Drosophila primary neuronal cultures as a useful cellular model to study and image axonal transport

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Running head: Transport studies in *Drosophila* primary neurons

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Abstract

The use of primary neuronal cultures generated from *Drosophila* tissue provides a powerful model for studies of transport mechanisms. Cultured fly neurons provide similarly detailed subcellular resolution and applicability of pharmacology or fluorescent dyes as mammalian primary neurons. As an experimental advantage for the mechanistic dissection of transport, fly primary neurons can be combined with the fast and highly efficient combinatorial genetics of *Drosophila*, and genetic tools for the manipulation of virtually every fly gene are readily available. This strategy can be performed in parallel to *in vivo* transport studies to address relevance of any findings. Here we will describe the generation of primary neuronal cultures from *Drosophila* embryos and larvae, the use of external fluorescent dyes and genetic tools to label cargo, and the key strategies for live imaging and subsequent analysis.

1 Introduction

Neurons extend axons to communicate and innervate all parts of the body. Axons are the longest cellular protrusions, with motorneurons reaching up to a meter in humans. Axons predominantly form output synapses which are in their majority located at the distant end of the axon. The extreme spatial separation between synaptic terminals and the neuronal cell body or soma poses a logistical problem in that soma-derived proteins, RNAs, lipids and organelles crucial for the sustainability and function of axons and their synapses, need to be transported over large distances [1-3].

Long distance axonal transport involves the coordinated action of motor proteins binding directly or indirectly (through adaptors) to the transported cargoes whilst walking along microtubules [4-7]. For this, axonal microtubules are arranged into loose parallel bundles that run all along the axon shaft [8,9]. Motor proteins involved in long distance axonal transport use ATP as energy source to step along microtubules; they include dynein/dynactin responsible for retrograde movements and certain members of the kinesin family for anterograde transport (kinesin-1, -2, -3, -4 [3,10,11]). Apart from antero- and retrograde cargo transport, this system must also ensure the controlled release, hence delivery, of cargoes at the appropriate target sites [12,13].

It is not surprising that many neurodegenerative diseases are linked to genetic defects in this transport machinery, thus highlighting the importance of this process for neuronal function and viability [5,14]. Decrease in axonal transport is also considered as a widespread ageing hallmark [15]. It is therefore

pivotal to understand the transport machinery, its regulatory mechanisms and the consequence of its dysfunction.

Despite the recent advances in understanding the mechanism of fast axonal transport (e.g. [16-19]), there are numerous unresolved issues in relation to the regulation of speed, directionality, selectivity of transport, targeted unloading of cargoes, and adaptation of transport to the specific cellular demands. Such complex regulatory processes are likely to involve the specific compositions of motor protein complex (e.g type of motor protein and cargo adaptors [20,21]), the interaction between different complexes and with other classes of microtubule binding proteins [22,23,2], posttranslational modifications (PTMs) of these complexes or of their microtubule tracks mediated by the orchestrating action of signalling pathways [24].

A powerful model to study transport mechanisms in its complexity, is the fruit fly *Drosophila melanogaster*, which has been used for this purpose for more than two decades (e.g. [25-30]. The fundamental function and structure of *Drosophila* and vertebrate neurons is comparable [31-35], including the organisation and regulation of microtubules which follow similar organisational principles as in vertebrates, in spite of the fact that neurofilaments are absent [9]. Furthermore, most motor proteins have counterparts in *Drosophila*, but there is less redundancy: while around 40 genes encode members of the kinesin super-family in vertebrates [36], only 18 kinesin coding genes cover a similar range of motor protein classes in *Drosophila* [3]. For example, kinesin light chain comprises 3 mouse members, but only one in *Drosophila* [3]. In addition, an arsenal of genetic tools for the manipulation of virtually every fly gene is readily available or can be efficiently generated through genomic engineering, and they can be easily targeted to specific neurons or combined to generate loss or gain of function of different genes or tools in the same neurons or organisms [37-39].

In its majority, research in *Drosophila* neurons makes use of *in vivo* studies in the whole organism. However, also the use of cultured *Drosophila* primary neurons, first used half a century ago (e.g. [40,41]) has proven a valuable complementary strategy. As is the case for mammalian primary neurons, also fly neurons give access to subcellular detail and robust and sensitive readouts and are accessible to external application of compounds such as fluorescent dyes and drugs [42-46]. Importantly, many of the observed subcellular features and dynamics are very similar to those of vertebrate neurons [47]. However, as an important advantage, primary fly neurons are accessible to the powerful combinatorial genetics mentioned above: as long as genetically manipulated embryos can generate neurons, these can be analysed in culture. Accordingly, primary *Drosophila* neuronal cultures have been used to successfully study various different aspect of neuronal physiology [48] [49], including cell division [50], axon guidance [51-53], cytoskeletal dynamics [54,55,43,56-58,42,59,60], neuronal activity [61,62], protein aggregation in neurodegenerative disease models [63], receptor signalling and trafficking [53,45] and for the study of axonal transport [64,44,65]. In this method paper we will explain primary neuronal cultures from *Drosophila* as cellular system where axonal transport can be studied efficiently.

2 Materials

1. Drosophila specimens: primary neuronal cultures can be performed from *Drosophila* embryos [66], late instar L3 larva [54] and late pupa [67] (see Note 1).
2. Sodiumhypochlorite solution (or household bleach), diluted 1:1 with water to 50%.
3. Ultrafine sieves and brushes for the collection and cleaning of embryos.
4. Agar plates: dissolve 2-4% agar in water, boil, pour into empty plastic petri dishes (50mm diameter), let cool down and harden.
5. 70 % Ethanol in ultrapure (Milli-Q) autoclaved water.

6. Supplemented Schneider's medium pH 6.8 – 7.0: Combine Schneider's *Drosophila* medium with 20 % non-heat inactivated FBS. Filter-sterilise and protect from light with aluminium foil. To inactivate the serum's complement system incubate for 3 days at 26°C (alternatively use heat-inactivated FBS and skip the 3 days incubation at 26°C). Add 2 µg/mL insulin (2 mg/ml insulin stock can be aliquoted and kept at -20°C). Use a small aliquot of medium to test the pH and adjust to 6.8–7.0 with 1 N HCl or NaOH if needed. Aliquots of 1 to 2 mL medium can be stored at −80°C for 6 months. After thawing aliquots for use, filter-sterile with an 0.22 µm syringe filter and freshly add 10 U Penicillin / Streptomycin per mL medium.
7. HBSS buffer: 30 mL 10 x Hanks' Balanced Salt Solutions (GIBCO, no calcium or magnesium), 3ml Penicillin/Streptomycin (GIBCO, 10000 U/mL), 167 mL distilled water, 0.01g phenylthiourea; aid to dissolve by incubating at 37 °C for 30 minutes, sterile filter with 0.22 µm filter. This buffer can be stored in aliquots at 4°C for 6 months.
8. Dispersion or dissociation medium: dissolve 0.005 mg Dispase II and 0.001 mg Collagenase type V in 2 ml HBSS medium. Incubate at 37 °C for 30 minutes until dissolved. Filter through a 0.22 µm filter and store at 4°C. It can be used for up to a week.
9. Pestles: small autoclavable plastic pestles for 1.5 ml microcentrifuge tubes with a tight fit.
10. Custom-made culture chambers or 35-mm glass bottom dishes (MatTek) to grow primary neurons: culture chambers can be assembled from 1 lead-free glass microscope slide (Menzel Gläser) with a 15-mm hole (custom-made at a workshop) and 1 intact slide glued together with aquarium silicone. Dry (up to 5 days) and scrape off any excess silicone. Clean with 70% ethanol followed by 100% acetone. The chambers can be reused after cleaning with acetone and autoclaving. Alternatively, use 35-mm glass bottom dishes (for example from MatTek) with optical quality glass for microscopy. While incubation chambers and glass bottom plates can be used with inverted microscopes, incubation chambers are ideal for upright microscopes.
11. 4x concanavalin A stock solution: in a laminar flow hood, dissolve concanavalin A (conA) in ultrapure autoclaved water to a concentration of 20 µg / mL. This stock solution can be frozen in aliquots at -20 °C.
12. Treated coverslips: *Drosophila* primary neurons can grow on acetone-treated or conA -coated glass coverslip (Menzel Gläser, 24 x 24 mm, VWR international MENZBB024024A123). For acetone treatment, dip coverslips in 100% acetone, let dry and autoclave. For conA treatment, incubate coverslips with 150 µL conA at a concentration of 5 µg / mL at 37 °C for 1.5 hours. Remove conA solution, wash with 150 µL sterile water, remove and dry at ~50 °C overnight. Note that the morphology of neurons changes when growing on conA [66]. When using glass bottom dishes for live imaging, treat the glass coverslip that is attached to the bottom of the dish.
13. Vaseline for sealing chambers/dishes with coverslips.
14. 100 nM LysoTracker DND-99 / 50 nM MitoTracker Green FM/CMXRos in supplemented Schneider's medium.
15. Fixatives**:** We carry out standard fixations in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). To prepare 100 mL fixative, dissolve 4 g Paraformaldehyde in 36 mL 0.2 M NaH_2PO_4 (warm up slightly and stir), add 14 mL 0.2 M NaH_2PO_4 and fill up with water. Adjust pH if necessary.
16. PBT: 0.3 % Triton X-100 (v/v) in 1x PBS

3 Methods

<u>3.1 Drosophila embryonic primary neuronal cultures</u> (Fig. 1A-D)

141 *Drosophila* primary neuronal cultures can be prepared from several developmental stages (embryo, 142 larva and pupa) [66]. In this chapter, we will discuss our protocol for primary neuronal cultures derived 143 from whole embryos or third instar larval CNSs, in which our transport studies have been performed 144 (Fig. 1-2).

