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In vivo analysis of axonal transport in zebrafish

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Abstract

Intracellular transport of proteins and organelles in neurons plays an essential role in nervous system development and maintenance. Axon outgrowth, synapse formation, and synapse function, among other physiological processes, require active transport of these cargos between the neuronal soma and axon terminals. Abnormalities in this axonal transport are associated with a number of neurodevelopmental and neurodegenerative disorders, such as Charcot-Marie-Tooth disease, Alzheimer disease, and amyotrophic lateral sclerosis. Despite its importance for nervous system development and health, methods for visualizing axonal transport in an intact vertebrate have been lacking. Using the advantages of the zebrafish system, we have developed a straightforward approach to visualize axonal transport of various cargos and motor proteins in intact zebrafish embryos and larvae. Here, we describe this approach in detail and discuss how it can be applied to address questions related to cargo-specific transport regulation and its effects on axon morphology and function in the developing and mature nervous system.

INTRODUCTION

All cells employ intracellular transport of proteins and organelles to function and respond to environmental cues. Neurons are particularly reliant on this process because of their highly compartmentalized nature, large cell volume, and high metabolic demand. Axons, which can be up to a meter long in humans, are presented with the particular challenge of efficiently moving organelles and proteins between the cell soma and distant terminals to form and then maintain connections. This movement is primarily accomplished by active, microtubule-based transport, which is mediated by two types of molecular motors, kinesins and dyneins (Allen, Metuzals, Tasaki, Brady, & Gilbert, 1982; Brady, Lasek, & Allen, 1982; Paschal, Shpetner, & Vallee, 1987; Schnapp & Reese, 1989; Vale, Reese, & Sheetz, 1985). In axons, kinesins, a superfamily of motor proteins composed of 45 members in humans, are predominantly utilized to move cargo away from the cell body, while the single cytoplasmic dynein motor accomplishes the vast majority of cell body-directed transport. Various types of vesicles and organelles are moved by fast axonal transport both in anterograde and retrograde directions at speeds of 0.5–10 $\mu\text{m/s}$, with retrograde transport typically being slower due to its saltatory nature (Hirokawa, Niwa, & Tanaka, 2010). The most studied and

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SUPPLEMENTARY DATA

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potentially most utilized kinesin motor is kinesin-1 (also known as KIF5). This motor is a tetramer composed of two motor domain-containing heavy chains and a dimer of light chains responsible for binding adaptor proteins and cargo (Gyoeva, Sarkisov, Khodjakov, & Minin, 2004; Hirokawa et al., 1989). Work in numerous systems has confirmed a role for this motor in the anterograde movement of organelles and structural proteins and has shown it to be essential for axon outgrowth and maintenance. Cytoplasmic dynein, the single motor responsible for retrograde axonal transport, is a large multiprotein complex composed of two motor domain-containing heavy chains, two intermediate chains, two light intermediate chains, and a complement of light chains, named for their molecular weights (reviewed in Holzbaur & Vallee, 1994). In addition to this core complex, dynein is oftentimes bound to dynactin, itself a multiprotein complex (Schroer, 2004). Cargo is thought to bind to the core motor components directly or via unique adaptor proteins. How particular cargo is selectively bound to, moved, and deposited by this single motor is a topic of active investigation.

To study axonal transport in real time, live imaging techniques have been established in culture models as well as in the invertebrate systems, *Drosophila* and *Caenorhabditis elegans*. Imaging this process in a live vertebrate has, however, presented a particular challenge because established model systems, such as mouse and frog, lack transparency. In addition, visualizing cargo or motor movement in mouse requires the generation of transgenic lines that label specific axonal cargo. Moreover, this transgenic approach usually labels multiple neurons, precluding imaging at the resolution of a single axon. Zebrafish embryos and larvae have a number of properties that avoid these obstacles, making them an ideal model system for studying axonal transport in an *in vivo* context. Zebrafish embryos and larvae remain optically transparent throughout development, making them amenable to live imaging approaches. Also, transient transgenic approaches are well established in zebrafish; this allows expression of cargo protein fusions in a tissue-specific manner without the production of a stable transgenic line. Importantly, the mosaic nature of this transient expression allows visualization of cargo movement in a single axon.

