

ORIGINAL PAPER

The morphometrical analysis on the ultrastructure of A549 cells

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Abstract

Objective: To report the morphometric characteristics of ultrastructure inside A549 cells. **Methods:** A549 cells were processed for inverted microscopy and transmission electron microscopy (TEM). Cell images were obtained randomly using inverted microscopy and TEM. The morphometric parameters of ultrastructure were tested using precise morphometric techniques by Image-Pro Plus analysis software. **Results:** (1) The diameter of A549 cells from inverted microscopy and TEM images was 14.93 μm and 10.59 μm . (2) By defining cell as reference space the volume densities (V_V) of nucleus and cytoplasm were about 0.28 and 0.72; the surface densities (S_V) of nucleus were 0.19 μm^{-1} . By defining cell nucleus as reference space the V_V of nucleoli, euchromatin and heterochromatin were 0.076, 0.72 and 0.20 respectively; the S_V of nucleoli was 0.15 μm^{-1} . By defining cytoplasm as reference space the V_V of mitochondria, lamellar bodies and lysosomes were 0.046, 0.025 and 0.014; the S_V of mitochondria, lamellar bodies and lysosomes were 0.60 μm^{-1} , 0.36 μm^{-1} , and 0.18 μm^{-1} . (3) In individual A549 cell total volume and surface of mitochondria were 61.91 μm^3 and 1001.67 μm^2 ; Total volume and surface area of lamellar bodies were 76.82 μm^3 and 428.68 μm^2 ; Total volume and surface area of lysosomes were 21.69 μm^3 and 212.04 μm^2 . **Conclusions:** The morphometric parameters of some ultrastructures within A549 cells were established using precise morphometric techniques by Image-Pro Plus analysis software.

Keywords: morphometry, ultrastructure, A549 cells.

Introduction

A549 cells were derived from human alveolar cell carcinoma [1] and matched closely the type II alveolar cell phenotype and share many characteristics with human primary alveolar epithelial cells [2], so which were widely studied as models of not only lung cancer [3, 4] but also as human primary alveolar epithelial cells *in vitro* [5, 6]. According to the Public Medicine government webpage, more than 6200 research papers using A549 cells have been reported [7–9]. These studies were mainly focused on the changes of functions of A549 cells. However, it is well known that morphological changes are the basis of functional changes; in turn, functional changes would affect the morphological structure. Efforts to improve the understanding of function of these cells are dependent on a better understanding of their sub-cellular structures. In some of the previous studies the morphology of the A549 cells was qualitatively or semi-quantitatively measured using light or electron microscopy [10, 11], but no comprehensive study of the morphometric characteristics of the A549 cell line has been reported. In this paper, we reported characteristics of the ultrastructure of the cell's organelles using precise morphometric techniques.

Material and Methods

A549 cells' culture

A549 cells were obtained from the Institute of Cell Biology (Shanghai, China) and maintained for one week

in 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 units/mL of penicillin (Sigma) and 100 $\mu\text{g}/\text{mL}$ of streptomycin (Sigma). The cells were incubated at 37°C in a 97–98% humidified atmosphere containing 5% carbon dioxide. The growth medium was changed every 2–3 days.

Samples processing

Inverted Microscopy

After being cultured for one week the cells were harvested with trypsin, washed twice with 0.1 M phosphate buffer (PBS) (pH 7.4) and transferred to a 4°C isotonic Hank's solution in culture flask. Subsequently, the cells in culture flask were observed using a TE 2000-s inverted microscopy (Nikon).

Transmission Electron Microscopy (TEM)

In order to obtain good preservation and visualization of the cytoplasmic ultrastructures the procedures of fixation were modified in the processing for TEM [12]. Briefly, after harvesting with trypsin, washing twice with 0.1 M PBS (pH 7.4) and fixation in 2.5% glutaraldehyde for 24 hours, the cells were post-fixed in a 1% aqueous solution of OsO_4 for one hour, followed by in-block uranyl acetate staining overnight. Preservation and staining was enhanced with this longer than normal treatment with aqueous uranyl acetate. Subsequently, the cells were washed in cacodylate buffer, dehydrated in graded acetone solutions and embedded in Spurr's

low-viscosity resin. Ultrathin sections were cut with glass knives using a LKB ultra-microtome set at 80 nm section thickness. The sections were mounted on 200 mesh copper grids or on formvar-coated slot copper grids and stained with uranyl acetate and lead-citrate. Finally, sections were examined using a Hitachi-7500 TEM (Hitachi Ltd., Tokyo, Japan).