145 1. Collect embryos (Fig. 1A.1) in vials or cages for ~15 hours at 20-25 °C (the temperature can be 146 adapted to increase the yield of stage 11 to 12 embryos [68], see Notes 1 and 2).
147 2. Dechorionate embryos (Fig. 1A.2) with 50 % bleach for 90 s. Wash thoroughly with water in a 148 sieve. Note that extended incubations as well as the quality of the bleach can affect the 149 viability of the cultures. Carefully collect embryos with a thin paint brush from the sieve and 150 transfer them to an agar plate.
151 3. Select stage 11 to 12 embryos (Fig. 1A.3) by autofluorescence pattern (for embryo stages see 152 [68]; see Note 3 a-c). If mutant *Drosophila* stocks are used (with adequate GFP-balancers), 153 mutant embryos can now be selected based in the absence of GFP, using a fluorescent 154 dissection microscope. For cultures on acetone treated glass coverslip select 10 embryos per 155 culture (the number of embryos can be altered to achieve different densities of neurons in 156 culture). For cultures on conA coated glass coverslip select 5 embryos per culture.
157 4. Transfer the embryos (Fig. 1B.4-5) into a 1.5 mL tube with a 5 µL drop of supplemented 158 Schneider’s medium using clean forceps (with 70% ethanol). From this point on the next steps 159 will be performed in a laminar flow hood wearing gloves and with autoclaved materials 160 whenever possible.
161 5. Wash the embryos (Fig. 1B.4-5) with 70 % ethanol solution for 30 s by gently inverting the 162 tube several times.
163 6. Remove ethanol and wash embryos in 500 µL supplemented Schneiders medium (Fig. 1B.6). 164 Take care not to accidentally pipette the embryos. Pipetting against a black background can 165 help to visualise the embryos.
166 7. Replace Schneider’s medium with 100 µL HBSS-based dispersion medium at RT (Fig. 1B.7-9; 167 see Note 4)
168 8. Grind embryos in the tube with an autoclaved pestle to break down the embryos (Fig. 1B.7- 169 9), use one pestle per genotype (see Note 5). At this step there is the option to add a 170 preculture step to reduce maternal contribution of proteins or allow for extra time for 171 knockdowns to work, see section 3.2).
172 9. Incubate the tubes with the cell dispersion for 4 - 7 min at 37 °C (Fig. 1B.7-9). OPTION: This 173 step can be omitted leading to neurons with more complex morphologies and less isolated 174 single neurons.
175 10. Stop the dispersion reaction by removing from 37 °C and adding 200 µL of supplemented 176 Schneider’s medium (Fig. 1B.10).
177 11. Sediment cells at 600 - 750 x g for 4 min (Fig. 1B.11-12). Remember the orientation of the tube 178 during centrifugation since it may be difficult to see the pellet.
179 12. Aspirate and discard the medium (Fig. 1B.11-12). Make sure this step is performed promptly 180 to avoid cell pellets resuspending in the old medium. Add 31 µL of fresh supplemented 181 Schneider’s medium per culture/chamber. Adjust volume depending on the final number of 182 cultures (we usually prepare 3 cultures per condition at a time). Gently resuspend cells in 183 Schneider’s medium without generating bubbles.

184 13. Distribute 30 µL cell suspension to either glass bottom dishes (treated with acetone or coated 185 with conA) and cover with a regular coverslip using Vaseline as sealant; or to custom-made 186 culture chambers and cover with a lead-free coverslips (treated with acetone or coated with

conA) and seal with Vaseline (Fig. 1C.13). Note that the morphology of neurons changes when growing on conA [66].

14. Let the cells settle onto the acetone or conA treated surfaces for 2 hours at 26°C (Fig. 1C.14).
15. Flip over the incubation chambers / glass bottom dishes and grow primary neurons on top of the chamber as hanging drop cultures (Fig. 1C.15) until the desired time in vitro has been reached (see Note 6).

3.2 Pre-culture step for Drosophila embryonic primary neuronal

Cells can be cultured in a 1.5 mL tube for several days before being dispersed again and grown on coverslips in culture chambers. This step is useful in order to deplete healthy gene product deposited by heterozygous mothers in the egg cells (which otherwise carry mutations in a specific gene). Maternally contributed gene products can persist for several days and mask the homozygous mutant phenotype of embryos and larvae [69]. This step is also effective in allowing extra time for conditions of knockdowns.

1. Follow steps 1-8 of Section 3.1 (*Drosophila embryonic primary neuronal cultures*). For this protocol increase the number of embryos to 15 per slide and use ConA coated coverslip.
2. After grinding the embryos, immediately add 200 µL of supplemented Schneider's medium.
3. Sediment cells at 600 - 750 x g for 4 min.
4. Aspirate the medium. Add 100 µL of fresh supplemented Schneider's medium, gently resuspend cells and incubate in the tube for the desired time at 26°C (1-7 days).
5. After the incubation, sediment cells at 600 - 750 x g for 4 min.
6. Aspirate Schneider's medium and add 100 µL HBSS-based dispersion medium.
7. Gently resuspend the cell pellet and incubate 4 - 7 min at 37 °C.
8. Continue from step 10 of Section 3.1 (*Drosophila embryonic primary neuronal cultures*).

3.3 Third instar larval brain cultures

1. Collect 2-3 late stage 3 larvae per culture.
2. Wash larvae 3x in in 1.5 mL 70% ethanol.
3. Wash larvae 3x in 1.5 mL sterile PBS.
4. Transfer larvae into a sterile dissection dish with supplemented Schneiders medium.
5. Dissect out brains (use sterile forceps) and transfer to a 1.5 mL tube with supplemented Schneiders medium.
6. All following steps should be done in a laminar flow hood.
7. Wash brains 3x in 500 µL supplemented Schneider's medium.
8. Remove medium and replace with 100 µL dispersion medium.
9. Continue from step 8 of Section 3.1 (*Drosophila embryonic primary neuronal cultures*).

3.4 Labelling of cargo

The use of primary neuronal cultures to study transport allows the labelling of cargo by (a) genetically encoded tools, mostly tagged proteins or localisation sequences fused with GFP or other fluorophores and (b) fluorescent chemical compounds applied to the culture media such as LysoTracker, fluoro-dextranes and mito-tracker. Here we describe the methods and tools we have been experimenting with. However, there is an arsenal of further tools that can be used to label specific cargo, such as

230 dense-core vesicle labelled with atrial natriuretic factor-GFP (ANF-GFP), APP-positive vesicles labelled
231 with App-YFP, Golgi vesicles labelled with GalT-RFP, endosomes labelled with Rab4-RFP and
232 ribonucleoprotein granules labelled whit GFP-Imp to mention some [27,70,30].

233 ***3.4.1*** **LysoTracker staining** (see Note 7)**:**

234 LysoTracker is a fluorescent probe which, due to its hydrophobic nature, diffuses into cells, and
235 preferentially accumulates in vesicles with an acidic content. It is commonly used in vertebrate models
236 to label late endosomes/lysosomes and to study their trafficking within cells including neurons [71-
237 75]. LysoTracker has been used previously to label lysosomes/late endosomes in *Drosophila* primary
238 neuronal cultures [76], *Drosophila* tissue and cells lines [77]. Here we will describe its use in *Drosophila*
239 primary neuronal culture (Fig. 2A, Suppl. Movie 1). Using this method, we find LysoTracker-positive
240 vesicles move at ~0.6-0.9 µm/s in either direction (compared to reported average speeds for
241 lysotracker vesicles between 0.34 µm/s and 1.2 µm/s in vertebrate neurons) [78].

