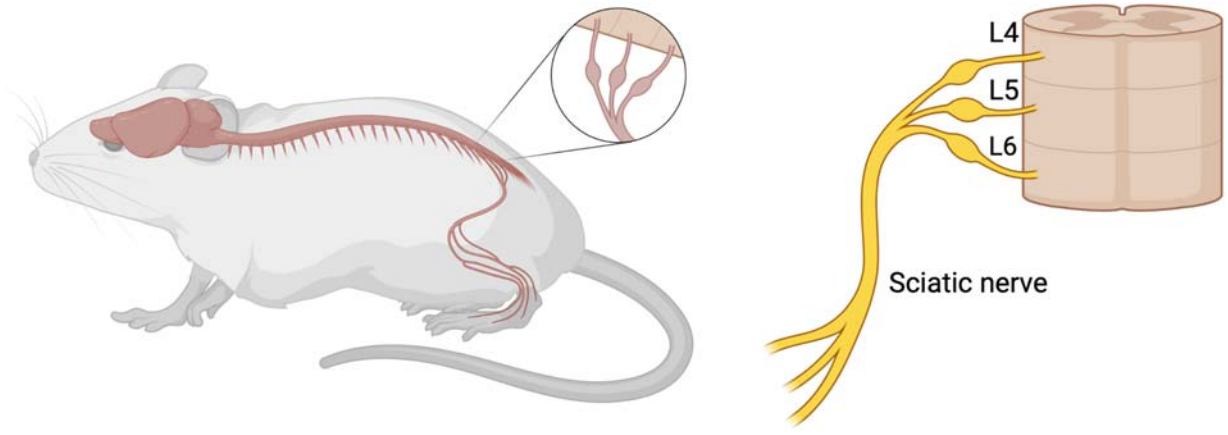


FIGURE 21.2 Rat spinal column with lumbar vertebral bodies illustration depicting the rat spinal column with lumbar vertebral bodies L4, L5, and L6. The sensory neuron cell bodies of rat sciatic nerve fibers in originate from the L4–L6 spinal segments. Also, variations are evident in which the L6 level fibers do not contribute to the sciatic nerve.

