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A novel method to study the local mitochondrial fusion in myelinated axons *in vivo*

Chuan-Li Zhang¹, Lance Rodenkirch², Justin R Schultz¹, and Shing Yan Chiu¹

¹Dept. of Neuroscience, 1300 University Avenue, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706

²W.M.Keck Laboratory for Biological Imaging 1300 University Avenue, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706

Abstract

Mitochondrial remodeling (replication, fission/fusion) is a dynamically regulated process with diverse functions in neurons. A myelinated axon is an extension from the cell soma of a fully differentiated neuron. Mitochondria, once synthesized in the cell body, enter the axon displaying robust trafficking and accumulation at nodes of Ranvier to match metabolic needs. This long-distance deployment of mitochondria to axons raises the issue of whether myelinated axons can function independently of the cell body to execute mitochondrial remodeling to match local demands. Mitochondrial fusion has been suggested to occur in axons in simple neuronal cultures *in vitro*. However, whether such events occur *in vivo* in an intact nervous system remains unanswered. Here we describe a novel technique which allows monitoring of mitochondrial fusion in intact sciatic nerve of frog (*Xenopus laevis*). Mitochondrial population was labeled by injecting two different mitotracker dyes (red and green), spatially apart along sciatic nerves surgically and then allow to “meet” *in vivo*. At 24 hrs post-surgery, the sciatic nerves were taken out for mitochondrial imaging at the half-way point. During the post-injection periods, the anterograde-directed Green mitochondria meet with the retrograde-directed Red mitochondria. If fusion occurs, the merged of Green and Red fluorophores in the same mitochondrion will produce a Yellow color in merged images. The labeled mitochondria were observed with a Nikon A1 confocal microscope. Our new mitochondrial imaging method opens an avenue to separately assess the role of local axonal mitochondrial fusion, independent of the cell body of nerve fibers.

Keywords

mitochondrial fusion; mitochondrial remodeling; mitochondrial mobility; multiple sclerosis; myelinated fibers; sciatic nerve

1. Introduction

Mitochondria are mobile organelles classically known to be the key energy producers within cells. However, over the last ten years mitochondrial research has identified an important

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Corresponding author: S.Y. Chiu, PhD, Department of Neuroscience, University of Wisconsin School of Medicine, Madison, WI 53706, USA, Tel: 608-262-6994; Fax: 608-262-6816; Cell: 608-334-6996; schiu1@wisc.edu.

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new function referred to as mitochondrial remodeling (Detmer and Chan, 2007; Baloh, 2008; Benard and Karbowski, 2009). Mitochondrial remodeling is a process collectively referring to the fusion and fission activities exhibited by these organelles. Advance in the field of mitochondrial remodeling has necessitated a conceptual transformation of the function of mitochondria from mere ATP production to the regulation of cell survival and rescue (Chen, McCaffery et al., 2007; Jahani-Asl, Cheung et al., 2007; Tondera, Grandemange et al., 2009; Jahani-Asl, Germain et al., 2010). In the area of cell rescue, a recent paper has suggested that mitochondrial fusion preserves mitochondrial integrity by stabilizing damaging mutation of mitochondrial DNA (Chen, Vermulst et al., 2010). Do myelinated axons have the ability to execute local, autonomous mitochondrial remodeling? Most of the studies on mitochondrial remodeling are performed on simple cell culture systems where it occurs under close range somal control (Detmer and Chan, 2007; Suen, Norris et al., 2008). While it is clear that protein synthesis and biogenesis occur at or near the soma (Kaplan, Gioio et al., 2009), it is unclear whether biogenesis or remodeling of mitochondria can occur locally in myelinated axons *in vivo*, which can be 6 feet away from the soma in large mammals. Indeed, mitochondrial replication has been suggested to occur mainly in the perinuclear region of PC-12 cells (Davis and Clayton, 1996). Further, even if the cell soma provides axons with mitochondria each fully armored with remodeling machinery comprising fusion (MNF1/2, OPA1) and fission (drp1) proteins, activation of this remodeling machinery still might involve additional signaling molecules and co-factor proteins (Benard and Karbowski, 2009) synthesized only in the neuronal soma. Does the soma direct axonal mitochondrial remodeling by synthesizing and sending necessary signaling proteins long-distance, or can axons autonomously trigger local mitochondrial remodeling?

