Analysis of Mitochondrial Dynamics in Adult *Drosophila* Axons

Daniel Maddison[1], Francesca Mattedi[2], Alessio Vagnoni[2] & Gaynor Ann Smith[1*]

[1] UK Dementia Research Institute, School of Medicine, Hadyn Ellis Building, Cardiff University, Cardiff CF24 4HQ, UK

[2] Department of Basic and Clinical Neurosciences, Maurice Wohl Clinical Neuroscience Institute, Institute of Psychiatry, Psychology and Neuroscience, King's College London, London SE5 9RX, UK

[*]Smithga@cardiff.ac.uk

ABSTRACT

Neuronal survival depends on the generation of ATP from an ever-changing mitochondrial network. This requires a fine balance between the constant degradation of damaged mitochondria, biogenesis of new mitochondria, movement along microtubules, dynamic processes, and adequate functional capacity to meet firing demands. The distribution of mitochondria needs to be tightly controlled throughout the entire neuron, including its projections. Axons in particular can be enormous structures compared to the size of the cell soma, and how mitochondria are maintained in these compartments is poorly defined. Mitochondrial dysfunction in neurons is associated with aging and neurodegenerative diseases, with the axon being preferentially vulnerable to destruction. *Drosophila* offer a unique way to study these organelles in fully differentiated adult neurons in vivo. Here we briefly review the regulation of neuronal mitochondria in heath, aging, and disease and introduce two methodological approaches to study mitochondrial dynamics and transport in axons using the *Drosophila* wing system.

BACKGROUND

We introduce two methodological approaches for the in-depth study of mitochondrial morphology and dynamics in adult axons in vivo (see Protocol: **Clonal Imaging of Mitochondria in the Dissected Fly Wing** [Maddison et al. 2022]; Protocol: **Live Imaging of Mitochondria in the Intact Fly Wing** [Mattedi et al. 2022]). These are useful for both hypothesis-driven approaches (Vagnoni and Bullock, 2018; Vagnoni et al., 2016) and can be used to discover new modulators of mitochondrial function through completely unbiased genetic screening (Lin et al., 2021; Smith et al., 2019b). For the latter, male flies can be fed ethyl methanesulfonate and crossed with females containing MARCM machinery. One wing can then be dissected from F_1 males and used to screen for axon or mitochondrial phenotypes resulting from the random mutagenesis specifically in clones. Isolated males with interesting phenotypes are saved and bred to recover the mutation of interest, which can be found using whole-genome sequencing and deficiency mapping if lethal (Lin et al., 2021; Neukomm et al., 2014, 2017; Smith et al., 2019b). These protocols are useful for independent evaluations of mitochondrial morphology and trafficking, respectively, to gain further knowledge of mitochondrion–neuron crosstalk.

Mitochondria are highly dynamic organelles that can form networks, continually changing their morphology and number in response to cues from the cellular environment. Neurons are maintained by many active processes, which require the generation of ATP through oxidative phosphorylation by a dynamic mitochondrial network. This requires the tight regulation of mitochondrial dynamics, the degradation of damaged mitochondria through mitophagy pathways, biogenesis of new mitochondria, and trafficking along processes to where demand is highest. The vast majority of research into mitochondrial maintenance has focused on non-polarized cell types; however, it is likely that unique compartmentalized processes exist in neurons, due to their high energy demands and polarized nature. The notion that differential cell body and axon mitochondrial regulatory mechanisms exist stems from evidence to suggest that mitochondrial-targeted proteins have a different abundance at polar ends of the neuron (Graham et al., 2017), and mitochondrial biogenesis can occur initially in the distal projections (Van Laar et al., 2018). Although the primary role for mitochondria is to generate ATP, these organelles are also necessary to maintain cellular Ca^{2+} levels, lipid profiles, and redox status, and to control programmed cell death; neurons seem to be partially vulnerable to homeostatic insults.