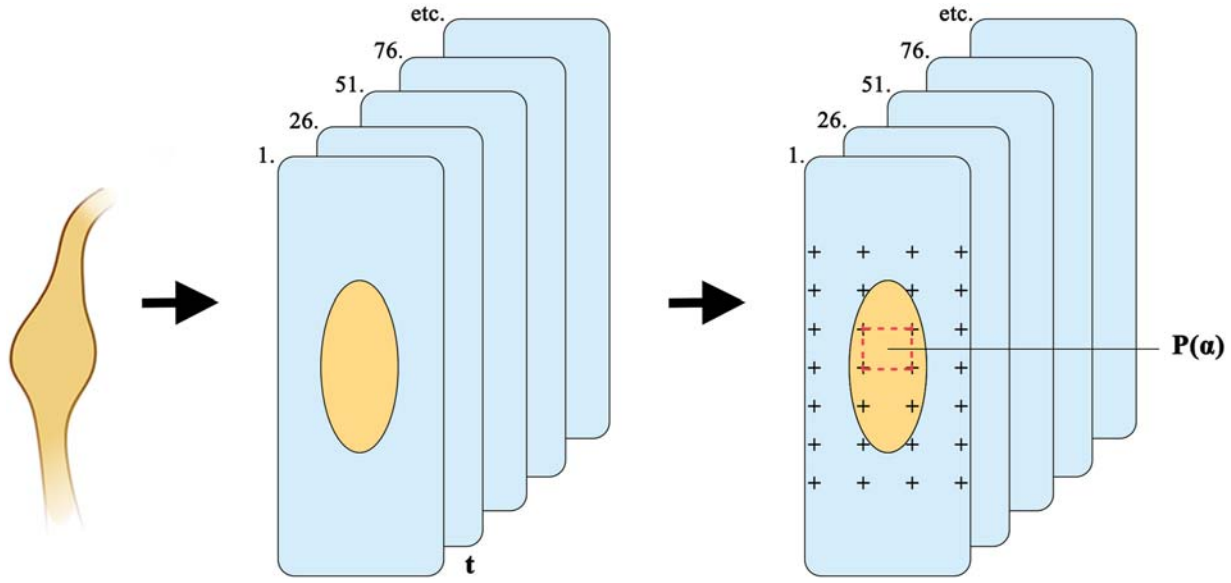


FIGURE 21.3 The illustration represents the usage of Cavalieri's technique in the estimation of DRG volume (it also can be applied to other tissues). An imaginary square unit area created by the centers of "+" signs is shown in point grids with P(α). Calculating the number of unit areas that hit the related surface will ultimately give the total volume using the following formula: $\mathbf{V_{(ganglion)} = 1/f_{(section)} .t.P(\alpha).\Sigma P}$; **V**: Volume; $\mathbf{1/f_{(section)}}$: Sampling of sections; **t**: Section thickness **P(α):** Area per point, **ΣP:** Total points counted.

is calculated using point grids in each section. The values obtained from each section are summed up, multiplied by the average section thickness, and the volume of the structure of interest is calculated. The point grid can be used to calculate the area of interest of the structure. This point grid consists of "+" signs placed equidistant from each other. Each point between the marks represents a unit square, and the area of this square is called the "area associated with the point" [P(α)] and is used to calculate the total volume Fig. 21.3.

One of the most important advantages of stereological techniques is that the type 2 error value of the study can be calculated before the analysis is performed. Using a measuring scale suitable for the structure planned to be evaluated is very important in terms of the accuracy and precision of the sampling and calculation. By calculating the coefficient of error (CE) from the data obtained as a result of a pilot study to be carried out before starting the main study, it can be ensured that sampling and point counts can be made most appropriately without increasing the workload.

4. Estimation of sensory neuron perikaryon volume

The DRG tissue has an elongated ovoid compact structure, and the neurons extend parallel to the long axis. To perform accurate estimations and minimize the variation, sections should be taken in a vertical plane parallel to the long axis. In sensory neurons, taking the nucleolus as the focal point of the cell in cell diameter calculations would be appropriate. The presence of multiple nucleoli, especially in neurons with small diameters, may cause confusion at this point. To solve this problem, the nucleolus with the largest diameter can be selected and considered as the focus of the measurement. However, due to the large differences in the volume and diameter of cells located in the dorsal root ganglion, it is often not possible to make high-precision measurements.

The sensory neuron volume estimation can be performed using cell diameter value. The nonamorphous round structure of sensory neurons facilitates measurement. Cell diameter can be measured in sections sampled at regular intervals using the nucleator method (discussed in the previous section) originally introduced by Gundersen et al. (Gundersen, 1988). Meanwhile, the perikaryon volume estimations can also be done using the rotator technique in vertical sections equally sampled for the optical disector method (Baddeley et al., 1986; Jensen & Gundersen, 1993). In applying this technique, a line parallel to the vertical axis is drawn based on the nucleolus as the focal point of the cell Fig. 21.4. The line is divided into two half-planes by the system, and then the top and bottom of the cell are marked by the researcher, and the volume is calculated using the following formula: $v = \pi/2.(h/3). \sum l^2$.