The concept of an autonomous axon functioning independently from a distant cell soma is rapidly emerging. Pioneering works on primary cultures have suggested that mitochondria-specific precursor proteins can be locally synthesized in axons (Kaplan, Gioio et al., 2009) and that mitochondrial fusion/fission occurs in axons (Amiri and Hollenbeck, 2008). However, the extrapolation from *in vitro* to *in vivo* is a major challenge, particularly in the case of myelinated axons. A fully differentiated axon in an intact nervous system is several orders of magnitude longer than its counterpart in culture, has undergone myelinogenesis and operates in a morphological, cellular and biochemical environment unlike any that *in vitro* conditions can mimic.

Here we demonstrate a new technique which allows monitoring the mitochondrial fusion in live sciatic nerve of frog *in vivo*. We believe that this technique will be useful in assessing the role of mitochondrial remodeling in neurological disorders involving axonal pathology.

2. Materials and Methods

Female adult frogs were obtained from Nasco (Fort Atkinson, WI). Mito-Tracker dyes, Rhod-2 and Dimethyl Sulphoxide (DMSO) were purchased from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade, obtained from Sigma (Sigma-Aldrich, St. Louis, MO).

2.1 Differential dye injection in sciatic nerve

All animal experiments were carried out according to the guidelines from Institutional Animal Care and Use Committee (IACUC). Sciatic nerves from 45 female adult frogs were used. All surgical procedures and dye injections were performed at room temperature. The fusion detection technique was modified from our previous studies on mitochondrial motility (Zhang, Ho et al., 2010). Briefly, frogs were anaesthetized by submersion in water containing ethyl 3-aminobenzoate, methanesulfonate (2 g/L water) for 10-15 minutes. Once

anaesthetized, the skin was cut and sciatic nerve was exposed near the sciatic notch (the site where the nerve exits the spinal cord). A sharp glass micropipette (2-5 μm), mounted to a holder and controlled by a fine 3-dimensional manipulator, was used to locally inject MitoTracker-Green-FM (MTG) into the sciatic nerve. At 4 cm downstream, MitoTracker-Red-CMXRos (MTR) was locally injected similarly. The injection pipette contained 0.2-0.4 μl of 0.5 mM Mito-Tracker (dissolved in DMSO), was gently injected under visual guidance through a microscope at 20 $\times$ magnification (Ghabriel and Allt, 1982). The frog was allowed to recover from surgery at 6, 24 hrs post-surgery. The frog was then killed and the sciatic nerves were taken out for mitochondrial imaging at the half-way point (i.e., 2 cm from each injection site).

2.2 Confocal microscopy

Time lapse imaging of individual nerves were performed using a Nikon A1 laser scanning confocal microscope (Nikon Instruments Inc. Melville, U.S.A.). Sciatic nerves, labeled with MitoTracker, were bathed in a frog Ringer solution containing (mM): NaCl, 100; KCl, 3; CaCl_2 , 2.0; MgSO_4 , 1.0; HEPES, 3; NaHCO_3 , 20; and glucose, 10; Sodium pyruvate 2.5; Malic acid 2.5. The solutions were fully oxygenated with 95% O_2 and 5% CO_2 . All mitochondrial imaging experiments were carried out at room temperature (20-22°C). The nerve was mounted on perfusion system on the stage of the microscope. A specimen insert system was installed on the stage of the microscope to keep the nerve submerged in Ringer's solution while being in a stationary horizontal position on the perfusion chamber on the stage. Sequential confocal scanning was performed with a variety of imaging modalities. Galvanometer and resonant scanners are both available on the Nikon A1. We felt the image quality and signal to noise levels were superior with the galvanometer configuration. Therefore the entire study employs this type of time series confocal acquisition. The 488 nm and 561 nm laser lights were used to excite MTG and MTR respectively using a Plan Apo VC 60X 1.4 N.A. Objective. The 488 nm line is generated from a 50 mW Argon laser and the 561 nm laser is a separate diode laser at 10 mW. The optical thickness was set at 0.47 μm which is thin enough to be smaller than the thickness of 1 μm for a typical mitochondrion. The pinhole was set to one airy unit (AU; 1 AU = 0.47 μm) and time lapse images were recorded at one image every second for 3 minutes for each axon. A variety of 2 dimensional time lapse movies and 3 dimensional volume scans (z-series) were recorded for analysis. All time lapse imaging, three-dimensional reconstructions and measurements were accomplished using the Nikon Elements software (Nikon Inc. Melville, New York).

