Detailed imaging of mitochondrial transport and precise manipulation of mitochondrial function with genetically-encoded photosensitizers in adult *Drosophila* neurons

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Method Article

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Abstract

Precise distribution of mitochondria is essential for maintaining neuronal homeostasis. Although detailed mechanisms governing the transport of mitochondria have emerged, it is still poorly understood how the regulation of transport is coordinated in space and time within the physiological context of an organism. How alteration in mitochondrial functionality may trigger changes in organellar dynamics also remains unclear in this context. Therefore, the use of genetically-encoded tools to perturb mitochondrial functionality in real time would be desirable. Here we describe methods to interfere with mitochondrial function with high spatiotemporal precision with the use of photosensitisers *in vivo* in the intact wing nerve of adult *Drosophila*. We also provide details on how to visualise the transport of mitochondria and to improve the quality of the imaging to attain super-resolution in this tissue.

1. Introduction

Mitochondria are highly dynamic organelles and their correct distribution is crucial to support many cellular functions. The remarkably long processes of neurons mean that these cells are particularly dependent on mechanisms for long-range cytoskeletal transport of mitochondria. Due to their peculiar architecture, and the stereotypical directionality of the transport in the axons, neurons are a particularly good model to understand the logic of intracellular trafficking.

The fundamental importance of axonal transport for achieving maturation and maintaining the functionality of neurons is well documented [1] and many models have been developed to visualise this process. High throughput, ease of gene manipulation and accessibility to pharmacological treatments make cultured neuronal cells, for example primary neurons or neurons derived from neuroblastoma and stem cells, arguably the mainstay of neuronal trafficking studies and have significantly contributed towards the mechanistic understanding of the transport process [2–13]. *Ex vivo* models, where a whole tissue is dissected and maintained in culture or imaged directly after dissection, are valid alternatives [14–20] with the advantage of potentially preserving a near-native environment while affording accessibility for pharmacological and electrophysiological studies.

In vivo animal models to study the transport process in neurons are now also widely used [6, 21–29]. A clear advantage of an *in vivo* system is the possibility of studying the transport process while retaining the full complexity of an organismal setting, which may be preferable when studying axonal transport during ageing and in animal models of age-dependent neurodegenerative disorders [30]. However, because the neurons of interest are often buried deep into the tissue that is being imaged, this advantage tends to be offset by the need of complex surgery required to visualize the neurons of interests. This is the case, for example, for the implantation of cranial windows for the observation of thalamo-cortical projections in mice [31] or for the exposure of the mouse sciatic nerve for trafficking studies [32]. In addition, the resolution at which dynamic subcellular components are resolved during *in vivo* imaging is often modest compared to the quality that can be obtained from *in vitro* cultured cells. Despite the current rapid development of super-resolution techniques, only few *in vitro* studies have focused on long-range

intracellular transport captured by live-cell super-resolution imaging [33, 34]. While challenging, achieving super-resolution of trafficked organelles *in vivo* would be desirable, and a tangible step forward, to better understand the intricacies of intracellular trafficking in this context.

Performing gene manipulations to interfere with the transport or function of specific cargoes might not be straightforward *in vivo*, especially in mammalian systems. Knockdown or knockout studies are time-consuming; acute modulation of transport, for instance by the addition of drugs, also presents challenges in an *in vivo* setting, limiting the possibility of gaining in-depth mechanistic insight of this process. This is problematic when studying mitochondrial dynamics which often relies on pharmacological manipulation to interfere with different stages of the oxidative phosphorylation.

We developed a system for detailed imaging of organelle transport in adult *Drosophila in vivo*, which exploits the accessibility of the wing neurons for microscopic observation and does not require surgical procedures [35]. In this chapter, we describe methods to image mitochondrial transport in different neuronal population of the wing using anaesthetized adult animals. We have previously showed the suitability of this tissue for structured illumination microscopy (SIM) of neuronal membranes and nuclear markers [35]. With the aim of improving the resolution for *in vivo* imaging of mitochondrial trafficking, here we describe the application of Super-Resolution Radial Fluctuation (SRRF) microscopy [36, 37] to live imaging in the wing nerve of adult *Drosophila*. Finally, we report the generation of new transgenic flies encoding the photosensitisers KillerRed and SuperNova targeted to the mitochondria and describe photostimulation protocols to induce mitochondrial damage rapidly, coupled to the imaging of the redox state and motility of the organelles. We believe this will expand the utility of the wing nerve system and provide additional tools for the study of mitochondrial function and transport *in vivo*.

