Journal of Electron Microscopy Advance Access published February 16, 2012

Journal of Electron Microscopy **0(0)**: 1–5 (2012)
doi: 10.1093/jmicro/dfs001

Biological: Technical Report

Efficient and accurate analysis of mitochondrial morphology in a whole cell with a high-voltage electron microscopy

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Abstract Mitochondria in all eukaryotes are essential organelles responsible for adenosine triphosphate synthesis, calcium homeostasis and steroidogenesis. Because the structure and distribution of mitochondria are highly diverse depending on their function and cellular conditions, it is important to develop a rapid and accurate method to assess their morphology. In this study, we visualize whole mitochondria in cultured cells using high-voltage electron microscopy (HVEM). Compared with conventional transmission electron microscopic approaches, the present method does not require thin sectioning and thus requires less time for image acquisition and processing. Furthermore, compared with fluorescence-based light microscopic approaches, our method provides more accurate size information. Thus, we propose that HVEM is a useful tool for rapid and accurate analysis of mitochondrial morphology and distribution in a cell.

Keywords mitochondria, morphology, HVEM

Received 17 August 2011, accepted 14 January 2012

Mitochondria are dynamic cell organelles which play critical roles in the synthesis of adenosine triphosphate and other cellular functions including steroidogenesis, calcium homeostasis and control of apoptosis [1,2]. One of the unique features of mitochondria is their highly dynamic morphology [1]. Under physiological conditions, mitochondria continuously undergo fusion and fission, thereby dynamically changing their morphology from granular forms to network-like tubular forms [3], which is highly related to the function and quality of the mitochondria [4]. For instance, pathological conditions in neurodegenerative diseases as well as cellular stresses markedly affect mitochondrial morphology in terms of length, thickness and subcellular distribution [5]. However, assessment and accurate measurement of mitochondrial morphology are technically challenging.

High-voltage electron microscopy (HVEM) is useful for obtaining high-resolution images from thick biological specimens [6,7]. The high penetrating power of HVEM is an advantage for observing thick biological samples such as cells cultured on a mesh grid and thick-sectioned tissues [8]. However, systematic morphological analysis of the mitochondria in a cell using HVEM has not yet been reported. In this study, we optimized a technique for HVEM-based imaging of mitochondria in cultured whole cells without physical sectioning, thus successfully identifying and measuring mitochondria.

For this method, we modified coverslips by punching holes in Thermanox® plastic coverslips (TPC, 13 mm in diameter, NUNC Brand Products, USA), and punched TPC were coated with carbon