Digital images

Cells prepared for inverted microscopy were imaged using a CCD Canon camera connected to a DELL Inspiron 530s computer. Ten images containing approximately 600 cells were taken randomly. Cells images for TEM were taken randomly in each of the four corners of the squares of the mesh grids under TEM (Figure 1a).

Definitions of A549 cells' components

The A549 cell was divided into several morphologically distinct components. The nucleus consisted of euchromatin, heterochromatin and nucleoli. The cytoplasm was subdivided into mitochondria, lamellar bodies, lysosomes and "remaining cytoplasm". Mitochondria had a double membrane. Lamellar bodies were defined as unique membrane bound structures containing parallel lamellar substructures. Lysosomes were characterized by a single membrane and containing electron dense materials. "Remaining cytoplasm" was defined as the cytoplasmic ground substances plus all organelles not mentioned above.

Morphometric analysis

Diameter and volume of A549 cell

One hundred cells were selected randomly in inverted microscopy and TEM images and the diameter of these cells was measured using Image-Pro Plus 6.0 software. The volume of cells measurements were performed using the equation:

$$\text{Volume}_{\text{cell}} = \frac{4\pi r^3}{3} \quad (1)$$

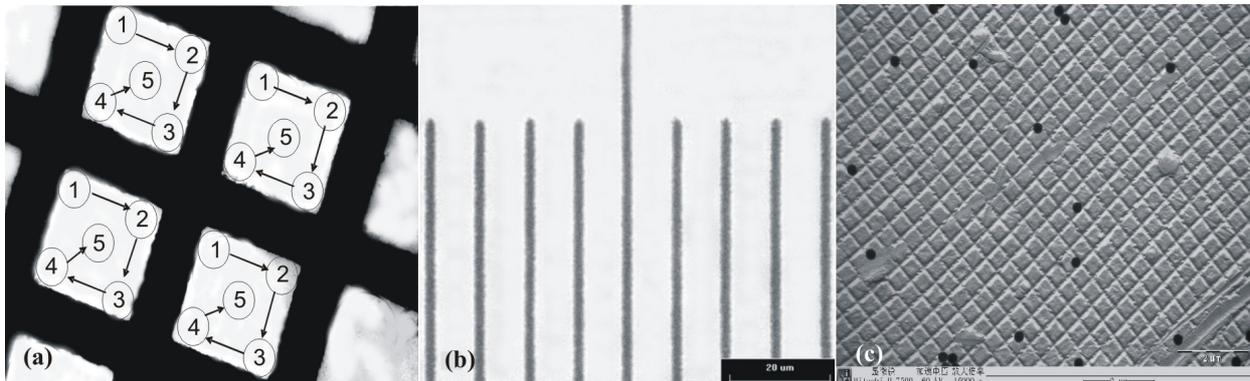


Figure 1 – The principle of obtaining images randomly (a). The size of test system was calibrated by an objective micrometer with 10 µm per small grid (b). The magnification of transmission electron micrographs were calibrated by means of carbon grating replica with 2160 lines per mm (c).

Application to TEM images

Micrographs ($\times 8000$) served to determine the V_V , S_V of the nucleus and the V_V nucleus and cytoplasm using a simple square lattice, which contained 266 test

where r was 1/2 the diameter.

Because cells in the transmission electron images were present as the profiles, so the diameter of the cell from TEM was obtained using the equation:

$$\text{Diameter}_{\text{cell}} = \frac{4\bar{d}}{\pi} \quad (2)$$

where \bar{d} was the diameter of the profile of cells in the TEM images.

Morphometric parameters

The volume and surface densities (V_V , S_V and N_V) of various organelles were achieved by application of morphometric point-counting procedure [13–17]. The counting grid was superimposed on cell images using Image-Pro Plus 6.0 software. The V_V was computed as the mean ratio estimates:

$$V_V = P_i/P_T \quad (3)$$

where P_i was the number of hit points falling within a given compartment and P_T was the number of points falling on the reference area. The S_V was directly derived from counts of the intersection points I_i of the surface contour of profiles with test lines of known length L_T :

$$S_V = \frac{(2 \times I_i)}{L_T} \quad (4)$$

Calibration of magnification and cell shrinkage

Magnification of inverted microscopy was calibrated using a slide micrometer with 10 µm per small grid (Figure 1b). Magnification of TEM was calibrated using a Carbon Line Grating Replica 2160 lines per mm (Figure 1c). Cell shrinkage was calculated using equation:

$$f_{\text{shrinkage}} = \frac{\overline{Da}}{\overline{Db}} \quad (5)$$

where \overline{Da} and \overline{Db} were the mean diameter of cells after and before processed.

points and a total line length of 3.021 µm (Figure 2a). For nucleus components (euchromatin, heterochromatin and nucleoli), a square lattice representing a length of 1.228 µm was employed.