As mitochondria are ancient in eukaryote evolution, almost all known mitochondrial molecules found in *Drosophila* are highly conserved in humans and other species. By utilizing the fly, we can therefore study the fundamental aspects of mitochondrial biology in the nervous system: they house well-defined neuronal cell types where axons, cell bodies, dendrites, and terminals can be visualized with genetically encoded tools, specific dyes, or antibodies and can be processed for electron microscopy to capture the mitochondrial ultrastructure. Work in *Drosophila* has led to the discovery and/or characterization of several key molecules required for mitophagy, biogenesis, and mitochondrial fission/fusion and motility pathways (Misgeld and Schwarz, 2017), and this insight has transformed our understanding of mitochondrial maintenance. For example, we know that mitophagy, a process of mitochondrial degradation in the lysosome compartment following rapid depolarization of the membrane, requires PTEN-induced kinase 1 (PINK1) and Parkin and their genetic interaction, discovered using *Drosophila* (Park et al., 2006). Recent work also suggests that another other parallel pathway may play a more prominent role in basal mitophagy (Lee et al., 2018a), and precise regulatory mechanisms await characterization. Nevertheless, more general autophagy pathways mediated through Atg5 and Atg7 have been found to account for the vast majority of selective mitochondrial

protein turnover (Vincow et al., 2019). Before mitophagy is induced, however, mitochondrial-derived vesicles can be released for signaling to late endosomes as the first line of defense against cellular stress (Cadete et al., 2016; Sugiura et al., 2014). Work from *Drosophila* also revealed that two key proteins, Miro and Milton, are needed to transport mitochondria (Russo et al., 2009; Stowers et al., 2002) and orchestrate their disengagement from the cytoskeleton in areas of high Ca^{2+} levels to enhance buffering (Misgeld and Schwarz, 2017). Mitochondria are highly dynamic organelles and undergo constant fission and fusion. The key factors Opa-1, Marf, fzo, and Drp1 were found to be tightly regulated by changes in mitophagy, induction of programmed cell death, reactive oxygen species, transport, and the cytoskeleton (Berman et al., 2009; Deng et al., 2008; Ding et al., 2016; DuBoff et al., 2012; Hales and Fuller, 1997; Insolera et al., 2021; Liao et al., 2017; Yang et al., 2008; Zhang et al., 2016). Fly genetics has given us a new perspective of fundamental mechanisms of mitochondrial regulation in neurons and the interconnectivity of dynamic changes.

Mitochondria are tightly linked to neuronal aging and neurological disease (Lin and Beal, 2006), prompting the need for new methods to study age-associated changes in mitochondria. Prominent neurodegenerative disorders such as Parkinson's disease (PD) (Park et al., 2018), amyotrophic lateral sclerosis (Smith et al., 2019a), Huntington's disease (Carmo et al., 2018), and Alzheimer's disease (Wang et al., 2020) have been associated with oxidative stress and morphological and functional abnormalities in mitochondria. While mitochondrial dysfunction is widely recognized as a unifying feature of neurodegeneration, the dopaminergic (DA) neurons that degenerate in PD seem to be particularly vulnerable. In fact, useful mammalian and *Drosophila* models of PD rely on systemic dosing with mitochondrial stressors (e.g., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or rotenone) that rapidly collapse mitochondrial membrane potential, causing DA neuron degeneration while other neuronal subtypes are preserved (Coulom and Birman, 2004; Whitworth, 2011). Autosomal dominant and recessive mutations in genes controlling mitochondrial degeneration and maintenance, such as those encoding PINK1, Parkin, DJ-1, and leucine-rich repeat kinase 2 (LRRK2), can lead to the development of PD (Cherian and Divya, 2020) and importantly can be modeled in flies (Whitworth, 2011; Xiong and Yu, 2018). In *Drosophila*, it was also discovered that Parkin, PINK1, and Tau function to modulate the activity of the mitochondrial fission protein Drp1 (Deng et al., 2008; DuBoff et al., 2012), and mitochondrial dynamics is now seen as a target for therapeutics in PD and beyond (Whitley et al., 2019).