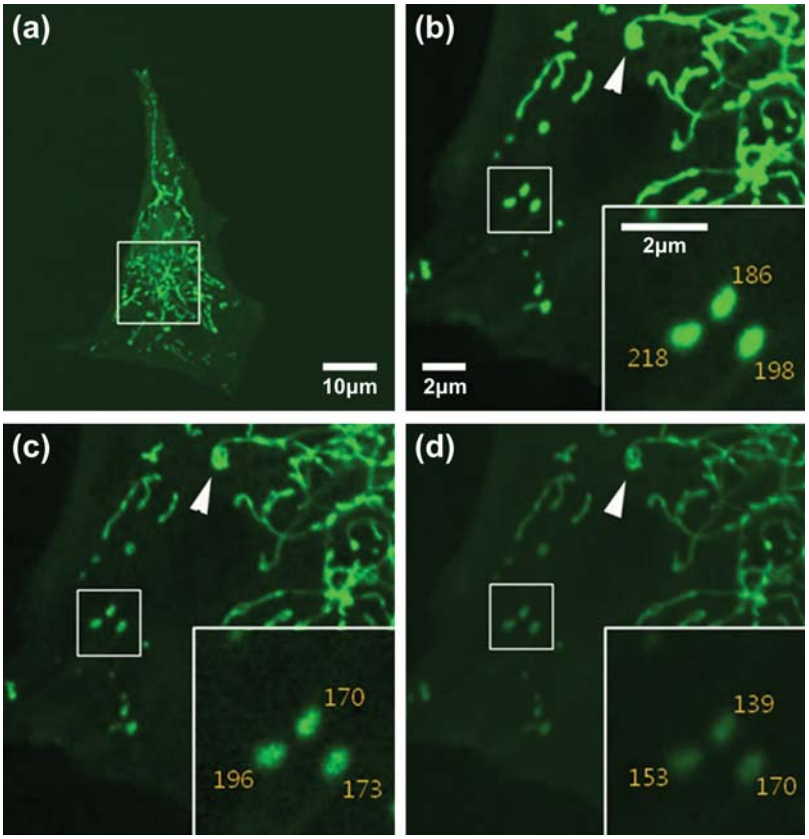

Fig. 1. CLSM images of mitochondria in COS7 cells transfected with pEGFP-mito plasmids. Mitochondria were easily identified by enhanced green fluorescent protein (EGFP) expression (a). However, acquisition of images with a different laser intensity showed differences in shape or size of mitochondria (b–d). Insets in b–d show the pixel intensity (counts) of three mitochondria under different scanning conditions. Arrowheads indicate an area which demonstrates the change in mitochondrial morphology depending on the scanning conditions.

in a vacuum evaporator after application of a thick Formvar membrane (3% Formvar 15/95 resin powder in chloroform, EMS #63450-15-7, USA). The TPC were then sterilized by ultraviolet irradiation for 20 min. The sterilized punched TPC were placed in a 12-well cell-culture dish, and HeLa or COS7 cells were seeded and then cultured at 37°C with 5% CO_2.

Cells at 50–70% confluency were rinsed with 0.1 M phosphate-buffered saline (pH 7.4) and fixed with 2.5% glutaraldyhyde (EMS #16310, USA) in 0.1 M cacodylate buffer (CB, room temperature) for 30 min. Post-fixation was executed with 1% osmium tetroxide in 0.2 M CB for 30 min at room temperature and then washed with 30% ethanol for 30 min. Fixed coverslips were stained with 0.1% uranyl acetate (EMS #22400, USA) in 30% ethanol for 30 min at room temperature. The coverslips were then dehydrated through an increasing series of ethanol and then freeze-dried. Single-slot grids (EMS #2010-NUM 100/vial, USA) were coated with 1% mesh cement (1% neoprene solution in toluene). The grids were laid over the punched holes on the TPC, lifting up the cells on the formvar membrane. The grids were stored in desiccators before observation. Specimens were observed under a Hitachi H-1250M (Tokyo, Japan) or a JEOL JEM-ARM 1300S (Tokyo, Japan) at an acceleration voltage of 1000 kV. We constructed cell montages from multiple images at 3000–5000 magnification, using Adobe Photoshop 7.0. The cell-montage images were analyzed using Scion image software (Scion Corporation, USA). We measured the parameters of mitochondria in each category as follows: The length of linear mitochondria was measured from tip to tip, the length of branching mitochondria was taken as the sum of all branch lengths, the median circumference was measured for circular mitochondria and the diameter of ball-shaped mitochondria was measured.

For expression of mitochondria-targeted enhanced green fluorescent protein (EGFP), pEGFP-mito