2. Materials

2.1 Generation of plasmids for the production of transgenic fly lines

1. Plasmids containing the mitochondrial targeting sequence (MTS) of the human cytochrome *c* oxidase subunit VIII (Cox VIII), KillerRed and SuperNova coding sequences. We used an 2x-MTS-mCherry (Vagnoni lab collection), #45761 and #53234 from Addgene for KillerRed and SuperNova, respectively.
2. PCR primers (underlined, sequences complementary to the coding sequences of interest; *italic*, overlapping sequences – see also section 3.1):

A) 2x-MTS Forward (FWD): 5'-GCGTTAGGTCCTGTTTCATTGgt accATGTCCGTCCTGACGCCGCTGCTGCTGCGG-3'; Reverse (RVS) with overlapping sequence to KillerRed: 5'-AGGGCGGGGCGGCCCTCGGAaggatc cCAACGAATGGATCTTGCGCGCGGGCACTGG-3'; RVS with overlapping sequence to SuperNova: 5'-GGGCCGACCTCTGAACCCATggatccCAACGA ATGGATCTTGCGCGCGGGCACTGG-3'

B) KillerRed

FWD: 5'-GCGCCAAGATCCATTGTTGggatcctcc_gagggcggccccgccctgttccagagc-3';

RVS: 5'-ACTCTAGCCACCGCGGTGGCggccgcctt_aatcctcgtcgcctaccgatggcgctggtg-3'

C) SuperNova

FWD: 5'-GCGCCAAGATCCATTGTTGggatccatg_ggttcagaggtcggccccgccctgttc-3'; RVS: 5'-

ACTCTAGCCACCGCGGTGGCggccgccttaatc_ctcgtcgcctaccgatggcgctggtg-3'.

3. pUASP plasmid for P-element mediated transgenesis.

2.2 Fly maintenance

1. Fly food: 70 mg/ml yeast, 55 mg/ml glucose, 7.7 mg/ml agar, 35 mg/ml organic plain white flour, 1.2 mg/ml Tegosept, 0.4% propionic acid (see **Note 1**).
2. Adult flies: adult transgenic animals expressing a fluorescent reporter to mark the mitochondria in the wing marginal nerve. The stocks used in this study are described in Table 1.
3. Incubator equipped with temperature and humidity control, and 12-h-light/12-h-dark cycle (see **Note 2**).

2.3 Sample preparation for imaging

1. Coverslips no. 0, 1 or 1.5 (see **Note 3**).
2. 10S halocarbon oil (see **Note 4**).
3. Dumont forceps #5.
4. Round tip artist paintbrush for *Drosophila*
5. Masking tape and double-sided tape.
6. Stereo microscope for *Drosophila*

2.4 Imaging axonal transport in the *Drosophila* wing and photostimulation

1. Inverted confocal microscope equipped with 40x, 60x or 100x oil immersion objective and with temperature control. For photostimulation experiments, the microscope should be equipped with a photostimulation module (see **Note 5**).
2. ImageJ or Fiji equipped with RatioProfiler plugin (<https://imagej.net/Cookbook>) and the 'Velocity measurement tool' (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Velocity_Measurement_Tool)

for kymographs generation (see **Note 6**).

3. NIS-element software (Nikon) for image processing.

4. Microsoft Excel and GraphPad Prism to analyse and plot the data.

2.5 SRRF analysis

ImageJ or Fiji equipped with NanoJ Core and NanoJ-SRRF plugins [38].

3. Methods

We use the GAL4-UAS binary system [39] to drive the expression of GFP in the mitochondria of the adult wing neurons. By using either a pan-neuronal driver, for example *nSyb-Gal4*, or a more restricted driver such as the cholinergic neuronal driver *ChAT-Gal4*, mitochondria can be visualised clearly in the neurons of the adult *Drosophila* wing (Figure 1B-D) (see **Note 7**). However, application of the SRRF algorithm to time series acquired by spinning disk microscopy allows to achieve a considerably improved resolution of both stationary and moving mitochondria (Figure 1D and Movie 1), which is useful to appreciate finer details of mitochondrial dynamics.

In order to damage mitochondria with spatiotemporal precision, we produced novel transgenic *Drosophila* lines expressing the photosensitisers KillerRed (KR) [40] and SuperNova (SN) [41] targeted to the mitochondrial matrix (mito::KR and mito::SN, respectively) (Table 1, Figure 2 and Figure 5A). Upon irradiation with green light, KR and SN produce a large amount of reactive oxygen species (ROS). As direct readout of the efficacy of our stimulation protocol, we measured the mitochondrial redox state with the roGFP2-Grx1 reporter (mito::roGFP2-Grx1), which exhibits fluorescence emission shift from 488 nm to 405 nm in more oxidised mitochondrial environments [42, 43]. Brief stimulations of mito::KR with a 561-nm laser light are sufficient to trigger a rapid and transient increase in mitochondrial oxidation (Figure 3). Our protocol allows to stimulate areas covering a large portion of the nerve (Figure 3) as well as achieving precise photostimulation of a single mitochondrion (Figure 4). *Mito::KR*⁻ neurons that do not express mito::KR do not show a detectable mitochondrial oxidative response when subjected to the same photostimulation regime, ruling out the possibility that the exposure to the 561-nm laser light alone is sufficient to strongly increase mitochondrial oxidation, at least for the brief irradiation times used here. No significant change in the mitochondrial redox state was observed after brief stimulation of mito::SN with a 561-nm laser light (Figure 5B-D). This suggests heightened sensitivity to mito::KR in our system which we find advantageous to be able to probe the early consequences of mitochondrial oxidation while minimising light-induced phototoxicity. SuperNova is a monomeric variant of KillerRed and was shown to generate a different proportion of singlet oxygen and superoxide species compared to KillerRed [41, 44]. This was not reported to diminish its phototoxic effect [41], although a robust effect of SuperNova stimulation might become clearer on longer timescales *in vivo* or after longer irradiation times [45]. SuperNova2, a variant of SuperNova with enhanced phototoxicity has been recently engineered [46] and could be a valid alternative to enhance the phototoxicity of the protein in an *in vivo* setting.