242 1. Follow sections 3.1 - 3.3. for the generation of *Drosophila* primary neuronal cultures.
243 2. Prepare supplemented Schneider's medium with 100 nM LysoTracker DND-99.
244 3. Shortly before use, spin down solution to remove any precipitates that might have formed
245 and could affect imaging.
246 4. When cells have reached the desired maturity (see Note 8), detach the coverslips from the
247 culture chambers or glass bottom dish and wash the cells (they will be attached to the
248 coverslips if using culture chambers or at the bottom of the glass bottom dish) and culture
249 chambers in supplemented Schneider's medium with 100 nM LysoTracker DND-99.
250 5. Remove all medium and add 50 - 60 µL supplemented Schneider's medium with 100 nM
251 LysoTracker DND-99 to the culture chambers/glass bottom dishes.
252 6. Reseal the culture chambers/glass bottom dishes with the same coverslip, a new Vaseline seal
253 may be required.
254 7. Incubate for 15 minutes at the desired temperature.
255 8. Replace labelling solution with 50-60 µL label-free supplemented Schneider's medium.
256 9. Let cells adjust to the condition in the imaging chamber for 15 minutes and proceed to image
257 the cells.

258 ***3.4.2*** **MitoTracker staining:**

259 Mitochondria can be labelled by both fluorescent mitochondrial dyes including MitoTracker and
260 genetically encoded fluorescently tagged mitochondrial proteins or tagged mitochondria-targeting
261 sequences (such as mito-GFP [79], mito-mCherry [70]). MitoTracker is a fluorescent dye which
262 covalently binds free sulfhydryls. It is frequently used for transport studies in vertebrate neurons [80]
263 [81] [74] and some MitoTracker variants have previously been used in *Drosophila* tissue and in
264 *Drosophila* cell lines to label mitochondria [82]. We experienced that MitoTracker dyes in *Drosophila*
265 primary neuronal cultures can diminish mitochondrial dynamics slightly, as has similarly been reported
266 for rat hippocampal neurons [83]. Nonetheless, MitoTracker easily and reliably labels mitochondria in
267 cultured neurons and in our studies, it is instrumental in determining the number and distribution of
268 mitochondria at the axon, cell body and synaptic terminals in fixed samples [44]. Here we will describe
269 how we use MitoTracker (Fig. 2B, Suppl. Movie 2), before explaining the use of genetically encoded
270 fluorescently tagged mitochondrial proteins in the next section.

271 1. Follow sections 3.1 - 3.3. for the generation of *Drosophila* primary neuronal cultures.
272 2. Prepare supplemented Schneider's medium with 50 nM MitoTracker Green FM or CMXRos
273 (note that not all MitoTracker variants will be retained after fixation).

3. Shortly before use, spin down solution to remove any precipitates that might affect the imaging.
4. Once primary neuronal cultures from the desired genotype have reach the preferred developmental stage, detach the coverslips from the culture chambers or glass bottom plates and wash the cells (they will be attached to the coverslips if using culture chambers or at the bottom of the glass bottom plates) and culture chambers in supplemented Schneider's medium with 50 nM MitoTracker Green FM/CMXRos.
5. Remove all medium and add again 50 - 60 µL supplemented Schneider's medium with 50 nM MitoTracker Green FM/CMXRos. Be careful as the coverslips are prone to break.
6. Replace Vaseline and reseal the culture chambers/ glass bottom plates back.
7. Incubate for 15 minutes.
8. Remove medium and replace with regular supplemented Schneider's medium.
9. Proceed to imaging. OPTION: MitoTracker CMXRos-labelled cultures can be fixed by following the steps below.
10. Prewarm the standard fixative to RT. With a black permanent pen, mark the region of the culture drop on the non-cell side of the coverslip. Remove the coverslip from culture chamber and position on a piece of plasticine or a rubber block with the cells facing up in a humid chamber. If glass-bottom dishes are used, detach the coverslips from the culture chambers and proceed to step 8.
11. Add 100–200 µL of 4% paraformaldehyde to the cells. Close the humid chamber and incubate for 30 min.
12. Remove fixative and discard adequately, wash the cultures three times with PBT. MitoTracker can be combined with immunocytochemistry, using standard protocols [84].

3.4.3 **Genetically encoded cargo markers (e.g. Synaptotagmin-GFP and mito-GFP/mCherry):**

Genetically encoded fluorescent tools are particularly useful to examine the dynamics of motile organelles and transport vesicles in neurons and have been used to this end during decades *in vivo* and *in vitro* studies and in various organisms including rodents, zebrafish, *Drosophila* and *C.* elegans [85] [86] [87] [88]. *Drosophila* examples of such tools include synaptic vesicle proteins such as GFP-fused Synaptotagmin, Synaptobrevin and Synaptogyrin to label synapses and synaptic transport, tagged Ras-associated binding GTPases and Autophagy-related proteins to label endosomes and autophagosomes respectively, and mitochondrial targeting sequences fused to fluorescent proteins (eg. mito-GFP) to label mitochondria [44,65,89,90,45]. We have used primary cultures derived from transgenic *Drosophila* embryos expressing mito-GFP/mCherry, Synaptobrevin-GFP and Synaptotagmin-GFP using the UAS/Gal4 system to study the impact mutations in microtubule regulators such as Tau and spectraplakins [44] and α1-tubulin84B have on axonal transport. Here we will describe how we use genetically encoded cargo markers (see Note 9), for examples see Fig. 2C and D; Suppl. Movies 3 and 4. Using this method, we find Synaptotagmin-positive vesicles move at a velocity of about 1.0-1.9 µm/s.

1. Generate *Drosophila* primary neuronal cultures (sections 3.1 - 3.3) from embryos or larvae expressing the genetically encoded cargo marker in the desired genetic background (see Note 10 for suitable drivers and transfection options).
2. Once primary neuronal cultures have reached the adequate developmental stage, detach the coverslips from the culture chambers or glass-bottom dishes and wash the cells (they will be attached to the coverslips if using culture chambers or at the bottom of the glass bottom

plates) and culture chambers in supplemented Schneider's medium (~60 µL). Be careful as the coverslips are prone to break. This step is important to remove debris from the cultures which may interfere with live imaging.

3. Remove all medium without disturbing the cells and add 50 - 60 µL fresh supplemented Schneider's medium to the chamber/dish.
4. Re-apply new Vaseline if needed and place the coverslip back in place.
5. Let the cells adjust to the imaging conditions for 30 minutes in the environmental chamber attached to the microscope.

3.5 Live imaging of neuronal transport

Imaging neuronal transport is a delicate balancing act between sufficiently high resolution to visualise small vesicles, short exposure times to allow visualisation of fast neuronal transport events and minimise exposure to avoid photo-cytotoxic effects and photo-bleaching. We make use of spinning disk microscopy as the systems have been specifically developed to reduce phototoxicity whilst providing provide high sensitivity.

1. Image neurons using a spinning disk confocal (SDC) microscope with environmental chamber and at least 60 x magnification objective (or equivalent equipment with good sensitivity and signal to noise ratio). Ideally, set the environmental chamber to the same conditions as the primary neurons were cultured at.
2. Use a microscope with automated z-focus control (e.g. Definite Focus system or similar), which is highly advantageous as any focus drift could shift vesicles/organelles out of focus and prevent successful tracking of their movement (see Note 11).
3. Use either eye piece widefield fluorescence, phase contrast or SDC imaging to locate suitable cells.
4. Make sure to take note of the position of the cell body and axon/neurite tips for all imaged cells / axons to determine antero- and retrograde directionality.
5. Image with the appropriate timeframes and acquisition intervals, depending on the observed organelle/vesicle speeds, mutant conditions, labels used in the assay and their intensity during imaging. For LysoTracker, Synaptobrevin-GFP, Synaptotagmin-GFP and mito-GFP/mCherry, imaging timeframes in the range of 1 - 5 minutes at 0.25 - 1 second intervals have been used successfully. Due to the frequency of events and the slow transport component of mitochondria, imaging intervals might be expanded, and intervals prolonged for the analysis of mitochondrial transport.
6. Adjust the imaging conditions to the moving, not the stationary objects.
7. The number of cells to image depends on the frequency/number of visualised transport events. For the most part we have imaged 10-20 cells per each of the 3 coverslips, for statistical analysis of organelle/vesicle movements (e.g. Synaptotagmin-GFP, LysoTracker).
8. Process the imaging time series for either direct object tracking or kymograph analyses.