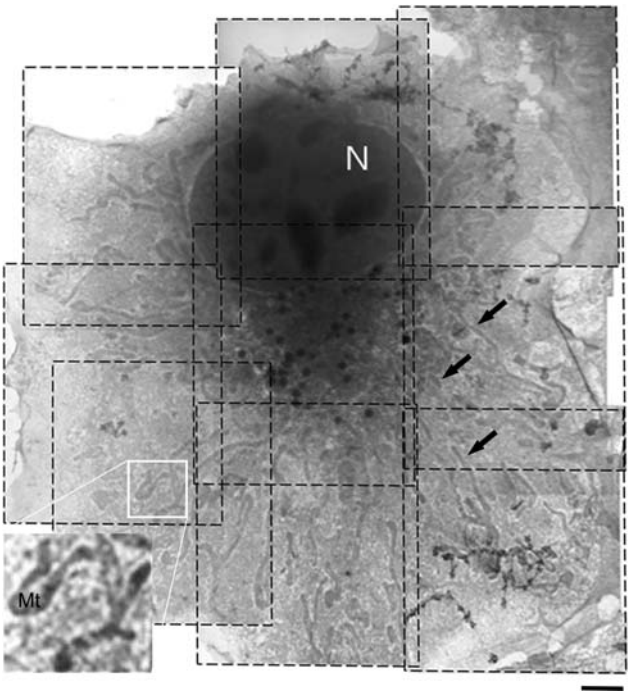

Fig. 2. Cell montages of higher-magnification HVEM images. Nine images were sufficient to cover an entire COS7 cell. Mitochondria (arrow) are readily identifiable (enlarged in the inset). Scale bar = 1 μm. N = nucleus; Mt = mitochondria.

(Clontech, Japan) was transfected with Lipofectamine 2000 (Invitrogen #11668-019, USA) according to the manufacturer's protocol. Thus, COS7 cells were placed onto 12-mm glass cover slips, and a mixture of plasmid DNA (0.8 μg) and lipofectamine (2 μl) in Opti-MEM (50 μl, Invitrogen #31985, USA) was added to the media. The cells were then incubated for 1 h. After replacement with fresh media, the cells were incubated for an additional 24 h. The cells were then fixed with 4% paraformaldehyde, and GFP signals from the mitochondria were observed by using confocal laser scanning microscopy (CLSM: LSM 510, Carl Zeiss, Germany).

Distribution of mitochondria in COS7 cells was readily detected by CLSM using mitochondria-targeted GFP proteins (Fig. 1). CLSM is useful for the rapid visualization of fluorescent probes or proteins which selectively label mitochondria. Time-lapse live imaging could be performed to understand the dynamics of mitochondrial morphology. However, the resolution of CLSM was inferior to that of conventional transmission electron microscopy (TEM), and it was difficult to discriminate different shapes and sizes of

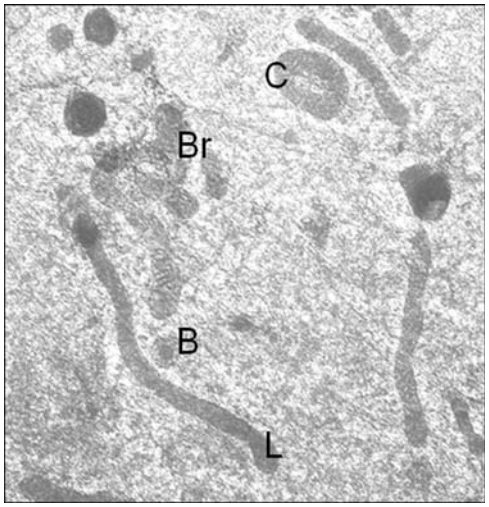

Fig. 3. Representative image exhibiting different shapes of mitochondria. Mitochondira are classed into four categories: L = *line* form; B = *branch* form; C = *circle* form and B = *ball* form.

mitochondria by CLSM because of the effect of different scanning parameters, in particular, the laser intensity (Fig. 1).

With TEM, high-resolution images suitable for observation of mitochondrial size and structure can be obtained [9]. However, a single mitochondrion must be cut into numerous ultrathin sections for TEM imaging, and alignment and reconstruction of the serial images are required to obtain shape information for a whole mitochondrion. Because HVEM has strong penetrating power, most monolayer-cultured eukaryotic cells can be examined as whole mounts, without sectioning. Thus, HVEM provides high-resolution imaging and increased depth information [6].