Micrographs ($\times 15\,000$) were used to measure the V_V of mitochondria, lamellar bodies and lysosomes with a square lattice representing a length of $0.5061\ \mu\text{m}$ and the N_V of mitochondria, lamellar bodies and lysosomes with a frame of known area ($102.45\ \mu\text{m}^2$) by counting profiles (Figure 2b).

The S_V of mitochondrial outer membrane, lamellar

bodies and lysosomes were obtained from TEM images of higher power magnification ($\times 30\,000$) using a multi-purpose grid with 24 lines of length $0.201\ \mu\text{m}$ (Figure 2c). Since the higher power TEM images were centered on cytoplasm, all measurements were referred to cytoplasmic volume.

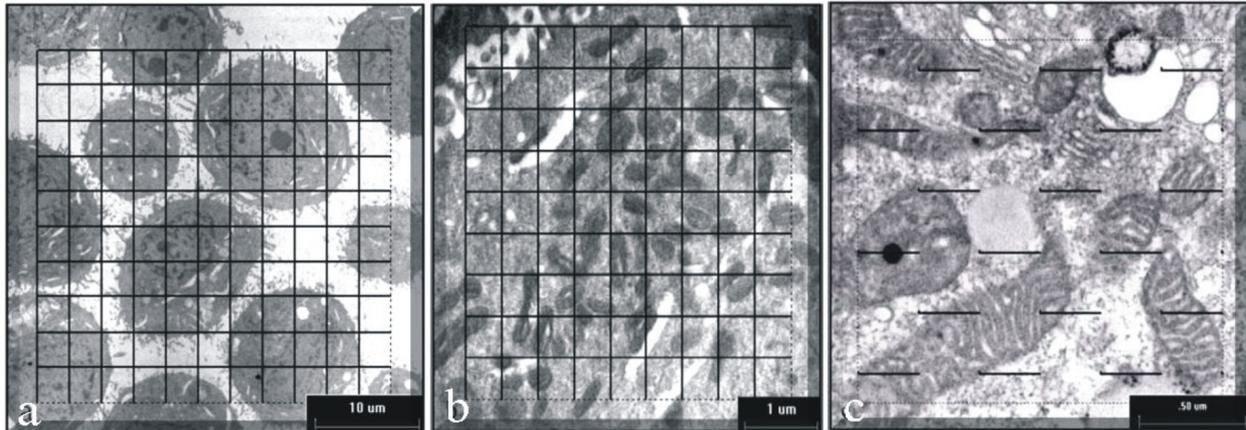


Figure 2 – The TEM images with the superimposed grid were used for morphometric analysis on ultrastructures of A549 cell: (a) was the analysis on the morphometric parameters of nucleus and cytoplasm; (b) was the analysis on the volume densities of the mitochondria, lamellar bodies and lysosomes; (c) was the analysis on the surface densities of mitochondria, lamellar body and lysosomes.

Statistical analysis

Results are expressed as the mean (\bar{X}), standard deviation (SD), coefficient of variation (CV) and 95% confidence intervals.

Results

A549 cells suspended in chilled isotonic Hank's solution had an overall spherical shape. A549 cells images from TEM showed that various cellular components such as mitochondria, lamellar bodies,

lysosomes, microcapsule as well as the nucleus within cytoplasm (Figure 3a). Most lysosomes surrounded by single membrane were autolysosomes containing residual organelles. The lamellar bodies were round or oval. There were parallel or thread-like plate structure inside lamellar bodies (Figure 3b). The mitochondria has an axial ratio of about 5:1. The cristae within mitochondria were perpendicular to the long or short axis of mitochondria and were irregular or polymorphic in shape (Figure 3c).

