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Mitochondrial imaging in dorsal root ganglion neurons following the application of inducible adenoviral vector expressing two fluorescent proteins

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Abstract

Mitochondrial morphology and dynamics are known to vary considerably depending on the cell type and organism studied. The objective of this study was to assess the potential application of adenoviral-fluorescent protein constructs for long-term tracking of mitochondria in neurons. An adenoviral vector containing two fluorescent proteins, the enhanced green fluorescent protein (eGFP) targeted to the cyto-plasm to highlight the neuronal processes, and the red fluorescent protein (RFP) directed to mitochondria under the control of an inducible promoter, facilitated an efficient and accurate method to study mitochondrial dynamics in long-term studies. Dorsal root ganglion neurons from rat embryos were cultured and infected. The infected neurons exhibited green fluorescence after 24 h, while 16 h following induction with doxycycline, red fluorescence protein began to localize within mitochondria. The red fluorescent protein was transported into mitochondria at the cell body followed by distribution within processes. As the neurons aged, the expression of red fluorescent protein was confined to cytoplasmic vacuoles and not mitochondria. Further analysis suggested that the cytoplasmic vacuoles were likely of lysosomal origin. Taken together, the current study presents novel strategies to study the life history of cellular organelles such as mitochondria in long-term studies.

Keywords

Mitochondria; Dorsal root ganglion (DRG); Adenovirus; Enhanced green fluorescent protein (eGFP); Red fluorescent protein (RFP); Lysosomes

1. Introduction

Mitochondria are double membrane tubular organelles routinely depicted as oval-shaped structures that are dispersed throughout the cytoplasm of all eukaryotic cells. They are believed to have many functions including aerobic respiration, calcium buffering and coordination of cell death (McBride and Wasiak, 2006; Brown et al., 2004; Kroemer et al., 1998; Nasr et al., 2003). Mitochondria play a critical role in cellular physiology and homeostasis and are implicated in several degenerative disorders (For review see Orth and Schapira, 2001). One of the earliest reports concerning mitochondrial dynamics was that of Lewis and Lewis (Lewis, 1914). Since then, the morphology and dynamics of mitochondria have been extensively

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investigated in various models under different conditions using numerous techniques. One such technique has been the application of fluorescent dyes; however, fluorescent dyes are susceptible to photobleaching phenomenon and cannot be utilized in living cells during long-term studies without compromising the normal physiological functions (Laurent et al., 1994; Ligon and Steward, 2000). Hence, the application of fluorescent proteins has increasingly become a vital tool to study organelle dynamics and morphology.

Neurons are one of few cells that display long cellular processes and provide an excellent model to study mitochondrial dynamics through the length of neuronal processes in cell culture. We were therefore prompted to reexamine mitochondrial distribution, dynamics and morphology utilizing an adenoviral vector containing two fluorescent proteins.

Due to their capacity to mediate a highly efficient gene transfer in non-dividing cells, viral vectors have emerged as the primary alternative in genetic manipulations (Davidson and Breakefield, 2003; Callaway, 2005). Since neurons are terminally differentiated cells and difficult to transfect using non-viral strategies, viral vectors have been widely utilized for gene expression in this population. Among the currently available viral vectors, adenoviral vectors have several attractive features. In particular, they can accept relatively large insertions of foreign DNA and allow the infection of a broad range of post-mitotic cells with high efficiency. Previous reports have successfully demonstrated the application of adenoviral vectors in cellular trafficking (Dinh et al., 2005), targeting expression of neurotrophic factors (Torres et al., 2005), as a marker to examine genetically altered neurons (Smith et al., 1997) and to analyze the functional genes in vivo (Romero and Smith, 1998; Wang et al., 2006). In addition, recombinant adenoviral vectors remain epichromosomal, which reduce the possibility of irreversible chromosomal alteration (Mitani and Kubo, 2002; Sato et al., 2002; Amalfitano and Parks, 2002). Furthermore, recent reports have demonstrated promising application for viral vectors to protect and/or reverse neurological impairment associated with lysosomal metabolic disorders such as metachromatic leukodystrophy in animal models (Consiglio et al., 2001; Brooks et al., 2002). These important features make adenovirus a suitable vector system for introducing fluorescent proteins into the neurons. Although the application of viral vectors in the study of subcellular trafficking and organization has also been extensively explored in short-term studies (Dinh et al., 2005; Chillon and Kremer, 2001; Ehrenguber et al., 2001; Serganova and Blasberg, 2005; Szebenyi et al., 2002), relatively little attention has been given to the fascinating insight of mitochondrial life history in long-term studies, from biogenesis to appropriate targeting in cellular environment and ultimately cellular degradation and recycling.