We have used these advantages to image transport of multiple cargos in the afferent axons of the posterior lateral line (pLL) sensory system. The pLL system is a mechanosensory system found in aquatic vertebrates that senses water currents and controls various swimming behaviors. The pLL axons relay sensory information from the mechanosensory organs (neuromasts) in the trunk to the central nervous system (Figure 1). These axons are especially convenient for our imaging studies because of the following reasons: (1) they are one of the longest axons in zebrafish at this stage (approximately 5 mm long at 4 days postfertilization (dpf)), (2) they are close to the surface (<20 μ m) and planar, and (3) the lateral line circuit develops rapidly with primary axon extension complete by 2 dpf and active synapses formed by 4 dpf.

Below, we describe a relatively straightforward method for imaging cargo in the pLL axons as well as co-transport of one of these cargos with the dynein motor complex. This method takes advantage of a neural-specific promoter that transiently marks pLL neurons, making visualization of transport in a single axon possible. This method can be adapted by any laboratory that has access to a standard confocal or a spinning disk confocal microscope

with a high-power water objective to image cargos of interest in an intact neural circuit with no surgical manipulation.

1. METHODS

1.1 PREPARATION OF DEOXYRIBONUCLEIC ACID FOR MICROINJECTION

1. Using a plasmid containing an appropriate promoter and cargo fusion open reading frame, inoculate a 5-mL culture (with appropriate antibiotic) from a single colony.
 - a. We use the Chien lab Tol2Kit, which is based on Gateway modular assembly (Life Technologies), to rapidly make cargo reporter constructs (Kwan et al., 2007).
 - b. Promoters appropriate for expressing cargo fusions to visualize axonal transport include neuronal promoters, such as that derived from the *neurod* locus (Drerup & Nechiporuk, 2013; Mo & Nicolson, 2011).
2. Prepare deoxyribonucleic acid (DNA) using the QIAprep Spin Miniprep kit.
 - a. Use the optional PB wash.
 - b. Elute in 30 μ L of deoxyribonuclease/ribonuclease-free water.
3. Confirm construct using restriction digest and quantify concentration using a spectrophotometer.
 - a. The 260:280 absorbance ratio should be between 1.8 and 2.0.
 - Higher or lower readings oftentimes result in embryonic toxicity.

1.2 ZYGOTIC MICROINJECTION

Microinjection is done according to established protocols (https://zfin.org/zf_info/zfbook/chapt5/5.1.html) using an Applied Scientific Instrumentation pressure injector equipped with a foot pedal, a Nikon SMZ745 (or similar) dissecting microscope, and a standard micromanipulator (e.g., the MX130 model from AppliedScientific Instrumentation).

1. Prepare a dilution of plasmid DNA in water.
 - a. Prior to generating transport data, identify the ideal amount of DNA for microinjection.
 - Typically, we find this is between 3 and 25 pg.
2. Using loading tips, backfill a pulled glass capillary with the diluted DNA.
 - a. Glass capillaries are pulled using a Flaming/Brown micropipette puller. Make sure that the capillary taper is long enough to reach the egg but still rigid enough to penetrate the chorion.
3. With forceps, break the tip of the capillary and adjust the pulse duration such that 0.5 nL of fluid is dispensed with every pulse.

