Commentary on Mitochondrial Stereology in Transmission Electron Microscopy

Comentario sobre Estereología Mitocondrial en Microscopía Electrónica de Transmisión

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SUMMARY: Mitochondria (m) are responsible for the energy availability of cells, and their analysis is indicated for example, in studies related to metabolism and oxidative stress. The direct measurement of mitochondria (morphometry) is biased because of the section obliquity and position relative to the mitochondria length (non-equatorial cut). Therefore, stereology is an appropriate technique to evaluate mitochondria. However, before beginning the study, it is necessary to consider the premises to obtain random and uniform samples to be analyzed stereology. Mitochondria must have the chance to appear in all the possibilities of cut and orientation in the micrographs. The number of micrographs to be analyzed will depend on the distribution and occupation of mitochondria in the cell. After this is resolved, a proposal is the estimation of the following stereological data: volume density (Vv), surface density (Sv), and mean cross-sectional area (A). Overlapping a known test area at each micrograph, the density by area of mitochondria is estimated (N_{AT}). Vv [m] can easily be estimated by point-counting ($Vv = Pp/P_T$; Pp are the points hitting the structure, P_T are the number of points of the test system). Sv is estimated overlaying a test-line (L_T) on the micrographs and counting the intersections of the lines (I) with the outer membrane (om), inner membrane (im), and crests (c), thus, Sv [om], Sv [im], Sv [c] ($Sv = 2I / L_T$). A [m] is obtained as the ratio: $A = Vv / 2N_{AT}$.

KEY WORDS: Mitochondria; Morphometry; Stereology; Microscopy; Cell biology.

INTRODUCTION

For over four decades, it has been speculated that mitochondria originated from a symbiosis between the eukaryotic cell and bacteria (Reijnders, 1975). Moreover, research in the last decade has highlighted that since its integration in eukaryote cells, this organelle of bacterial origin has not only been tolerated by immunity but has also been placed as a central regulator of cell defense, which reinforces the interest of mitochondrial research (Meyer *et al.*, 2018).

The mitochondria have multiple essential features and have attended in contemporary literature (Picard *et al.*, 2016; van der Bliek *et al.*, 2017) because of its performance in generation of reactive oxidation species (Schwerzmann *et al.*, 1989; Ray *et al.*, 2014), apoptosis regulation (Yee *et al.*, 2014), activation of endoplasmic reticulum stress response (Kim *et al.*, 2016), and others. For example, the content of

mitochondria differs in various species ranging between 22.0-37.0 % with a close correlation between the mitochondrial volume density, heart rate and the rate of basal oxygen consumption in any group of animals (Barth *et al.*, 1992).

Mitochondrial disorders represent a heterogeneous group of multisystem diseases with extreme variability in clinical phenotype. Ultrastructural analysis revealed a heterogeneous mixture of mainly abnormal, partially swelling mitochondria with unusual and sparse cristae, which highlights the importance of morphological analysis in diagnostics of mitochondrial disorders (Brantová *et al.*, 2006). Therefore, the shape and size of mitochondria may be varied in different tissues and when subjected to metabolic, physiological, and pathological changes. For example, when the inner mitochondrial membrane had lost

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its selective permeability originates a swelling in the mitochondrial matrix, which distends the outer membrane until it ruptures (Sesso *et al.*, 2012), which is relevant information associated to mitochondrial size and shape.

However, the way mitochondria appear in electron micrographs is varied because mitochondria can be sectioned in different planes, which gives different obliquities in mitochondrial profiles (Fig. 1). This possibility of altering the profile related to the section plane in which the mitochondria are sectioned makes it challenging to diagnose changes in size and shape in the mitochondria. Because of the irregular shape of the mitochondria, stereology may give objective quantitative data and allows easy comparisons between mitochondria of different experimental and pathological situations (Mandarim-de-Lacerda, 2011). Therefore, measuring organelle boundaries (morphometry) to determine diameters, lengths, shapes, is often misleading (distorted) and does not help in mitochondria assessment.
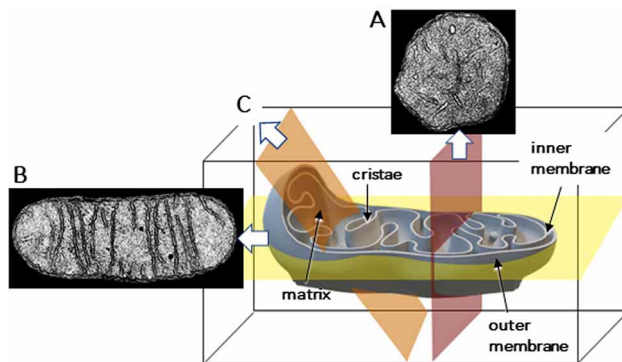

Fig. 1. Mitochondria may appear sectioned in various orientations: (A) round shape when sectioned transversely, (B) elongated shape when sectioned longitudinally, and (C) all intermediate possibilities.