3.6 Analysing axonal transport

1. Process the imaging time series for either direct object tracking (manual tracking plugin for ImageJ, TrackMate [91]) or kymograph generation and analyses (e.g. KymoResliceWide & Velocity measurement (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Velocity_Measurement_Tool [92]) or KymoAnalyzer plugins for ImageJ [93]).

2. Suitable parameters to analyse are: the number of moving objects vs stationary objects, flux (number of moving particles through a defined length of the axon over a set time), directionality of movements (fraction of anterograde vs retrograde movement), average object track velocity (total speed over the whole kymograph track of a moving object including pauses, changes in speed and in direction), transport segment velocity (speed calculated for each individual segment of a kymograph track of a moving object. A new segment within the trach is defined each time there are changes in speed or direction of movement), transport run length (average length of a particle's runs), number of pauses per transport track, number of direction switches.

NOTES:

1) Any genetic combination can be used for primary cultures, these can include specimens with specific mutations, and/or expressing fluorescently tagged markers to visualise cargos. In the case of specimens carrying embryonic lethal mutations the mutant stock will need to be balanced over a balancer chromosome which allows for the selection of mutant stage 11 embryos. For this we use the TwistGal4-UAS-GFP balancers (available in the Bloomington stock centre #6873, #6662, #6663)

2) Collection times must be adapted when changing temperatures. e.g. 15 hours at 25 °C will favour stage 16 but hardly yield stage 11 embryos, while 15 hours at 21-23 °C will be enriched with stage 11 embryos (for stages and precise timing see [68]).

3) There are several factors to consider when selecting embryos:

   a. Embryonic stages 11 would be ideal for primary neuronal cultures, at this stage most neurons in embryos are post-mitotic and are beginning axogenesis. [66]. However, depending on the experimental setting, stage 15 / 16 might be useful, e.g. when the fluorescent signal from specific cargo is too week at younger stages (elav-Gal4 driven expression of synaptic markers is difficult to detect at stage 11).
   b. When collecting for embryos expressing synaptic markers, select embryos with weak expression as this will facilitate imaging (strongly expressing cells will have more stationary objects that can negatively impact imaging). An inducible expression system and a short expression time might be desirable to reduce the number of labelled stationary objects.
   c. When collecting mutant embryos against fluorescent balancers, make sure the balancer fluorescence is visible at the desired embryonic stage.

4) When using more embryos / more larval brains, adjust all volumes proportionately after grinding and before incubating at 37°C in HBSS-based dispersion medium at RT. Do not exceed 100 µL dispersion medium in a 1.5 mL tube for the grinding step as an increased volume can lead to spillage. We use approximately 100 µL dispersion medium per 15-30 embryos.

5) The grinding step is a key point in the protocol. Make sure to have a tight fit between tube and pestle. Using too much force might adversely affect the cultures though. However, be sure to dissociate all embryos. Any intact embryos will survive, develop into larvae, and disrupt the rest of the cultured cells. An alternative for grinding is to harvest cells from the ventral region of the embryo using a glass capillary and a micromanipulator [66], this method is considerably more laborious but may lead to purer cultures.

6) 26 °C would be the standard incubation temperature for *Drosophila* primary neurons. However, *Drosophila* primary neurons can be cultured at a range of temperatures ranging from 12 to 29 °C. For instance, incubation at 29 °C can boost UAS-Gal4 expression. Make sure controls are incubated at the same temperature. If imaging of the initial outgrowth is required, the seeding and attachment time can be reduced to 30 minutes. However, to get the same cell density, a 2-3 times increased amounts of embryos will be required.

7) The same protocol can be used to treat neurons with drugs and other live cell imaging fluorescent probes such as SiR-tubulin. We used 50 - 100 nM SiR-tubulin to label microtubules. Note that the higher end of SiR-tubulin concentrations can have effects on microtubule dynamics. A minimum of 30-minute incubation will ensure microtubules are robustly labelled, and can optionally be followed by a washing step with supplemented Schneider's medium but is not required, longer incubation will yield stronger labelling. Microtubules can be visualised from 45-50 minutes onwards. SiR-tubulin labelling of *Drosophila* primary neurons does not require Verapamil treatment. We have successfully visualised neurite outgrowth and development from primary neurons as early as 45 minutes *in vitro* up to 22 hours *in vitro* (for long-term imaging setup, see Fig. 1D). For this, the cell pellet is resuspended in supplemented Schneider's medium that contains 50 nM SiR-tubulin. Other compounds (e.g. microtubule stabilisers or destabilisers, inhibitors of kinesins, kinases and phosphatases, oxidative stress inducers amongst others) can be added to the cell culture medium keeping in mind that each drug will need a different incubation time.

8) Axonal transport of synaptic components in *Drosophila* primary neurons, can be observed already at 6 h *in vitro* [44], however we frequently image neurons at 2 to 3 days in vitro so that synaptic components are robustly established and long-range transport can be observed. Primary neurons cultured form larval brains allow imaging of lysotracker after one day in vitro.

9) The same protocol can be used to visualise EB dynamics in neurons. Both expression of EB1-GFP or EB1-mCherry via elav-Gal4 or sca-Gal4 and transfection of neurons with pAc-EB1-mCherry have been successfully used in our laboratories [42,43,54,55].

10) For UAS-Gal4-based expression of fluorescently tagged proteins in primary neuronal cultures, suitable driver lines are: sca-Gal4, elav-Gal4, tubulin-Gal4, nSyb-Gal4. Note that sca-Gal4 expression is strong in young neurons (6HIV) but decreases over time and nSyb-Gal4 is weakly expressing in young neurons but increases over time and can be robustly detected after 2-3 days in vitro (Ines Hahn, personal communication). An alternative to UAS-Gal4-based expression is transfection of neuronal cultures. We find that the actin-promotor in the pAc5.1/V5 vector variants is suitable for expression of constructs upon transfection of primary neurons (for protocol see [55]). Alternatively, Gal4 expressing neurons can be transfected with UAS-constructs.

11) Be aware that due to the neurites/axons/dendrites being dynamic, they might move and leave the focal plane. ConA treatment of coverslips can be an advantage here since it increases the attachment axons.

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Figure legends

Fig. 1 Workflow to generate Drosophila primary neuronal cultures for short- and long-term imaging. The numbers in the figure correspond to the protocol steps in section 3.3 (A) Diagram showing the steps for embryo collection, removal or the chorion, and selection of embryos for primary neuronal cultures. (B) Diagram showing the main steps to generate a suspension of primary cells from Drosophila embryos. Note that the protocol can be modified to use late stage 3 larval brains as well (see section 3.3 for more detail). (C) Diagram showing the hanging drop culturing technique. Drosophila primary cells are allowed to attach to coverslips before inverting the sealed culture chambers (upper row) / glass bottom dishes (lower row). Cells are then grown the desired time in vitro (hours to days). Cultures can optionally be incubated with drugs and/or dye-based organelle/vesicle markers before medium is exchanged for imaging. (D) For long-term imaging, cells can be allowed to either (a) directly attached to coated or uncoated glass bottom dishes or (b) grown as hanging drop cultures in glass bottom dishes, then unsealed and vaseline removed (see C). Cells can then be treated with drugs and/or dye-based organelle/vesicle labels. The dish is then filled up with Schneider's medium and sealed with a coverslip to prevent evaporation of the medium during long-term imaging.

Fig. 2 Transport of vesicles and organelles in *Drosophila* primary neuronal cultures. Single frame from time-lapse movies from *Drosophila* primary neuronal cultures showing different labelled cargo (cell bodies are at the bottom of the image), and kymograph plot showing the trajectory of cargoes. (A) Larval primary cultures treated with the dye-based fluorescent label LysoTracker DND-99. (B-D) Embryonic primary neuronal cultures (B) treated with the dye-based fluorescent label MitoTracker Green FM, (C) expressing Synaptotagmin-GFP using the tubulin-Gal4 driver and (D) expressing mito-mCherry using the elav-Gal4 driver. Magenta dashed lines mark the axon. Panels on the right in A-D show corresponding kymographs. Lines with positive slopes in kymographs indicate anterograde transport, lines with negative slopes retrograde transport and horizontal lines indicate stationary objects. Scale bar in overview images is 5 µm in length.

Supplementary material

Suppl. Movie 1

Live movie of larval Drosophila primary neurons grown for 1 day in vitro and incubated for 15 minutes with 100 nM LysoTracker DND-99. Scale bar represents 5 µm, time is given in seconds.

