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Quantification of Mitochondrial Morphology in situ

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Abstract—The structural organization of mitochondria reflects their functional status and largely is an index of cell viability. The indirect parameter to assess the functional state of mitochondria in cells is the degree of their fragmentation, i.e., the ratio of long or branched mitochondrial structures to round mitochondria. Such evaluations requires an approach that allows to create an integral pattern of the three-dimensional organization of mitochondrial reticulum using confocal images of mitochondria stained with a fluorescent probe. In the present study, we tested three approaches to analyzing the structural architecture of mitochondria under normal conditions and fission induced by oxidative stress. We revealed that, while the most informative is a three-dimensional reconstruction based on series of confocal images taken along the Z-dimension, with some restrictions it is plausible to use more simple algorithms of analysis, including one that uses unitary two-dimensional images. Further improvement of these methods of image analysis will allow more comprehensive study of mitochondrial architecture under normal conditions and different pathological states. It may also provide quantification of a number of mitochondrial parameters determining the morphofunctional state of mitochondria—primarily, their absolute and relative volumes—and give additional information on three-dimensional organization of the mitochondrion.

Keywords: mitochondria, fragmentation, confocal microscopy, 3D modeling

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INTRODUCTION

In addition to ATP generation, mitochondria perform many different functions, such as the regulation of intracellular redox status, biosynthesis of a number of metabolites and regulators, thermogenesis, regulation of programmed cell death, detoxication, etc. (Zorov et al., 1997). Mitochondria are involved in pathological processes, such as ischemic damage and diseases, which are the subject of mitochondrial medicine (Zorov et al., 2013), as well as yet not belonging to mitochondrial medicine, such as Alzheimer's and Parkinson's diseases (Friedland-Leuner et al., 2014; Picone et al., 2014; Wang et al., 2014; Benek et al., 2015; Silachev et al., 2015; Cenini and Voos, 2016). Protection of mitochondria will be in the near future a clinically relevant approach to treatment of these pathologies. The analysis of mitochondria functional state and structural organization is required to understand their role in normal and pathological cellular processes. Mitochondrial size and shape are not stable, and a balance exists between mitochondria fragmentation and fusion processes (Frederic and Chevremont, 1952; Bakeeva et al., 1978; Smith and Ord, 1983). The balance is drastically shifted toward fission (fragmentation) under pathological conditions, in which almost the entire population of mitochondria takes the form of small round organelles (thread-grain transition) (Vorobjev and Zorov, 1983; Skulachev et al., 2004; Chan, 2006).

These structural transitions are determined by the cellular redox state. It has been demonstrated that oxidative damage to kidney both in culture and in the organ is accompanied by mitochondrial fission, which may be the first sign of cell degradation (Plotnikov et al., 2007, 2008). The dynamic balance is critical not only for normal cell functioning, but is a key mechanism underlying the reparation of damaged mitochondria. Evaluation of the mitochondrial fission and recovery rate may help to find the mechanisms of pathology development and confirm the efficiency of therapy.

The current technology for assay based on use of images captured by conventional fluorescent or laser-scanning microscopy allows one to quantify the parameters determining mitochondrial morphology

Abbreviations: IR—ischemia–reoxygenation, SkQ1—10-(6'-plasto-quinonyl) decyltriphenylphosphonium, TMRE—tetramethylrhodamine ethyl ester.

and state. Moreover, software analysis provides qualitatively new information that is difficult to obtain by visual observation. Computer-based methods of analysis also avoid subjectivity and significantly save time.

There are two main approaches to the quantification of mitochondrial fission in cells: two- and three-dimensional. Both are based on fluorescent labeling of mitochondria. This can be done either by using fluorescent probes accumulated in mitochondria depending on the membrane potential or genetically encoded fluorescent proteins with targeted (mitochondrial) localization. For the two-dimensional method, a single fluorescent image of the cell taken with a high resolution is sufficient for the analysis. Three-dimensional methods require a so-called "Z stack": a set of confocal frame images of cells along the Z axis corresponding to the cell thickness collected with the laser scanning confocal microscopy.

In this work, we applied both algorithms to evaluate mitochondrial morphology in cells after ischemia and compared them using the widest possible set of parameters of the mitochondrial morphofunctional state.

