Visualization of mitochondrial membrane potential and reactive oxygen species via double staining

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Abstract

Quantitative and qualitative analysis of both generated reactive oxygen species (ROS) and mitochondrial membrane potential cannot be detected simultaneously. We here introduce a simple, new double staining method. We have successfully used this for several years utilizing cerium for ROS detection and JC-1 staining to assess the mitochondrial membrane potential. The resultant signals on laser confocal images can be localized in the same cells and can easily quantify them. We used a confocal microscope along with our new, combined staining method to both visualize mitochondrial membrane potential (ΔΨm) and imaged ROS. These were quantified by JC-1 staining and by cerium ions with reflectance in a method modified in our laboratory. To test this double labeling technique we used PC 12 cells subjected to 1 h hypoxia and 24 h re-oxygenization. We are able to produce a quantitative analysis of red/green signals of JC-1 that reflected the energy state of the cells. Cerium reflectance correlates with the amount of ROS release in the same cells. Significant differences have been calculated after hypoxia and re-oxygenization in both modality of the cell staining. The red/green ratio was 18.2 ± 9.3 (n = 30) in normoxic cells versus 1.65 ± 0.9 (n = 30) in the hypoxia/re-oxygenation group (p < 0.05). In the same randomly selected cells the average cerium reflectance signal intensity was 2.5 ± 1.2 (n = 30) in the control group while 5.8 ± 3.1 (n = 30) in the hypoxia/re-oxygenation group (p < 0.05). This assay, by characterizing hypoxic injury and re-oxygenization induced ROS production, offers a qualitative and quantitative method to detect the consequences of oxidative stress in experimental conditions and to detect different cell protective strategies.

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Reactive oxygen species play a pivotal role in different neurodegenerative diseases and in ischemic/hypoxic conditions. The reactive oxygen species (ROS) produced by mitochondria is an important factor in cell necrosis and/or apoptosis. By the detection and quantification of ROS we are able to identify an energy crisis of the affected cells and evaluate the efficacy of a range of possible drug intervention strategies. ROS detection is technically difficult, since they react quickly and readily with other molecules. Chemiluminescence’s assays [3], fluorescence-based analysis, enzymatic assays [1], and electron spin trapping methods [14] are frequently used, but they fail to provide a good visualization in individual cells. A recently developed method of ROS analysis [9], offers a high standard visualization by use of a confocal microscope. Utilizing another fluorescence mitochondrial staining method could provide a greater amount of information, which reflects the membrane potential of mitochondria [12]. This combined staining method provides both qualitative and quantitative data on the cellular level.

To test our new, combined staining method, we used a PC 12 cell culture. PC 12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum, 5% horse serum, 2 mM l-glutamine, and antibiotics. Nerve growth factor (NGF) was also a component in the tissue culture medium. The cells were grown on glass (d = 12 mm) on collagen.

In vitro hypoxia was produced by Argon gas using the method published by Kusumoto et al. [6]. Following 1 h of oxygen deprivation the cultures (n = 6) were returned to the normal condition for 24 h (re-oxygenization) [5]. Control cultures (n = 6) were maintained in the incubator. A blood-gas analyzer (ABL Radiometer, Copenhagen) was used to measure oxygen pressure in the medium fluid. The partial oxygen pressure was 154.65 ± 1.35 mmHg (n = 6) in normoxic, and it...
In (D) the high intensity of red signal correspond to the high ROS production. (A, B, C, D) shows identical PC 12 cells from hypoxia/re-oxygenation group. In (B) there is an overlay image of JC-1. The green dominance reflects the collapsed mitochondrial membrane potential. (C) Cerium reflectance red signal is contrasted by green signal of JC-1 monomers for better visualization. (B, D) control. (A) demonstrates the JC-1 overlay image of 522 and 585 nm fluorescence. The red dominance reflects the aggregated form of JC-1, the consequence of mitochondrial oxidation. In both the experimental and control group there were 30 individual cells were measured in six identical cultures. All data was statistically evaluated using the Mann–Whitney test and the Kolgomorov–Smirnov test. The intensity curve was made by SPSS 12.0 (LEAD Technologies, US) statistical program. Data was considered to be statistically significant if \( p < 0.05 \).

The aggregated form of JC-1 molecules (red fluorescence) accumulated in functional mitochondria. On the other hand, the green fluorescence signal from JC-1 monomers is evenly distributed in the cytoplasm. The red/green ratio was 18.2 ± 9.3, while in the hypoxia/re-oxygenization group this ratio was as low as 1.65 ± 0.9. This difference was significant \(( p < 0.05 )\). The cerium reflectance appeared as fine red signals in the cells. The distribution and location of ROS was evaluated in the same cells where JC-1 signals were detected (Fig. 1C and D). The average signal intensity was 2.5 ± 1.2 \(( n = 30)\) in the control group while it was as high as 5.8 ± 3.1 \(( n = 30)\) in the hypoxia/re-oxygenation group \(( p < 0.05 )\).

There was an inverse relationship between ROS signal and JC-1 ratio. A low JC-1 ratio means that there will be a low amount of the aggregated form of JC-1 in the mitochondria and this correlates with a high amount of ROS (Fig. 2). Our double-labeling data correlated well with the PI staining which represents the cell injury level. The percentage of the PI positive cells after hypoxia/re-oxygenization was about double as it was in the normoxia group (43.5 ± 3.2% versus 18.7 ± 2.3% \(( n = 12, p < 0.05 )\)).

We used a combination of non-toxic JC-1 staining to detect mitochondrial membrane integrity and cerium to visualize ROS. The new, combined staining procedure here follows. DMEM was removed and 300 \( \mu l \) of 20 mmol/l CeCl\(_3\) solution (dissolved in lactated Ringer) for 2 min. The CeCl\(_3\) solution was removed, and then the cell culture was rinsed with saline again. Next, the cells were fixed in a 0.25% buffered glutaraldehyde solution for 2 min. The fixed cells on glass were covered with the Vectashield mounting medium for fluorescence study (Vector Laboratories, Inc. Burlingame, CA) and put on glass slides.

Fig. 1. High power confocal micrographs represent cerium reflectance and JC-1 fluorescence in PC 12 cells. (A, C) images visualized identical cells from normoxic control. (A) demonstrates the JC-1 overlay image of 522 and 585 nm fluorescence. The red dominance reflects the aggregated form of JC-1, the consequence of well-preserved mitochondrial membrane potential. (C) Cerium reflectance red signal is contrasted by green signal of JC-1 monomers for better visualization. (B, D) shows identical PC 12 cells from hypoxia/re-oxygenated group. In (B) there is an overlay image of JC-1. The green dominance reflects the collapsed mitochondrial membrane potential. In (D) the red intensity of red signal correspond to the high ROS production. (A, B, C, D × 420).
The reduction of membrane potential is accompanied by a decrease in H2O2 production in isolated mitochondria. On the other hand, in cells after hypoxia/re-oxygenation the mitochondrial membrane potential falls and ROS production increases. This could occur by complex I inhibition or oxidant-mediated disruption of membrane integrity.

In our experiment we were able to simultaneously measure these two modalities in the same cells. This relationship has an inverse feature described with this equation: $y = 7.41e^{-0.47}$, where $y$ is the cerium signal and $x$ is the JC-1 ratio.

This novel double staining method is easily reproducible. Furthermore, it is a good tool for screening neuroprotective, ROS blocking, drug-candidate molecules.

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References