To investigate the consequences of acute mitochondrial damage on the motility of the organelle, we present two specular approaches. In the first approach, KillerRed is bleached in *mito::KR mito::GFP* neurons and the damaged mitochondria undergo a yellow-to-green colour switch (Figure 6A and Movie 2). An alternative approach is to use *mito::KR* in combination with a mitochondrially-targeted photoactivatable GFP (*mito::PA-GFP*). In this case, simultaneous stimulation with 405-nm and 561-nm lasers leads to quenching of the red signal and concomitant activation of the green fluorophore, with mitochondria undergoing a red to green transition (Figure 7A and Movie 3). Co-expression of the two mitochondrial markers does not affect the motility and morphology of the organelles and, with both approaches, irradiated (i.e., dysfunctional) mitochondria can be tracked through colour switch. However, the use of PA-GFP is preferable when the aim is to study the dynamics of the irradiated mitochondria, as only one laser line (488 nm) is required to directly follow the mitochondria after photostimulation. To study the dynamics of both irradiated and non-irradiated mitochondria, the simultaneous use of both 488-nm and 561-nm laser lines for the whole duration of image acquisition is recommended.

3.1 Generation of plasmids for the production of transgenic lines

1. The Cox VIII MTS, KillerRed and SuperNova are PCR amplified using the Q5® Hot-Start Fidelity 2X Master Mix (NEB) and FWD and RVS primers reported in section 2.1, which include overlapping sequences complementary to adjacent fragments and necessary for the assembly as described in step 3.
2. The PCR products are purified through solid-phase purification using spin columns (we routinely use Monarch® PCR&DNA Cleanup kit, NEB).
3. The NEBuilder® HiFi assembly method is used to introduce the KillerRed and SuperNova in the pUASP vector, downstream of two MTS from the human cytochrome *c* oxidase subunit VIII (see **Note 8**).

3.2 Fly maintenance

1. Cross males and virgin females to obtain flies of the desired genotype. In our protocol, the Gal4 drivers and UAS responders are heterozygous in all genotypes.
2. Keep the crosses at 25°C with a 12h-light/12h-dark cycle.
3. Remove the flies after 4-5 days and keep the vial with wandering larvae.
4. Collect newly emerged flies and select the desired genotype. Put the selected flies in new vials, maintain them at 25°C with a 12h-light/12h-dark cycle and image them after 24-48h, when approximately 30% of mitochondria are motile [47].

3.3 Sample preparation

1. Anesthetise the flies on a CO₂ pad (see **Note 9**), while preparing the imaging chamber (Figure 1A).
2. On each of the short sides of the coverslip, make a stack of three layers of masking tape.
3. Add a piece of double-sided tape in the middle of the coverslip and on each stack of masking tape.
4. Apply a thin layer of 10S halocarbon oil on one side of the double-sided tape in the middle of the coverslip.
5. Mount one fly on the coverslip. Pick the fly up by the legs and place it on the oil, with the ventral side up and with the head on the double-sided tape in the centre of the coverslip.
6. Dip a fine paintbrush in oil and use it to spread out the wings. Make sure that the wings are flat on the coverslip and most air bubbles are removed (see **Note 10**).
7. Place a second coverslip on top to seal the chamber and image the fly immediately. To minimise delay, we recommend mounting the fly in proximity of the microscope used for live imaging.

3.4 Imaging axonal transport in the *Drosophila* wing

1. Set the microscope temperature controller at 25°
2. Mount the flies expressing a fluorescence marker (for instance, GFP) targeted to neuronal mitochondria.
3. Find the region of interest in the *Drosophila* wing by using the transmitted light (through eyepieces).
4. Inspect the whole wing for damage (for instance, small cuts). If the wing is damaged, discard the chamber and mount another fly.
5. Switch to fluorescence mode to check the fluorescent signal (through eyepieces) and then to confocal mode (through microscope software) to acquire the images.
6. Adjust exposure time and laser power to obtain a good signal, clearly visible above background. Here, we used exposure time 300-500 ms and 15-25% laser power.
7. Image mitochondrial transport at 1 fps (Figure 1C) (see **Note 11**).