MATERIALS AND METHODS

Two- and semi-three-dimensional analysis. For two-dimensional analysis, we applied standard algorithms for "particle analysis" of the ImageJ (NIH, United States) and Fiji (LOCI, United States) software. We also used a specially designed algorithm for analysis of mitochodrial fission (Herbert et al., 2014; Lautenschlager et al., 2015). This algorithm distinguishes mitochondria in two-dimensional coordinates and estimates the mitochondria length and cytoplasm area (excluding the area occupied by the nucleus). The algorithm allows one to evaluate the average length of mitochondria, as well as the average number of mitochondria per cytoplasm area. Using the same program, we performed a similar analysis for images along the cell thickness (Z stack) (semi-three-dimensional analysis). It allows one to collect the information on the length and quantity of allocated mitochondria within the cell volume rather than within the cell area.

Three-dimensional analysis. Mitochondrion configuration was analyzed with an independent Fiji software module (Bolte and Cordelières, 2006). It allocates all fluorescent objects in cells into a single object. Total volume of mitochondria and their integral surface area are analyzed. The total surface area allows to indirectly evaluate the mitochondrial shape: the bigger object is (and its shape closer to a sphere), the bigger is the ratio of its volume to its surface area. The smaller the are objects occupying the same integral volume are, the larger their total surface area and the smaller this ratio is. Thus, a decreased ratio of the volume ratio to the area indicates mitochondrial fission.

For three-dimensional analysis with automatic segmentation, i.e., allocation of single mitochondria, we applied a "spot segmentation" tool (Ollion et al., 2013). It is essential to gain information on the size, shape, fluorescence intensity, and other parameters of single mitochondria, in particular, parameters analyzed with the algorithms described above.

Experiments using cultured cells of kidney epithelium. Kidneys were aseptically removed from 3- to 7-day-old rats, minced, and placed into Hank's solution, pH 7.4. The tissue was washed and incubated with 0.1% collagenase in Hank's solution for 30 min at 37°C. Large tissue fragments were discarded, and the supernatant was centrifuged at 200 *g* for 3 min. The pellet was resuspended in DMEM/F12 (1 : 1) medium and kept in the tube for 10–12 min. Then, the pellet with kidney tubule cells was collected. The cells were cultivated in DMEM/F12 (1 : 1) medium with 10% fetal bovine serum until 60–70% confluence was achieved and used in experiments. Ischemia was induced by nitrogen purged Hank's solution to make it anoxic. The cells were cultivated in this anoxic solution for 24 h at 37°C. Then, the solution was repalced by the standard culture medium, causing reoxygenation. To evaluate the mitochondrial transmembrane potential, the cells were incubated with 200 nM tetramethylrhodamine ethyl ester (TMRE) for 30 min. Mitochondria-targeted antioxidant SkQ1 (Bakeeva et al., 2008) was used as a protective agent. The cells were incubated with 120 nM SkQ1 for 6 days before the onset of ischemia. Images were taken by an LSM510 laser scanning microscope (objective Plan-Neofluar 63×/1.25 Oil, excitation at 543 nm, emission collected within 560–590 nm).

Statistics. About 500 mitochondria were counted in each image. The results are presented as mean and its standard error (SEM).

RESULTS

Two- and semi-three-dimensional analysis of mitochondrial fission. Three image series were captured using confocal microscopy: for control cells, cells that underwent ischemia reoxygenation (IR), and cells that underwent IR with SkQ1 treatment (Fig. 1). Mitochondria were visualized using a TMRE probe. It was found that, in control cells mitochondrial reticulum, was mostly presented by filaments and rods (Fig. 1a); i.e., structures with a high length/width ratio. IR caused visual mitochondria fission with the transition transition into a "grain" state (Fig. 1b). Pretreatment with SkQ1 inhibited this transition. Visual observations do not yield quantitative information on the fission process, and so images were assessed with various software methods. Two-dimensional approach demonstrated that ischemia induced mitochondrial fission. Average mitochondrial length was diminished (Fig. 2a), whereas the number of short fragments was increased (Fig. 2b). Mitochondria-targeted antioxi-