Peripheral neuropathies manifest as part of mitochondrial disease or are the only manifestation in mitochondrial disease, associated with mutations in *MFN2* and *GDAP1* that impact mitochondrial dynamics or with mutations in *POLG* and *TYMP* that regulate mitochondrial DNA (mtDNA) replication (Pareyson et al., 2013). These can be modeled in the fly and used for mechanistic insight or screening for therapeutic targets (Foriel et al., 2015). Several other newly discovered mitochondrial targets have also been discovered in *Drosophila* models of amyotrophic lateral sclerosis (Layalle et al., 2021); the predominant pathways were revealed to be mitochondrial transport (Baldwin et al., 2016) and turnover (Baek et al., 2021). Although genetic models based on human associations are the foundation of *Drosophila* neurodegenerative disease research, an important consideration is the fact that mitochondrial dysregulation even manifests in idiopathic forms, and there is still a considerable amount of basic regulatory mechanisms that we do not fully understand in neurons. It is not surprising, therefore, that a forward genetic screen for genes that control axon maintenance and integrity in *Drosophila* revealed that a significant proportion of genes were linked to mitochondrial function and dynamics and, in turn, rare disease (Yamamoto et al., 2014). Another unbiased genetic screen to find molecules that control mitochondrial dynamics in *Drosophila* axons has also found that mitochondrial perturbations often precede cell death (Lin et al., 2021; Smith et al., 2019b). Despite the wealth of genetic and cell biological data that link mitochondria with neurodegeneration, age remains the biggest risk factor for developing these neurological disorders, and perhaps mitigating age-dependent mitochondrial changes would improve outcomes in patients more generally.

During the aging process, mtDNA volume and integrity decrease due to accumulation of somatic mutations and oxidative damage induced by reactive oxygen species. Results from *Drosophila* studies have been intriguing. mtDNA mutations caused by proofreading-deficient flies, harboring a POLγA (*tamas*) loss-of-function mutation, have no effect on longevity (Kauppila et al., 2018), despite significant indels and point mutations in the mtDNA pool. However, expression of the mitochondrially targeted cytidine deaminase APOBEC1, which specifically induces C:G>T:A transitions, significantly shortens lifespan (Andreazza et al., 2019). The reduction in mitochondrial volume has been found to be in part due to decreased mitochondrial biogenesis with age. In the fly, overexpressing PGC-1 increases mitochondrial activity and extends life (Rera et al., 2011), and PGC-1 was found to play a key role in 'I'm not dead yet' (Indy) mutant flies (Rogers and Rogina, 2014). In response to the accumulation of damaged mitochondria with age, basal mitophagy may increase in highly metabolic tissues such as muscle and DA neurons (Cornelissen et al., 2018), a process that can be facilitated by further stimulating mitochondrial fission (Rana et al., 2017) and autophagy induction (Aparicio et al., 2019). Longitudinal studies in *Drosophila* have also revealed a remarkable age-dependent decline in mitochondrial transport (Vagnoni et al., 2016), mediated through reduced levels of kinesin-1 motor protein (Vagnoni and Bullock, 2018). To understand the synergism between fly genetics and mitochondrial perturbations in adult life, it is important to have the ability to visualize mitochondria directly in neurons in vivo.