Fluorescent proteins are exceedingly superior tools in real-time analysis of intracellular protein localization and are ideal markers to study mitochondrial dynamics in long-term studies. Here we are presenting a new approach by using two fluorescent proteins within a human adenovirus serotype 5, which is rendered replication defective by the deletion of essential genes for the assembly of infectious virus. In this construct, CMV promoter drives enhanced green fluorescent protein (eGFP) expression, while red fluorescent protein (RFP) is under the control of an inducible tetracycline response element (TRE). The purpose of such an approach is to highlight the axonal processes of neurons over several millimeters with eGFP, while utilizing mitochondrial specific RFP expression to reveal the dynamics of mitochondria within eGFP-highlighted axons. Rat dorsal root ganglion (DRG) neurons were monitored up to 25 days in vitro (DIV) following the introduction of eGFP and RFP utilizing such a construct. Following 48 h of induction with doxycycline, the cells were washed and incubated in doxycycline-free media, and RFP was traced from its expression and localization in the mitochondria to its movement within the neuronal processes and ultimately degradation and recycling in the cell body. RFP-tagged mitochondrial localization signal (MLS) enters mitochondria in the cell body of neurons prior to the mitochondrial entrance in the axons. RFP localization is specific to mitochondria and is stable up to 9 days post-induction at which point the expression of RFP

starts to decline. As aged mitochondria are degraded and recycled, RFP localization is confined within the secondary and ultimately primary vesicles, possibly of lysosomal origin. The localization of RFP-positive lysosomal vesicles in the cell body suggests the cell body as the ultimate sight of degradation and recycling of aged/damaged mitochondria.

2. Experimental procedure

2.1. Generation of adenovirus containing eGFP and MLS-RFP

Plasmids were constructed by subcloning PCR products of MLS (5-ATG CTG AGC CTC CGG CAG AGC ATC CGC TTT TTT AAG CCC GCC ACC CGC ACC CTG-3) fused to RFP from pDs Red2-mito (Clontech Laboratories, Mountain View, CA) that targets the recombinant protein to the mitochondrial matrix using targeting sequence from subunit VIII of human cytochrome c oxidase, with forward 5-CCG GAC TCA GAT CTC GAG ATT ATG CTG-3 and reverse 5-GCT CTA GAG TCG CGG CCG CTA CAG GAA CAG-3 primers. The PCR product was cloned in the XhoI and XbaI sites of a shuttle vector (pShuttle, Stratagene, La Jolla, CA). The resulting construct was linearized with PmeI and cotransformed into BJ5183 (Stratagene, La Jolla, CA) together with pAd-trac-eGFP-TRE for ligation. The transformants were selected for kanamycin resistance, and recombinants were identified by restriction digestion. The recombinant was digested with PacI to expose its inverted terminal repeats (ITRs) and used to transfect HEK293 cells (~70% confluence, Stratagene, La Jolla, CA) using lipofectamine (Invitrogen, Carlsbad, CA) for virus amplification. Successful recombination (pAd-eGFP-TRE-RFP-mito-tag) produced infectious virions that induce the lyses of HEK293 cells. This cytopathic effect (CPE) was monitored microscopically. The total cell lysis in the wells occurred within 10-14 days. Viral DNA isolated from 200 μ l of supernatant from the wells displaying CPE was examined by PCR to determine if virions contain the cDNA of interest (Zhang et al., 1993). Virus containing the DNA inserts was purified and grown in HEK293 cells to produce large amounts of adenovirus. Cell culture supernatant containing adenovirus was concentrated by centrifugation over cesium chloride using the method of Prevec and colleagues (1991). The final concentration of viral particles was measured spectrophotometrically and determined to be approximately 1.9×10^7 vpu/ μ l. The final construct contained eGFP under the control of CMV promoter and MLS-RFP under the control of a TRE (Fig. 1).