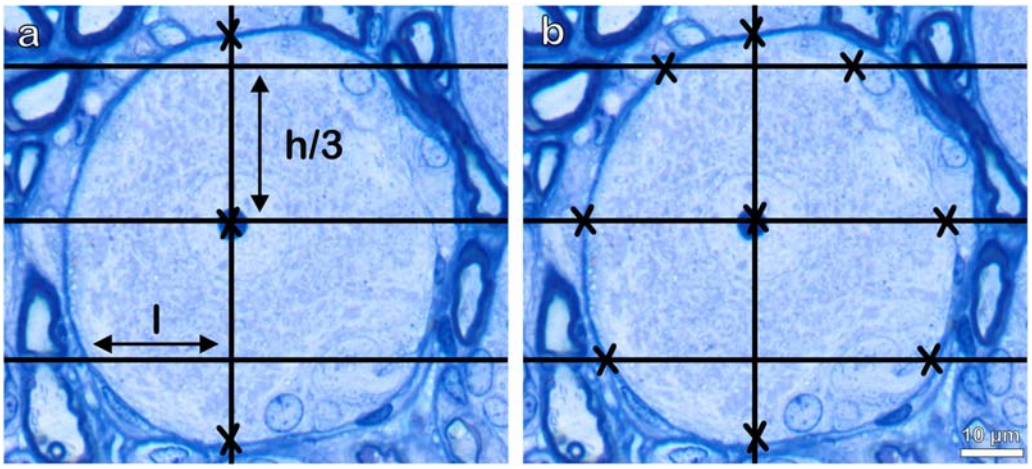


FIGURE 21.4 The application of the vertical rotator technique. (A) The nucleus of the cell is tagged by the user, and (B) two parallel lines are generated by the software at a perpendicular angle to the vertical direction. *h*, height of the cell, *l*, length between the intersection points and vertical axis.

5. Stereological estimations in a cell or cell component on electron microscopic images

It is also possible to use stereological techniques in electron microscopic images besides light microscopic images (Brown et al., 2002; Hunter et al., 2020; Knudsen et al., 2021). In addition to volume estimations of macro structures such as whole organ volume, we frequently encounter studies in which volume calculations are made in micron-level cells (liver cell, Purkinje cell, motor neuron, sensory neuron, etc.). In a study by Santuy et al., the volume fraction occupied by mitochondria and their distribution between dendritic, axonal, and nonsynaptic processes were evaluated using Cavalieri's principle via stereological grids (Santuy et al., 2018). In another study, skeletal muscle mitochondrial content and lipid droplet content density calculations were performed on images obtained using transmission electron microscopy (Broskey et al., 2013). The logic of the conducted studies was the calculation of the mitochondrial volume rate to the whole tissue volume and stereological grids used in these studies (West, 2012). In another study dealing with the calculation of mitochondrial volume in motor neurons, mitochondrial volume was calculated in serial sections taken at 100 nm intervals using the Cavalieri technique without using grids (mitochondrial areas were manually traced) (Fogarty et al., 2021). Similar to these studies, it is possible to calculate various organelle volumes in peripheral and central nervous tissues (Santuy et al., 2018; Tang & Nyengaard, 1997). Moreover, it is possible to estimate the number of unmyelinated nerves via electron microscopic images (Delibas et al., 2021; Larsen, 1998). To obtain an acceptable CE value, an average of 250–300 unmyelinated nerve fibers needs to be evaluated per a cross-section of a nerve in approximately 60 photomicrographs obtained from transmission electron microscopy (Brown et al., 2020). The point grid counting technique can calculate the number of points that hits the unmyelinated fibers. Meanwhile, the usage of the 2D fractionator technique is also possible in unmyelinated fiber number estimations. The meshes of the copper grids used during imaging in TEM can guide the researcher in sampling the tissue. Ultimately, the counting can be performed using unbiased counting frames placed at regular intervals (Fig. 21.5).

6. Estimation of the sensory neuron number in ganglion tissue: A combination of fractionator and disector techniques

Quantitative information on the number of particles in a three-dimensional reference volume is unavailable in single thin (almost two-dimensional) microscopic sections. To overcome this problem, the disector method was developed by Stereo in 1984 (Sterio, 1984). Using the disector method, particles (cells, etc.) in a tissue or organ can be unbiasedly calculated, reducing the type 2 error. The disector principle is a 3D stereological probe for generating particle number data from 2D dimensional morphological analyses independent of sample size, shape, and orientation which is divided into two as physical and optical (Orive, 1980; Sterio, 1984). The physical disector technique is based on the comparison of the same areas in two consecutive sections on high magnification Fig. 21.6. The

