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Protocol 2

Live Imaging of Mitochondria in the Intact Fly Wing

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ABSTRACT

Detailed mechanisms governing the transport of mitochondria in neurons have recently emerged, although it is still poorly understood how the regulation of transport is coordinated in space and time within the physiological context of an organism. Here, we provide a protocol to study the intracellular dynamics of mitochondria in the wing neurons of adult *Drosophila* in situ. The mounting and imaging procedures that we describe are suitable for use on most microscopes, and they can be easily implemented in any laboratory. Our non-invasive mounting procedures, combined with the translucency of the wing cuticle in adult animals, makes the wing nervous system accessible to advanced microscopy studies in a physiological environment. Combining the powerful genetics of *Drosophila* with time-lapse live imaging, the users of this protocol will be able to analyze mitochondrial dynamics over time in a subset of sensory neurons in the wing. These cells extend long axons with a stereotypical plus-end out microtubule orientation that represents a unique model to understand the logic of neuronal cargo transport, including the mitochondria. Finally, the neurons in this tissue respond to mechanical and chemical stimulation of the sensory organs of the wing, opening up the possibility of coupling the study of mitochondrial dynamics with the modulation of neuronal activity in aging *Drosophila*. We anticipate that the unique characteristics of this in vivo system will contribute to the discovery of novel mechanisms that regulate mitochondrial dynamics within an organismal context with relevant implications for the pathogenesis of age-dependent neurological disorders.

MATERIALS

Reagents

Drosophila of the desired age and genotype

To observe robust mitochondrial transport, we recommend using flies that are 1–2 d old.

Halocarbon oil (10S; for example, VWR 24627.188)

Immersion oil for microscope objectives

Equipment

Equipment needed for sample preparation only (see Steps 2–15)

Artist paintbrush (round, fine-tip, for *Drosophila* work; for example, Pro Arte Prolene Plus size 1)

CO₂ pad (for example, Flystuff 59-114)

Coverslips (22 × 64 mm, no. 0, 1, or 1.5; Gerhard Menzel)

Diamond blade for cutting glass (optional; see Step 14; CK Tools T5087)

Forceps (Dumont #5)

Microscope slide (1 mm thick, for example, 75 × 25-mm)

Side clips (stage-mounted) or slide holders (see Steps 10 and 15)

Stereo microscope for *Drosophila* work

Tape (masking and double-sided)

Equipment needed for live imaging and analysis only (see Steps 16–26)

Computer

ImageJ/Fiji with the appropriate plugins

We routinely analyze mitochondrial transport manually by using the MTrackJ (Meijering et al., 2012) plugin for Fiji/ImageJ. The Manual Tracking plugin developed by Fabrice Cordelières (Bordeaux University) is a good alternative. StackReg (Philippe Thévenaz, EPFL, Switzerland) or the Image Stabilizer plugin (Kang Li, Carnegie Mellon University, PA) are needed for drifting correction. For kymographs, the Velocity Measurement Tool, KymoClear and KymoDirect, KymoAnalyzer, and KymoButler may be used.

Inverted confocal microscope suitable for live-imaging studies with an incubator for temperature-controlled experiments, a 60× or 100× oil-immersion objective with high numerical aperture, and software (NIS-Elements, Nikon)

We favor the use of a spinning-disk system (e.g., the Nikon Ti-E inverted system equipped with a Yokogawa CSU-X1 spinning-disk head and an Andor iXon Ultra DU-897 EMCCD camera). Alternatively, we also often use a Nikon A1RHD confocal microscope equipped with GaAsP detectors (for enhanced sensitivity to green and red signals) and multi-alkali and spectral detectors.

METHOD

1. Set the microscope temperature controller to 25°C.

Sample Preparation

2. Using a stereomicroscope, anesthetize flies of the desired age and genotype on a CO₂ pad for 2–3 min at room temperature (i.e., the time it takes to prepare the imaging chamber, as described below).

See Troubleshooting.

Prepare the imaging chamber by following Steps 3–10 for lightweight chambers or Steps 11–15 for heavier chambers (Fig. 1A).

Lightweight Chamber

3. On each of the short sides of the coverslip, make a stack of three layers of masking tape.
4. Add a piece of double-sided tape on each stack of masking tape and in the middle of the coverslip.
5. Apply a thin layer of 10S halocarbon oil in the middle of the coverslip by one side of the double-sided tape.