3. Results

The nature of *in vivo* mitochondrial fusion is illustrated Fig. 1A. The fused mitochondrion in a myelinated axon could originate from the soma, where two separate mitochondria fuse to produce a single fused mitochondrion prior to traveling down the axon. Alternatively, the two separate mitochondria could first travel down the axon, and then locally fuse into one in response to local stimuli. Obviously, both mechanisms might contribute to the presence of fused mitochondria in axons, but the latter mechanism of local fusion might be a better adaptive response against local demands.

3.1. Mitochondrial labeling in axons *in vivo*

We are not aware of any published methodology to examine possible mitochondrial fusion in fully differentiated axons *in vivo*. We used the long axons of adult frog *in vivo* where two spatially distinct populations of mitochondria can be labeled to track subsequent mitochondrial fusion events. Fig. 1B represents a schematic diagram of our mitochondrial labeling and imaging method. MTG and MTR were locally injected as indicated at an inter-injection distance of 4 cm along a sciatic nerve under surgery. Each injection site produces a

pure population of uni-color mitochondria that travels retrogradely and anterogradely along axons. Green and Red mitochondria will meet in between the injection sites as the animal recovers from surgery. The key of this technique is to image the consequence of these mitochondrial encounters in freshly isolated nerves taken out at various times (6, 24hrs) after surgery.

All measurements in this work were obtained from morphologically identifiable single myelinated axons (typically 10-20 μm in diameter). Data are reported as Mean $\pm$ SD. Unpaired Student's t-test is applied. $P < 0.05$ is considered significantly different. Fig. 1C shows a typical image of an identifiable myelinated axon with mitochondria labeled with MTG and MTR.

3.2. Mitochondrial fusion in between injection sites

Since qualification of morphological data is difficult, we performed data analysis of mitochondrial fusion with three different methods. First, we count the number of green, red and yellow mitochondria of each axon from 2 dimensional time lapse movies 24 hours after injection. In 94 axons from 25 nerves (one per frog) analyzed, we counted 2420 Green mitochondria (using only the Green fluorescence channel) and 2833 Red mitochondria (using only the Red fluorescence channel). When the two fluorescence channels are overlapped, a total of 494 mitochondria are Yellow (i.e., harbor both Red and Green dyes), yielding a fusion index of $10.4 \% \pm 14 \%$ (Mean $\pm$ SD). Even though many axons are labeled with the red and green mitotrackers, only axons harboring both mitotrackers are selected for fusion analysis. The average number of axons containing both mitotrackers are 3.76 ± 1.2 per sciatic nerve (mean $\pm$ SD; range is 2 to 7 axons per nerve). Second, we confirmed that a yellow mitochondrion is not due to overlap of two moving Red and Green mitochondria by Kymograph analysis (as in Fig. 2C). This method was applied to 9 randomly selected axons (3 nerves) out of the 94 axons. Third, we validate that the yellow mitochondria represent complete fusion by 3 dimensional volume scans (as in Fig. 4). This method was applied to 13 randomly selected axons (4 nerves) out of the 94 axons.

A typical observation of mitochondrial fusion is shown in Fig. 2A. One day after injection, live imaging of freshly isolated nerves in between injection sites reveals, as expected, robust movement of mostly retrograde-directed Red and mostly anterograde-directed Green mitochondria in single myelinated axons. The exciting observation is the presence of Yellow mitochondria in between injection sites (see supplemental movie #1). Fig. 2B shows an elongated Yellow mitochondrion (*) moving into the field of view from the left. Kymograph analysis of mitochondrial movement for this Yellow mitochondrion (Fig. 2C) using combined and separate fluorescence channels revealed complete overlap of Red and Green images as the mitochondrion moves in successive frames. This suggests that the Yellow mitochondrion is not due to overlap of two moving but separate Red and Green mitochondria, but rather results from fusion.