In *Drosophila* and other organisms, the morphology of the individual mitochondrion and the whole mitochondrial network often reflects key dynamic and functional changes and gives the first meaningful insight into phenotypic origins. Broadly speaking, mitochondrial length in neurons correlates with fission/fusion dynamics, and the number of organelles correlates with the balance between mitophagy and biogenesis. There have been a number of successful techniques to investigate mitochondria in adult neurons. One of the most commonly used techniques is to probe mitochondrial interconnectivity in DA neurons in the dissected *Drosophila* head. Typically, DA neurons are labeled with an anti-TH antibody or a genetically encoded marker driven by *ple-Gal4*. *UAS-mito::GFP* can be expressed by the same driver, allowing for clear mitochondrial resolution under a confocal microscope. DA neurons have distinct clusters and large cell bodies, which allow for analysis of mitochondrial morphology, length, and volume (Cackovic et al., 2018; Esposito et al., 2013; Klein et al., 2014; Liu and Lu, 2010; Park et al., 2006; Whitworth et al., 2005). A useful technique often used in parallel to study adult mitochondrial ultrastructure is to examine the stereotypical mitochondrial morphology within the indirect flight muscle through toluidine blue staining or TEM (Klein et al., 2014; Liu and Lu, 2010; Park et al., 2006). Analysis of mitochondria within neuronal projections in the brain is not a common practice due to their complexity, arborization and high variation in morphology, even within the same neuronal subtype.

Here we discuss the *Drosophila* wing system as a highly effective tool to study adult mitochondria in neurons. The translucent nature of the wing and availability of genetically encoded fluorescent markers for neurons and mitochondria in this region allow for in vivo and ex vivo imaging through the cuticle. Using this approach, it is possible to perform detailed mitochondrial measurements within the cell body, dendrites, and axon compartments for phenotypic analysis in different genetic backgrounds. The stereotypical organization of sensory neurons in the wing facilitates excellent visualization of mitochondria throughout the whole neuron, with axons bundled together in the proximal region of the L1 vein. We introduce two methods in which the wing system can be used to study adult mitochondria with particular focus on the axon compartment (see Protocol: **Clonal Imaging of Mitochondria in the Dissected Fly Wing** [Maddison et al. 2022]; Protocol: **Live Imaging of Mitochondria in the Intact Fly Wing** [Mattedi et al. 2022]).

OVERVIEW OF METHODS

Clonal Imaging of Mitochondria in the Dissected Fly Wing

Individual mitochondria can be quantified in neuronal clones for the in-depth study of morphology and neuronal integrity. Mosaic analysis with a repressible cell marker (MARCM) allows for only a subset of these wing neurons to be fluorescently labeled (Lee and Luo, 1999), making it much easier to determine the location of mitochondria within individual axons. A number of flippase (FLP) transgenes under control of the *asense* gene promoter have been engineered into the *Drosophila* genome (Neukomm et al., 2014), allowing for the efficient generation of wing neuronal clones after recombination at *FRT* sites on chromosome I, II, or III. When combined with a ubiquitously expressed *Gal80* transgene (e.g., *tubulin-Gal80*) situated distally to an *FRT* site on the same chromosome arm, FLP activity during the development of neuronal progenitors results in the production of a subset of mature neurons that are GAL80 negative, thus expressing the fluorescent markers present in the experiment genotype, whereas the majority of the other neurons in the wing are unlabeled. Fly lines that can be used for fluorescent labeling and for routine clonal analysis are indicated in Table 1. This is partially useful for studying homozygous lethal mutations, as any unmarked cells are wild type, enabling the animal to survive into old age. Visualization of mitochondria can be achieved using freshly dissected wings, allowing for rapid imaging of multiple animals. Clones and the residing mitochondria can be readily detected using conventional confocal microscopy methods (Lin et al., 2021; Neukomm et al., 2014, 2017; Smith et al., 2019b). There are several ways to measure individual mitochondria to gain insight into their morphology and measure total mitochondrial volume and number within a given length of axon. Several clones are typically generated per wing, depending on the combination of *FLP* and *FRT* sites used (Neukomm et al., 2014), and mitochondria quantified in each clone can be averaged to give numeric values per wing or animal using a standardized method. This method can also be adapted to measure changes in mitochondrial and neuronal physiology using biochemical reporters.