Suppl. Movie 2

Live movie of embryonic Drosophila primary neurons precultured in a 1.5 mL tube, grown for 1 day in vitro and incubated for 15 minutes with 50 nM MitoTracker Green FM. Scale bar represents 5 µm, time is given in seconds.

Suppl. Movie 3

Live movie of *Drosophila* embryonic primary neurons expressing UAS-Synaptotagmin-GFP under control of the tubulin-Gal4 driver line. Cells were grown for 3 days in vitro. Scale bar represents 5 um, time is given in seconds.

Suppl. Movie 4

Live movie of embryonic *Drosophila* primary neurons expressing UAS-mito-mCherry under control of the elav-Gal4 driver line. Cells were grown for 1 day in vitro. Scale bar represents 5 µm, time is given in seconds.

References

1. Terenzio M, Schiavo G, Fainzilber M (2017) Compartmentalized Signaling in Neurons: From Cell Biology to Neuroscience. Neuron 96 (3):667-679. doi:10.1016/j.neuron.2017.10.015
2. Franker MA, Hoogenraad CC (2013) Microtubule-based transport - basic mechanisms, traffic rules and role in neurological pathogenesis. Journal of cell science 126 (Pt 11):2319-2329. doi:10.1242/jcs.115030
3. Prokop A (2013) The intricate relationship between microtubules and their associated motor proteins during axon growth and maintenance. Neural Development 8 (1):17. doi:10.1186/1749-8104-8-17
4. Hirokawa N, Niwa S, Tanaka Y (2010) Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. Neuron 68 (4):610-638. doi:10.1016/j.neuron.2010.09.039
5. Sleigh JN, Rossor AM, Fellows AD, Tosolini AP, Schiavo G (2019) Axonal transport and neurological disease. Nature reviews Neurology 15 (12):691-703. doi:10.1038/s41582-019-0257-2
6. Guedes-Dias P, Holzbaur ELF (2019) Axonal transport: Driving synaptic function. Science 366 (6462):eaaw9997. doi:10.1126/science.aaw9997
7. Franker MAM, Hoogenraad CC (2013) Microtubule-based transport – basic mechanisms, traffic rules and role in neurological pathogenesis. Journal of cell science 126 (11):2319-2329. doi:10.1242/jcs.115030
8. Kevenaar JT, Hoogenraad CC (2015) The axonal cytoskeleton: from organization to function. Front Mol Neurosci 8:44. doi:10.3389/fnmol.2015.00044
9. Prokop A (2020) Cytoskeletal organization of axons in vertebrates and invertebrates. The Journal of cell biology 219 (7). doi:10.1083/jcb.201912081
10. Hirokawa N, Noda Y, Tanaka Y, Niwa S (2009) Kinesin superfamily motor proteins and intracellular transport. Nature Reviews Molecular Cell Biology 10 (10):682-696. doi:10.1038/nrm2774
11. Schiavo G, Greensmith L, Hafezparast M, Fisher EM (2013) Cytoplasmic dynein heavy chain: the servant of many masters. Trends in neurosciences 36 (11):641-651. doi:10.1016/j.tins.2013.08.001
12. Horiuchi D, Collins CA, Bhat P, Barkus RV, Diantonio A, Saxton WM (2007) Control of a kinesin-cargo linkage mechanism by JNK pathway kinases. Current biology : CB 17 (15):1313-1317. doi:10.1016/j.cub.2007.06.062
13. Guedes-Dias P, Nirschl JJ, Abreu N, Tokito MK, Janke C, Magiera MM, Holzbaur ELF (2019) Kinesin-3 Responds to Local Microtubule Dynamics to Target Synaptic Cargo Delivery to the Presynapse. Current biology : CB 29 (2):268-282.e268. doi:10.1016/j.cub.2018.11.065
14. Guo W, Stoklund Dittlau K, Van Den Bosch L (2020) Axonal transport defects and neurodegeneration: Molecular mechanisms and therapeutic implications. Seminars in Cell & Developmental Biology 99:133-150. doi:https://doi.org/10.1016/j.semcdb.2019.07.010
15. Mattedi F, Vagnoni A (2019) Temporal Control of Axonal Transport: The Extreme Case of Organismal Ageing. Frontiers in Cellular Neuroscience 13 (393). doi:10.3389/fncel.2019.00393
16. Hill SE, Colón-Ramos DA (2020) The Journey of the Synaptic Autophagosome: A Cell Biological Perspective. Neuron 105 (6):961-973. doi:https://doi.org/10.1016/j.neuron.2020.01.018

17. Corradi E, Dalla Costa I, Gavoci A, Iyer A, Roccuzzo M, Otto TA, Oliani E, Bridi S, Strohbuecker S, Santos-Rodriguez G, Valdembri D, Serini G, Abreu-Goodger C, Baudet ML (2020) Axonal precursor miRNAs hitchhike on endosomes and locally regulate the development of neural circuits. The EMBO journal 39 (6):e102513. doi:10.15252/embj.2019102513
18. Cheng X-T, Sheng Z-H Developmental regulation of microtubule-based trafficking and anchoring of axonal mitochondria in health and diseases. Developmental neurobiology n/a (n/a). doi:https://doi.org/10.1002/dneu.22748
19. Zhao J, Fok AHK, Fan R, Kwan P-Y, Chan H-L, Lo LH-Y, Chan Y-S, Yung W-H, Huang J, Lai CSW, Lai K-O (2020) Specific depletion of the motor protein KIF5B leads to deficits in dendritic transport, synaptic plasticity and memory. eLife 9:e53456. doi:10.7554/eLife.53456
20. Maday S, Twelvetrees Alison E, Moughamian Armen J, Holzbaur Erika LF (2014) Axonal Transport: Cargo-Specific Mechanisms of Motility and Regulation. Neuron 84 (2):292-309. doi:https://doi.org/10.1016/j.neuron.2014.10.019
21. Verhey KJ, Hammond JW (2009) Traffic control: regulation of kinesin motors. Nature Reviews Molecular Cell Biology 10 (11):765-777. doi:10.1038/nrm2782
22. Kelliher MT, Saunders HAJ, Wildonger J (2019) Microtubule control of functional architecture in neurons. Current Opinion in Neurobiology 57:39-45. doi:https://doi.org/10.1016/j.conb.2019.01.003
23. Harterink M, Edwards SL, de Haan B, Yau KW, van den Heuvel S, Kapitein LC, Miller KG, Hoogenraad CC (2018) Local microtubule organization promotes cargo transport in C. elegans dendrites. Journal of cell science 131 (20). doi:10.1242/jcs.223107
24. Gibbs KL, Greensmith L, Schiavo G (2015) Regulation of Axonal Transport by Protein Kinases. Trends in Biochemical Sciences 40 (10):597-610. doi:https://doi.org/10.1016/j.tibs.2015.08.003
25. Gindhart JG, Jr., Desai CJ, Beushausen S, Zinn K, Goldstein LSB (1998) Kinesin Light Chains Are Essential for Axonal Transport in Drosophila. Journal of Cell Biology 141 (2):443-454. doi:10.1083/jcb.141.2.443
26. Reis GF, Yang G, Szpankowski L, Weaver C, Shah SB, Robinson JT, Hays TS, Danuser G, Goldstein LS (2012) Molecular motor function in axonal transport in vivo probed by genetic and computational analysis in Drosophila. Mol Biol Cell 23 (9):1700-1714. doi:10.1091/mbc.E11-11-0938
27. Weiss KR, Littleton JT (2016) Characterization of axonal transport defects in Drosophila Huntingtin mutants. J Neurogenet 30 (3-4):212-221. doi:10.1080/01677063.2016.1202950
28. Vijayakumar J, Perrois C, Heim M, Bousset L, Alberti S, Besse F (2019) The prion-like domain of Drosophila Imp promotes axonal transport of RNP granules in vivo. Nature Communications 10 (1):2593. doi:10.1038/s41467-019-10554-w
29. Duncan JE, Goldstein LSB (2006) The Genetics of Axonal Transport and Axonal Transport Disorders. PLOS Genetics 2 (9):e124. doi:10.1371/journal.pgen.0020124
30. Medioni C, Ephrussi A, Besse F (2015) Live imaging of axonal transport in Drosophila pupal brain explants. Nature Protocols 10 (4):574-584. doi:10.1038/nprot.2015.034
31. Sánchez-Soriano N, Bottenberg W, Fiala A, Haessler U, Kerassoviti A, Knust E, Löhr R, Prokop A (2005) Are dendrites in *Drosophila* homologous to vertebrate dendrites? Developmental Biology 288 (1):126-138. doi:https://doi.org/10.1016/j.ydbio.2005.09.026
32. Bellen HJ, Tong C, Tsuda H (2010) 100 years of Drosophila research and its impact on vertebrate neuroscience: a history lesson for the future. Nature reviews Neuroscience 11 (7):514-522. doi:10.1038/nrn2839
33. McGurk L, Berson A, Bonini NM (2015) Drosophila as an In Vivo Model for Human Neurodegenerative Disease. Genetics 201 (2):377-402. doi:10.1534/genetics.115.179457
34. Sánchez-Soriano N, Tear G, Whitington P, Prokop A (2007) Drosophilaas a genetic and cellular model for studies on axonal growth. Neural Development 2 (1):9. doi:10.1186/1749-8104-2-9
35. Sanes JR, Zipursky SL (2010) Design principles of insect and vertebrate visual systems. Neuron 66 (1):15-36. doi:10.1016/j.neuron.2010.01.018