3.3. Live fusion in axons captured at one z-plane

We captured live events fusions in 5 out of 94 axons examined at a fixed z-plane (optical thickness 0.47 μm). The 5 axons are from 5 different nerves of 5 frogs. Fig. 3 shows the evolution (white dotted line) of a mitochondrion that started out Green (in merged images) then underwent fusion and turned Yellow (see supplemental movie #2 and supplemental material).

3.4. Complete fusion demonstrated by 3-D reconstruction

Since we cannot anticipate which mitochondrial encounter will produce fusion, we performed a 3-D reconstruction on mitochondria that are already "Yellow" and stationary

(see supplemental movie #3). Fig. 4A shows several Yellow mitochondria inside an axon that had been differentially injected (MTR/MTG) 4 cm apart 6 hrs earlier. The Image was taken at the half-way point between the injection sites. Fig. 4B shows 3-D reconstructions of the group of Yellow mitochondria viewed at different angles. The reconstructions show Identical Red and Green volume images, suggesting complete fusion (i.e., complete exchange of cell content).

3.5. Validation of fusion with other Red/Green dye combinations

Most of our fusion studies were performed with Green (carbocyanine-based MTG-FM) and Red (Rosamine-based MTR (CMXRos) MitoTrackers. We also demonstrated fusion using another Green/Red dye pair: MTG and Rhod-2 (N = 3 nerves). A kymograph of MTR and Rhod-2 labeled mitochondria after 10 hrs of injection (inter-injection distance 4 cm) is shown in Fig. 4 C. We selected one particular elongated Yellow mitochondrion (Fig. 4 D white arrow), and separately viewed its 3-D reconstructions at different rotation angles (Fig. 4E-G) at high magnifications. Complete mixture of the Red and Green fluorophores is seen, suggesting complete fusion.

3.6. Fused mitochondria are generated by local axonal fusion

3.6.1. Somal contribution excluded by travel time analysis and *in vitro* experiments—In separate studies, we estimated that at an inter-injection distance of 4 cm, the “front” of two mitochondrial populations started to meet at 6 hrs in the middle (Fig. 5A; see legends for experimental details). From this, we infer that at 24 hrs after injection, the two populations of labeled mitochondria will merge significantly in axons (produce fusions), but will not have time to travel to the soma and re-enter axons. For example, the most distal injection site (MTR) is about 7 cm from the nearest DRG soma. For a Red mitochondrion at that injection site to back travel to the soma, undergo fusion there with another Green mitochondrion (back travel from the MTG site), then re-enter the axon and reach the imaging axonal site, would involve a total distance of 12 cm. According to the travel time analysis in Fig. 5A would take ~36 hrs. Hence, any fusion (yellow mitochondria) detected between injection sites up to 36 hrs post-injection should originate from axons. Further, we have observed Yellow mitochondria in between injection sites as early as 6 hrs after injection.

The most convincing evidence to rule out somal contribution to the observed mitochondrial fusion in between injection sites came from *in vitro* experiments performed on “soma-less”, excised sciatic nerves. In these studies, a long sciatic nerve (6-7 cm) was taken out, desheathed and embedded in agar. This “soma-less” and “synapse-less” nerve was differentially injected with MTR/MTG dyes at 4 cm apart, then incubated in the dark at room temperature with oxygenated Frog ringer solution for 1 day. At the end of the incubation, we imaged in the middle of the nerve segment and computed the percentage of Yellow mitochondria. The results show that $30 \pm 14\%$ (N = 10 axons in 3 experiments from 3 nerves) of the mitochondria are Yellow mitochondria. Since these nerves are without a soma, the yellow mitochondria must have originated from local axonal mitochondrial fusion. While these *in vitro* studies have excluded somal contribution, it is unclear whether the increased axonal mitochondrial fusion over the *in vivo* studies ($10.4 \pm 14.6\%$, N = 94 axons in 25 experiments from 25 nerves, $P < 0.005$) reflects a nerve response to severing the soma, or increased frequency of mitochondrial encounter in an excised nerve. Future studies will resolve this issue.