2.2. Cell culture preparation

All experimental protocols involving animals were approved by the University of Kentucky Institutional Animal Use and Care Committee and are in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals and the Society for Neuroscience Guidelines for the Use of Animals in Neuroscience Research. Dorsal root ganglion cells were obtained using previously described methods (Smith et al., 1997). Briefly, anesthetized P1 newborn Sprague-Dawley rat pups were decapitated, and their dorsal root ganglion were removed and immediately placed in chilled Hanks solution containing 1% collagenase (Invitrogen, Carlsbad, CA). Following 25 min incubation at 37 °C in a CO₂ incubator, DRGs washed, trypsinized and incubated at 37 °C in a CO₂ incubator for an additional 7 min. Next, DRGs were washed (2 $\times$) with 10% fetal bovine serum (FBS) in DMEM, treated with Dnase (2 mg/ml) and 0.5% gentamycin and dispersed to single neuronal cells by triturating in the presence of nerve growth factor (β -NGF; 100 ng/ml). Cell suspension was centrifuged for 5 min at 4 °C and 1200 rpm. To remove the majority of Schwann cells, cells were preplated twice at 37 °C in 60 mm culture dish for 45-60 min. The supernatant was centrifuged at 4 °C to pellet the neurons. The neurons were resuspended in chilled N₂ media (Invitrogen, Carlsbad, CA) containing β -NGF (50 ng/ml), Penstrep (1%), 50 μ g/ml BSA and FBS (1%), counted with hemocytometer and plated within a cloning cylinder in the center of 35 mm graded culture dish precoated with Matrigel (BD Biosciences, San Jose, CA) at a final

density of approximately 1000 cells/dish. The neurons were allowed to grow for 5 days to extend their processes over long distances prior to infection with $1-1.2 \times 10^7$ vpu/ml of purified adenovirus for 24 h. Cells were washed, refed and incubated at 37 °C for 48 h, at which time the cells were treated with 2 µg/ml of doxycycline (SIGMA) to induce RFP production. After 48 h, cells were washed and replenished with fresh medium without doxycycline. Cells were thereafter incubated at 37 °C under 6% CO₂ for the duration of experiments.

To examine the dynamics of mitochondria in DRG neurons, the neuronal cultures (5 DIV) were treated with equal molar adenoviruses pAd-eGFP-TRE-RFP-mito-tag and pAd-Tet-on vectors. Green fluorescence in neurons was first detected in the cell body of neurons within 24 h following infection, spreading along processes by 48 h at which time neurons were treated with 2 µg/ml doxycycline to induce the expression of RFP for 48 h after which the cultures were washed and replaced with doxycycline-free medium and followed throughout the course of study.

To examine the possible neurotoxicity associated with GFP/RFP expression, the cultures were examined with phase-contrast microscopy at 400×. Random regions of cultures were marked at 400× with a marker and photographed before and up to 7 days after transgene expression. The photographs were compared and changes in the number of viable neurons in each field was calculated and compared to the control cultures that were only transfected with pAd-Tet-on. Only cells with distinct neuronal morphology were included in the analysis.

2.3. 5-Bromo-2-deoxy-uridine (BrdU) labeling and immunocytochemistry

Staining of mtDNA in cultured DRG neurons were made using previously described method by Davis and Clayton (1996). Briefly, DRG neurons were treated with BrdU (Boehringer Mannheim Corp., Indianapolis, IN) to a final concentration of 20 µM for 24 h at 37 °C, and the incorporated BrdU was chased for up to 5 days to identify and track down the recently synthesized mtDNA. Before fixation, cells were treated with Mitotracker Orange CMT (Invitrogen, Carlsbad, CA) at a final concentration of 100 nM diluted in pre-warmed Dulbecco's modified Eagles medium (DMEM) with FBS (5%) for 45 min, followed by three washes with PBS, pH 7.4. After the final wash, the pre-warmed DMEM plus serum containing 3.7% formaldehyde was added, and cells were stored at 37 °C for 15 min. Following fixation, cells were rinsed with PBS three times, permeabilized with ice-cold acetone for 5 min, and rehydrated at 25 °C in PBS and were incubated in 2N HCl at 37 °C for 1 h to denature the DNA. Acid was neutralized with 100 mM borate buffer, pH 8.5, changing the buffer every 10 min (2×). Next, cells were incubated with anti-BrdU, 1:50 (Boehringer Mannheim Corp., Indianapolis, IN) in PBS containing 0.1% BSA overnight at room temperature in a humidified chamber. The next day, after four washing cycles with PBS, the sections were incubated with the secondary antibody (FITC-anti mouse IgG, 1:500) for 1 h, rinsed three times in PBS and coverslipped with Vectashield mounting medium (Vector Laboratories Ltd., Burlingame, CA), and were stored in the dark at 4 °C prior to examination. Fluorescence images of the cell body and processes of identical size were captured and analyzed using ImageJ software (rsb.info.nih.gov/ij). The images of neuronal processes were collected at least 5 µm distal to the cell body. The mean fluorescence intensity of all pixels was calculated and normalized against the background and qualitative analyses were determined as the ratio of fluorescent intensity in the cell body to that of processes over 5 days.

