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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 cytoplasm 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.



## 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 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.,

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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; Ehrengruber 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 μl 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 spectrophotmetrically and determined to be approximately $1.9 \times 10^7$ vpu/μl. 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 $CO_2$ incubator, DRGs washed, trypsinized and incubated at 37 °C in a $CO_2$ incubator for an additional 7 min. Next, DRGs were washed (2×) 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 $N_2$ media (Invitrogen, Carlsbad, CA) containing β-NGF (50 ng/ml), Penstrep (1%), 50 μg/ml BSA and FBS (1%), counted with hemocytometer and plated within a


**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.