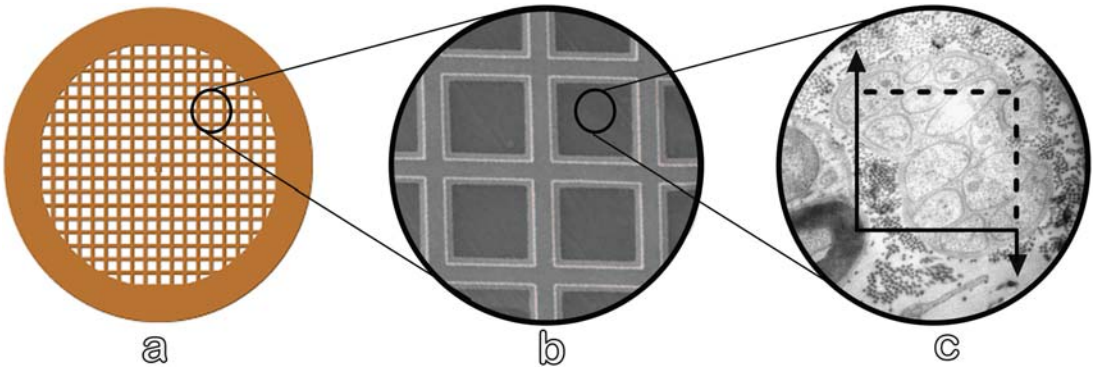


FIGURE 21.5 The figure represents the estimation of unmyelinated fiber number using TEM images. The meshes of the copper grid can be used as a guide for systematic random sampling. The counting can then be performed using an unbiased counting frame in one area per mesh.