2.4. Lysosomal and mitochondrial staining

To view the older neuronal population that did not further express eGFP, DRG neurons were treated with 20 nM MitoTracker Green FM (Molecular Probes, Eugene, OR) for 45 min prior to microscopic examination. To detect lysosomal vesicles, cells were treated with 50 nM

LysoTracker blue (Molecular Probes, Eugene, OR) for 1 h followed by two washes with pre-warmed medium without the dye prior to the microscopic inspection.

2.5. Movement analysis

Mitochondrial movement was measured as previously described (Ligon and Steward, 2000). Briefly, velocities of mitochondrial movement were determined from images recorded at 20-s intervals 30-60 min of real time. A minimum of five mitochondria was randomly selected per axon from the first frame. The approximate center of each mitochondrion was determined, and the remaining frames of time-lapse were examined. A mitochondrion was considered to have moved if the center was displaced greater than 1.0 μm from its initial position in 60 s (1 min of real time). Only velocities of mitochondria undergoing linear movement for at least three consecutive frames in one direction were included in the. Results are expressed as mean $\pm$ standard error of means (S.E.M.) and statistical analysis were performed by the Student's *t*-test and significant differences were considered at the $p < 0.05$ levels.

2.6. Image analysis

Neurons were viewed under an Axiovert 100/TV microscope (Zeiss) or Leica confocal microscope under constant CO₂ stream on a heated stage (37 °C) and the images were recorded digitally using the Axiovision Imaging System (Zeiss) interfaced through a Pentium computer using Axiovision Software (Carl Zeiss Vision GmbH-v 4.1, Germany). The exposure time was limited to 40 ms and the light path was shuttered between exposures. Both eGFP and RFP were excited with the 488-nm line argon laser and imaged sequentially. Unless otherwise indicated, only morphologically distinct eGFP-positive neurons containing distinct RFP-positive mitochondria were selected for data analysis. Captured images were processed using software ImageJ (NIH; <http://rsb.info.nih.gov/ij>).

3. Results

3.1. DRG neurons in culture

General morphological features of neonatal rat DRG neurons prior to infection (5 days DIV) appeared bipolar with an evident heterogeneity in size and complexity of processes. The shape of neuronal cell bodies varied, ranging in diameter from $20 \pm 6 \mu\text{m}$ ($n = 20$), and appeared as nearly round to an oval shape, with a phase-contrast halo and extensive bipolar neurite outgrowth (Fig. 2A). As neurons aged, the unique bipolar characteristics were altered, and the cells acquired a multipolar morphology (Fig. 2B). The parallel lines incised along the diameter of culture dish promoted the growth of long processes only in one axis that extended over several millimeters away from the cell body.

3.2. Expression of eGFP and mitochondrial specific RFP in dorsal root ganglion neurons

To examine the dynamics of mitochondria in living neurons during long-term experiments, DRG neuronal cultures (5 DIV) were treated with equal molar adenoviruses pAd-eGFP-TRE-RFP-mito-tag and pAd-Tet-on vectors. Adenoviral application routinely resulted in 70-80% transfection efficiency. Green fluorescence in neurons was first detected in the cell body of neurons within 24 h following infection, spreading along the processes by 48 h at which time neurons were treated with 2 $\mu\text{g}/\text{ml}$ doxycycline to induce the expression of RFP for 48 h after which the cultures were washed and incubated in doxycycline-free medium. Red fluorescence was visible in a small region of perikarya approximately 6 h post-induction, gradually increasing in intensity by 16 h. Although a direct diffusion of RFP into mitochondria within the neuronal processes cannot be ruled out, our observations indicate the cell body as the primary site of RFP incorporation into mitochondria (Fig. 3). It should be noted that only 40-50% of all eGFP-positive neurons expressed RFP. This observation was likely due to

contamination from mismatched constructs that were not separated from the viable vectors during purification process. The localization of RFP was specific to mitochondria with negligible cytoplasmic signal, while eGFP signal was maintained in the cell body and processes of neurons (Fig. 4A-C). The maximum induction of eGFP was reached 72 h following infection, gradually decreasing in intensity thereafter likely due to nonspecific loss of transgene expression over time. RFP expression reached maximum levels 9 days following induction at which point the levels gradually declined but were detectable up to 18 days post-infection. By the 19-day post-infection, eGFP levels were not detectable microscopically at which time, if needed we utilized mitochondrial dye for mitochondrial localization purposes. RFP signal was only present sparsely within processes and the cell body as described later (Fig. 5A and B).