3.5 SRRF imaging

1. Repeat steps 1-5 reported in section 0.
2. Adjust exposure time and laser power to obtain a good signal, clearly visible above background. Here, we used 30 ms exposure time and 50-60% laser power (see **Note 12**).
3. Change the size of the field of view so that the time to acquire a single frame is shorter than the chosen exposure time (see **Note 13**).
4. Acquire a series of 33 frames by continuous imaging, i.e., where the interval between each frame is limited by the exposure time, in this case 30 ms. Each series of 33 frames will be used to create a

single SRRF timepoint (see section 3.6).

5. Repeat the 33-frame acquisition with an interval of 1 or 2 s, depending on the desired time lapse between acquisitions (0.5 fps and 0.33 fps, respectively) (see **Note 14**).

3.6 SRRF analysis

1. Open the raw data in Fiji having installed the Nano-SRRF plugin (Update > Manage Update Sites: add NanoJ-Core and NanoJ-SRRF and complete update process).
2. Calculate the dataset drift using the NanoJ-Core Drift estimation tool in Fiji which computes the sample drift and yields a table later applied to SRRF processing (Plugins > NanoJ-Core > Drift Correction > Estimate Drift). Set the time averaging for drift estimation to the number of frames taken per SRRF time point (in this case 33 frames) with the reference frame set to 'previous frame'.
3. Configure SRRF Temporal Analysis setting to Temporal Radiality Pairwise Product Mean (TRPPM) (Plugins > NanoJ-SRRF > SRRF Configure Advanced Settings). Here, the SRRF analysis settings were set as follows: Ring Radius = 0.3 and a Radiality Magnification = 2 (see **Note 15**).
4. For easier presentation, the results can be imported to NIS-Elements and converted from 32-bit float to 16-bit images. With our settings, the final result is an image sequence of 30 SRRF frames ('SRRF' in Movie 1).
5. To create a movie of the original data with the same frame number to the one obtained through SRRF analysis, generate the mean of 33 frames for each acquisition series, which corresponds to a single SRRF time point. With our settings, this results in a final image sequence composed of 30 frames ('Original' in Movie 1).

3.7 Photostimulation in the *Drosophila* wing: monitoring mitochondrial redox state upon acute mitochondrial damage

1. Repeat steps 1-5 of section 3.4. In this case, select *mito::KR mito::roGFP2-Grx1* or *mito::SN mito::roGFP2-Grx1* Flies expressing only *mito::roGFP2-Grx1* can be used to control for potential light-dependent, photosensitiser-independent, mitochondrial damage.
2. Set up two different optical configurations to image simultaneously roGFP2-Grx1 (A) and the photosensitisers (B):

(A) create two channels with different excitation lasers (405-nm laser and 488-nm laser) and same emission filter set (525/50 bandpass filter).

(B) Use the 561-nm excitation laser and collect the emission signal with a 595/50 bandpass filter.

3. Define the stimulation area by drawing a stimulation ROI (Figure 3A-B) and save it (see **Note 16**).

4. Set up an imaging protocol with three steps: 'before stimulation', 'stimulation', 'after stimulation'.
5. Before stimulation: acquire 2-5 frames using optical configurations (A) and (B) from step 2.
6. Stimulation: to efficiently and precisely bleach *mito::KR* and *mito::SN*, use a 561-nm laser with 95% (3.98 mW) laser power, 16 fps scan speed and 31 loops of stimulation for the cell bodies and axons (ROI area: 65 μm^2) or 15 loops for single mitochondria (ROI area: 2.9 μm^2). With this configuration, the total duration of the stimulation step never exceeds 3 s (see **Notes 17 and 18**).
7. After stimulation: acquire 1 frame every 30 s for 5 min using optical configurations (A) and (B) from step 2.

3.8 Photostimulation in the *Drosophila* wing: assessing mitochondria motility upon acute mitochondrial damage

1. Repeat steps 1-5 reported in paragraph 3.4. In this case, select *mito::KR mito::PA-GFP* or *mito::KR mito::GFP*.
2. Define the stimulation area by drawing a stimulation ROI and save it (see **Note 19**).
3. Set up an imaging protocol with three phases: 'before stimulation', 'stimulation', 'after stimulation'.
4. Before stimulation: acquire 1 frame every 2.5 s for 15-20 s.
5. Stimulation: set a scanning speed of 16 fps. Use 561-nm laser with 95% (3.98 mW) laser power and 62 loops to bleach *mito::KR* (ROI area: 141 μm^2). For simultaneous *mito::KR* bleaching and *mito::PA-GFP* activation, use 561-nm laser with 80% (3.39 mW) power and 405-nm laser with 100% (0.388 mW) power and scan simultaneously with 79 loops (ROI area: 90 μm^2). With these configurations, the total amount of time for the stimulation step never exceeded 8 s.
6. After stimulation: acquire a frame every 2.5 s for 2 min.