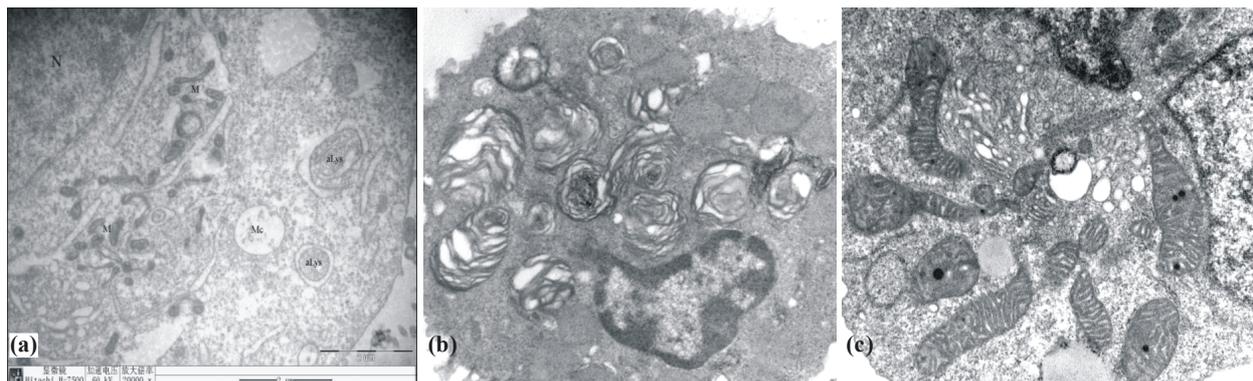


Figure 3 – (a) The mitochondria, autolysosomes, microcapsule and nucleus can be observed within A549 cell cytoplasm ($\times 20\,000$). (b) The lamellar bodies were round or oval, in which there were the parallel or thread-like plate structures ($\times 50\,000$). (c) The mitochondria were characterized by cristae arranging perpendicularly to the long or short axis and were irregular and polymorphic in shape ($\times 50\,000$).

The mean diameter of A549 cells suspended in chilled isotonic Hank's solution was $14.93\ \mu\text{m}$. The axial ratio of these cells was 1.04:1 indicating the cells are spherical in shape. Therefore, according to the equation (1) the volume of the A549 cell could be estimated to be $1670\ \mu\text{m}^3$, which formed the basis for computation of the absolute values of organelles within

cells. The mean diameter of A549 cells in the TEM images was estimated to be $10.59\ \mu\text{m}$.

The morphometric data of the average A549 cells including components of nucleus and cytoplasm were calculated considering cell shrinkage prepared for TEM and magnification of TEM images. All parameters were summarized in Tables 1–4.

Table 1 – The morphometric parameters of nucleus and cytoplasm

Parameters	Units	Means	SD	CV	95% Confidence intervals
V_{Vn}	–	0.2789	0.0611	0.2191	0.2451~0.3129
$V_{V_{cyt}}$	–	0.7211	0.0611	0.0847	0.6871~0.7459
S_{Vn}	μm^{-1}	0.1934	0.0388	0.2004	0.1785~0.2075
V_n	μm^3	465.76	102.04	0.2091	409.25~522.27
V_{cyt}	μm^3	1204.2	102.04	0.0847	1147.7~1260.7
S_n	μm^2	322.92	64.712	0.2004	298.76~347.08

Table 2 – The morphometric parameters of nucleoli, heterochromatin and euchromatin

Parameters	Units	Means	SD	CV	95% Confidence intervals
V_{Vnu}	–	0.0761	0.0043	0.0571	0.0739~0.0781
S_{Vnu}	μm^{-1}	0.1508	0.0228	0.1518	0.1424~0.1596
V_{Vhet}	–	0.2012	0.0234	0.1164	0.1883~0.2137
$V_{V_{euc}}$	–	0.7225	0.0236	0.0327	0.7097~0.7363
V_{nu}	μm^3	35.623	2.0333	0.0571	34.497~36.749
S_{nu}	μm^2	70.517	10.703	0.1518	66.521~75.513
V_{het}	μm^3	94.114	10.954	0.1164	88.047~100.18
V_{euc}	μm^3	337.85	11.052	0.0327	331.73~343.97

Table 3 – The morphometric parameters of mitochondria, lamellar bodies and lysosomes