3.6.2. Contamination by free diffusion of MitoTracker dye excluded—At the half-way point between the two injection sites (4 cm apart), a Green mitochondrion may turn yellow, not by fusion with another Red mitochondrion, but by taking up free MTR that

diffuses 2 cm from the other injection site, either by intracellular or extracellular diffusion pathways. This type of cross contamination of dyes can be ruled out. First, intracellular diffusion of free dyes along axons as an artifactual means of producing yellow mitochondria can be ruled out as follows. Intracellular diffusion of free MitoTrackers (MT) along axonal cytoplasm (even if it existed) is slower than the speed of mitochondrial movement. For example, even assuming the best case scenario that free MT (not taken up by mitochondria at the injection site) undergo free diffusion along axons with a free diffusion coefficient of $D = 10^{-5} \text{ cm}^2/\text{sec}$, it would only travel ~1 cm in 10 hrs. This is not fast enough to catch up with the fast moving mitochondria, which would reach a longer distance (2 cm) in less time (6 hrs; Fig. 5A).

Second, extracellular diffusion of free dyes can also be ruled out as a source of artifactually producing yellow mitochondria by the experiment in Fig. 6A & B. We performed a single, local MTG injection into sciatic nerves. At various times (1, 3, 6, 12 and 24 hrs) after injection we performed line scan of fluorescence intensity axially along the nerve bundle with a 4× objective lens ($N = 3$ nerves, Fig. 6A). The normalized profiles peak at the injection site declines with a narrow space of constant of ~1 mm that is quite independent of times after injection. Presumably, any residual extracellular dye at the injection site quickly binds to immobile structure (such as myelin) right after the one-time injection, leaving a permanent spatial record of the limited extracellular free diffusion. This conclusion was verified by high magnification images where we used the labeling of the outer myelin of a single myelinated fiber as the “footprint” of extracellular dye diffusion and followed these “footprint” at increasing distance from the injection site. Fig 6B shows a bright “footprint” (i.e., outermost myelin stain) at 1.0 mm from the injection site. When this same fiber is imaged at 3.0 mm from the injection site, the extracellular “footprint” disappears but not the intra-axonal labeled mitochondria. Thus, both “footprint” and line scan analysis suggest a limited diffusion of free dyes in the extracellular space. The most likely scenario is at the site of the one-time injection, a small fraction of the injected dye (0.2-0.4 μl) enters the axon and labels moving mitochondria over a narrow 2 mm region, while the rest of the extracellular MTG are quickly permanently bounded to fixed structures like myelin. The crucial point is that the site of imaging of the Yellow mitochondria is at a much larger distance of 20 mm, thus ruling out extracellular diffusion of free dyes to artifactually produce yellow mitochondria at such a distance. Besides extracellular diffusion of free dyes, dyes at the injected site could also be carried by blood flow to great distance to artifactually produce yellow mitochondria. We regard this scenario as unlikely, since we also observed yellow mitochondria in an *in vitro* preparation (Fig. 5B; excised, desheathed nerves) where blood flow is essentially non-existence.

Collectively, the various arguments in this section, together with our observation of live fusion events (Fig. 3) in a previous section, make it unlikely that artifacts of dye diffusion could explain our results.

4. Discussions

Using a simple differential dye injection method, this paper provides the first *in vivo* evidence for local mitochondrial fusion in myelinated axons of an adult animal (frog). What is the frequency of mitochondrial fusion in normal myelinated axons *in vivo*? We typically observe about 10 % of the mitochondrial population labeled yellow (fusion) from 94 axons at 24 hrs after dye injection. Using this method, it is possible to examine local mitochondrial fusion in myelinated axons under various pathologies. In this method of dye labeling, two types of artifacts might be possible. First is that a mitochondrion can turn yellow by taking up Red and Green dyes from the opposite injection sites. However, this is unlikely since it is reasonably well documented that the mitochondria we observed travel faster than dye