3.9 Analysis of mitochondrial redox state upon acute mitochondrial damage

1. Open the ".nd2" file in Fiji and split the channels.
2. Select the *mito::roGFP2-Grx1* channels (Channel 1 and Channel 2) and run "Subtract background" (Process > Subtract background). Define the rolling radius according to the pixel size (in this case, it was set to 20 px). Channel 1 and Channel 2 represent the fluorescent signal after excitation with the 405-nm and the 488-nm laser, respectively.
3. Merge Channel 1 and 2, in this order, and save the file as ".tiff".
4. Open the stimulation ROI used to bleach the *mito::KR* signal (Section 3.7, step 3) in the ROI manager.
5. Overlay the stimulation ROI on the ".tiff" file from step n.3.

6. Run “Ratio profiler” (Cookbook > Image Intensity Processing > Ratio Profiler). A “Results” tab will open and report the ratio between Channel 1 and Channel 2 for each frame. This value is the 405 nm / 488 nm ratio for the mito::roGFP2-Grx1 probe, used to quantify the mitochondrial redox state.
7. Produce ratio images for the visualisation of mitochondrial redox changes (Figure 3A-B, 4A-B, 5B) (see **Note 20**).
8. To control for the specificity of the irradiation to the region defined by the ROI, run “Ratio profiler” again on an area that has not been stimulated (Figure 3C-D, 5C-D)(see **Note 21**).
9. In order to obtain the fold change of mitochondrial redox state over time, normalise the 405 nm / 488 nm ratio at each time point to the mean 405 nm / 488 nm prior to stimulation (Figure 3C-D, 4C, 5C-D).

3.10 Analysis of mitochondria motility upon acute mitochondrial damage

1. Open the “.nd2” file in Fiji and split the channels.
2. Run “Subtract background” on each channel and apply a filter to reduce the noise, if needed. For instance, use “Despeckle” (Process > Noise > Despeckle) or “Smooth” (Process > Smooth) in Fiji.
3. Crop the image to analyse only the area of interest. Here we consider a nerve portion of 70 µm in length (see **Note 22**).
4. Open the “Velocity measurement tool” (use the >> button on the Fiji/ImageJ launcher to select this toolset).
5. Draw a straight line along the axons of interest. Define the thickness of the line to include all the axons of interest for kymograph generation. Here, we used 20-px-thick line, running along the whole length of the selected portion of the nerve (Figure 6B and 7B).
6. Press the “k” button to generate the kymograph for each channel.
7. Merge the two kymographs (Figure 6C and 7C).

4. Notes

1. Flies were grown on ‘Iberian’ food, although other recipes are also suitable for visualisation of cargo transport in wing neurons. It is crucial, however, to be consistent with the recipe used as changes in the diet can influence the outcome of the experiments. We found that inconsistent use of the yeast and flour can be the source of experimental variability.
2. To minimise variability in the experimental outcome, it is important that temperature, humidity and light-dark cycle are maintained constant.
3. The shorter working distance afforded by the coverslips n. 0 means that acquiring clear images is straightforward. However, these coverslips are thin and very flexible, which makes stabilising the sample in the imaging chamber more difficult and might lead to drifting while imaging. We find coverslips n. 1 to be a good compromise between quality of images obtained, sample stability and

maintenance of an appropriate optical configuration. However, coverslips n.1.5 are recommended for live super-resolution imaging (e.g., instant SIM).

4. We compared the 10S halocarbon oil from both VWR (cat. no. 24627.188) and the halocarbon oil 700 from Sigma Aldrich (cat. no H8898). We found the former to be more viscous and to better aid sample stabilisation. However, using the Sigma oil does not prevent obtaining high-quality images.
5. Different microscopes can be used to this purpose. We find spinning disk confocal to be the best option for live imaging, as it is gentler on the sample (i.e., less phototoxicity) and affords both high-resolution and high-speed. We routinely use two types of confocal settings: a Nikon Ti-E inverted system equipped with a Yokogawa-CSU-X1 Spinning Disk head, Andor iXon Ultra DU-897 EMCCD Camera (for axonal transport studies and SRRF) and an inverted Nikon A1RHD confocal microscope equipped with both GaAsP, multi-alkali and spectral detectors equipped with a photostimulation unit (for light-induced activation of the photosensitisers). With both systems, imaging was performed with a Nikon 60X/1.4NA Plan Apochromatic oil immersion objective.
6. Other kymograph analysis bundles can also be used effectively in this system, for example, KymoClear and KymoDirect [48, 49], KymoAnalyzer [50] or KymoButler [51]. Alternatively, a kymograph of only motile mitochondria could be generated from manually annotated tracks. This would eliminate the background associated with the numerous stationary mitochondria in the wing neurons.
7. The *ChAT-Gal4* driver can be used effectively to visualise mitochondria in the wing neurons using different fluorophores. The mitochondria in *ChAT-Gal4⁺* neurons display robust motility and invariably good fluorescent signal in both the L1 and L3 wing veins. *ChAT-Gal4* is therefore an excellent alternative to pan-neuronal and more restricted drivers currently used in this system for live imaging of axonal transport [35, 52–55]. We do not recommend the use of ubiquitous drivers as the fluorescent signal from non-neuronal cells in the wing would mask the signal from the axons of the marginal nerve.
8. We found that fusing KillerRed and SuperNova to one MTS results in diffuse cytoplasmic distribution of the proteins in *Drosophila* S2R+ cells, while using two MTS is sufficient for efficient mitochondrial targeting.
9. Make sure that the selected fly does not carry markers that affect wings morphology (e.g., CyO). Carefully check the wings at the stereo microscope. If damaged, discard the fly.
10. Wings not being flat on the coverslip will decrease the precision of the photostimulation and the clarity of the signal. The wing arch region (which is closer to the body) might not be flat on the bottom coverslip as a result of head and the thorax of the fly becoming unstuck from the coverslip, for instance if the head of the animal is not well attached on the double-sided tape. In this case, the application of little oil could be used to further restrain the animal. We found that finer round tip paintbrushes (for example, n.1) are particularly suitable to carefully position the fly.
11. It is possible to image mitochondrial transport in the wing for up to 30 minutes [35]. However, when imaging for longer periods of time, we recommend using a slower frame rate (e.g. 0.5 or 0.33 fps) to