Parameters	Units	Means	SD	CV	95% Confidence intervals
V_{Vmi}	–	0.0460	0.0032	0.0883	0.0443~0.0477
V_{Vlam}	–	0.0250	0.0156	0.6242	0.0162~0.0338
V_{Vlys}	–	0.0139	0.0067	0.5177	0.0091~0.0119
V_{mi}	μm^3	61.910	5.4680	0.0883	58.882~64.938
V_{lam}	μm^3	76.820	26.068	0.0883	62.383~91.257
V_{lys}	μm^3	21.694	11.230	0.5177	15.474~27.914

Table 4 – The surface densities and surface area of mitochondria, lamellar bodies and lysosomes

Parameters	Units	Means	SD	CV	95% Confidence intervals
S_{Vmi}	μm^{-1}	0.5988	0.2530	0.5638	0.5045~0.6935
S_{Vlam}	μm^{-1}	0.3565	0.2293	0.6432	0.2715~0.4425
S_{Vlys}	μm^{-1}	0.1763	0.2071	1.1744	0.0987~0.2533
S_{mi}	μm^2	1001.67	304.21	0.5638	888.09~1115.3
S_{lam}	μm^2	428.68	275.74	0.6432	325.73~531.63
S_{lys}	μm^2	212.04	249.03	1.1744	119.06~305.02

Results showed that the individual A549 cell contained 72% cytoplasm and 28% nucleus. The nucleus contained about 8% nucleoli and about 92% chromatin. About 76.9% of chromatin was presented as the form of euchromatin. About 4.6% of cytoplasm was made up of mitochondria. The lamellar bodies' space amounted to 2.5% of cytoplasm, while lysosomes contributed 1.39% of cytoplasm.

Discussion

In order to obtain morphometric parameters of A549 cells composition, cell culture conditions must be limited strictly. In this study, we cultured A549 cells according to the conventional methods commonly used in scientific research. In addition, the processing of cells for TEM required a series of procedures including fixing, dehydrating, etc. These processing could cause the shrinkage of cells. The present results also showed that the cell diameter from TEM images was reduced by 18% compared with that from inverted microscopy. Thus, the final morphological parameters of the A549 organelles in this study were calibrated according to cell shrinkage.

A great deal of short and small microvilli were present on the surface of A549 cells and lamellar body were within cytoplasm, which were consistent with its characteristics which closely matched type II alveolar cell phenotype [2]. Some differences were observed mainly on lysosomes. Most of lysosomes within A549 cell belonged to autolysosomes containing cellular debris. Autolysosomes might imply active metabolism and fast growth of cells [19, 20]. Many large vacuoles were observed in A549 cells. It has been suggested that vacuoles were indication of abnormal or delayed differentiation [21].

Although A549 cells has been widely used as research models regarding lung epithelial structure and the occurrence, development, and treatment of lung cancer. However, to our knowledge, the morphometric parameters about A549 cell in this paper were firstly reported. More morphometric characteristics about the ultrastructure of A549 cells could be comprehensively understood by application of some new morphometric parameters, for example regular form factor [22, 23] and even some the structures of molecules, such as protein and mRNA could also be accurately analyzed if combining other molecular biology techniques to morphometric techniques [24–26].

What was A549 cell like? This question has no any answers. The present study found that (1) The diameter of A549 cells from inverted microscopy and TEM images was 14.93 μm and 10.59 μm . (2) By defining cell as reference space the volume densities (V_V) of nucleus and cytoplasm were about 0.28 and 0.72; the surface densities (S_V) of nucleus were 0.19 μm^{-1} within an average A549 cell. By defining cell nucleus as reference space the V_V of nucleoli, euchromatin and heterochromatin were 0.076, 0.72 and 0.20 respectively; the S_V of nucleoli was 0.15 μm^{-1} . By defining cytoplasm as reference space the V_V of mitochondria, lamellar bodies and lysosomes were 0.046, 0.025 and 0.014; the S_V of mitochondria, lamellar bodies and lysosomes were 0.60 μm^{-1} , 0.36 μm^{-1} and 0.18 μm^{-1} ; the number densities (N_V) of mitochondria, lamellar bodies and lysosomes were 0.30 μm^{-3} , 0.56 μm^{-3} and 0.12 μm^{-3} respectively. (3) In individual A549 cell total volume and surface of mitochondria was 61.91 μm^3 and 1001.67 μm^2 ; Total volume and surface area of lamellar body was 76.82 μm^3 and 428.68 μm^2 ; Total volume and surface area of lysosome was 21.692 μm^3 and 12.04 μm^2 .

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