See Troubleshooting.

6. Mount one fly on the coverslip. Pick the fly up by the legs using the Dumont forceps and place it on the oil, with the ventral side up, the wings outwards and the head pressed against the double-sided tape in the center of the coverslip.
7. Ensure that the wings are flat on the coverslip and most air bubbles are removed, if present. Dip a fine paintbrush in oil and use it to spread out the wings.
8. Seal the chamber by placing a second coverslip on top.
Make no delays in the following steps (Steps 9 and 10) to image the fly as soon as possible. Mounting the fly in the proximity of the microscope used for live imaging will minimize delay and keep the environmental conditions consistent.
9. Add a drop of immersion oil on the microscope objective and position the imaging chamber on the microscope stage.

The chamber is lightweight, and it is important that it does not become dislodged by the pressure applied by the objective.

See Troubleshooting.

10. Secure the chamber with stage-mounted side clips to facilitate imaging.
Proceed immediately to Step 16.

Chamber with Glass Slide

11. On each of the short sides of the coverslip, make a stack of four to five layers of masking tape.
The extra layers of masking tape help prevent the fly from being crushed (Fig. 1B).
12. Repeat Steps 4–7.

13. Seal the chamber by placing a glass microscope slide (1 mm thick) on top.
14. (Optional): Cut the glass slide using a diamond blade to match the length of the bottom coverslip.
15. Add a drop of immersion oil on the microscope objective, position the imaging chamber on the microscope stage and secure it with slide holders to improve stability.
Proceed immediately to Step 16.

Live Imaging

16. Find the region of interest in the *Drosophila* wing by using the transmitted light through the microscope eyepieces.
17. Inspect the whole wing for damage (for example, small cuts along the margin) that went undetected during sample preparation (Steps 2–15).
 - If the wing is damaged, discard the chamber and mount another fly, starting from Step 2.
 - If the wing is undamaged, proceed immediately to Step 18.

See Troubleshooting.

18. Switch to fluorescence mode to check the fluorescent signal through the microscope eyepieces. Next, switch to confocal mode and visualize the wing neurons using the microscope software. Use laser lines (488 nm, 561 nm, 640 nm), depending on the fluorophores to image.
We prefer to use fluorophores that can be excited with a 488-nm or 561-nm laser line as the fluorescence intensity in the wing neurons can be rapidly assessed through the eyepieces. We avoid UV light for long-term imaging due to phototoxicity.
19. Adjust exposure time and laser power to obtain a good signal, clearly visible above background.
For time-lapse imaging of mitochondrial transport, an exposure time of 300 ms and 15% laser power is sufficient to obtain good-quality movies.

See Troubleshooting.

20. Image mitochondrial transport at 0.3–1 frames per sec.
If the aim of the experiment is to capture finer details of mitochondrial dynamics, we recommend increasing the frame rate.

See Troubleshooting.

Quantitative Analysis

The wing nerve has a high density of mitochondria, the majority of which are stationary; therefore, automatic tracking of transport is particularly challenging. The variable shape of the mitochondria is also an issue for feature recognition, a typical step of automatic analysis packages, and makes accuracy problematic. Analyze mitochondrial transport manually by using the MTrackJ or Manual Tracking plugin.

21. Crop the image to select only a region of interest, which stays in focus for the duration of the whole experiment.

The wing nerve might not be in focus throughout the whole field of view, and this is particularly unlikely in the axons of the arch region. We usually analyze a 50- μ m-long region.

22. Make a note of the orientation of the axons with respect to the position of the cell bodies and presynaptic terminals.

This is essential to determine the directionality of transport. If drifting correction is needed, use StackReg (Philippe Thévenaz, EPFL, Switzerland) or the Image Stabilizer plugin (Kang Li, Carnegie Mellon University, PA).

23. Generate single tracks by marking the XY coordinates of the start and end points of each mitochondrial run (“two-point tracking”). Stop tracking when the organelle exits the field of view or goes out of focus.

Mitochondrial movements generally follow a straight line in the wing nerve. Two-point tracking generates average run lengths and velocities. Frame-by-frame tracking is recommended if the aim of the study is to calculate instantaneous velocities.