To examine possible neurotoxicity associated with GFP/RFP induction, cultures were examined before and after virus infection with phase-contrast microscopy. GFP/RFP expression did not result in significant increase in neuron death as compared to control cultures, which averaged $83.0 \pm 1.7\%$ live cells. The 17% cell death in control cultures includes cell death that occurred 7 days following transfection with pAd-Tet-on vector. During the same time period, neuronal viability in GFP/RFP groups, which were infected with pAd-eGFP-TRE-RFP-mito-tag and pAd-Tet-on vectors was $77.7 \pm 2.8\%$ live cells, indicating that GFP/RFP expression does not significantly alter the survival rate in the current model.

3.3. Mitochondrial movement

The movement of mitochondria in the processes varied considerably. Three patterns of mitochondrial mobility were observed. First, the majority of mitochondria containing RFP (~77%) were stationary with occasional back and forth movement without any net displacement (group A). This population was not included in the measurements of the average mitochondrial velocity as described in the experimental procedure. Second, the mobile mitochondria (~23%) were traveling in both anterograde and retrograde directions (group B) with an average anterograde velocity of $0.93 \pm .03 \mu\text{m/s}$ S.E.M. and retrograde velocity of $0.82 \pm 0.37 \mu\text{m/s}$ S.E.M. (Fig. 6A and B). Although the average anterograde movement of mitochondria was higher than that of the retrograde movement, the statistical analysis did not indicate any significant difference in the velocity of mitochondria in each direction ($p = .15$). Third, by far the smallest group (group C) which was occasionally seen traveling at a very fast velocity in either anterograde or retrograde directions. Due to unpredictability of such a movement, we were unable to precisely determine the velocity of this group of mitochondria; however, the average velocity of six different mitochondria that displayed such movement ranged from 5 to 10 $\mu\text{m/s}$ (4 anterograde, 2 retrograde).

3.4. Mitochondrial shape, length and distribution

The shape of mitochondria within axons varied from elongated slender and sausage-shape, to oval, to round with the length ranging from less than 0.4 to greater than 20 μm with an average size $2.99 \pm 0.74 \mu\text{m}$ (average $\pm$ S.E.M., $n = 204$ mitochondria) (Fig. 7A and B). Mitochondrial distribution within neurons was generally divided into three patterns, (1) mitochondrial clusters in the cell body, axon terminals and some axonal branches, (2) uniform and distinct mitochondrial distribution in long axonal processes, (3) areas devoid of mitochondria. Although we were not able to analyze the mitochondria individually within the highly dense regions, the microscopic analysis of mitochondria within long neuronal processes revealed that within each axon, neighboring mitochondria were capable of independent movement, some occasionally changing direction, while others alternate between periods of movement and immobility.

3.5. Mitochondrial DNA replication

We utilized BrdU labeling as described in the experimental procedure to identify the site of mtDNA replication. The pattern of incorporation of BrdU into mtDNA was characterized by higher fluorescence staining in the cell body 24 h following BrdU incorporation. Chasing BrdU up to 5 days post-staining revealed a broader pattern of BrdU distribution that was characterized by a decreased intensity in the cell body and an increased distribution within cellular processes. The qualitative analyses of BrdU staining within the cell body and the processes suggest a decreased ratio of fluorescent intensity as a factor of time (Fig. 8).

3.6. Mitochondrial degradation

By the 19-day post-induction, the RFP levels were not detectable by spectrophotometry, although microscopically, RFP was localized in small spherical vesicles moving toward the cell body. There was no RFP expression in axon terminals or distal neuronal processes. Since eGFP expression was not detectable at this time-point, we utilized Mitotracker Green FM, which produces fluorescence once accumulated in the mitochondria, to determine whether RFP is localized within mitochondria. The results show RFP localization within spherical vesicles distinct from mitochondria. Lysosomal staining indicated that the vesicles containing RFP were likely of lysosomal origin (Fig. 9). In addition to several large RFP-positive vesicles, many smaller RFP-positive vesicles were also present. Although we were unable to determine the exact nature of such vesicles, it is likely that these vesicles were autophagosomes, which subsequently fused to form larger lysosomal vesicles, in turn, facilitating mitochondrial degradation through the lysosomal machinery.

