

Kymographic Analysis of Transport in an Individual Neuronal Sensory Cilium in *Caenorhabditis elegans*

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Abstract

Intraflagellar Transport (IFT) is driven by molecular motors that travel upon microtubule-based ciliary axonemes. In the single-celled alga *Chlamydomonas reinhardtii*, movement of a single anterograde IFT motor, heterotrimeric kinesin-II, is required to generate two identical motile flagella. The function of this canonical anterograde IFT motor is conserved among all eukaryotes, yet multicellular organisms can generate cilia of diverse structures and functions, ranging from simple threadlike non-motile primary cilia to the elaborate cilia that make up rod and cone photoreceptors in the retina. An emerging theme is that additional molecular motors modulate the canonical IFT machinery to give rise to differing ciliary morphologies. Therefore, a complete understanding of the trafficking of ciliary receptors, as well as the biogenesis, maintenance, specialization, and function of cilia, requires the characterization of motor molecules.

Here, we describe in detail our method for measuring the motility of proteins in cilia or dendrites of *C. elegans* male-specific CEM ciliated sensory neurons using time-lapse microscopy and kymography of green fluorescent protein (GFP)-tagged motors, receptors, and cargos. We describe, as a specific example, OSM-3::GFP puncta moving in cilia, but also include (Fig. 1) with settings that have worked well for us measuring movement of heterotrimeric kinesin-II, IFT particles, and the polycystin TRP channel PKD-2.

Key words *Caenorhabditis elegans*, Kymograph, Cell biology, In vivo, Sensory non-motile cilia, PKD-2, Polycystins, OSM-3, Kinesin-2, KLP-6, Kinesin-3, Male

1 Introduction

In this chapter, we describe how to measure the speed of fluorescently marked motors, IFT complex components, and receptors, moving in an individual dendrite or sensory cilium of a *C. elegans* male-specific neuron. The method requires a compound microscope equipped with a GFP filter set and a sensitive camera, but does not require confocal microscopy, and is therefore a technique that can be used by most biology laboratories.

After the movement of heterotrimeric kinesin-II was described in *Chlamydomonas* [1–4], elaborations of the basic IFT machinery by accessory motors were discovered. First, the monomeric kinesin-2 OSM-3 was found to cooperate with the canonical IFT

kinesin to build amphid and phasmid cilia in *C. elegans* [5–7]. The mammalian homolog of OSM-3, called KIF17, has been reported to be involved in ciliary targeting of olfactory channels [8] and photoreceptor development and maintenance [9], although this conclusion is controversial [10]. Our work further supports this model of the origin of ciliary diversity; we have found an interplay between the canonical kinesin-II, the kinesin-2 OSM-3/KIF17, and a kinesin-3, KLP-6/KIF28 [11], in male-specific neuronal sensory cilia in *C. elegans* [12]. Kymographic analysis of ciliary motor motility was invaluable in testing the modification of IFT by accessory motors.

We will describe the acquisition and analysis of streaming video using Metamorph software, but free ImageJ software (<http://imagej.nih.gov/ij/>) is an effective substitute. We describe the technique in the study of male-specific neuronal cilia and dendrites, but the method is easily adapted to study transport in axons or in other cell types, using other molecular markers or using other cell-type-specific promoters. We will explain how we grow transgenic *C. elegans* nematodes, mount them for imaging, immobilize them, acquire time-lapse images at high magnification on a compound upright microscope, and how to convert the images into kymographs that are used to measure the *in vivo* velocity of proteins.

This technique has been used fruitfully and extensively to track movement of tagged proteins in the amphid channel cilia [4, 6, 7], which comprise a bundle of ten cilia originating from eight different neurons [13]. In such studies, a GFP-tagged molecule is expressed in all amphid channel neurons. Because the bundled cilia are spatially very close together, all ten cilia are analyzed *en masse* using kymography [4, 6, 7]. Any differences that might exist between cilia are therefore undetectable. Differences in IFT have been found in individual cilia types [12, 14]. The kymographic analysis of molecular movement in individual CEM neuronal cilia, as described here, confers the advantages of studying molecules in a single cilium [12].