reduce phototoxicity. On the other hand, if the study focuses on the analysis of instantaneous velocities, recording at higher frame rates for a shorter period is more appropriate.

12. For good quality SRRF movies, a strong fluorescent signal and acquisition at high fps are essential. Therefore, we recommend against using dim fluorophores.
13. To obtain a single SRRF image, a series of frames needs to be acquired in a short time interval (e.g. 1 s) and exposure times between 10 ms and 100 ms are recommended [37].
14. The effective elapsed time between time series is defined by the combination of both the time needed to acquire 1 loop of 33 consecutive frames (1 second, with our settings) and the interval between each loop (2 seconds in our case). Our effective acquisition time is therefore of 0.33 fps. In an imaging period of 90 s, this equals to 30 acquisition loops for a total of 990 frames. These settings allow to follow mitochondria reliably without significant photobleaching.
15. The settings were chosen by adjustment and comparison of several images. Ring Radius, used to calculate the intensity gradients of nearby subpixels, was chosen by iterative trials of different radii, until an optimal response of the analysis was observed, typically judged by the best resolution improvement and minimal patterning. Radiality Magnification, which determines the number of subpixels each pixel is split into, was not increased to more than 2 as improvements were not evident at greater values.
16. The size of the stimulation ROI, the scan speed and the laser intensity can influence the density of photons delivered per unit area. For each experiment, the stimulation ROI size, the number of loops, scan speed and laser intensity should be empirically optimised and then the settings kept constant throughout the experiment. In our experience, keeping the scan speed and laser intensity constant while adjusting the number of iterations, gives the most reliable results.
17. At constant laser power, high scanning speed and high number of loops result in more precise bleaching than a low scanning speed and low number of loops. For experimental design, it is useful to consider the time it takes to switch between optical configurations or stimulation loops, which may vary considerably depending on the microscope setup.
18. Our stimulation protocol is sufficient to trigger a rapid change in the redox state of the mitochondria. However, this recovers almost to pre-stimulation levels within 5 min (Figure 3C-D). If desired, longer and more frequent stimulation loops can be used which may result in sustained oxidation of the irradiated area. In this case, however, it is important to carefully control for potential light-induced mitochondrial oxidation.
19. The size of the ROI can be chosen depending on the specific experimental aim. To bleach moving mitochondria, the bigger the stimulation ROI the higher the probability that motile mitochondria will cross it at any given time. In the wing neurons, average mitochondria speed is approximately 0.2 $\mu\text{m/s}$ and 0.4 $\mu\text{m/s}$ for anterograde and retrograde transport, respectively [47]. With a stimulation step of 10-15 s, the displacement of irradiated motile mitochondria could be of up to approximately 2-6 μm . We found that using a 15-25- μm -long stimulation ROI is sufficient for reliable bleaching of mito::KR in motile mitochondria.

20. Ratiometric images were generated with the “RatioView” modality of NIS-element software. Alternatively, the Image Calculator command in Fiji can also be used.
21. Some areas immediately outside the bleached ROI are likely to be reached by the scattering of the excitation light and may respond to the stimulation protocol. This can be ascertained empirically and the extent of the irradiated sample outside the boundaries of the ROI minimised. When quantifying control regions (i.e., areas that have not been stimulated), it is therefore preferable to choose an area not adjacent to the irradiated ROI. Alternatively, using two-photon microscopy to perform the photostimulation experiments is likely to reduce the scattering of light in the tissue.
22. The wing nerve does not always appear in focus in the whole field of view when live flies are mounted. To reliably analyse mitochondria motility, select an area displaying minimal drifting where the nerve is flat on the coverslip.

Declarations

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Competing interests

The authors declare no competing interests.