4. Discussion

Recent advances in molecular biology and the latest technical developments in fluorescence microscopy and image analysis have enabled investigators to perform detailed microscopic investigation within living cells and develop a better understanding of the nature of subcellular structures. While the fluorescent dyes have been routinely utilized and have provided a sensitive tool to study subcellular structures in short-term studies, a serious drawback of using such dyes in long-term studies are potential problems with leakage and photobleaching and altered cellular metabolism (Laurent et al., 1994; Legion, 2000). However, the discovery and cloning of genes encoding the various colors of fluorescent proteins have revolutionized the techniques used to study subcellular components of living cells. Fluorescent proteins, such as RFP and GFP, are natural proteins capable of autofluorescence and can be targeted to various subcellular regions by fusion of their cDNA with a number of short sequencing signals that target the proteins to specific locations, in turn, allowing a distinct visualization of subcellular compartments. A number of reports have suggested that there are neurotoxic effects associated with the application of reporter genes such as eGFP in neurons (Detrait et al., 2002; Hanazono et al., 1997). However, in the present study, the expression of eGFP or RFP did not result in neurotoxicity in rat DRG cell culture model. This apparent inconsistency may be due to the differences in the models that were utilized in each report (e.g. cortical vs. dorsal root ganglion neurons). Furthermore, other reports have also demonstrated that using viral vectors containing fluorescent protein have no detectable toxic effects in vivo (Ghosh et al., 2006; Kelkar et al., 2004; Szebenyi et al., 2002) or in vitro (Vereecque et al., 2004; Smith et al., 1997). A careful assessment of previous reports, in concert with the current findings, suggests that the neurotoxicity effects of fluorescent proteins, which have been reported by others, may only be present under certain conditions in certain cell types.

Neurons are asymmetrical in morphology with long processes extending from the cell body over long distances, thus the subcellular structures that are manufactured within the cell body require traveling long distances before reaching their destination. Although studies of

subcellular structures in living neurons bring with it a host of biological challenges not usually found in other cell types, their unique morphology presents an excellent opportunity to investigate the dynamics of subcellular organelles such as mitochondria. Here, we have demonstrated a combined application of adenoviral vectors and multiple fluorescent proteins to study mitochondrial dynamics within living-cultured DRG neurons up to 29 days in vitro (25 days post-transfection; 23 days post-doxycycline induction).

Since neurons are exceptionally difficult to transfect, the production of a single recombinant adenoviral vector containing two fluorescent markers provides a versatile tool for the expression of two marker proteins in a single neuron concomitantly. The adenoviral vector pAd-eGFP-TRE-RFP-mito-tag contained two fluorescent marker proteins: eGFP was directed to the cytosol and labeled the long neuronal processes and cell body, and RFP that was fused to a mitochondrial translocation signal and was utilized to trace mitochondria within the eGFP-positive processes. A single CMV promoter drove the expression of eGFP, while the expression of RFP within mitochondria was controlled by a Tet-On inducible system, which was switched “on” and “off” to allow chasing the intracellular localization and dynamics of mitochondria over the course of experiment.

Mitochondria have various shapes. The significance of the ability of mitochondria to undergo morphological transformation has been well studied. A comprehensive review of previous reports suggest that changes in mitochondrial morphology may have various causes ranging from a normal physiological response to create an energy efficient mechanism of organelles transportation (Hollenbeck, 1996), to a protective mechanism in order to isolate cellular stress (Szabadkai et al., 2004), to a mode of response or outcome of cellular injury and toxicity (Brocard et al., 2003; Rintoul et al., 2003; Reynolds et al., 2004). Furthermore, cellular injuries may result in the opening of voltage-dependend calcium channel, in turn allowing calcium influx into the neurons (Zhou and Jones, 1996). Massive calcium influx has been closely associated with mitochondrial dysfunction and cellular ATP decline (Nasr et al., 2003), and reduction in cellular ATP is likely to affect both mitochondrial mobility and morphology since both processes are high-energy depended (Rintoul et al., 2006; Chen and Chan, 2006). This explanation is in agreement with previous findings, which have demonstrated a decrease in mitochondrial motility and size due to calcium overloading (Dubinsky and Levi, 1998). In addition, Rintoul and colleagues have demonstrated that the neurotoxicity caused by calcium influx following glutamate treatment significantly affects mitochondrial morphology and mobility (Rintoul et al., 2003). Furthermore, there is convincing evidence suggesting that mitochondrial polymorphism in neurons is likely a response to various physiological demands such as bioenergetic status of cells (Malka et al., 2005; Okamoto and Shaw, 2005; Jensen et al., 2000), Calcium buffering requirements (Szabadkai et al., 2004) or efficient methods of ATP conservation (Skulachev, 2001; Hollenbeck, 1996), which may differ at various time-points throughout the life cycle of cells. Thus, it is likely that such variability in mitochondrial shapes may play a role in maintenance of physiological and homeostatic balance depending on the local bioenergetic demands. Moreover, it has previously been shown that mitochondria exist in two conformational states, orthodox and condensed, and changes in morphology are caused by changes in such conformational states (Bereiter-Hahn, 1990). If this hypothesis is correct, the elongated mitochondria may represent an orthodox state during which the expanded matrix may increase the ATP production and/or reduce energy loss associated with respiration (Bereiter-Hahn, 1990; Skulachev, 2001). Therefore, it is reasonable to suggest that any perturbation in mitochondrial homeostasis that interferes with the mitochondrial energy production is likely to affect both the shape and the movement of mitochondria, and it seems realistic to consider mitochondrial morphology and movement as a reliable indicator for general health, physical condition and cellular energy states of cultured neurons.