- a. Typically, we calibrate such that 0.5 nL of fluid contains 3–25 pg of DNA.
 - The amount is quantified based on fluid droplet size in mineral oil using a stage micrometer.
 1. A 100 μm -diameter droplet is roughly 0.5 nL of fluid.
 - Air pulses of 100–200 ms are typical.
 - Backpressure should be corrected such that a very small fluid leak is observed.
4. Collect eggs within 20 min after fertilization and arrange in wells of a 1.5% agarose plate (Figure 1).
 - a. Agarose plates for injection can be made as described in https://zfin.org/zf_info/zfbook/chapt5/5.1.html.
5. Orient the eggs such that the cell cytoplasm is facing the needle.
6. Inject 0.5–1 nL into the cytoplasm, and wash zygotes into a fresh 100 mm petri dish with embryo media.
 - a. Only inject embryos at the single- or two-cell stage.
7. Grow embryos (50 per dish) at 28.5 °C in a standard incubator.
8. At blastula stage (~4 h postfertilization at 28.5 °C), remove any unfertilized or abnormal embryos.
9. Sort for expression of your fluorescently labeled cargo in your cell population of interest at the appropriate stage using a compound microscope equipped with epifluorescence.
 - a. To anesthetize embryos and larvae, replace embryo media with new media containing 0.02% tricaine.
 - b. For our work, we sort on a Zeiss Axio Imager compound microscope with a 10X objective in a depression slide.
 - c. Using embryos derived from a transgenic strain that marks a tissue of interest greatly facilitates sorting of embryos that express cargo fusions in the target cell population. We utilize *TgBAC(neurod:egfp)ⁿ¹¹* BAC (bacterial artificial chromosome) embryos, which express cytoplasmic enhanced green fluorescent protein (EGFP) in neurons of developing embryos and larvae (Obholzer et al., 2008).
10. Transfer embryos/larvae of interest into fresh embryo media without tricaine and keep them in the 28.5 °C incubator until they are ready to image.

1.2.1 Materials—20X embryo media (4.9 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.41 g KH_2PO_4 , 0.12 g Na_2HPO_4)

2.9 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.75 g KCl, 17.5 g NaCl in 1000 mL H_2O)

500X sodium bicarbonate (3.0 g NaHCO_3 in 100 mL H_2O)

Embryo media (100 mL embryo media stock, 4 mL sodium bicarbonate stock in 2000 mL H_2O)

20X tricaine (0.4 g ethyl-*M*-aminobenzoate methane sulfonate, 0.08 g Na_2PO_4 in 100 mL H_2O)

QIAprep Spin Miniprep kit (Qiagen, #12123)

Loading tips (Eppendorf microloaders, #5242956003)

Thin-walled glass capillaries with a filament (World Precision Instruments, #TW100F-4)

Agarose

100 mm petri dishes (Fisher Scientific, #09-757-100D)

Injection plate mold (<http://www.adaptivesciencetools.com/>)

MPPI-3 pressure injector with foot pedal and micromanipulator (Applied Scientific Instrumentation)

Nikon SMZ745 dissecting microscope

Zeiss Axio Imager (or equivalent) compound microscope equipped with epifluorescence and a 10X objective

1.3 MOUNTING ZEBRAFISH FOR CONFOCAL IMAGING

1. Prepare 1.2% low-melting-point agarose in embryo media by microwaving on 50% power.
 - a. Aliquots of low-melting-point agarose can be prepared in 5-mL glass tubes and stored at room temperature for several months when sealed with parafilm.
2. After the agarose is in liquid form, cover with parafilm and place it in a 42 °C water bath next to a standard dissecting microscope.
 - a. Allow agarose to cool to 42 °C before use to avoid scalding the larvae.
3. Anesthetize one individual at a time in fresh embryo media with 0.02% tricaine.
4. When the larvae stop responding to gentle nudges, use a glass pipette to transfer them in a small droplet of media into the vial of agarose.
5. Dispense any extra media into the waste and pull the larva back into the pipette with a small amount of agarose.

6. Place the larva in a droplet of agarose onto the underside of the coverslip mounted to the lid of a 35-mm petri dish (Figure 2(A) and (B)).
 - a. These modified petri dishes are made by drilling a small hole (~1 cm diameter) in the lid of the dish and attaching a coverslip (22 × 22 mm; #1.5) to the top using a thin layer of vacuum grease.
7. Using a larva manipulation capillary, gently position the individual at the correct angle for imaging.
 - a. For imaging pLL axons, this is accomplished by laying the larva on its side, perfectly flat.
 - b. Position the larva as close to the coverslip as possible to minimize working distance.
8. Allow the agarose to harden for ~2 min.
 - a. Time will vary based on the ambient temperature.
9. Invert the larva-bearing lid and place it on its accompanying petri dish, which should be filled with embryo media containing 0.02% tricaine.
 - a. Larvae can be housed like this for more than 30 min, although care should be taken to control for the time in anesthetic prior to imaging.
10. Mount dish in a custom-made brass slide appropriate for use on a standard stage slide holder (Figure 2(C and (D))).