This method relies on tracing a line over the cilium of interest in a stack of sequential images and then plotting all the pixel values along that line for each frame of the video, generating a “kymograph” in which the horizontal axis is the distance along the line and the vertical axis is time. If fluorescently marked particles are stationary, a vertical stripe is seen. However, moving particles will appear as diagonal lines in the kymograph. The characteristics of these lines can be used to determine the *in vivo* velocity, direction, frequency, and run length of motor complexes. This *in vivo* method will not necessarily elucidate single-molecule characteristics of motors, such as processivity or binding affinity, which may be best studied *in vitro*. However, this method will report accurately the activity of the tagged molecules inside cells in a living organism.

Once you reliably detect the characteristic movement of a tagged molecule of interest, you can then assess the function of

associated proteins by using genetic crosses to mate the transgene or extrachromosomal array encoding your tagged molecule into various mutant backgrounds and looking for changes in velocity, frequency, distance, or direction. It is important to use the same transgene or array when making comparisons between different genetic backgrounds, developmental stages, or conditions. For example, we used this technique to show that posttranslational glutamylation of microtubules regulates activity of OSM-3 kinesin-2 motors in CEM cilia [15]. Combination of the imaging of motor activity with the powerful genetics toolkit of *C. elegans* can be used to facilitate study of the function of ciliary transport regulators such as IFT components, transition zone components, microtubule-associated proteins, and many others.

2 Materials

2.1 Materials and Equipment Needed to Culture *C. elegans*

1. Incubator.
2. Bunsen burner.
3. 2-l Erlenmeyer flask.
4. Aluminum foil.
5. 55 °C water bath.
6. Autoclavable ingredients for NGM agar plates: NaCl, agar, peptone, H₂O.
7. Sterile ingredients to add to autoclaved NGM agar: sterile 1 M CaCl₂, cholesterol in ethanol (5 mg/ml concentration; not autoclaved), 1 M MgSO₄, and filter-sterilized 1 M KPO₄ buffer pH 6.0 (108.3 g KH₂PO₄, 35.6 g K₂HPO₄, H₂O to 1 l).
8. 60 mm sterile petri dishes.
9. OP50 *E. coli* strain: obtained from *Caenorhabditis* Genetics Center (cgc.cbs.umn.edu). This strain is the standard food for *C. elegans*.
10. LB broth for growing bacteria: tryptone, yeast extract, NaCl, 1 N NaOH.
11. 250 ml autoclavable bottles.
12. Transgenic *C. elegans* strain expressing ciliary marker of interest: In this protocol, we will use the strain PT2098 of genotype *pha-1(e2123ts) III; him-5(e1490) V; myEx685 [klp-6p::osm-3::gfp + pBX]* [12] as an example (see **Note 1**).

2.2 Mounting Worms for Microscopy

1. Heating block at 65 °C (with adaptor for 16 × 100 mm glass tubes).
2. Agar.
3. Deionized water.
4. Borosilicate glass 16 × 100 mm culture tubes.

5. Worm pick (Fig. 1) Flatten the end of a 3 cm long ~30 G platinum wire by tapping lightly with a hammer, and trim to spatula shape under a stereodissecting microscope using a razor blade. Break about 1 cm off the tapered end of a glass