Although varied in size and shape, the distribution of mitochondria within neuronal processes was relatively uniform with a higher density of mitochondria in the cell body, axon terminals and some axon branches. Due to the high density of mitochondria within the cell body and nerve terminals, we were unable to analyze the physical connectivity among mitochondrial population in those regions, however, within the neuronal processes, mitochondria appeared as heterogeneous population, capable of sudden changes in morphology and direction of movement.

The higher BrdU incorporation into mtDNA in the cell body supports the previous reports, which identified the proximity of the nucleus as the site of mtDNA synthesis (Davis and Clayton, 1996). However, our observation does not exclude the processes of neurons as a secondary site of mtDNA replication since the higher density of BrdU-labeled mtDNA in the cell body may simply be the result of a higher number of mitochondria in that region. Nonetheless, the reduction in BrdU-labeled mtDNA from the cell body to the processes overtime demonstrates the dynamic distribution of mitochondria from the cell body to the processes. This observation is in agreement with the hypothesis that mitochondrial life cycle starts in the cell body followed by transportation to regions with high-energy demand, and ultimately returning to the cell body for recycling and degradation. This hypothesis was also supported by the observation that in aged neurons, RFP localization shifted from mitochondria to small, spherical vesicles that were consistently found in or moving toward the cell body. As these small vesicles reached the cell body, some of them fused and form larger vesicles. Co-localization of RFP-positive vesicles with lysosomal marker suggests that the degradation of aged/damaged mitochondria occurs within the lysosomes by autophagy. Autophagy is a process of self-digestion of cellular structures initially involving the formation of autophagosomes that subsequently deliver their contents to lysosomes for degradation (Lemasters, 2005; Brunk and Terman, 2002; Klionsky and Emr, 2000; Mortimore et al., 1996). Additionally, reports by Xue and colleagues suggest that mitochondria with low or collapsed membrane potential are likely to release signals that lead to mitochondrial degradation by means of lysosomal machinery (Xue et al., 2001). Our observation is also consistent with previous report indicating that 89% of mitochondria moving toward axon terminals have high membrane potential, while 81% of mitochondria transported toward the cell body display a low membrane potential (Miller and Sheetz, 2004). In the present study, the distinct presence of both morphologically normal mitochondria and lysosomal RFP-positive vesicles suggests that mitochondrial recycling is a normal cellular function utilized by cells to rid themselves of aged and damaged mitochondria. Therefore it is reasonable to speculate that any interruption of such processes may severely compromise the cellular homeostasis, resulting in accumulation of damaged/aged mitochondria in aging. This hypothesis is in accordance with the mitochondria-lysosomal axis theory of aging (Brunk and Terman, 2002).

In Summary, here, we have described an overview of mitochondrial life cycle from its origin in the cell body, to transportation in the cellular processes, and ultimately degradation in the cell body by the lysosomal machinery. Furthermore, we have demonstrated that expressing multiple fluorescent proteins via adenoviral vectors directly targeted to subcellular structure such as mitochondria is a potentially useful technique for identifying cellular cues responsible for subcellular dynamics, shape and distribution under various conditions.

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Fig. 1.