36. Hirokawa N, Tanaka Y (2015) Kinesin superfamily proteins (KIFs): Various functions and their relevance for important phenomena in life and diseases. Experimental cell research 334 (1):16-25. doi:10.1016/j.yexcr.2015.02.016
37. Ejsmont RK, Hassan BA (2014) The Little Fly that Could: Wizardry and Artistry of Drosophila Genomics. Genes 5 (2):385-414. doi:10.3390/genes5020385
38. Hahn I, Ronshaugen M, Sánchez-Soriano N, Prokop A (2016) Functional and Genetic Analysis of Spectraplakins in Drosophila. Methods in enzymology 569:373-405. doi:10.1016/bs.mie.2015.06.022
39. Bier E, Harrison MM, O'Connor-Giles KM, Wildonger J (2018) Advances in Engineering the Fly Genome with the CRISPR-Cas System. Genetics 208 (1):1-18. doi:10.1534/genetics.117.1113
40. Donady JJ, Seecof RL (1972) Effect of the Gene Lethal (1) Myospheroid on Drosophila Embryonic Cells In vitro. In Vitro 8 (1):7-12
41. Seecof RL, Alléaume N, Teplitz RL, Gerson I (1971) Differentiation of neurons and myocytes in cell cultures made from Drosophila gastrulae. Experimental cell research 69 (1):161-173. doi:https://doi.org/10.1016/0014-4827(71)90321-1
42. Alves-Silva J, Sánchez-Soriano N, Beaven R, Klein M, Parkin J, Millard TH, Bellen HJ, Venken KJ, Ballestrem C, Kammerer RA, Prokop A (2012) Spectraplakins promote microtubule-mediated axonal growth by functioning as structural microtubule-associated proteins and EB1-dependent +TIPs (tip interacting proteins). J Neurosci 32 (27):9143-9158. doi:10.1523/jneurosci.0416-12.2012
43. Szikora S, Földi I, Tóth K, Migh E, Vig A, Bugyi B, Maléth J, Hegyi P, Kaltenecker P, Sanchez-Soriano N, Mihály J (2017) The formin DAAM is required for coordination of the actin and microtubule cytoskeleton in axonal growth cones. Journal of cell science 130 (15):2506-2519. doi:10.1242/jcs.203455
44. Voelzmann A, Okenve-Ramos P, Qu Y, Chojnowska-Monga M, Del Caño-Espinel M, Prokop A, Sanchez-Soriano N (2016) Tau and spectraplakins promote synapse formation and maintenance through Jun kinase and neuronal trafficking. Elife 5. doi:10.7554/eLife.14694
45. Zschätzsch M, Oliva C, Langen M, De Geest N, Ozel MN, Williamson WR, Lemon WC, Soldano A, Munck S, Hiesinger PR, Sanchez-Soriano N, Hassan BA (2014) Regulation of branching dynamics by axon-intrinsic asymmetries in Tyrosine Kinase Receptor signaling. Elife 3:e01699. doi:10.7554/eLife.01699
46. Qu Y, Hahn I, Webb SED, Pearce SP, Prokop A (2017) Periodic actin structures in neuronal axons are required to maintain microtubules. Mol Biol Cell 28 (2):296-308. doi:10.1091/mbc.E16-10-0727
47. Sánchez-Soriano N, Gonçalves-Pimentel C, Beaven R, Haessler U, Ofner-Ziegenfuss L, Ballestrem C, Prokop A (2010) Drosophila growth cones: A genetically tractable platform for the analysis of axonal growth dynamics. Developmental neurobiology 70 (1):58-71. doi:https://doi.org/10.1002/dneu.20762
48. Bai J, Sepp KJ, Perrimon N (2009) Culture of Drosophila primary cells dissociated from gastrula embryos and their use in RNAi screening. Nature Protocols 4 (10):1502-1512. doi:10.1038/nprot.2009.147
49. Küppers-Munther B, Letzkus JJ, Lüer K, Technau G, Schmidt H, Prokop A (2004) A new culturing strategy optimises Drosophila primary cell cultures for structural and functional analyses. Developmental Biology 269 (2):459-478. doi:https://doi.org/10.1016/j.ydbio.2004.01.038
50. Ceron J, Tejedor FJ, Moya F (2006) A primary cell culture of Drosophila postembryonic larval neuroblasts to study cell cycle and asymmetric division. European Journal of Cell Biology 85 (6):567-575. doi:https://doi.org/10.1016/j.ejcb.2006.02.006
51. Katsuki T, Ailani D, Hiramoto M, Hiromi Y (2009) Intra-axonal Patterning: Intrinsic Compartmentalization of the Axonal Membrane in Drosophila Neurons. Neuron 64 (2):188-199. doi:https://doi.org/10.1016/j.neuron.2009.08.019
52. Slováková J, Speicher S, Sánchez-Soriano N, Prokop A, Carmena A (2012) The actin-binding protein Canoe/AF-6 forms a complex with Robo and is required for Slit-Robo signaling during axon

640 pathfinding at the CNS midline. J Neurosci 32 (29):10035-10044. doi:10.1523/jneurosci.6342-
641 11.2012
642 53. Oliva C, Soldano A, Mora N, De Geest N, Claeys A, Erfurth ML, Sierralta J, Ramaekers A, Dascenco
643 D, Ejsmont RK, Schmucker D, Sanchez-Soriano N, Hassan BA (2016) Regulation of Drosophila Brain
644 Wiring by Neuropil Interactions via a Slit-Robo-RPTP Signaling Complex. Developmental cell 39
645 (2):267-278. doi:10.1016/j.devcel.2016.09.028
646 54. Hahn I, Voelzmann A, Parkin J, Fuelle J, Slater PG, Lowery LA, Sanchez-Soriano N, Prokop A (2020)
647 Tau, XMAP215/Msps and Eb1 jointly regulate microtubule polymerisation and bundle formation
648 in axons. bioRxiv:2020.2008.2019.257808. doi:10.1101/2020.08.19.257808
649 55. Qu Y, Hahn I, Lees M, Parkin J, Voelzmann A, Dorey K, Rathbone A, Friel CT, Allan VJ, Okenve-Ramos
650 P, Sanchez-Soriano N, Prokop A (2019) Efa6 protects axons and regulates their growth and
651 branching by inhibiting microtubule polymerisation at the cortex. eLife 8:e50319.
652 doi:10.7554/eLife.50319
653 56. Prokop A, Beaven R, Qu Y, Sánchez-Soriano N (2013) Using fly genetics to dissect the cytoskeletal
654 machinery of neurons during axonal growth and maintenance. Journal of cell science 126 (Pt
655 11):2331-2341. doi:10.1242/jcs.126912
656 57. Sanchez-Soriano N, Travis M, Dajas-Bailador F, Gonçalves-Pimentel C, Whitmarsh AJ, Prokop A
657 (2009) Mouse ACF7 and drosophila short stop modulate filopodia formation and microtubule
658 organisation during neuronal growth. Journal of cell science 122 (Pt 14):2534-2542.
659 doi:10.1242/jcs.046268
660 58. Stephan D, Sánchez-Soriano N, Loschek LF, Gerhards R, Gutmann S, Storchova Z, Prokop A, Kadow
661 IC (2012) Drosophila Psidin regulates olfactory neuron number and axon targeting through two
662 distinct molecular mechanisms. J Neurosci 32 (46):16080-16094. doi:10.1523/jneurosci.3116-
663 12.2012
664 59. Kraft R, Escobar MM, Narro ML, Kurtis JL, Efrat A, Barnard K, Restifo LL (2006) Phenotypes of
665 Drosophila Brain Neurons in Primary Culture Reveal a Role for Fascin in Neurite Shape
666 and Trajectory. The Journal of Neuroscience 26 (34):8734-8747. doi:10.1523/jneurosci.2106-
667 06.2006
668 60. del Castillo U, Müller H-AJ, Gelfand VI (2020) Kinetochore protein Spindly controls microtubule
669 polarity in Drosophila axons. Proceedings of the National Academy of Sciences 117
670 (22):12155-12163. doi:10.1073/pnas.2005394117
671 61. Yao W-D, Rusch J, Poo M-m, Wu C-F (2000) Spontaneous Acetylcholine Secretion from Developing
672 Growth Cones of Drosophila Central Neurons in Culture: Effects of cAMP-Pathway
673 Mutations. The Journal of Neuroscience 20 (7):2626-2637. doi:10.1523/jneurosci.20-07-
674 02626.2000
675 62. Küppers B, Sánchez-Soriano N, Letzkus J, Technau GM, Prokop A (2003) In developing Drosophila
676 neurones the production of gamma-amino butyric acid is tightly regulated downstream of
677 glutamate decarboxylase translation and can be influenced by calcium. Journal of neurochemistry
678 84 (5):939-951. doi:10.1046/j.1471-4159.2003.01554.x
679 63. West RJH, Sharpe JL, Voelzmann A, Munro AL, Hahn I, Baines RA, Pickering-Brown S (2020) Co-
680 expression of C9orf72 related dipeptide-repeats over 1000 repeat units reveals age- and
681 combination-specific phenotypic profiles in Drosophila. Acta neuropathologica communications 8
682 (1):158. doi:10.1186/s40478-020-01028-y
683 64. Narayanareddy BR, Vartiainen S, Hariri N, O'Dowd DK, Gross SP (2014) A biophysical analysis of
684 mitochondrial movement: differences between transport in neuronal cell bodies versus processes.
685 Traffic (Copenhagen, Denmark) 15 (7):762-771. doi:10.1111/tra.12171
686 65. Liao P-C, Tandarich LC, Hollenbeck PJ (2017) ROS regulation of axonal mitochondrial transport is
687 mediated by Ca2+ and JNK in Drosophila. PLoS One 12 (5):e0178105.
688 doi:10.1371/journal.pone.0178105
689 66. Prokop A, Küppers-Munther B, Sánchez-Soriano N (2012) Using Primary Neuron Cultures of
690 Drosophila to Analyze Neuronal Circuit Formation and Function. In: Hassan BA (ed) The Making