Live Imaging of Mitochondria in the Intact Fly Wing

We also introduce a protocol for looking at mitochondria in intact flies, conducive to live cell imaging (see Protocol: **Live Imaging of Mitochondria in the Intact Fly Wing** [Mattedi et al. 2022]). Through imaging of many axons simultaneously, it is straightforward to design a study with enough statistical power for meaningful analysis of mitochondrial trafficking. We typically image mitochondrial transport in the wing arch region, where the axons of most wing neurons are collected in a bundle. Because the neurons are typically marked by a Gal4 driver expressed throughout the wing nerve (Table 1), imaging the axonal bundle has the added advantage of providing a general readout of mitochondrial transport functionality at the whole-wing level. Samples are prepared in a bespoke, easy-to-assemble imaging chamber, and a set-up is used that we find suitable for obtaining high-quality movies of neuronal mitochondrial dynamics in situ. We have shown this system to be compatible with conventional confocal microscopy as well as with super-resolution microscopy, including structured illumination microscopy (Vagnoni and Bullock, 2016) and super-resolution radial fluctuations of mitochondrial axonal trafficking (Mattedi et al., 2021). Our procedures are also suitable for rapid photoactivation/photoconversion of mitochondrion-targeted fluorophores during time-lapse imaging of mitochondrial axonal transport (Mattedi et al., 2021). Lastly, transport data are analyzed, including examples of representative kymographs that can be obtained from the time-lapse acquisition of mitochondrial movements.

FURTHER REMARKS

By substituting *UAS-mito::GFP* with other fluorescent marker elements, these protocols can be easily adapted to investigate other important internal structures such as lysosomes, autophagosomes, other

vesicles, the endoplasmic reticulum, or the cytoskeleton (see Protocol: **Clonal Imaging of Mitochondria in the Dissected Fly Wing** [Maddison et al. 2022]; Protocol: **Live Imaging of Mitochondria in the Intact Fly Wing** [Mattedi et al. 2022]). Using the same progeny from genetic crosses indicated here, it is also possible to study synapse-residing mitochondria by dissecting and mounting whole *Drosophila* legs. *OK371-GAL4* is able to drive reporters of interest within motor neurons down to the neuromuscular junction (Mahr and Aberle, 2006; Sreedharan et al., 2015).

While, for initial experiments, the general visualization of mitochondria or other organelles is recommended to give vital information on number, morphology, localization, and trafficking deficits between different experimental groups, it is often necessary in subsequent experiments to study physiological responses and specific signaling pathways. These protocols also allow for adaptation to use genetically encoded biochemical-based reporters so that physiological changes can also be measured in vivo. Reporters existing for mitochondrial research in *Drosophila* are largely based on adaptations of GFP that are engineered to change conformation depending on redox balance, time after synthesis, or laser activation, while other tools rely on the quenching of GFP as a signal for sequestration into highly acidic compartments (Table 2).

Mitochondria regulate a multitude of signaling and metabolic events in neurons, and knowledge of how their function and regulation impact the aging process and disease states is increasingly becoming known. Recent technologies to study these dynamic organelles in adult tissues and organisms are key to broadening our understanding of how neurons are maintained. The fly represents a unique opportunity to study the dynamic regulation of the ancient eukaryotic organelle in fully differentiated adult neurons. We can utilize sophisticated molecular–genetic approaches available only in this system to study mitochondrial biology in an aging context in vivo and ex vivo. The wing system in particular overcomes challenges of studying mitochondria in elaborate neuronal projections in the brain, offering a standardized system in which axons are similarly oriented and structured, analogous to microfluidic chambers used for in vitro studies. Better ways to study mitochondria in axons are much needed, as evidence seems to suggest that the axon is particularly vulnerable to pathological insults such as disease or injury (Johnson et al., 2013; Medana and Esiri, 2003). It is hoped that these fruit fly tools and methods will lead to a better fundamental understanding of mitochondrial biology in neurons, which may in turn lead to new therapeutic targets for neurodegenerative disease.