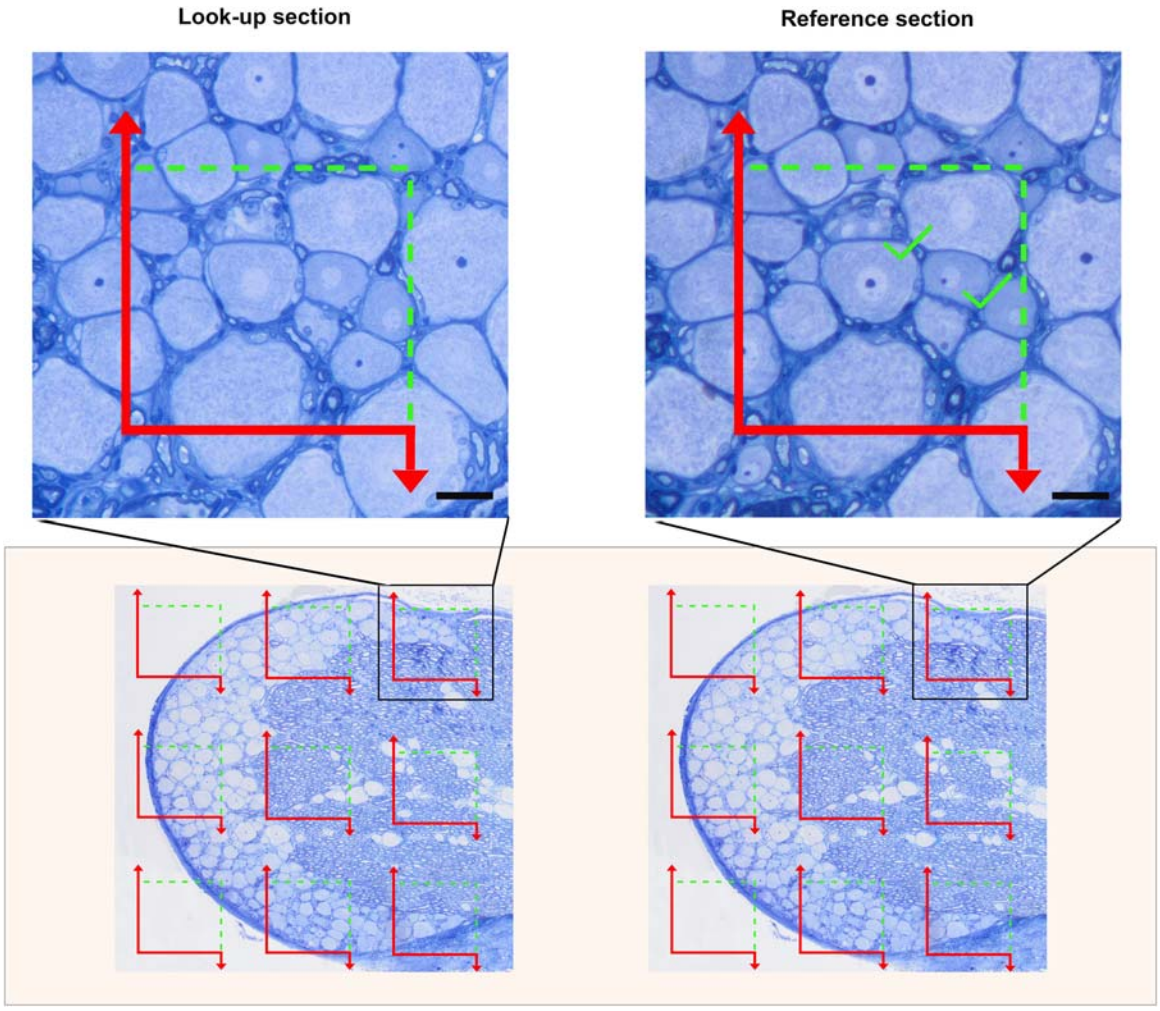


FIGURE 21.6 Application of the 2D physical disector technique is presented. The lower image shows the consecutive sections taken on a slide. The photographs are obtained from the same areas of both sections and compared with each other. The presence of nucleolus is determined as a counting criterion. By this if the nucleolus is counted if it presents in the reference section but not in the look-up section.

counting criterion is the existence of the target particle in one of the consecutive sections but not in the other pair (Look-up/reference) (Geuna, 2005). On the contrary, in the optic disector method, the particle number is calculated in a virtual volume limited by the section thickness. In line with the specified counting criteria (nucleus or nucleolus), the z-axis is moved in the virtual volume at high magnification, and the number of particles coming into focus is calculated (Howard et al., 1985; Laroye & Taylor, 1992).

The calculation of the total number of cells in the ganglion is considered an important parameterfor evaluating the success of nerve regeneration through morphometric analysis. The physical disector method is frequently used to calculate the particle number, and this technique is also frequently used to calculate the number of sensory neurons located in the ganglion (Muratori et al., 2015; Tandrup, 2004). The combination of the disector method with the fractionator method is an unbiased method used to make an estimate close to the real number (Pakkenberg & Gundersen, 1988). The method can be summarized as a combination of random systematic uniform sampling and fragmentation methods. During the application of these methods, the structure's orientation and morphology must be considered. The structure to be studied is systematically and equally divided. Particle counting is performed in the areas determined as a result of the sampling, with the help of the pilot study, by using consecutive optical sections in a virtual single thick section volume.

There are some critical steps that need to be considered in applying the disector technique. The researcher should determine the section thickness and the distance of consecutive disector pair before commencing the analyses and apply the same procedure to all tissues. Additionally, careful attention is required during the tissue processing stage. The rodent dorsal root ganglia (DRG) tissues, which are well-defined, small, and oval-shaped, must be oriented along their long axis during the embedding process in resin.

The section thickness needs to be determined using a pilot study. According to the principles of the physical disector technique, the section thickness should be less than one-third of the average height of the particle counted. Considering the diameter of the sensory neurons in Wistar albino rats, it will be suitable to take sections in approx. 1−2 μm to give all particles an equal chance to be sampled. Another important parameter to be determined is the distance between two disector pairs. Considering the acceptable CE value, the acceptable distance should be less than 60 μm. In agreement with other research groups (Delaloye et al., 2009), our laboratory experienced that the error value increases dramatically if the distance exceeds 60 μm. It will be sufficient to obtain an average of 15−17 sections from each block for correctly applying the method. The obtained disector pairs are counted using an unbiased counting frame.