1.3.1 Materials—35 mm petri dishes (Fisher Scientific, #08–757-100A)

- Drill a 1-cm hole in the lid
High-vacuum grease (Fisher Scientific, #14–635-5D)
22 × 22 mm/#1.5 cover glass (Fisher Scientific, #12–541B)
Low-melting-point agarose (ISC BioExpress, #E–3126–125)
Embryo manipulation capillaries
- These capillaries are made using a thin-walled capillary (World Precision Instruments, 3" Borosil 1.0 mm OD, #1B100–3) with a 2-lb fishing line inserted through the center and secured with superglue.
Brass slide (7.5 × 4 cm; dimensions: inner diameter 30 mm, outer diameter 40 mm)
- This can be made in a university machine shop, such as that at the University of Oregon (<http://tsa.uoregon.edu/>).

1.4 IMAGING MONOMERIC RED FLUORESCENT PROTEIN-TAGGED DYNEIN LIGHT INTERMEDIATE CHAIN 1 TRANSPORT IN ZEBRAFISH POSTERIOR LATERAL LINE AXONS

Axonal transport in the superficial pLL axons can be imaged using a standard laser scanning confocal microscope. We use an Olympus FV1000 upright confocal microscope with a 60X/NA1.2 water objective.

1. Place a mounted larva on the stage and, using the 10X objective, find your sample under transmitted light.
2. Switch objectives to 60X water, and add a droplet of sterile water to the surface.
 - a. It should be ensured that prior to system initiation, the stage has been placed (manually) in the lowest possible position so that the working distance is great enough to allow the petri dish to fit.
3. Using epifluorescence, find the EGFP-positive (in the *TgBAC(neurod:egfp)^{nll}* transgenic) pLL ganglion, which is situated just behind the ear (Figure 3(A)).
 - a. Confirm that one to two cells express monomeric red fluorescent protein (mRFP)-tagged dynein light intermediate chain 1 (Dync1li1) using red epifluorescence.
4. Under red epifluorescence, trace the expressing axon to the middle segment of the trunk, between neuromasts 3 and 4 (Figure 3(B) and (C)).
 - a. This region is typically the flattest, which is ideal for imaging.
5. Identify a region of the axon that can be seen without changing the focal plane.
6. In the software control panel, adjust the scan area size to 1028 × 1028 pixels to visualize a roughly 150 μm-wide region of the trunk.
7. Using low laser power (<10%), high HV (~750–800), and 2X gain, scan rapidly with the 568-nm laser to find the flat axonal region observed through the eyepieces.
 - a. Do this as quickly as possible to limit bleaching.
8. Once a 40- to 100 μm region is identified, use the region of interest tool to isolate the axon of interest.
 - a. Make the region as narrow and long as possible to include little surrounding area but a long stretch of axon.
9. Adjust the scanning parameters such that mRFP-Dync1li1-positive puncta movement is visible with limited background fluorescence.
 - a. Make sure that only the 568 nm channel is active.
 - Ensure sequential scanning is engaged and Kalman corrections are not.
 - b. Keep the laser power as low as possible to avoid bleaching and damage.
 - This is typically 5–15% for our purposes.

- c. Adjust the HV to compensate for low signal.
10. Set the scanning rate to image for a fixed number of frames (600–1000) at the highest possible rate (Freerun on Olympus software).
 - a. It should be remembered that according to the Nyquist sampling theorem (Nyquist, 1928) the scanning frequency must be at least twice the cargo velocity. For example, if cargo velocity is 1 $\mu\text{m}/\text{sec}$ the scanning frequency needs to be 2 Hz.
 - Typically, we scan at two to five times per second.
 - b. Ensure that the pixel dwell time is minimized.
 - Typically, it should be 2 $\mu\text{s}/\text{pixel}$.
11. Acquire the time series (in one plane) to visualize mRFP-Dync1li1 transport (Figure 3(B); Movie 1).
 - a. Make sure to turn off depth scanning and turn on time series scanning modes.
 - b. Observe run to ensure that no movement in the z-axis occurs and the signal remains strong.
 - If necessary, adjust laser acquisition settings (with care!) to compensate for bleaching.
12. Save the file as a .oib or comparable file type.
13. Using EGFP epifluorescence, move the stage so the pLL ganglion can be visualized, and take a depth scan of this structure using the 488 nm (neuronal marker) and 568 nm (mRFP-Dync1li1) channels.
 - a. This allows determination of orientation to confirm anterograde (terminal directed) versus retrograde (cell body-directed) movement.
14. If necessary, larvae can be released from agarose and either raised or lysed for genotyping.
15. Repeat this procedure for individual larvae until the appropriate sample size is reached.
 - a. Typically, we use 10 larvae per condition.
 - b. Petri dishes can be reused, but replace the coverslip with a fresh one for each larva.