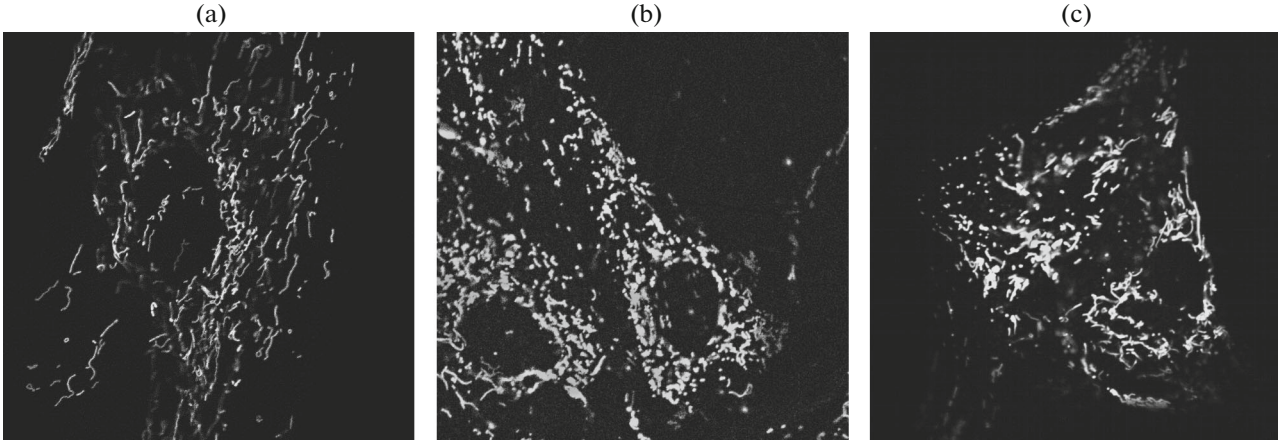

Fig. 1. Confocal image analysis of primary culture of kidney cells loaded with 200 nM TMRE. (a) Control cells, (b) cells exposed to ischemia–reoxygenation (IR), and (c) cells pretreated with 120 nM mitochondria-targeted antioxidant SkQ1 before IR.

dant SkQ1 prevented reduction in mitochondrial length. It should be noted that the total number of fragments in cells treated with the protective agent was diminished (Fig. 2b). Similar changes were revealed using the semi-three-dimensional method and by analyzing of confocal images of single cells along its thickness (Z stack). It was also found that IR notably reduced the average length (Fig. 2c) and number (Fig. 2d) of mitochondrial fragments. SkQ1 treatment prevented this process in cells.

Three-dimensional analysis of mitochondrial reticulum. Using this approach, we found that the total mitochondrial volume normalized to the cell volume dropped after IR. SkQ1 treatment had no effect on this process (Fig. 3a). These results differ from the data gained gained by two- and semi-three dimensional analysis according to which the average mitochondrial length was higher after SkQ1 treatment (Figs. 2a, 2c). The ratio of the mitochondria volume to the mitochondria surface area was diminished after IR (Fig. 3b) but was not changed in cells protected with SkQ1. This suggests that, in cells that had undergone IR, mitochondria are smaller than in control cells or cells protected with SkQ1.

Even without comprehensive analysis, it is clear that the ratio of mitochondrial volume to surface increases, although SkQ1 has not augmented the total mitochondrial volume. This suggests that the mitochondria-targeted antioxidant initiates the mitochondrial fusion.

Three-dimensional analysis with software segmentation of objects showed that average mitochondrial volume had dropped in cells after IR exposure (Fig. 4a) as well as mitochondrial length (Fig. 4b); SkQ1 treatment prevented these changes.

DISCUSSION

In live cells, the mitochondria are usually in the state of a dynamic balance resulting from permanent fission and fusion processes. It corresponds to the name of the structure (*mitos*, thread; *chondros*, grain). The phenomenon of global mitochondrial fragmentation was described in 1983 (Vorobjev and Zorov, 1983). It has been found that, in cells exposed to oxidative stress, the entire population of mitochondria (mitochondrion) is transformed into small fragments. The mechanism of the mitochondrial fission is quite complicated and involves many proteins activated as a response to mitochondrial damage (Poliakova et al., 1995; Skulachev et al., 2004; Chan, 2006; Plotnikov et al., 2007; Zorov et al., 2015). We have suggested that the mitochondrial fragmentation (fission) is the general cellular and mitochondrial response resulting in a drastic increase of mitochondrial fragments. It is an indicator of accelerated segregation of mitochondrial content and utilization of damaged components by autophagy (mitochondrial quality control) rather than a characteristic of cellular degradation.