Particle counting is performed in the virtual volume created between the look-up and the reference section through the unbiased counting frame. It would be more accurate to evaluate two different neuron types separately instead of using a cumulative approach in calculating the number of neurons located in ganglions (Brown, 2017). Type A or large-sized neurons are characterized by containing Nissl granules and having field-bright staining, whereas Type B neurons are typically smaller and darkly stained. Among these 2 cell types with different functions, type B neurons are much more dominant in number than type A neurons (Tandrup, 2004). The small size and high number of B-type neurons necessitate counting these neurons with the help of a smaller counting frame (Brown et al., 2020). Finally, the total number is estimated by multiplying the obtained number with the previously applied sampling fractions (section sampling, area sampling, tissue sampling) (Gundersen, 1988).

7. The estimation of the total myelinated nerve fiber number, axon area, and myelin sheath thickness

Stereological estimations of the myelinated fiber size can be performed in a randomly selected single cross-section obtained from a nerve resin block. To obtain reliable results from the nerve tissue the transverse sample taken from the cross-section must be in a straight position. At this point, it is important to treat with the fixative, keeping the nerve tissue straight after the resection. Following the processing and embedding stages, a section taken in 500 nm thickness will be sufficient for stereological estimations.

After outlining the complete cross-section of the entire nerve, the two-dimensional fractionator technique can be effectively used to estimate the number of myelinated fibers. (Gundersen, 1986). Using the fractionator technique, the entire nerve can be sampled with unbiased counting frames placed at a known distance (determined by the pilot study). The countings will be performed in each counting frame by strictly following the unbiased counting frame estimation rules with the ultimate goal of counting approximately 150−200 myelinated nerve fibers in total per nerve. The final result is obtained by multiplying the total number of fibers counted by the inverse of the sampling fraction (Fig. 21.6).

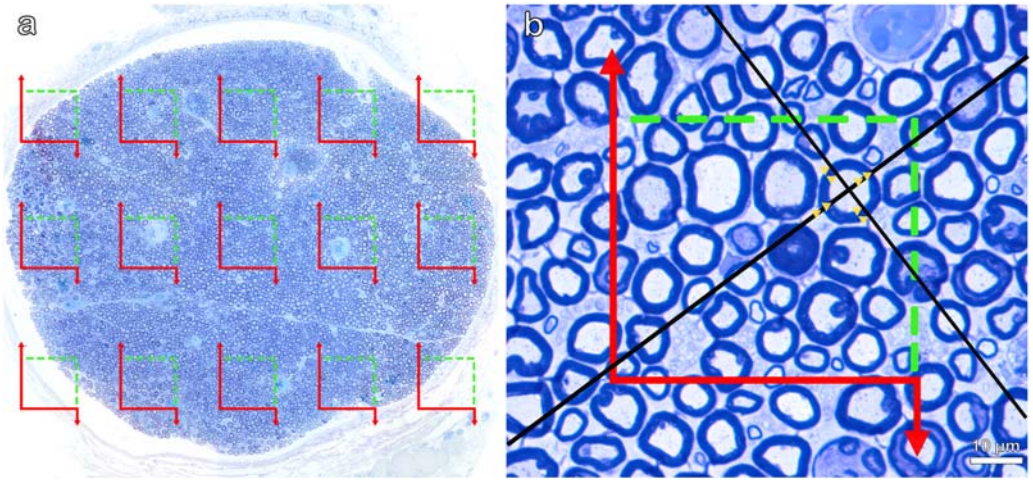


FIGURE 21.7 The application of the 2D physical disector technique in nerve tissue. (A) The myelinated nerve fiber counting's can be performed inside the counting frame. The working rules of the counting frame should be considered during counting (forbidden *red*) and allowed (*green lines*). (B) Application of the nucleator principle can also be performed in a randomly selected nerve fiber inside of the counting frame. The center of the fiber is tagged by the user, and two isotropic lines are created by the software. The intersections of the lines between the boundaries of the myelin sheath are tagged, and ultimately, the myelin sheath thickness is estimated by the software.