diffusion. Second is dye toxicity. The Red MitoTracker used in our study (Rosamine-based) has been suggested to induce photo-damage to mitochondria, causing cells swelling and apoptosis in extensive photo-irradiation (Minamikawa, Sriratana et al., 1999). However, our observed fusion is unlikely an artifact of photo-damage for three reasons. First, most of the fusion presumably occurred *in vivo* inside the animal in the absence of light; once the nerve is taken out (when most of the fusions have already occurred), we used very low laser intensity and did not observe mitochondrial swelling. Further, the earliest time point that we observed fusion was at 10 min after taking the nerve out. This suggests that fusion occurs *in vivo*, prior to probable laser induced damage during the observation phase. Second, we used another Red dye (Rhod-2) with totally different chemistry than MitoTracker. Rhod-2 is sequestered inside mitochondria after de-esterization, thus exhibiting a different entrapment mechanism than MT. The fact that we observed fusion using various dye-pairs with different chemistry (MTG/MTR; MTG/Rhod-2) further attests to the fact that this observed fusion is not an artifact.

Conclusion

We have demonstrated a very simple and innovative technique which allows imaging of local axonal mitochondrial fusion in fully differentiated nerve fibers of an adult animal (frog). We believed that this technique could be very useful to examining the mitochondrial dynamics and remodeling in animal models of axonal pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. We present an innovative method to study the mitochondrial fusion/fission in *in vivo*.
2. Injecting two different Mitotracker dyes (green and red) spatially apart along sciatic nerve allows imaging of two color mitochondria in myelinated axons.
3. Live fusion of green and red mitochondrial population produce yellow color and can be visualized using time lapse confocal microscopy.
4. This innovative technique could be very useful in monitoring the mitochondrial dynamics and remodeling in animal model of axonal pathology.

Fig. 1. The nature of mitochondrial fusion in a fully differentiated myelinated axon *in vivo*

A. Two models for origin of fused mitochondria in axons *in vivo*. Top – fusion in soma precedes transport to axons. Bottom - transport to axon precedes local fusion.

B. Experimental design to monitor the mitochondrial fusion in frog sciatic nerve. MTG and MTR are locally injected (0.2-0.4 µl) 4 cm apart in sciatic nerves of anaesthetized frogs to label two populations of uni-color Green and Red mitochondria in axons. This is followed by imaging of encounters of Red and Green mitochondria in single axons in between injection sites of freshly isolated nerves at 6, 24 hrs after injection. Fusion produces Yellow mitochondria.

C. A typical, identifiable myelinated axon with green, red and yellow mitochondria labeled with MTG and MTR, 60x objective lens.

Fig. 2. Red, Green and Yellow mitochondria in between injection sites

A. Red, Green and Yellow mitochondria in a single myelinated axon mid-point between injection sites (4 cm apart) 24 hrs after MTG/MTR injection. (See supplemental movie #1).

B. Selected portion of the axon in A, showing a moving Yellow mitochondrion (*) in successive frames. Fluorescent images from Merged, Green and Red channels are shown.

C. Kymographs of B and surrounding areas. The Yellow mitochondrion (*) displays a thick Yellow kymographic trajectory (merged image). Separate fluorescent channels reveal identical shape and movement of Green and Red images, suggesting the Yellow mitochondrion is a fused mitochondrion. It initially moves then becomes stationary (horizontal trajectory). Also present are numerous anterograde-directed Green mitochondria (positive slope) and retrograde-directed Red mitochondria (negative slope).

Fig. 3. Live fusion captured at one z-plane in a single myelinated axon

Time-lapsed images show live fusion. In merged image column, the evolution of fusion indicated by the dashed line.

A. The evolution began with a Green mitochondrion moving anterogradely.

B. Fusion initiated at tip with another Red mitochondrion lying mostly out of the z-plane except at the tip.

C, D, E. Fusion intensifies as Red fluorophores flowed into the Green mitochondrion, producing Yellow.

F. Fully Yellow mitochondrion splitting away and moving retrogradely.

G. At the end of the evolution, a fully fused Yellow mitochondrion resulted, which can be compared to the original fully Green mitochondrion (A). Also shown are separate Green and Red channels for the same time-lapsed images. Optical section 0.47 μm used at one fixed z-plane (note: a typical mitochondrion is $\sim 1\text{-}2\ \mu\text{m}$ in thickness). (See supplemental movie #2).