It becomes clear that three-dimensional organization of mitochondrion is a “reporter” of normal or pathological cell functioning. Given this, it is necessary to assess the three-dimensional mitochondrion organization in an objective and maximally comprehensive way. It can be done using computerized image analysis.

The first obvious advantage of software image analysis is a quantification of various parameters. It may be useful, for example, in the study of protective agents, as was done in the present work. It is possible to distinguish cells with fragmented and not fragmented mitochondria visually, but to distinguish and, moreover, to prove the difference in fragmentation in dozens of percent is impossible without quantitative methods.

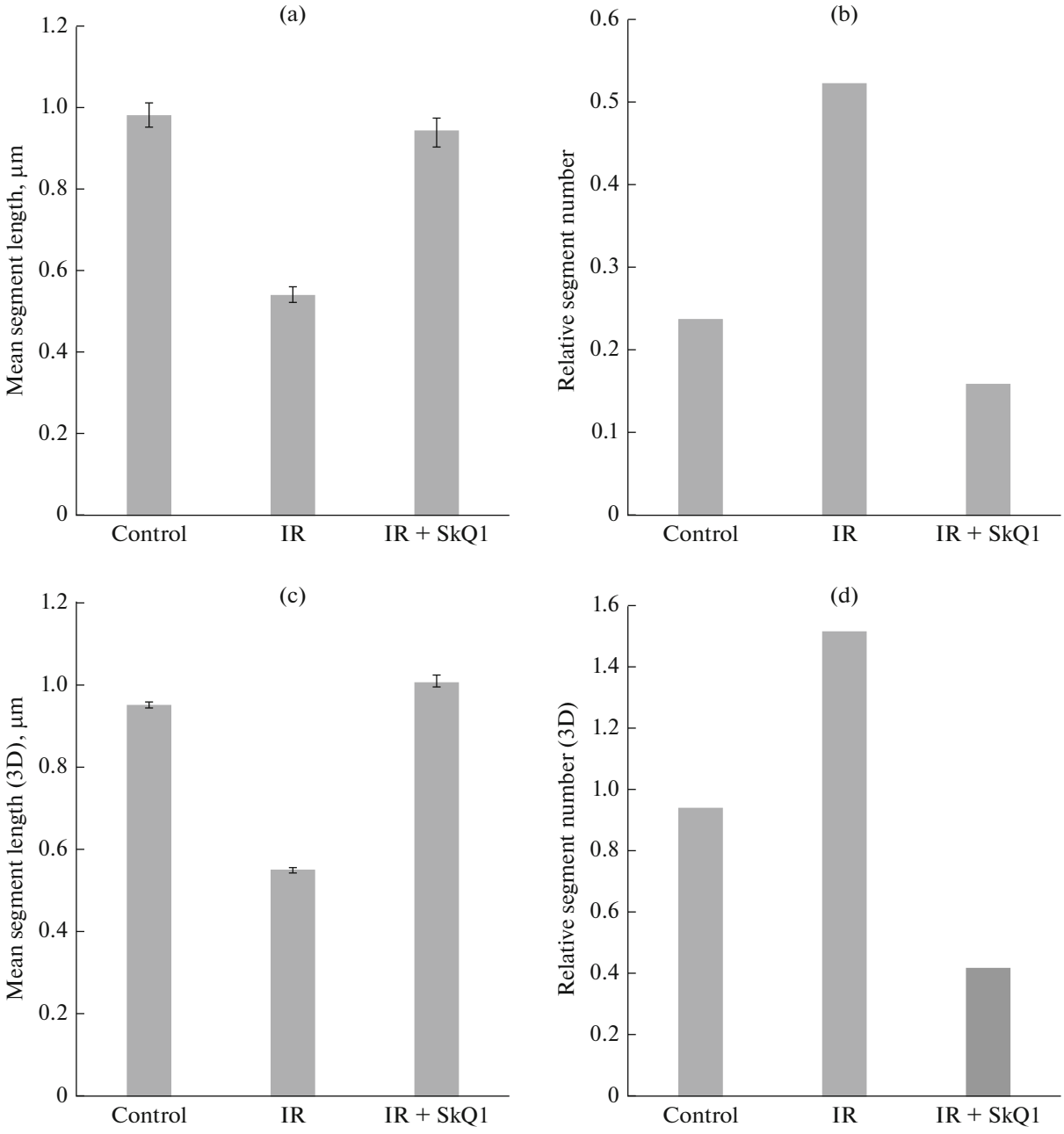

Fig. 2. Parameters of mitochondria defined with algorithms of (a–b) two- and (c–d) three-dimensional analysis of fragmentation in control cells after cell exposure to IR and after IR with SkQ1 pretreatment (120 nM). (a) Mean segment length, (b) segment number relative to cell area (nucleus excluded), (c) mean segment length, and (d) segment number relative to cell volume (nucleus excluded) calculated with an algorithm of semi-three-dimensional analysis (Lautenschlager et al., 2015).