The two-dimensional nucleator technique allows area calculation in biological objects with irregular shapes (Gundersen, 1988). The logic of the technique relies on a well-known mathematical formula used in calculating the area of a circle: $a = \mu r^2$. This formula can be used in conditions when "r" is varying. If we determine an *arbitrary* point inside the target biological sample and create isotropic lines originating from the point, the boundaries of the lines will guide us in finding the mean "r" value. The stereological software can automatically calculate the resulting size when the nerve fiber boundaries (hitting with isotropic lines) are tagged by the user. The application of this technique in axon area and myelin sheath thickness is shown in the figure (Fig. 21.7).

8. Conclusion

Stereological methods have been an essential tool in many fields of biology and medicine for decades, and their importance is unlikely to diminish in the future. With the rapid development of imaging technologies, stereological methods are becoming increasingly important for the analysis of three-dimensional structures in a wide range of biological and medical applications. The future of stereological techniques will be the integration of automated Image processing and AI technologies into stereological assessments.

In the future, AI integrated stereological methods are expected to play an increasingly vital role in neuroscience. As researchers continue to investigate the complex structure of the brain and nervous system, these techniques will be crucial for accurately measuring the number, size, and distribution of neurons, synapses, and other essential structures. Additionally, as new strategies for regenerating injured or damaged tissues emerge, stereological methods will be indispensable for assessing the extent and quality of tissue regeneration and evaluating the effectiveness of various treatment approaches.

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Proteomics, Multi-Omics and Systems Biology in Optic Nerve Regeneration

Edited by

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Proteomics, Multi-Omics and Systems Biology in Optic Nerve Regeneration is a comprehensive reference covering all vistas of standardization of axon regeneration, as well as all multiomics and system-level data and integration tools. By adopting a translational approach, this book bridges current research in the field to clinical applications, and readers can expect to learn standardization approaches for axon regeneration, multiomics datasets, different databases, search engines, multiple dataset integrative tools, pathway convergence approaches, and outcome measures that unify bench research with clinical outcomes.

Axon regeneration from existing neurons in the central nervous system (CNS) has become a possibility in the last decade. The potential of long-distance axon growth has opened the prospect of reconnectivity of axons of retinal ganglion cells with the neurons that are in the lateral geniculate nucleus in the brain. Long-distance axon regeneration and reconnectivity promises to restore lost function in the optic nerve, and thus vision. Further, long-distance regeneration and reinnervation is equally helpful for other fields such as spinal cord injuries. Proteomics and other functional omics (metabolomics and lipidomics) are becoming increasingly important to understand and normalize data. This is because transcriptomics is not often a mirror image of other omics, and functional repertoire of molecules is amenable to more rapid modifications. Alteration of proteins, lipids, and metabolites result in a shift in equilibrium from disease or disorder to normalcy. Such interventions may hold the equilibrium to prevent undergoing deeper pathologic states.

Proteomics and multiomics allow the evaluation of concerted pathway convergence and identification of molecules for treatment. With respect to identification of molecules, their placement, and systems biology, this book will have a wide swath of appeal, including 1) students; 2) basic researchers; and 3) clinicians in ophthalmology treating glaucoma and other optic neuropathies (including traumatic optic neuropathy). This book will also be interesting to clinicians and translational scientists seeking new treatments for peripheral and central system neuropathies.

Key features

- Uses multiomics datasets for selecting molecules for axon regeneration
- Bridges the preclinical and clinical world, from selection of molecules to outcomes leading to IND filing and their use
- Includes system-level knowledge needed for understanding central nervous system axon and dendrite regeneration and standardizes the system-level biology for axon regeneration
- Explores the current state of multiomics in axon and dendrite regeneration in the optic nerve, and its comparisons to other CNS regeneration
- Evaluates multiomics data tools to integrate and identify convergent pathways and molecules promoting axon regeneration.

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