In this text we intend to indicate procedures and techniques that can help the morphologist and the pathologist and study the quantitative variations of mitochondria in any tissue, contributing to a better understanding of mitochondrial alterations of various standard and pathological causes.

1. Technical preparation of samples (transmission electron microscopy). It is crucial to prepare the material accurately for observation by transmission electron microscopy, which beginning with the fixation, resin embedding, ultramicrotomy, and contrast with a previously established and tested protocol. Also, the images to be analyzed must be at the same magnification and be obtained at random. The electron microscope should be calibrated and free of magnetic lens aberrations (which can deform

mitochondrial images). Typically, several resin blocks should be got for each individual, each block having a random and varied orientation of the tissue under analysis, which will allow images of mitochondria to be attained at all cutting possibilities (that is essential for the execution of stereology).

2. Estimating mitochondrial stereology. Here we propose to estimate some parameters that we find useful for studying quantitative changes in mitochondria: volume density, numerical density per area, cross-sectional area, and surface density (for cristae and inner and outer membranes). Before proceeding, it is essential for the reader to be familiar with the stereology (jargon and principles) that are easily found in review articles (Gundersen *et al.*, 1988; Cruz-Orive & Weibel, 1990; Mandarim-de-Lacerda, 2003; Mandarim-de-Lacerda & Del-Sol, 2017), and textbooks (Weibel, 1979; Elias *et al.*, 1983; Russ & Dehoff, 2000; Howard & Reed, 2005; Mouton, 2011).

For data acquisition and execution of stereological formulas, it is necessary to overlay a test system that contains test area, points, and test lines. With transmission electron microscope images in JPEG format (Joint Photographic Experts Group, a process of digital image compression) you can use the test systems generated by the Web-based Stepanizer to superimpose the images (www.stepanizer.com) (Tschanz *et al.*, 2011).

Figure 2 illustrates an image of tissue (liver) containing mitochondria with a superimposed test system and will be mentioned in the following descriptions.

a) Volume density of mitochondria in a tissue (Vv [mit, tissue]). The simplest way to make this estimate is by counting points. In Figure 2, test points are indicated by incomplete circles (Pp) (in this case, 25 points). Only 10 points focus on mitochondria, and, therefore, we have a density of Vv [mit, tissue] = 10/25 = 0.4 or 40 %.

To know how many images and how many points should be counted for the results to be statistically significant, we suggest consulting a nomogram established for this purpose, as, for example, in previous publications (Weibel; Aherne & Dunnill, 1982; Mandarim-de-Lacerda, 2011; Mandarim-de-Lacerda & del-Sol).

b) Numerical density per area of mitochondria in a tissue (N_A [mit, tissue]). Mitochondria contained in a test area should be counted as long as they are not hit by the "forbidden lines" (exclusion lines) (this is well demonstrated in a Gundersen historical publication) (Gundersen, 1977).