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Table

Table 1. *Drosophila* stocks used in this study

Drivers and reporters	Purpose	Source
<i>nSyb-Gal4</i>	To express Gal4 pan-neuronally in the wing sensory neurons	BDSC (#68222) [58]
<i>ChAT-Gal4</i>	To express Gal4 in the cholinergic neurons of the wing	BDSC (#56500)[59]
<i>UAS-mito::KillerRed</i>	To mark mitochondria and trigger light-dependent ROS production	This study.
<i>UAS-mito::SuperNova</i>	To mark mitochondria and trigger light-dependent ROS production	This study
<i>UAS-mito::roGFP2-Grx1</i>	To mark and measure the oxidation state of the mitochondria	BDSC (#67664)[42]
<i>UAS-mito::GFP</i>	To mark mitochondria	BDSC (#8442) [60]
<i>UAS-mito::PA-GFP</i>	To mark mitochondria after activation with the 405 nm laser line	R. Rikhy (Indian Institute of Science Education and Research, Pune, India) [61]

Figures

Figure 1

Imaging mitochondria in vivo in the neurons of the adult *Drosophila* wing. (A) Schematic of the chamber where the flies are mounted for imaging. Each of the short sides of a bottom coverslip are occupied by three layers of masking tape (yellow). A layer of double-sided tape (light blue) is attached onto the masking tape and in the centre of the coverslip. A second coverslip is used to seal the chamber. (B) Schematic of the wing with areas where live imaging is typically performed highlighted in colours.

Rectangular selections represent the areas imaged in C. The arch of the L1 vein (magenta) and the L3 vein (orange) are areas of high axonal density with relatively low number of cell bodies and ideal for imaging studies of axonal transport. (C) Mitochondria marked by GFP expressed pan-neuronally (nSyb+ neurons, left panels) and in cholinergic neurons (ChAT+ neurons, right panels). Arrows indicate neuronal cells bodies. Scale bar: 10 μ m. (D) Selected frames of a time-lapse movie (Movie 1) acquired from the wing arch before ('Original') and after ('SRRF') SRRF processing. The degree of resolution improvement was measured to range between 2.4 and 3.1 fold, with the average diameter of the mitochondria measured to be approximately 470 nm and 180 nm before and after SRRF analysis, respectively, which is in line with previous estimates [56, 57]. White arrow: moving mitochondrion. Green arrow: stationary mitochondrion. Genotype: ChAT-Gal4 > UAS-mito::GFP. In (C) and (D): white lines, outline of the wing nerve. Scale bar: 10 μ m.

Figure 2

Mito::KR is localised to mitochondria in the wing nerve. Representative images of a 50- μ m long region from the arch (A) and margin (B) of the adult *Drosophila* wing. The vast majority of mitochondria are double stained with GFP and KillerRed. A'-A''' and B'-B''' show magnified insets (blue rectangles) from A and B, respectively. Arrows indicate neuronal cell bodies. In (A) and (B): white lines, outline of the wing nerve. Scale bars: 10 μ m.

Figure 3

Photostimulation of mito::KR results in a local increase in mitochondrial ROS. (A, B) Left panels: mito::KR signal before and after 561-nm irradiation (dashed green line) shows efficient bleaching of mito::KR in the axons of the marginal wing nerve (A) and arch region (B). Middle panels: ratiometric images reveal a rapid local increase in mitochondrial ROS shortly after irradiation. Mitochondrial oxidation returns to pre-bleach levels within 5 minutes. Right panels: control neurons lacking mito::KR do not display an increase in mitochondrial ROS. In all panels, the bleached area is marked by white rectangles and the white lines mark the outline of the wing nerve. Arrows indicate neuronal cell bodies. Scale bars: 10 μ m. (C)

Quantification of the mitochondrial redox state in the wing arch before mito::KR bleaching, 15 seconds after bleaching and at the end of the experimental series, as shown in (B). Repeated Measures One-Way Anova with Tukey's post-hoc test. ns = not significant ($p > 0.05$), *** $p < 0.001$. (D) Quantification of the mitochondrial redox state in the wing arch for the entire experimental series. The 405/488 nm ratio was measured at two timepoints before bleaching (dashed green line) and at 30-s intervals after that for five minutes. In (C) and (D), 'baseline' is the mean 405/488 nm ratio prior to stimulation. Quantifications of non-irradiated areas ('- Irradiation') are performed on the same UAS-mito::KR flies that were subject to irradiation ('+ Irradiation'), as described in Section 3.9. Error bars are mean $\pm$ s.e.m. N: number of wings, one ROI per wing was selected for bleaching.