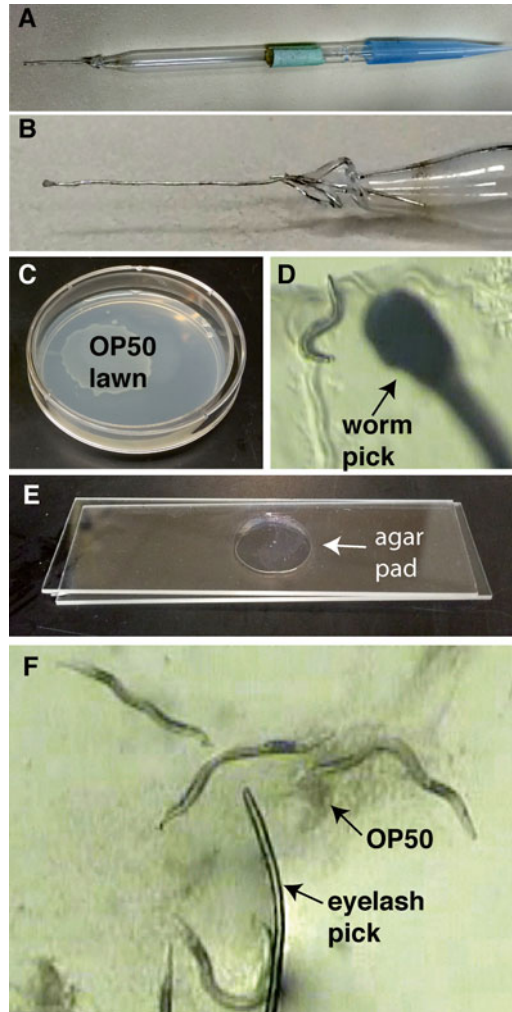


Fig. 1 Tools for nematode handling and growth. (a) The worm pick is made from a glass Pasteur pipette with a platinum wire fastened in the tapered end. (b) Detail of platinum wire in worm pick. The tip is shaped like a spatula. (c) NGM (nematode growth media) plate seeded with the *E. coli* strain OP50 to grow a bacterial lawn that serves as food for growing nematodes. Plate shown is 60 mm diameter. (d) View of a NGM plate with worms through the stereodissecting microscope. The visible L4 larval hermaphrodite has left tracks in the OP50 lawn. The spatula-shaped tip of the worm pick is visible. (e) An agar pad made by sandwiching a drop of agar between two slides. (f) Mounting male worms for imaging, viewed through stereodissecting microscope. Worms have been picked by dabbing with OP50 and transferred into the levamisole + M9 solution on the agar pad. The eyelash pick (eyelash or eyebrow hair glued to a toothpick) is used to sweep away the dark OP50 bacteria to improve image quality and to position the animals

Pasteur pipette. Use a tweezer to hold the platinum wire above the flame of a Bunsen burner while softening the end of the Pasteur pipette in the same flame. When wire glows red and glass is soft enough, insert wire into pipette and allow glass to harden around it. The spatula end of the wire is used to pick up worms either to place worms on OP50-seeded NGM plates or to pick worms off of plates to mount for imaging.

6. Glass slides and coverslips (we use thickness “1”).
7. Eyelash pick use Elmer’s Glue or similar to fasten an eyelash or eyebrow hair to the end of a toothpick.
8. Stereodissecting microscope (Zeiss Stemi SV 6 or similar).
9. M9 Buffer 3 g KH_2PO_4 , 6 g Na_2HPO_4 , 5 g NaCl, 1 ml 1 M MgSO_4 , H_2O to 1 l. Sterilize by autoclaving.
10. Levamisole (sigmaaldrich.com), 10 mM in M9 buffer. We keep a 10× solution (100 mM levamisole in M9) frozen at -20°C , thaw, and dilute to working concentration of 10 mM in M9 the day of the experiment.

2.3 Worm Imaging

1. Compound microscope equipped with 100× oil immersion objective, camera, and acquisition software: Streaming video is acquired using either a Zeiss Axioplan 2 microscope and Photometrics Cascade 512B EMCCD camera or a Zeiss Imager DIM microscope with a Retiga-SRV Fast 1394 Q-Imaging digital camera with 100× 1.4NA oil Zeiss Plan-APOCHROMAT objective. We use Metamorph software (Version 7.6.1.0, MDS Analytical technologies) to control image acquisition.

2.4 Analysis

1. Metamorph software: Includes kymograph module, which we use to generate and analyze kymographs made from streaming video. Velocities and other data are stored by data logging in Excel (Microsoft Office) software.
2. Prism 5 software: We perform all statistical analyses with Prism 5 (www.graphpad.com). Velocity measurements are typically subjected to one-way ANOVA analysis with a Bonferroni post-hoc test to compare all genotypes in pairwise fashion.