We demonstrated that comprehensive analysis of images can yield fundamentally new information. Quantification of various parameters demonstrated that the mitochondrial morphology recovered after cell treatment with the protective agent (Figs. 2a, 2c, 4a, 4b). However, the agent did not prevent a total drop of the mitochondrial relative volume (or number of mitochondria) (Figs. 2b, 2c, 3a). It gives the details of the protection mechanisms which we would not be able to assume without conducting such an analysis. By analyzing the sample, it can be proposed that the protective agent accelerates mitochondria fusion after the damage but is unable to prevent the damage by itself, because the reduced mitochondrial volume was the same as without the agent protection.

In addition, the parameters applicable for the analysis with algorithms using the mean fluorescence of a single mitochondria and various parameters of mito-

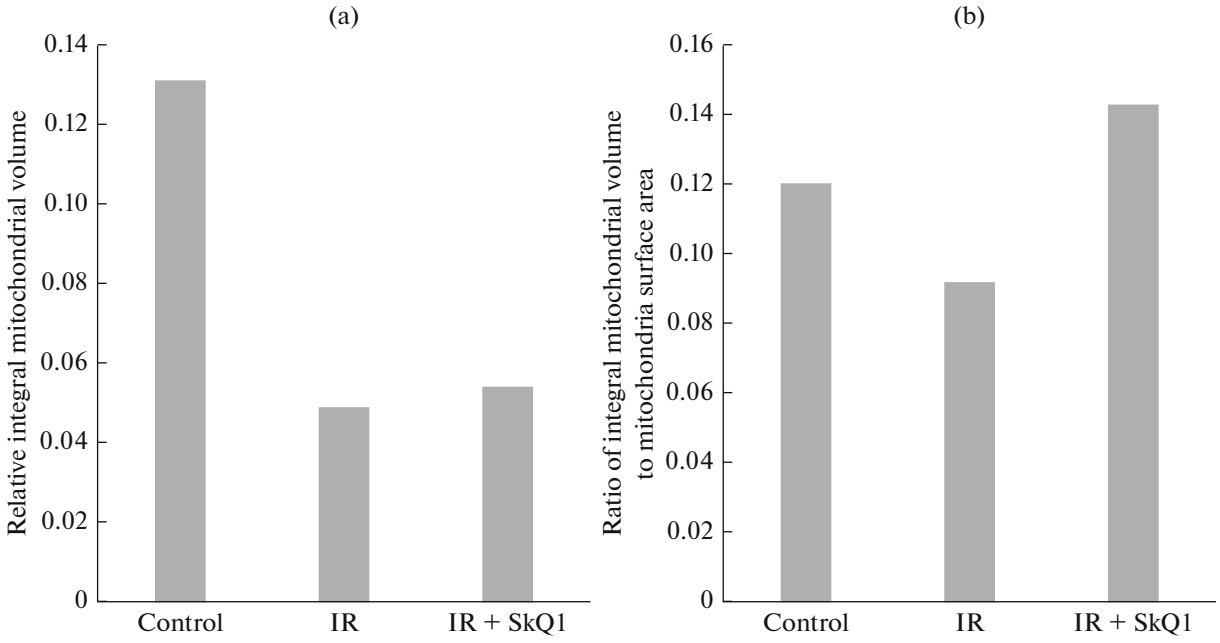

Fig. 3. (a) Integral mitochondrial (MT) volume normalized to the cell volume and (b) ratio of MT total volume to MT integral area occupied with MT profiles calculated with the three-dimensional method (Bolte and Cordelières, 2006). Designations are the same as in Fig. 2.