For motility parameters, a single ‘run’ is defined as a continuous movement of at least 2 μ m without any change of direction. Runs toward the cell body are retrograde, runs in the opposite direction toward the fly thorax are anterograde.

24. Count stationary mitochondria using the Cell Counter plugin in Fiji/ImageJ on the computer.

This is generally calculated by averaging the stationary mitochondria over two frames of the movies. This is essential to calculate the fraction of moving mitochondria.

25. Express the fraction of moving mitochondria as the number of motile mitochondria in the movie divided by the number of total (i.e., motile and stationary) mitochondria.

26. Generate kymographs using several Fiji/ImageJ bundles (Fig. 1C).

Although possible, kymograph analysis of mitochondrial motility in the wing nerve is difficult due to the complex tracks obtained from tracking abundant cargoes. The Velocity Measurement Tool has a dedicated function for making kymographs. KymoClear and KymoDirect, KymoAnalyzer, and KymoButler are valid options to produce and analyze kymographs.

TROUBLESHOOTING

Problem (Step 2, 5, and 9): The fly moves or drifts prior to or during imaging.

Solution: Mount a new fly. If the movement is due to the fly becoming awake during imaging, increase the time of exposure to CO₂ (Step 2). We found no difference in the amount of mitochondrial transport after 30 sec and 5 min of anesthetization (Vagnoni and Bullock, 2016). Also, using the sturdier imaging chamber setup (Steps 11–15 and Fig. 1B) can help restrain the fly during imaging. If the sample suffers from major drifting, ensure that the amount of halocarbon oil applied onto the bottom coverslip (Step 5) is not excessive enough to cause sliding of the wing during imaging. If minor drifting occurs during imaging, the StackReg or the Image Stabilizer plugins can be used to stabilize the movie before tracking the organelles so that the quantification of motility is consistent across experiments.

Problem (Step 17): There is no mitochondrial signal in the axons.

Solution: Ensure that the wing is not damaged by looking for any injuries, including small cuts and patches of melanized, potentially necrotic, wing cells. The appearance of melanized tissue is more common in older flies and often leads to degeneration of the underlying wing nerve.

Problem (Step 19): The signal/noise ratio is low.

Solution: When mounting the fly, ensure that the wing is flat on the coverslip, otherwise imaging quality will be compromised, with mitochondria frequently moving in and out of focus. Optimize settings (e.g., increase exposure time). If the signal is still suboptimal, consider using a different combination of driver and fluorescent reporter.

Problem (Step 20): Poor mitochondrial movement is observed.

Solution: There are multiple solutions to this problem. Consider the following:

- Select a different region of the wing. The arch region is ideal for trafficking studies because it contains the bundle of axons emanating from the cell bodies located along the wing margin. The portion of the marginal wing nerve closer to the arch is also suitable for trafficking studies as almost as many axons are present in this region. In young flies, mitochondria show robust movement in these areas. The number of organelles transported in the L3 vein is significantly lower because fewer sensilla are present there than in the L1 vein, and the axonal bundle is made up of only few axons. We found that our mounting procedures are also suitable to image the short neuronal dendrites that connect the cellular somas to the bristles of the wings (Vagnoni and Bullock, 2016; Vagnoni et al., 2016). In this case, imaging is carried out at the single-cell level, and repeated measurements of transport are required to obtain a snapshot of dendritic mitochondrial transport at the whole-wing level.
- Age of the flies is another consideration. Mitochondrial transport rapidly declines with age in this tissue. If the flies are older than 2 wk, little mitochondrial movement is expected (Vagnoni et al., 2016). Select younger flies. If lack of movement is also accompanied by a typically round and fragmented mitochondrial morphology, carefully inspect the wing for damage, as injury of neurons is known to have dramatic consequences on mitochondrial trafficking and morphology, including in the wing nerve (Vagnoni and Bullock, 2016).