Fig. 4. Fusion demonstrated by 3-D reconstruction using different dye combinations

Dye combination: MTG and MTR

A. A cluster of immobile yellow mitochondria in internodal region of a single myelinated axon 6 hrs after injection. The bright upper and lower boundaries represent myelin in light microscopy. Differential MTG/MTR injection; inter-injection distance 4 cm; imaging in between injection sites.

B. 3D reconstructions (Merged, Green and Red) viewed at three different angles.

Reconstructions were performed from a series of z-stack images. Each z-step is taken at 0.2 μm with a total of 17 steps.

Dye combination: MTG and Rhod-2

C. Kymographs of anterograde-directed mitochondria (Green; MitoTracker-Green) and retrograde-directed mitochondria (Red; Rhod2) without fusion.

D. 3D reconstructions at mid-point between MTG and Rhod2 (red) injections. Differential *in vivo* injections (MTG upstream, Rhod2 downstream; 4 cm inter-injection distance) performed as in Fig. 1B. Sciatic nerve was taken out 9 hrs after injection. Images were collected after 15 minutes of nerve isolation. Note merged images reveal three Yellow mitochondria (fusion; a long one indicated by white arrow). Separate Green (MTG) and Red (Rhod2) channels are shown (See supplemental movie #3).

E, F and G. The elongated Yellow mitochondrion (white arrow in D) was magnified and viewed at three different angles at merged and separate Red and Green channels.

Fig. 5. Exclusion of somal contribution in axonal mitochondrial fusion

A. Spatial distribution of axonal mitochondrial number at 6 and 24 hrs after injection. MTG was locally injected in sciatic nerves at a proximal site (near the DRG), or at a site 4 cm downstream. Nerves were taken out at 6 (3 experiments from 3 nerves) and 24 hrs (3 experiments from 3 nerves), and labeled mitochondrial numbers were counted from all individual axons at various distances from the injection site from various z-optical planes that cover the entire nerve trunk. Mitochondrial counts were performed both in the anterograde direction (from the proximal injection site; at 6 hrs: number of mitochondria = 2572; at 24 hrs: number of mitochondria = 1847) and in the retrograde direction (from the distal injection site; at 6 hrs: number of mitochondria = 3451; at 24 hrs: number of mitochondria = 2104). Distributions were plotted normalized to the mitochondrial number at the respective injection sites.

B. Plot of percentage of Yellow mitochondria in *in vivo* and *in vitro* sciatic nerves. Axons *in vitro* and *in vivo* injected with MTG/MTR were imaged at 1 day after injection. For *in vitro* assays, the nerve was first taken out and desheathed and embedded in agar and injected with MTG/MTR. The injected nerves were incubated in oxygenated ringer solutions for 1 day before fusion assay was performed in between injection sites. For the *in vitro* nerves, the percentage of Yellow mitochondria is $30 \pm 14.0\%$ (Mean $\pm$ SD, N = 10 axons from 3 nerves) compared with $10.4 \pm 14.6\%$ (Mean $\pm$ SD, N = 94 axons from 25 nerves) *in vivo* assays. ***: $p < 0.005$.

Fig. 6. Exclusion of free dye diffusion in producing Yellow mitochondria

A. Extracellular dye diffusion – A single injection of MTG was performed in sciatic nerves of frogs. At various times (1, 3, 6, 12 and 24 hrs) after injection, line scan of fluorescence intensity (average fluorescence intensity) was performed axially along the nerve bundle with a 4x objective lens (3 experiments from 3 nerves). The intensity profile was normalized relative to the peak intensity at the injection site. The space constant is around 1 mm on both sides of the injection site. This is much shorter than 20 mm, the site at which we imaged mitochondrial fusion.

B. Verification of limited extracellular diffusion of MTG by monitoring myelin stain from the injection site. High magnification of a myelinated axon at 1 mm from the injection site reveals both intracellular mitochondria and extracellular myelin stain. This myelin stain can be regarded as “footprint” left by the extracellular diffusion of MTG. At 3 mm of the same axon, the “footprint” disappears and only intracellular mitochondria are observed. This is consistent with (A) that extracellular MTG diffusion does not extend beyond 2-3 mm.