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In Figure 2, the exclusion lines are red and section the two lower mitochondria in the image and those on the left (indicated by x). Therefore, in this image, only the mitochondria indicated by numbers 1 and 2 should be considered. If we know the value of the test area (this is determined when we calibrate the system in Stepanizer), N_A [mit, tissue] is easily calculated.
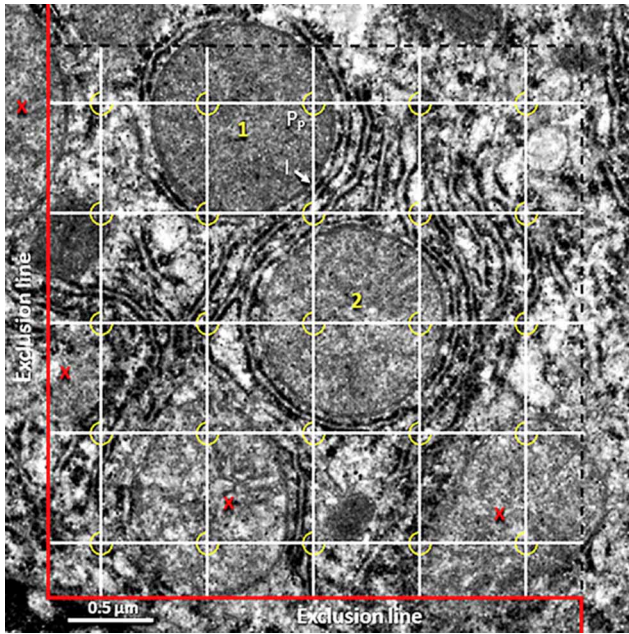

Fig. 2. This figure illustrates an electron micrograph of mitochondria having superimposed a test system for stereological data acquisition. Points (Pp) and intersections (I) of the test line with mitochondria (or their structures) can be counted. The number of mitochondria in the test area can also be computed (#1 and #2) if the exclusion lines do not cut them (in this case, mitochondria marked with X are not totaled).

c) Mitochondrial cross-sectional area (A [mit, tissue]). A [mit, tissue] is defined by the following formula:

$$A\ [mit, tissue] = \frac{Vv\ [mit, tissue]}{2 \times N_A[mit, tissue]}$$

moreover, we already learned how to estimate Vv [mit, tissue] and N_A [mit, tissue].

d) Surface density (Sv [structure, mit]). Sv is a stereological concept taught by Buffon (1777) in his game of needles [the history of this concept can be read in a previous publication (Mandarim-de-Lacerda, 2003)]. In practical terms, Sv is calculated as indicated in the following formula:

$$Sv\ [structure, mit] = \frac{2I}{L_T}$$

(I is the number of intersections of the test line -- L_T -- with the mitochondrial structure). Sv can be estimated for the outer membrane (om), the inner membrane (im), or cristae (c). Therefore, it is possible to estimate Sv [om,mit], Sv [im, mit] or Sv [c, mit] independently.

Intersections (I) can happen with vertical or horizontal lines, maximizing the count in the same image. In Figure 2, an intersection is indicated with a vertical line.

CONCLUSION

Although it is not common to find stereological quantification of mitochondria and their membranes in the studies, we intended here to provide some useful tools for anyone who wants to add this quantification to their work, valuable information when studying tissue changes due to physiological or pathological causes.

RESUMEN: Las mitocondrias (m) son responsables de la disponibilidad de energía de las células, y su análisis está indicado, por ejemplo, en estudios relacionados con el metabolismo y el estrés oxidativo. La medición directa de las mitocondrias (morfometría) está sesgada debido a la oblicuidad de la sección y la posición relativa a la longitud de las mitocondrias (corte no ecuatorial). Por lo tanto, la estereología es una técnica apropiada para evaluar las mitocondrias. Sin embargo, antes de comenzar el estudio, es necesario considerar las premisas para obtener muestras aleatorias y uniformes para analizar estereológicamente. Es esencial que las mitocondrias tengan la posibilidad de aparecer en todas las posibilidades de corte y orientación en las micrografías. El número de micrografías que se analizarán dependerá de la distribución y ocupación de las mitocondrias en la célula. Una vez resuelto esto, una propuesta es la estimación de los siguientes datos estereológicos: densidad de volumen (Vv), densidad de superficie (Sv) y área de sección transversal media (A). Superponiendo un área de prueba conocida en cada micrografía, se estima la densidad por área de mitocondrias (N_{AT}). Vv [m] se puede estimar fácilmente contando puntos (Vv = Pp / P_T; Pp son los puntos que llegan a la estructura, P_T son el número de puntos del sistema de prueba). Sv se estima superponiendo una línea de prueba (L_T) en las micrografías y contando las intersecciones de las líneas (I) con la membrana externa (om), la membrana interna (im) y las crestas (c), por lo tanto, Sv [om], Sv [im], Sv [c] (Sv = 2I / L_T). A [m] se obtiene como la relación: A = Vv / $2N_{AT}$.

PALABRAS CLAVE: Mitocondria; Morfometría; Estereología; Microscopía; Biología celular.

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