and Un-Making of Neuronal Circuits in Drosophila. Humana Press, Totowa, NJ, pp 225-247. doi:10.1007/978-1-61779-830-6_10

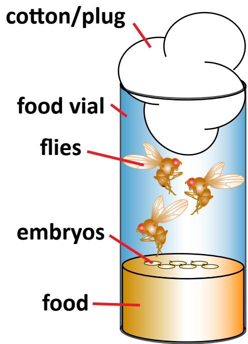
67. Sicaeros B, Campusano JM, O'Dowd DK (2007) Primary neuronal cultures from the brains of late stage Drosophila pupae. J Vis Exp (4):200-200. doi:10.3791/200
68. Campos-Ortega JA, Hartenstein, Volker. (1997) The embryonic development of Drosophila melanogaster. Berlin: Springer Verlag. Berlin: Springer Verlag,
69. Roote J, Prokop A (2013) How to design a genetic mating scheme: a basic training package for Drosophila genetics. G3 (Bethesda, Md) 3 (2):353-358. doi:10.1534/g3.112.004820
70. Vagnoni A, Bullock SL (2016) A simple method for imaging axonal transport in aging neurons using the adult Drosophila wing. Nature Protocols 11 (9):1711-1723. doi:10.1038/nprot.2016.112
71. Lee S, Sato Y, Nixon RA (2011) Lysosomal Proteolysis Inhibition Selectively Disrupts Axonal Transport of Degradative Organelles and Causes an Alzheimer's-Like Axonal Dystrophy. The Journal of Neuroscience 31 (21):7817-7830. doi:10.1523/jneurosci.6412-10.2011
72. Maday S, Wallace KE, Holzbaur ELF (2012) Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons. Journal of Cell Biology 196 (4):407-417. doi:10.1083/jcb.201106120
73. Moughamian AJ, Osborn GE, Lazarus JE, Maday S, Holzbaur EL (2013) Ordered recruitment of dynactin to the microtubule plus-end is required for efficient initiation of retrograde axonal transport. J Neurosci 33 (32):13190-13203. doi:10.1523/jneurosci.0935-13.2013
74. Evans CS, Holzbaur ELF (2020) Degradation of engulfed mitochondria is rate-limiting in Optineurin-mediated mitophagy in neurons. eLife 9:e50260. doi:10.7554/eLife.50260
75. Perrot R, Julien JP (2009) Real-time imaging reveals defects of fast axonal transport induced by disorganization of intermediate filaments. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 23 (9):3213-3225. doi:10.1096/fj.09-129585
76. Beaven R, Dzhindzhev NS, Qu Y, Hahn I, Dajas-Bailador F, Ohkura H, Prokop A (2015) Drosophila CLIP-190 and mammalian CLIP-170 display reduced microtubule plus end association in the nervous system. Mol Biol Cell 26 (8):1491-1508. doi:10.1091/mbc.E14-06-1083
77. DeVorkin L, Gorski SM (2014) LysoTracker staining to aid in monitoring autophagy in Drosophila. Cold Spring Harbor protocols 2014 (9):951-958. doi:10.1101/pdb.prot080325
78. Zhao X, Chen XQ, Han E, Hu Y, Paik P, Ding Z, Overman J, Lau AL, Shahmoradian SH, Chiu W, Thompson LM, Wu C, Mobley WC (2016) TRiC subunits enhance BDNF axonal transport and rescue striatal atrophy in Huntington's disease. Proc Natl Acad Sci U S A 113 (38):E5655-5664. doi:10.1073/pnas.1603020113
79. Pilling AD, Horiuchi D, Lively CM, Saxton WM (2006) Kinesin-1 and Dynein are the primary motors for fast transport of mitochondria in Drosophila motor axons. Mol Biol Cell 17 (4):2057-2068. doi:10.1091/mbc.e05-06-0526
80. Moutaux E, Christaller W, Scaramuzzino C, Genoux A, Charlot B, Cazorla M, Saudou F (2018) Neuronal network maturation differently affects secretory vesicles and mitochondria transport in axons. Scientific Reports 8 (1):13429. doi:10.1038/s41598-018-31759-x
81. Sheng Z-H (2014) Mitochondrial trafficking and anchoring in neurons: New insight and implications. Journal of Cell Biology 204 (7):1087-1098. doi:10.1083/jcb.201312123
82. Da-Rè C, Franzolin E, Biscontin A, Piazzesi A, Pacchioni B, Gagliani MC, Mazzotta G, Tacchetti C, Zordan MA, Zeviani M, Bernardi P, Bianchi V, De Pittà C, Costa R (2014) Functional characterization of drim2, the Drosophila melanogaster homolog of the yeast mitochondrial deoxynucleotide transporter. J Biol Chem 289 (11):7448-7459. doi:10.1074/jbc.M113.543926
83. Wang X, Schwarz TL (2009) Imaging axonal transport of mitochondria. Methods in enzymology 457:319-333. doi:10.1016/S0076-6879(09)05018-6
84. Budnik V, Gorczyca M, Prokop A (2006) Selected methods for the anatomical study of Drosophila embryonic and larval neuromuscular junctions. International review of neurobiology 75:323-365. doi:10.1016/s0074-7742(06)75015-2