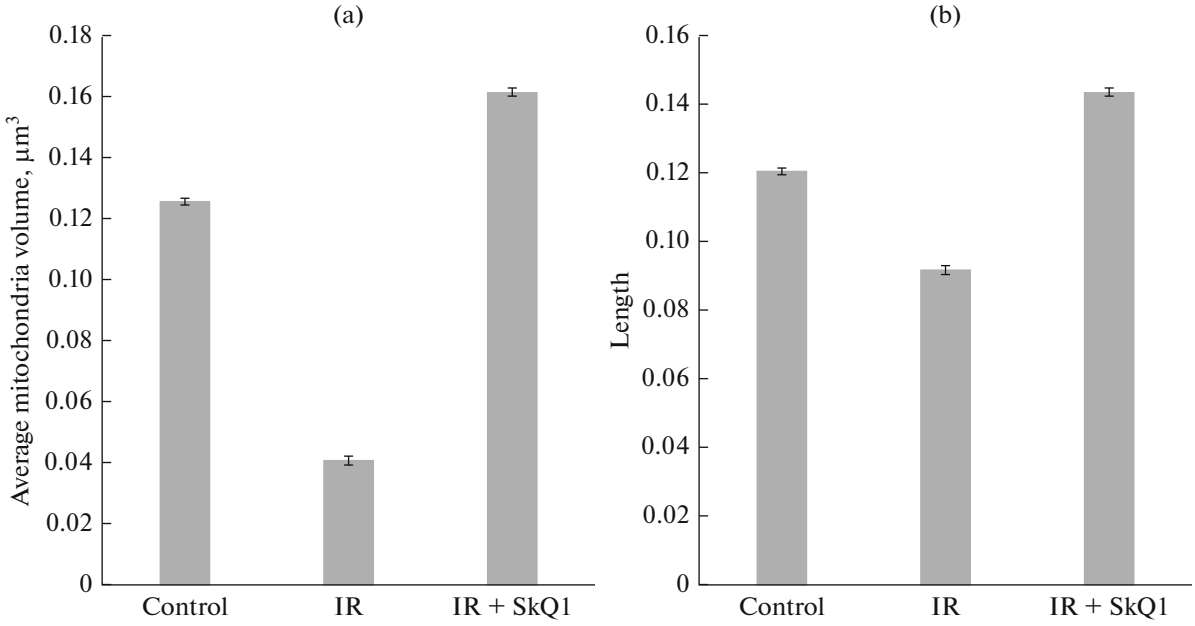

Fig. 4. Mitochondrial average (a) volume and (b) length calculated with a three-dimensional algorithm with segmentation (Ollion et al., 2013). Designations are the same as in Fig. 2.

chondrial "texture" (which could correspond to heterogeneity of the internal mitochondrial content) may be assessed with these algorithms. Thus, it is possible to evaluate the relationship between the mitochondrial size and transmembrane potential (using TMRE) or the level of reactive oxygen species (ROS) assessed with dichlorofluorescein. It is also possible to examine the distribution of these parameters in the mitochondrial population. For example, analyzed cells may have the same mean value of some parameters, but

Properties of analytical approaches to morphometric and functional assessment of mitochondria in cultured cells

Approach	Measured parameters of mitochondria (MT)	Advantages	Defects
Two-dimensional analysis with a single cut (semi-three-dimensional with stacks)	Average length, number per cell area (volume)	Precise identification	Conditional parameters
Three-dimensional analysis of mitochondria	MT integral volume and surface area	Accuracy, biological correctness of parameters	Low level of information, indirect estimation of MT shape
Three-dimensional analysis of single mitochondria	Single MT size, shape, fluorescence	Informative, biological correctness	Inaccuracy
Manual three-dimensional mitochondria outline	Single MT size, shape, fluorescence	Biologically correct, informative, precise	High time and labor consumption

show wider or even bimodal distribution in a particular cell indicating the existence of different mitochondrial populations.