Figure 4

Restricted photostimulation of mito::KR results in ROS increase in single mitochondria. (A) Representative images of the neurons in the wing margin before stimulation. White box: stimulation ROI, yellow box: magnified insets in B. Arrows indicate neuronal cell bodies. Scale bar: 10 μ m (B) Left panels: *mito::KR* signal before and after 561-nm irradiation (dashed green line) shows precise and efficient bleaching of *mito::KR* in a single mitochondrion of the marginal wing. Right panels: ratiometric images reveal a rapid local increase in mitochondrial ROS shortly after irradiation. Mitochondrial oxidation returns to pre-bleach levels within 5 minutes. In all panels, the bleached area is marked by white rectangles. Despite the precise bleaching of the *mito::KR* signal, a change in the 405/488 nm ratio in the immediate surrounding of the bleached area was also observed 15 seconds post-bleaching (white arrowhead). This might be due to light scattering during the bleaching protocol that could activate *mito::KR* in close proximity of the

stimulated ROI (see Note 21). In (A) and (B): white lines, outline of the wing nerve. (C) Quantification of the redox state in single mitochondria of the wing margin. The 405/488 nm ratio was measured at two timepoints before bleaching (dashed green line) and at 30-s intervals after that for five minutes. Scale bars are mean $\pm$ s.e.m. N=3 mitochondria.

Figure 5

Mito::SN is localised to mitochondria in the wing nerve. (A) Representative images of a 50- μ m long region from the arch (top panels) and margin (bottom panels) of the adult *Drosophila* wing. The vast majority of mitochondria are double stained with GFP and SuperNova. Arrows indicate neuronal cell bodies. Scale bar: 10 μ m. (B) Left panels: mito::SN signal before and after 561-nm irradiation (dashed green line)

shows efficient bleaching of mito::SN in the axons of arch region. Middle panels: ratiometric images show no significant increase in mitochondrial ROS after irradiation. Right panels: control neurons lacking mito::SN do not display an increase in mitochondrial ROS. In all panels, the bleached area is marked by white rectangles and the white lines mark the outline of the wing nerve. Scale bars: 10 μ m. (C) Quantification of the mitochondrial redox state in the wing arch before mito::SN bleaching, 15 seconds after bleaching and at the end of the experimental series, as shown in (B). (D) Quantification of the mitochondrial redox state in the wing arch for the entire experimental series. The 405/488 nm ratio was measured at two timepoints before bleaching (dashed green line) and at 30-s intervals after that for five minutes. Error bars are mean $\pm$ s.e.m. N: number of wings, one ROI per wing was selected for bleaching. In (C) and (D), 'baseline' is the mean 405/488 nm ratio prior to stimulation. Quantifications of non-irradiated areas ('- Irradiation') are performed on the same UAS-mito::SN flies that were subject to irradiation ('+ Irradiation'), as described in Section 3.9. Repeated Measures One-Way Anova with Tukey's post-hoc test. Ns = not significant ($p > 0.05$).

Figure 6

Assessing mitochondrial motility after acute mitochondrial damage using mito::KR and mito::GFP. (A) The cartoon illustrates the experimental design. After bleaching KillerRed with the 561-nm laser, mitochondria undergo a yellow-to-green transition, indicated by the change in the colour of the mitochondrial matrix in the cartoon: the red KillerRed signal is quenched and only the green GFP fluorescence is retained in the mitochondria. Bleached (i.e., dysfunctional) mitochondria can therefore be followed by illumination with both 488-nm and 561-nm lasers. (B) Neurons in the margin of the wing nerve before and after irradiation (dashed black line). The white rectangles indicate the photostimulated ROI. The area shaded in the bottom panels was used to generate the kymograph in (C). Scale bar: 10 μm . (C) Kymograph indicating a representative portion of stationary and motile mitochondria before and after KillerRed bleaching. The white and black rectangles (top and bottom panels, respectively) delimit the bleached area, with the dashed line indicating the time of bleaching. Only mitochondria that intersect the dashed line were bleached. Bottom panel: orange-to-green tracks: bleached mitochondria, while in motion or stationary. Orange tracks: unbleached mitochondria.

Figure 7

Assessing mitochondrial motility after acute mitochondrial damage using mito::KR and mito::PA-GFP. (A) The cartoon illustrates the experimental design. After simultaneously bleaching KillerRed and photoactivating the GFP, mitochondria undergo a red-to-green transition, indicated by the change in the colour of the mitochondrial matrix in the cartoon: the red KillerRed signal is quenched while the green GFP fluorescence appears in the mitochondria. Bleached (i.e., dysfunctional) mitochondria can therefore be followed by illumination with the 488 nm laser only. (B) Neurons in the margin of the wing nerve before and after irradiation with the 405 nm and 561 nm lasers (dashed black line). The white rectangles indicate the stimulated ROI. The area shaded in the bottom panels was used to generate the kymograph in (C). Scale bar: 10 μ m. (C) Kymograph indicating a representative portion of stationary and motile mitochondria before and after KillerRed bleaching and PA-GFP activation. The white and black rectangles (top and bottom panels, respectively) delimit the bleached area, with the dashed line indicating the time

of bleaching. Only mitochondria that intersect the dashed line were bleached and photoactivated. Bottom panel: red-to-green tracks: bleached mitochondria, while in motion or stationary. Red tracks: unbleached mitochondria.

Supplementary Files

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