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FIGURE LEGENDS

Figure 1. Chamber setup for live cell imaging and analysis of mitochondrial transport. A) Lightweight imaging chamber made up of two #1 coverslips (0.13–0.16 mm) that are spaced apart by three layers of masking tape. One fly is positioned in the center of the bottom coverslip, ventral side up, on a thin layer of halocarbon oil and with the head secured on a layer of double-stick tape. B) Imaging chamber made up of a glass slide (top, 0.9–1.1 mm) cut to the same length as the bottom #1 coverslip (0.13–0.16 mm). The glass slide is supported by four layers of masking tape. The fly is mounted following the same procedures used for the lightweight chamber in A. Both chambers are suitable for imaging on an inverted microscope. C) Representative kymograph of mitochondrial transport in the axons of the wing neurons from the arch region of a 2-d-old fly. Right, descending lines (cyan arrows) represent mitochondria moving in the anterograde direction. Left, descending lines (magenta arrows) indicate retrograde-moving mitochondria. Vertical lines correspond to stationary mitochondria.

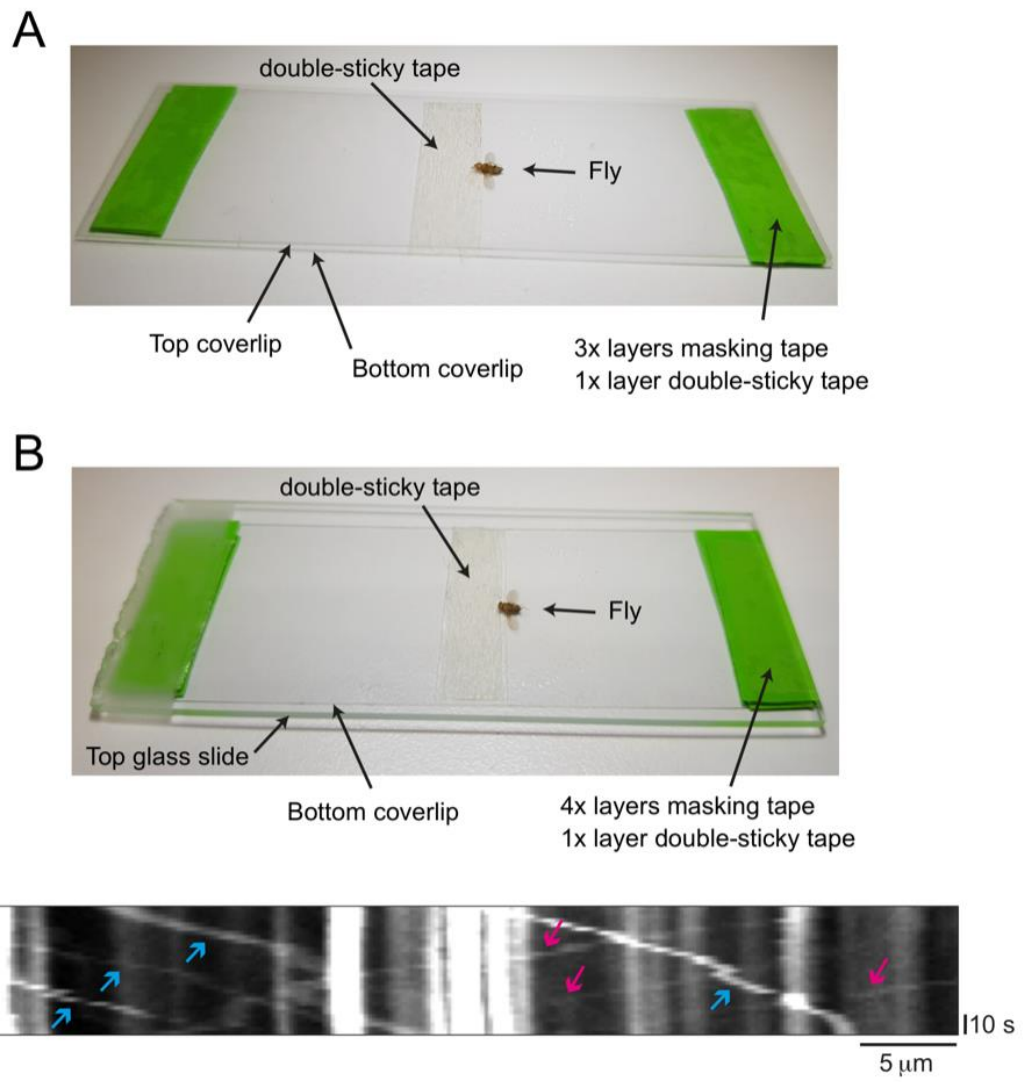


Figure 1.