1.4.1 Materials—Olympus FV1000 with a 60X/NA1.2 water objective

1.5 ANALYSIS OF MONOMERIC RED FLUORESCENT PROTEIN-TAGGED DYNEIN LIGHT INTERMEDIATE CHAIN 1 AXONAL TRANSPORT

Axonal transport of various cargos can be analyzed using kymograph analysis. Several software packages can accomplish this type of analysis, which consists of following the

brightest pixels along an assigned line; this generates traces in two dimensions that correspond to the movement of a unique, cargo-positive puncta (Figure 4). These traces can then be used to determine parameters of transport including distance, time, and velocity of individual movement. The example described below utilizes the MetaMorph (Molecular Devices) program to generate and analyze kymographs of mRFP-Dync1li1 movement.

1. Open a time series file in MetaMorph.
2. Open several accessory windows to help with analysis:
 - a. Kymograph tool
 - b. Region Statistics tool
 - c. Calibrate Distances tool
 - d. Custom built taskbar, which contains a Loop XY journal to record the X and Y coordinates for each trace, that is, individual cargo movement.
 - To make the journal:
 1. First create a journal to log the X and Y coordinates for a single trace.
 - a. Open the Journal Editor.
 - b. Create a new journal
 - c. Drag functions into the journal from the Built in Functions options to the left.
 - Open Data Log (select Excel).
 - Configure Log (select Region Name, X1, X2, Y1, Y2).
 - Configure Linescan Log (disable all).
 - Linescan (change Image selection to “current at start”).
 2. Save as Log XY in the journals folder.
 3. Create a second journal to record the X/Y coordinates for all traces.
 4. Create a new journal.
 5. Drag “Loop for all regions” built-in function into the journal from the built-in functions on the left.
 - a. Choose the Log XY journal file.
 - b. Ensure the Image option is “current at start.”
 6. Save as Loop XY for all regions.

- Create a Taskbar and include the Loop XY journal as a function.
 1. This taskbar can also include the other useful windows if desired, that is, Kymograph, Region Statistics, and Calibrate Distances tools.
- 3. Prior to analysis, determine (using the Calibrate Distances tool) if the software is calibrating the X/Y units based on metadata associated with the file.
 - a. This will become relevant during the data conversion in Step 17 of this section.
- 4. Using the Multisegment Line tool, trace the axon for analysis (Figure 4(A)).
 - a. Make sure to use only regions where individual axons can be identified.
- 5. Adjust the width (line width; dotted outline in Figure 4(A)) of the area to be analyzed to encompass the entire axon in the Kymograph tool window.
- 6. Move individual points to center the path exactly on the axon.
- 7. Record the length of the axon being imaged.
 - a. Found in the Region Statistics tool (Figure 4(B)).
- 8. Record the time of imaging based on the metadata associated with the original file.
- 9. Initiate the Kymograph function in the Kymograph tool window.
 - a. Make sure that all planes are being analyzed.
 - b. Default kymographs are generated such that time is on the Y-axis (top is frame 1) and distance is on the X-axis.
- 10. Once the kymograph is generated (Figure 4(C)), anterograde and retrograde mRFP-Dync1li1 movement can be traced.
 - a. Each direction needs to be recorded separately.
- 11. For anterograde transport, first determine the side (right or left) of the neuronal cell bodies based on the image of the pLL ganglion.
 - a. Typically, we place the ganglion on the left during imaging.
- 12. Trace individual lines using the Single Line tool from the upper left to lower right. This represents anterograde movement when the cell bodies are to the left (Figure 4(C); blue traces).
 - a. Trace only sloped lines that will be separated by vertical lines.
 - Vertical lines represent pauses in movement (no change in X over time).