3 Methods

3.1 Preparing NGM (Nematode Growth Media) Plates

1. Mix 3 g NaCl, 17 g agar, and 2.5 g peptone in a 2 l Erlenmeyer flask.
2. Add 975 ml H_2O . Cover mouth of flask with aluminum foil.
3. Autoclave on liquid cycle for 50 min.
4. Cool flask in 55°C water bath for 15 min.
5. Then add 1 ml sterile 1 M CaCl_2 , 1 ml 5 mg/ml cholesterol in ethanol (not autoclaved), 1 ml 1 M MgSO_4 , and filter-sterilized

25 ml 1 M KPO_4 buffer pH 6.0 (108.3 g KH_2PO_4 , 35.6 g K_2HPO_4 , H_2O to 1 l). Swirl to mix well.

6. Using sterile procedures, dispense the NGM agar solution into Sterile Disposable Petri Plates. Plates should be about 2/3 full of agar. Typically, we dispense ~9 ml into 60 mm plates. Optionally, use a peristaltic pump to dispense.
7. Leave plates at room temperature for 2–3 days before use to allow for detection of contaminants and to allow excess moisture to evaporate. Plates stored in an air-tight container at room temperature will be usable for several weeks.

3.2 *OP50 Bacterial Culture*

1. Prepare LB broth by combining 10 g tryptone, 5 g yeast extract, 10 g NaCl, 1 ml 1 N NaOH, and H_2O to 1 l. Stir and distribute into ten separate 250 ml bottles. Autoclave and store at room temperature.
2. Pick a single colony from a streak of OP50 bacterial culture as supplied to inoculate 100 ml bottle of sterile LB broth. Grow overnight at 37°C, and store at 4°C.

3.3 *Nematode Culture*

1. Seed NGM plates with ~100 μl of OP50 culture in LB. Dry overnight to form a bacterial lawn upon which the nematodes will feed.
2. Use the worm pick to transfer worms to a fresh NGM plate containing an OP50 lawn (Fig. 1), and incubate at 20°C for 2–3 days (see Note 2). Picking worms is most easily done by placing the spatula-shaped tip of the worm pick onto the edge of the OP50 bacterial lawn. A blob of bacteria will adhere to the bottom of the pick and serve as “stickum” with which to pick up *C. elegans*. To pick up an animal, gently dab the stickum onto it and quickly withdraw. A single or several worms will stick to the OP50 stickum. Then gently touch the tip to a fresh NGM plate and wait for animals to crawl off the pick onto the agar. The platinum tip of the pick is flamed in the Bunsen burner to glow red, thus sterilizing it, allowed to cool for a few seconds, and dipped in the OP50 bacterial lawn before each new attempt at picking.

3.4 *Preparing Slides and Mounting Worms for Imaging*

The day before you plan to image motility of GFP-tagged proteins, use the worm pick to transfer only L4 (fourth larval stage) males (Fig. 2) to a new OP50-seeded NGM plate (see Note 3).

When animals are in their first day of adulthood (18–24 h after picking), image animals as follows:

1. Make a 4% agar solution by gently melting 0.125 g of agar in 3 ml of water in a glass culture tube over the Bunsen burner. Keep the culture tube moving to avoid both rapid boiling (which can propel hot agar out of the tube or crack the tube)