741 85. Bercier V, Rosello M, Del Bene F, Revenu C (2019) Zebrafish as a Model for the Study of Live in vivo
742 Processive Transport in Neurons. Frontiers in Cell and Developmental Biology 7 (17).
743 doi:10.3389/fcell.2019.00017
744 86. Gunawardena S, Her LS, Brusch RG, Laymon RA, Niesman IR, Gordesky-Gold B, Sintasath L, Bonini
745 NM, Goldstein LS (2003) Disruption of axonal transport by loss of huntingtin or expression of
746 pathogenic polyQ proteins in Drosophila. Neuron 40 (1):25-40. doi:10.1016/s0896-
747 6273(03)00594-4
748 87. Nonet ML (1999) Visualization of synaptic specializations in live C. elegans with synaptic vesicle
749 protein-GFP fusions. Journal of neuroscience methods 89 (1):33-40. doi:10.1016/s0165-
750 0270(99)00031-x
751 88. Surana S, Villarroel-Campos D, Lazo OM, Moretto E, Tosolini AP, Rhymes ER, Richter S, Sleigh JN,
752 Schiavo G (2020) The evolution of the axonal transport toolkit. Traffic (Copenhagen, Denmark) 21
753 (1):13-33. doi:https://doi.org/10.1111/tra.12710
754 89. Lu W, Del Castillo U, Gelfand VI (2013) Organelle transport in cultured Drosophila cells: S2 cell line
755 and primary neurons. J Vis Exp (81):e50838. doi:10.3791/50838
756 90. Dey S, Banker G, Ray K (2017) Anterograde Transport of Rab4-Associated Vesicles Regulates
757 Synapse Organization in Drosophila. Cell reports 18 (10):2452-2463.
758 doi:10.1016/j.celrep.2017.02.034
759 91. Jean-Yves Tineveza NP, Johannes Schindelin, Genevieve M. Hoopes, Gregory D. Reynolds,
760 Emmanuel Laplantine, Sebastian Y. Bednarek, Spencer L.Shorte, Kevin W. Eliceiri (2017)
761 TrackMate: An open and extensible platform for single-particle tracking. Elsevier Methods 115
762 92. E K (2020) KymoResliceWide plugin for ImageJ v0.5. Zenodo
763 93. Neumann S, Chassefeyre R, Campbell GE, Encalada SE (2017) KymoAnalyzer: a software tool for
764 the quantitative analysis of intracellular transport in neurons. Traffic (Copenhagen, Denmark) 18
765 (1):71-88. doi:10.1111/tra.12456

766

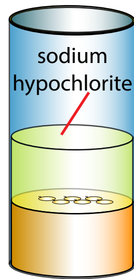
A Embryo collection, dechorionation and sorting

1

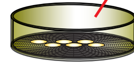


let flies lay eggs; remove flies

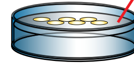
2



add sodium hypochlorite 90 sec



decant embryos into sieve wash with water



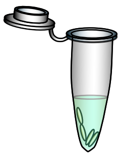
transfer to agar plate

3

sort embryos by fluorescence and stage

B Preparation of cell suspension

4 - 5

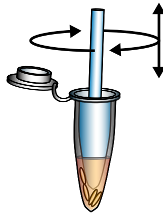


transfer embryos to tube; wash in 70% EtOH

6

wash embryos in Schneider's medium

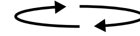
7 - 9



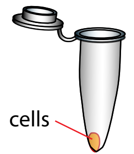
replace with Dispersion medium; grind embryos; incubate at 37°C

10

stop reaction by adding Schneider's medium



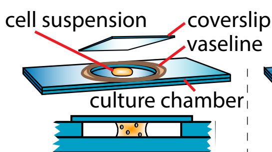
11 - 12



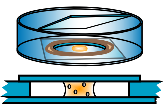
spin down cells; remove solution; resuspend in Schneider's medium

C Growing *Drosphila* primary neurons in hanging drop culture for short-term imaging

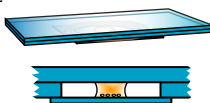
13



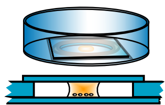
load cells into well & seal with coverslip



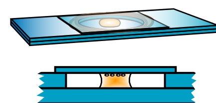
14



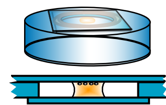
let cells settle & attach

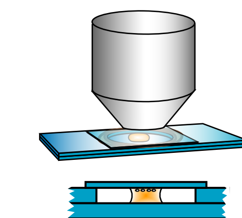


15

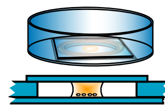


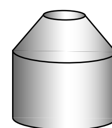
turn chambers; grow as hanging drop





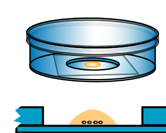
label; wash away detritus; re-seal and image





D Long-term imaging

a) load cells into well & let attach
b) unseal older cultures



wash cells; fill with medium; seal dish with vaseline & large coverslip

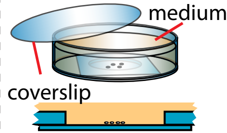
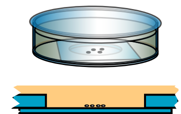
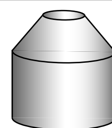
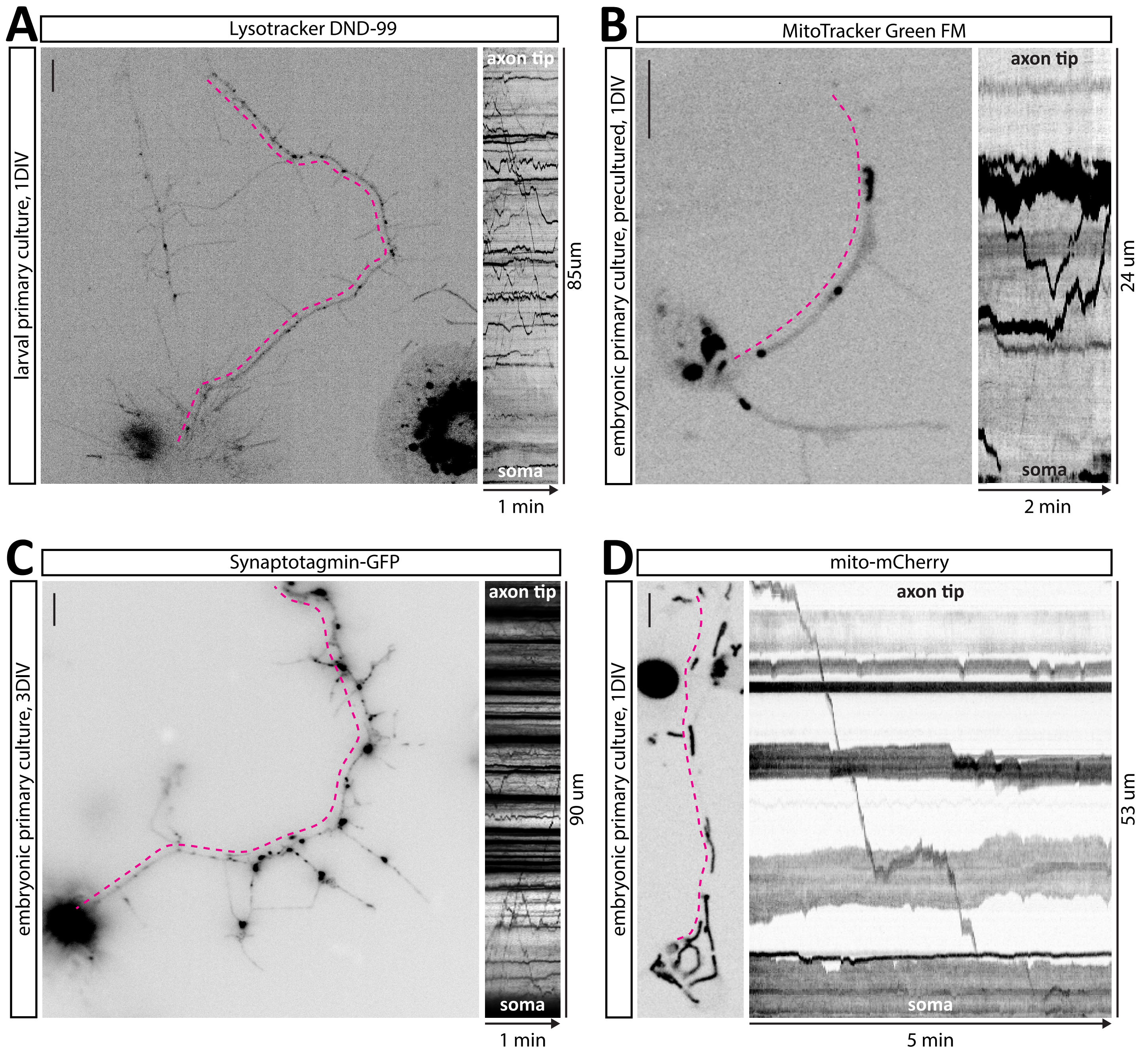


image cells






A
Lysotracker DND-99
larval primary culture, 1DIV
axon tip
soma
85um
1 min
B
MitoTracker Green FM
embryonic primary culture, precultured, 1DIV
axon tip
soma
24 um
2 min
C
Synaptotagmin-GFP
embryonic primary culture, 3DIV
axon tip
soma
90 um
1 min
D
mito-mCherry
embryonic primary culture, 1DIV
axon tip
soma
53 um
5 min