13. Once all regions for anterograde transport are selected, run the loop X/Y journal and export the coordinates as an excel file.
14. Count and record the number of mRFP-Dync1li1 cargo moving in the anterograde direction (left to right sloping separated by pauses) on the kymograph.
 - a. This requires care as puncta may pause for a large amount of time or move past one another.
 - For example, the kymograph in Figure 4(C) has five puncta moving in the anterograde direction.
15. Clear all regions and repeat this for the retrograde traces, which will be from the upper right to lower left when the pLL ganglion is to the left during imaging (pink traces in Figure 4(C)).
16. At completion, you will have 20 excel sheets (if there are two experimental groups, N = 10 each), representing separate anterograde and retrograde mRFP-Dync1li1 movement for each individual.
17. For each excel sheet, convert the X/Y coordinates for each trace to distance/ time information.
 - a. This is only necessary if the conversion has not been done automatically by the software using the metadata.
 - b. Distance is calibrated by multiplying the change in X by the micrometer per pixel.
 - c. Time is calibrated by multiplying the change in Y by the sec/frame.
18. Similarly, determine the velocity of individual particle movements ($\mu\text{m/s}$).
19. Average these values within each larva for both anterograde and retrograde movement.
 - a. It may also be of interest, depending on the question, to use each bout of particle movement as an N.
 - For instance, individual bouts can be used to analyze variations in distances and/or velocities for a given cargo within as well as between larvae; for example, we may ask if there are different populations of Dync1li1 particles, which may be differentially affected by a particular manipulation. This type of question requires histogram analyses of all individual puncta movements rather than a within-larva average.
20. Transpose averages to a suitable software package for analysis.
21. Anterograde and retrograde mRFP-Dync1li1 frequency can also be determined using particle counts generated from the kymographs.

- a. Correct for distance analyzed and total imaging time to control for variability between imaging sessions.
22. Analyses of wild-type mRFP-Dync1li1 transport across three developmental stages revealed changes in the distance but not velocity of transport as a function of time (Figure 5).

1.6 IMAGING AND ANALYSIS OF MONOMERIC RED FLUORESCENT PROTEIN-TAGGED DYNEIN LIGHT INTERMEDIATE CHAIN 1 AND LYSOSOME-ASSOCIATED MEMBRANE PROTEIN 1-EGFP CO-TRANSPORT

Two-color imaging analyses can be utilized in this system to determine frequency of cargo–cargo and cargo–motor co-transport (Figure 6; Movie 2). Technically, this approach is similar to the analysis of single cargo transport, and it can be applied to answer a number of questions ranging from inquiries into the mechanisms of cargo attachment to the impact of cargo interaction on motor movement parameters. Changes in the protocol are outlined below for imaging and analyzing co-transport using mRFP-Dync1li1/Lysosome-associated membrane protein 1 (Lamp1-EGFP) as an example of motor protein–cargo pair.

1. During microinjection, co-inject plasmids encoding mRFP-Dync1li1 and Lamp1-EGFP driven by the same promoter into wild-type zygotes.
2. During larva selection, check both the red and green channels to confirm that both cargos are expressed in the neurons of interest.
3. For imaging, after optimizing the settings for the 488 and 568 nm channels, confirm that sequential imaging is enabled and that the region is small enough that the frame rate is no less than that required by the Nyquist sampling theorem (Nyquist, 1928), typically two frames per second.
4. For kymograph analysis, each channel must be analyzed separately.
5. To separate channels, use the Color Separation tool in MetaMorph.
6. Generate individual kymographs from each channel so that individual movements parameters can be quantified for each cargo.
 - a. The impact of co-transport on transport distance and velocity parameters may be of interest in certain circumstances but will not be addressed here. Rather
 - b. we used this dual transport analysis to determine the frequency of motore cargo co-transport.
7. To calculate co-transport frequency of lysosomes with Dync1li1, trace individual movements of Lamp1-EGFP puncta and transfer the regions (using the Transfer Regions tool) to the mRFP-Dync1li1 kymograph.
8. Count the mRFP-Dync1li1 traces that align with the Lamp1-EGFP regions that were transferred.
 - a. This is the number of lysosomes that are co-transported with Dync1li1.