ACKNOWLEDGMENTS

Work in our laboratory was funded through an MRC Momentum Award (MC_PC_16030/1) to G.A.S., a Leverhulme Trust project grant to G.A.S., a van Geest Fellowship in Dementia and Neurodegeneration to A.V., a van Geest PhD Studentship from King's College London to A.V., and an Academy of Medical Sciences Springboard Award (SBF004\1088) to A.V. A.V. and F.M. thank the Wohl Cellular Imaging Centre at King's College London for help with light microscopy.

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TABLES

Table 1. Transgenic fly lines used to fluorescently label mitochondria in wing neurons

Strain	Purpose	BDSC no.	References
Drivers			
OK371-Gal4	GAL4 expression in glutamatergic neurons	26160	(Meyer and Aberle, 2006)
Chat-Gal4	GAL4 expression in cholinergic neurons	56500	(Salvaterra and Kitamoto, 2001)
nSyb-Gal4	GAL4 expression pan-neuronally	68222	(Verstreken et al., 2009)
Appl-Gal4	GAL4 expression pan-neuronally	32040	(Torroja et al., 1999)
nSyb-lexA	LexA expression pan-neuronally	52817	(Pfeiffer et al., 2012)
Fluorescent markers			
5xUAS-mito::GFP	Label the mitochondrial inner membrane with GFP	8442/8443	(Rikhy et al., 2003)
5xUAS-mito::tdTomato	Label the mitochondrial inner membrane with tdTomato	N/A	(Smith et al., 2019b)
14xUAS-mCherry::mito.OMM	Label the mitochondrial outer membrane with mCherry	66532/66533	(Vagnoni and Bullock, 2016)
lexAop-mCherry::mito.OMM	Label the mitochondrial outer membrane with mCherry	66530/66531	(Vagnoni and Bullock, 2016)
5xUAS-mCD8::GFP	Label cell membrane with GFP	32192	(Lee and Luo, 1999)
10xUAS-IVS-myr::tdTomato	Label cell membrane with tdTomato	32222	(Wong et al., 2002)
MARCM machinery			
asenseFLP^{2e}	Mediate homologous recombination between *FRT* sites to induce MARCM clones	N/A	(Neukomm et al., 2014)
FRT2A	FLP recognition site on chromosome arm 3L for homologous recombination	1997	(Golic, 1991)
FRT82B	FLP recognition site on chromosome arm 3R for homologous recombination	2035	(Xu and Rubin, 1993)
tubulin-Gal80	Represses GAL4 in all cells. Can be removed by FLP-mediated recombination to label a subset of cells.	5135	(Lee and Luo, 1999)

BDSC, Bloomington Drosophila Stock Center. N/A, not currently available from the stock center and must be requested from the laboratory directly.

Table 2. Transgenic fly lines that can be used to detect biochemical mitochondrial changes in wing neurons

Tool	Mitochondrial measurements	Tool development	Initial use in *Drosophila*
5xUAS-mito-roGFP2-Grx1	Glutathione-specific redox reporter	(Gutscher et al., 2008; Ostergaard et al., 2001)	(Albrecht et al., 2011)
5xUAS-mito-roGFP2-Orp1	H_2O_2-specific redox reporter	(Gutscher et al., 2009)	(Albrecht et al., 2011)
20xUAS-mito-GCaMP5	Ca^{2+} levels	(Dana et al., 2019; Nakai et al., 2001)	(Smith et al., 2019b)
5xUAS-mitoTimer	Age/dynamics reporter	(Terskikh et al., 2000)	(Laker et al., 2014)
5xUAS-mitoQC	Mitophagy reporter	(Allen et al., 2013)	(Lee et al., 2018b)
5xUAS-mito-keima	Mitophagy reporter	(Katayama et al., 2011; Kogure et al., 2006)	(Lee et al., 2018b)
5xUAS-mito-Dendra2	Photo-convertible for dynamic studies	(Gurskaya et al., 2006)	(Hwang et al., 2014)
UAS-mito-PA-GFP	Photo-activatable for dynamic studies	(Karbowski et al., 2004)	(Chowdhary et al., 2017)