The pAd-eGFP-TRE-RFP-mito-tag vector. A tetracycline response element (TRE) regulates the expression of mitochondrial localization signal (MLS) fused to red fluorescent protein (RFP), while a CMV promoter controls a continuous cytoplasmic eGFP expression. In this construct, eGFP and MLS-RFP genes are combined with adenoviral-mediated gene transfer for high-level transient protein expression in neurons.

Fig. 2. Phase-contrast image of DRG neurons (A) (5 DIV) and (B) (15 DIV) indicates general morphological characteristics of neurons prior and post-infection. The shapes of neuronal cell bodies varied from a nearly round to an oval profile, with a phase-contrast halo and extensive bipolar neurite outgrowth. The parallel lines incised along the diameter of culture dish promoted bipolar growth of long processes in one axis that extended over several millimeters (A). However, as the neurons aged, the processes were extended in many directions, and neurons displayed a multipolar rather than a bipolar morphology (B) (Axiovert 100/TV (A), 40× and (B) 100× oil objective).

Fig. 3. RFP progression following induction with doxycycline. Red fluorescence first appeared at the periphery of nucleus, gradually diffusing throughout the cell body and localizing within mitochondria before entering into the processes (Axiovert 100/TV, 100 $\times$ oil). Scale bar 20 μ m.

Fig. 4.

Photomicrographs of live DRG neurons displaying cytoplasmic green fluorescence and specific mitochondrial red fluorescence following transfection with pAd-eGFP-TRE-RFP-mito-tag. The expression of eGFP highlighted the neuronal cell bodies and processes over several millimeters (A), while 48 h following induction with doxycycline, RFP expression was specifically confined within mitochondria with negligible cytoplasmic signal as mitochondria transported away from the cell body (B). Overlay of eGFP and RFP demonstrates the specific localization of RFP within mitochondria and eGFP to the cytoplasm (C) (Axiovert 100/TV: (A, B) 40 $\times$; (C) 100 $\times$ oil). Scale bar 20 μ m (A), 10 μ m (C).

Fig. 5.

Fluorescent protein expression in DRG neurons. The expression of eGFP gradually increases following infection, reaching its maximum levels 3 days postinfection at which point eGFP levels gradually declines. Twenty days following infection, eGFP expression is not detectable. RFP expression reaches its maximum levels 9 days post-induction and gradually declines thereafter (A). In aged neuron (23-25 DIV), RFP expression is confined to small vesicles that do not co-localize with mitochondria (B). Since the expression of eGFP was not detectable in the older neurons, MitoTracker Green FM was utilized to identify the mitochondria (Leica confocal microscope, 100× oil). Scale bar, 20 μm.

Fig. 6. The movement of mitochondria in the processes varied considerably. The majority of mitochondria remain stationary with occasional back and forth movement (arrowhead). The mobile mitochondria were traveling in both anterograde (long arrow) and retrograde directions (short arrow). Representative pictures were taken 10 min apart (Axiovert 100/TV, 50× oil objective).

Fig. 7.

Mitochondrial shape and length. (A) The shape of mitochondria within axons varied from slender (a), to cylindrical (b), to oval (c), and to round (d). (B) Graph depicts the percentage of mitochondrial length within each indicated population range. The length of mitochondria was determined within ranges less than 0.4 μm to greater than 10 μm with an average length of 2.99 ± 0.74 μm (average $\pm$ S.E.M., $n = 204$ mitochondria; Axiovert 100/TV, 100 $\times$ oil). Scale bar, 10 μm.

Fig. 8.

Mitochondrial DNA staining with BrdU. mtDNA was stained with BrdU for 24 h, and the cells were incubated up to 5 day post-incorporation. One day following BrdU, the maximum intensity was primarily seen in the cell body of neurons (A and B). The intensity of BrdU staining in the cell body gradually decline, while the fluorescent intensity in the processes increased (C, day 3). Graph indicates the ratio of fluorescent intensity in the cell body to that of processes over 5 days. The results suggest that tagged-mitochondrial DNA has redistributed from the cell body to the processes (Axiovert 100/TV, 100 $\times$ oil).

Fig. 9.

RFP localization in aged neurons. RFP is localized in many small and few large vesicles that were distinct from mitochondria. (A) (MitoTracker green), (B) (LysoTracker blue), (C) (RFP) and (D) (overlay of all pictures) indicate that RFP is likely localized within lysosomal vesicles that are distinct from mitochondria (Leica confocal microscope, 100× H₂O). Scale bar, 20 μm.