We obtained mitochondrial information from whole-cell montages of 8–9 higher-magnification images (Fig. 2). Individual mitochondrial morphology was readily identifiable in the cellular peripheral zone, and we analyzed only these mitochondria. In the perinuclear region, mitochondria are packed closer together, and it is difficult to identify the morphology of an individual mitochondrion. Furthermore, mitochondria lying in the projection direction above or below the nucleus are masked by the shadow of the nucleus. This limitation may be overcome by applying serial-section electron tomography with HVEM [10]. However, serial-section electron tomography requires much more time and effort than our current method. Another limitation of whole-cell HVEM is difficulty

Table 1. Morphometric characteristics of mitochondria (Mt) in two kinds of culture cells

Parameter		HeLa cell		COS7 cell (n = 15)	
		#/cell	(%)	#/cell	(%)
Number of Mt/percentage of Mt	L	55.87 ± 6.03	61.59 ± 1.76	73.4 ± 8.99	60.75 ± 2.11
	Br	11.07 ± 1.87	11.85 ± 1.38	14 ± 2.98	10.75 ± 1.56
	C	3.47 ± 0.64	3.99 ± 0.69	6.27 ± 1.35	7.10 ± 1.92
	B	19.53 ± 1.71	22.57 ± 1.33	24.27 ± 2.37	21.39 ± 1.00
Percentage of total Mt area/measured cell area (%)			5.51 ± 0.608		6.115 ± 0.447
Ave. length of Mt (μm)	Total Mt	1.399 ± 0.1094		1.399 ± 0.0025	
	L	1.42 ± 0.12		1.53 ± 0.1	
	Br	2.62 ± 0.23		3.23 ± 0.35	
	C	1.31 ± 0.13		1.50 ± 0.11	
	B	0.30 ± 0.02		0.36 ± 0.2	
Ave. width of Mt (μm)		0.15 ± 0.002		0.20 ± 0.003	

There were no significant difference in the distribution ratio of the mitochondria shapes between HeLa cell and COS7 cells (L, line form; Br, branch form; C, circle form; and B, ball form). All data are represented as mean ± SD.

in investigating details of internal mitochondrial structure (cristae, the matrix and the inner membrane).

We were able to classify mitochondria into four categories based on their shapes: *line*, *branch*, *circle* and *ball forms* (Fig. 3). Previously, mitochondria in human diploid fibroblasts were classified by HVEM into three categories: *branched*, *linear* (non-branched) and *circular* [11]. However, we found ball-shaped mitochondria with both CLSM and HVEM , so we added this category. The *linear* form was most abundant in HeLa cells (61.10 ± 6.85%) and COS7 cells (60.75 ± 8.16%), whereas the *circular* form comprised the smallest fraction in both cell types (3.40 ± 2.54% in HeLa; 7.10 ± 7.43% in COS7; Table 1). In addition to morphological classification of the mitochondria, we could also be able to accurately measure their width and length on the HVEM images as a pilot approach (Table 1). In this study, we presented preliminary data on mitochondria in whole-mount cells. The volume proportions of mitochondria were 5.51 ± 0.608 and 6.115 ± 0.447% in HeLa and COS7 cells, and the average mitochondrial width was 0.15 ± 0.002 and 0.2 ± 0.003 μm in HeLa and COS7 cells, respectively. This is consistent with a previous report in human fibroblasts [11]; however, the average length of mitochondria in HeLa and COS7 cells was substantially smaller than that in human fibroblasts [11], but similar to pancreatic beta cells [12]. The proportion of the branched type of mitochondria was 30–55% in human fibroblasts [11], but we found that mitochondria in this category represented only 10% of the mitochondria studied in HeLa and COS7 cells, similar to that of pancreatic beta cells evaluated by serial-section electron tomography (6–10%) [12]. These results emphasize that mitochondrial morphologies are different depending on the cell type.

In summary, we successfully applied HVEM techniques to the characterization of mitochondrial shape and the measurement of mitochondrial size, using whole-mount cells. This technique required less time and effort than TEM. In addition, compared with CLSM, HVEM does not require labeling procedures and, therefore, the images are independent of the quality of flouorophore and the setting of scanning intensity. This method may be applicable to mitochondrial morphometrical studies related to physiological and pathological conditions.

Acknowledgement

We thank Dr K. Hama for critical comment on experimental design and comments on the manuscript.

Funding

This work was supported by Korea University Grant (to I.J.R.), KOSEF grant 2009-0077120 (to I.J.R.) and KOSEF grant 20100000143 (to W.S.)

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