A major advantage of computerized image analysis is that it is fast. Simple and accurate approaches that do not require scrutiny for each image allow hundreds of images to be analyzed in a day. Many images can be handled in a single hour even with careful result testing of complex algorithms. Similar analyses done in the manual mode require tens of hours for a single image.

These methods are not without certain defects. They should be considered in the analysis, although some may be eliminated by a large statistics, which it is easy to obtain automatically. Image quality is an important bottleneck. It seriously affects automatic algorithms and should be stable. It must be remembered that automatic algorithm operations cannot replace the work of the researcher. Artifacts are a great challenge, and researchers should ensure that the processing is carried out correctly. The goal of the research is very important. Some issues can be resolved with conventional relative parameters to compare experimental groups; others require methods for measurements of actual parameters of biological objects. In this study, we used standard software algorithms with different levels of informativeness. Unfortunately, loss of the simplicity of the approach results not only in obtaining more information, but in low processing reliability and increased probability of error. In a table we summarize the main features of each approach.

A two-dimensional algorithm accurately defines the mitochondria configuration in a single section (Lautenschlager et al., 2015). However, two mitochondria visible on a particular cutoff as two separate profiles potentially can fuse above or below the optical slice. This is the main problem of this approach. The cell may have only single highly branched motochondrion with multiple profiles in each optical slice. Thus, this method is adequate to measure relative mitochondria parameters rather than absolute mitochondria parameters on the particular optical section. However, it equally applicable for all experimental groups and, thereby, can be employed for comparison.

Semi-three-dimensional analysis of mitochondria length is an extension of the previous approach to several optical sections. In this approach, the problem mentioned above is the most obvious. Mitochondria in each section are regarded as individual ones; i.e., a mitochondrion that occupies multiple slices is considered as a new one on each slice. Fortunately, in cells attached to the substrate, the mitochondria are located mostly horizontally. It almost excludes the presence of long, vertically arranged mitochondria, which would be analyzed as multiple short ones because they occupy each slice in the cross section. As a whole, both two-and three-dimensional approaches assess an abstract mitochondrion branching rather than absolute mitochondria parameters. Thus, it is correct to apply them only for group comparison.

Analysis of the entire mitochondrion is the most adequate for absolute parameters and precise outline, but is less informative. It allows accurate estimation of the total mitochondria volume but can assess geometrical characteristics of mitochondria only on the basis of circumstantial evidence (the ratio of volume to surface area, which is measured less precisely).

Three-dimensional segmentation of single mitochondria is the most informative and biologically correct, but the least reliable. Its main defects are significant fluctuations and precise identification of single mitochondria, which depend on the image quality, cellular geometrical characteristics, relative mitochondria location, and settings of the processing program. This algorithm can correctly distinguish and analyze single mitochondria, but it is not accurate enough if the distance between mitochondria has diminished. Depending on the settings, it can split a single mitochondrion, as well as combine closely located single mitochondria into one structure. Thereby, it is necessary to reexamine what the algorithm had determined. A certain degree of inaccuracy

will be present in any case, but it must be similar to all experimental groups. For this purpose, the quality of images and parameters of their obtaining should be equal for different groups. It is more correct to have it both groups in not optimal, but identical, quality.

Clearly, the mostly biologically correct and informative approach is identification of each individual mitochondrion in the manual mode. The only objective disadvantage of this approach is the dozen hours that should be spent on the treatment of each mitochondrion image. It practically rules out application of this approach for real research.

In conclusion, automatic analysis of images has significant advantages. It is not time-consuming, are not tedious, are devoid of subjectivity, and allow the information available to be evaluated, as well as providing qualitatively new information that it is impossible to gain visually. Many analogous algorithms are incorporated in free software packages. They are fairly simple to use and, thereby, are readily available to a wide range of researchers. These approaches are applicable not only for analysis of mitochondrial fission in cells, but for many other tasks based on image analysis of biological objects as well.

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