- b. The number of lysosome- and Dync1li1-only traces can be recorded by excluding those that align between the two kymographs.
 - c. Again, this type of analysis is not for individual movements (sloped lines separated by pauses) but individual cargo that is represented by these intermittent movements.
9. Transfer this information to a statistical program, correct for distance and total imaging time, and determine the appropriate statistic for analysis.

1.6.1 Materials—MetaMorph (Molecular Devices)

Excel (Microsoft)

Statistical analysis software (such as JMP- <http://www.jmp.com/>)

CONCLUSIONS

Here, we describe a straightforward approach that uses the advantages of the zebrafish system to visualize and analyze the movement of cargos and motors in axons of an intact vertebrate. Our method capitalizes on pioneering work done in other systems, including cultured neurons and invertebrates. It utilizes the unique and innate qualities of zebrafish embryos and larvae which allow us to perform subcellular analyses in the context of an intact animal with an unadulterated neural circuit. This approach, which in principal could be applied to other types of axons in developing peripheral and central nervous system of the zebrafish, allows us to address questions regarding cargo–motor interaction and movement and to determine how alterations in this process affect both the formation and function of a circuit.

In this study, we used our approach to assay developmental changes in the movement of the dynein motor complex containing the core protein Dync1li1. As we have noted in our previous work, there are developmental changes in axonal transport of some cargo, that is, vesicles containing activated c-Jun-N-terminal kinase (JNK) and a generalized vesicular marker. The distance moved by each of these cargos during individual movement bouts decreases from 2–5 dpf (Drerup & Nechiporuk, 2013). Whether this was due to changes in motor movement or, rather, due to changes in cargo–motor attachment was unknown. In this study, we used live imaging of dynein movement (labeled by mRFP-Dync1li1) to determine that the distance moved by the dynein motor changes with the developmental stage. The observed change in dynein motility during axon development mirrors the changes previously observed in retrograde movement of certain cargo. This similarity argues for developmental transport regulation at the level of the dynein motor itself rather than cargo–motor attachment. The underlying mechanistic reason for this variation, likely due to changes in dynein–microtubule interaction, across axon development is unclear. Potentially, it could be due to the differences in microtubule modification or occupation of microtubules by associated proteins during distinct developmental stages (Hammond et al., 2010). Indeed, microtubule modifications have been shown to modulate kinesin-mediated movement in cultured neurons (Hammond, Cai, & Verhey, 2008; Konishi & Setou, 2009; Reed et al.,

2006; Sirajuddin, Rice, & Vale, 2014). Deciphering how dynein–microtubule interaction is regulated will enhance our understanding of cargo movement during axon development and maintenance.

Additionally, our approach to visualize co-transport can be used to determine how distinct experimental conditions or mutations in specific proteins affect cargo–motor interaction. For example, in our previous study, we analyzed co-transport frequencies of the dynein motor (mRFP-Dync1li1 fusion) and lysosomes (Lamp1-EGFP fusion) in wild-type and JNK-interacting protein three (*jip3*) mutant larvae to assay whether Jip3 was required for lysosome attachment to the dynein motor. Loss of Jip3 resulted in a decreased frequency of dynein–lysosome retrograde co-transport, demonstrating a novel role for Jip3 in this process (Drerup & Nechiporuk, 2013).

Finally, similar to other systems, our approach to visualize cargo transport and cargo–motor co-transport relies on the use of overexpression of tagged proteins. Care should be taken to ensure that this expression represents the endogenous cargo population and does not induce artifacts. High expression levels may nonspecifically affect transport, potentially by titrating motors from other cargo or disrupting the microtubule cytoskeleton. These types of artifacts can be avoided by performing a number of controls. First, antibody labeling should be done when possible to confirm that the overexpressed protein is localizing similarly to endogenous protein and both are normally distributed. Second, the health and wiring of the axons should be closely monitored to ensure that the overexpression of fusion proteins does not adversely affect neuronal health. Finally, when possible, fusion constructs should be expressed in a corresponding mutant background to ensure that fusion proteins can rescue mutant phenotypes and, therefore, are fully functional. We envision that in the near future, modern knock-in techniques, mediated by clustered regularly interspaced short palindromic repeats (CRISPRs) or transcription activator-like effector nucleases (TALENs), will be used to introduce fluorescent tags into the zebrafish genome to create fusions with endogenous proteins (Auer & Del Bene, 2014; Auer, Durore, Concordet, & Del Bene, 2014; Gagnon et al., 2014; Shin, Chen, & Solnica-Krezel, 2014). This approach will decrease the likelihood of possible artifacts associated with protein overexpression in these highly sensitive cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Schematic illustrating injection of zygotes for *in vivo* imaging. One-cell stage zebrafish zygotes are mounted in a 1.5% agarose plate and microinjected with 3–25 pg of DNA encoding a promoter driving expression of a cargo fusion protein. Mosaic inheritance of this DNA results in the ability to titrate doses to express cargo fusions in a small number of cells. Amount of the injected DNA is titrated such that one to two cells of the pLL ganglion (pLLg; left inset) express an mRFP-tagged cargo. Neuromasts (NMs; sensory organs of this system; blue (light gray in print versions)) are innervated by two axons (right inset; red (dark gray in print versions)-cargo-fusion-expressing axon).

FIGURE 2.

Mounting zebrafish larvae for *in vivo* imaging. (A) Using vacuum grease, attach a 22×22 mm (#1.5) coverslip to the top of a 35-mm petri dish lid with a 1-cm hole drilled in it. Mount a larva in a drop of 1.2% agarose on the underside of that coverslip. (B) Photo of a 4 dpf larva embedded as described. (C) Brass holder for imaging; inner diameter, 30 mm; outer diameter, 40 mm. (D) Fully assembled imaging setup that includes the brass slide, petri dish (filled with embryo media; 0.02% tricaine), and the larva mounted on the inside of the lid in contact with the media. Arrow indicates larva.

FIGURE 3.

Monomeric red fluorescent protein (mRFP)-tagged dynein light intermediate chain 1 transport in the zebrafish pLL. (A) Expression of mRFP-tagged Dync1li1 in two neurons of the pLL ganglion (pLLg; outlined) at 4 dpf. (B) Individual images from a time series (see Movie 1) demonstrating anterograde (arrows) and retrograde (arrowheads) Dync1li1 movement. (C) Innervation of a neuromast (NM) by a single axon expressing mRFP-Dync1li1. Note that only half of the neuromast is labeled as each half of the sensory organ is innervated by a single axon at this stage (arrowhead). Neurons and axons are marked by the *TgBAC(neurod:egfp)^{nl1}* transgene in A and C. Scale bars are 10 μm. (See color plate)

FIGURE 4.

Kymograph analysis. (A) Screenshot of a representative MetaMorph window showing the region of interest for analysis of this axon. (B) Region statistics for this axonal segment. For determining the width of region analyzed (boxed) ensure that the active region is chosen (arrowhead). (C) Kymograph generated from this region for 600 frames (~78 s). (Left) Kymograph generated with time on the Y-axis and distance on the X-axis. (Middle) Anterograde (blue (light gray in print versions)) and retrograde (pink (dark gray in print versions)) traces from this kymograph. Overlay is on the right. Note, only traces where the initiation and termination of movement are clearly defined are used for analysis. Pauses (vertical lines) are similarly excluded. Anterograde traces are numbered to illustrate number of Dync1li1-positive puncta analyzed.

FIGURE 5.

Developmental changes in mRFP-Dync1li1 transport. (A,B) Anterograde transport distance and velocity are similar across developmental stage. (C,D) Retrograde transport distance decreases from 48 to 96 h postfertilization (hpf), with 48 (green (light gray in print versions) circle) and 96 hpf (red (dark gray in print versions) circle) being significantly different from one another but not from 72 hpf. Velocity remains constant. Axon extension is complete at 48 hpf, and functional synapses are formed by 96 hpf. Blue (light gray in print versions) bars represent standard error and deviation.

FIGURE 6. LysosomeeDync1li1 co-transport. Images from a time series showing retrograde co-transport (yellow arrowhead), lysosome-only retrograde transport (arrow), and lysosome-only anterograde transport (white arrowhead). See also Movie 2. (See color plate)