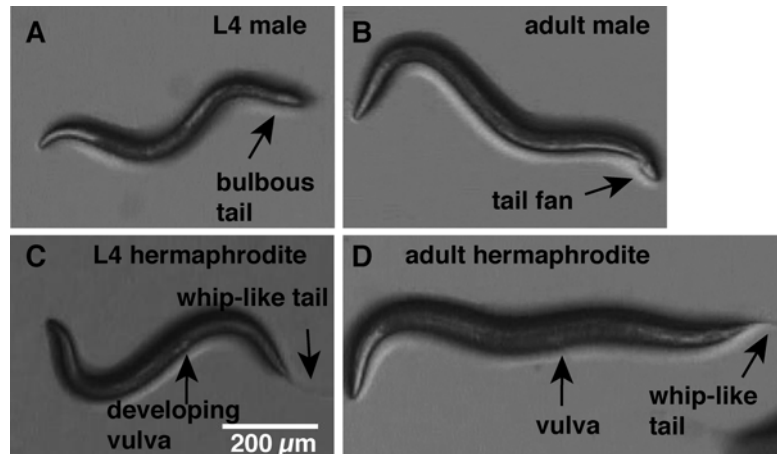


Fig. 2 Morphological features of male and hermaphrodite nematodes viewed through the stereodissecting microscope. **(a)** The day before imaging, pick L4 males. The L4 male is identified by the bulbous tail and lack of a developing vulva. **(b)** L4 males should uniformly be young adult males on the day of imaging. Confirm that all animals picked the previous day are young adult males, characterized by a fully developed tail fan and lack of a vulva. **(c)** L4 hermaphrodites can be distinguished from males by a clear “spot” that marks the developing vulva and a whip-like tail. **(d)** If you have accidentally picked any hermaphrodites the day before imaging, you will find adult hermaphrodites among your males. Adult hermaphrodites are characterized by a visible vulva and a whip-like tail. They are larger than adult males, and eggs may be visible inside the animal (not shown). If any adult hermaphrodites are visible on plates with your adult males, the males should not be imaged, as exposure to hermaphrodites might influence male neuronal activity and change the characteristics of ciliary motility, leading to erroneous data. All animals are positioned with anterior pointing to *left*

and burning of agar. Place tube with melted agar solution in heating block at 75 °C.

2. Lay out 12 glass slides in two rows on the bench.
3. Slightly enlarge the opening of a 200 μ l plastic pipette tip by trimming the end with a pair of scissors or blade (to reduce clogging as the agar solidifies), and draw up \sim 125 μ l of hot agar solution using a P200 pipetman.
4. Moving quickly, place a drop of hot agar on a glass slide, cover with a second glass slide, and apply gentle finger pressure to create a thin agar pad. Repeat for the rest of slides. Avoid trapping air bubbles in agar. With a little practice, you can make five or six agar pad slides before the pipette becomes clogged.
5. Slide the top glass slide off the “sandwich” carefully to avoid tearing the agar pad. This can be done soon after the agar solidifies, or top slides can be left in place for up to an hour to prevent agar pads from drying out before use. Place the

slide with agar side facing up on the stereodissecting microscope stage.

6. Add 6 μ l of M9 plus levamisole (10 mM) solution to the agar pad on a slide. Levamisole is an acetylcholine receptor agonist that immobilizes nematodes by causing hypercontraction of body wall muscles [16]. Use the platinum worm pick to transfer ~10 male animals into the liquid on the agar pad. With practice, all ten males can be picked up at once. Then, place the tip of the pick into the M9 plus levamisole solution on the agar pad, and wait for males to leave the pick (or shake them off gently). Be careful not to tear the agar pad, which results in worms pooling in regions that cannot be imaged. Once animals are in the levamisole solution, wait 8 min with slides uncovered for the anesthetic to fully paralyze animals (*see Note 4*).
7. Use the eyelash pick to move remaining bacteria away from animals for better image quality, and line the worms up in a row for convenience in finding them on the compound microscope. Gently place coverslip over the preparation. If possible, avoid trapping bubbles. Image within 30–40 min of mounting, because worms may become less healthy and will certainly become starved (*see Note 5*).
8. Mount the slide on the compound microscope stage for imaging.

3.5 Acquiring Streaming Video

Only CEM cilia that reside completely within the plane of focus should be imaged. CEM cilia normally curve slightly outward to reach the outside of the animal's cuticle; this curved morphology should be apparent if the cilium is completely within the plane of focus. Some animals may not have any acceptable CEM cilia completely in focus. Therefore, it is likely that you will not collect data from all of the worms you mount for imaging.

1. Set excitation power to 25–50%; set power higher only if necessary to visualize fluorescent puncta (*see Note 6*).
2. The following imaging parameters in Metamorph permit you to capture high-quality images of OSM-3::GFP puncta for the duration of the streaming image acquisition. Open the “Acquire” panel using the “Acquire” dropdown menu in Metamorph. Select “GFP” illumination under the “Acquire” tab; then, select “5 MHz Digitizer” and “1 \times Gain” in the “Special” tab, and set “Exposure Time” to 100 ms (*see Note 7*). Select “100 \times oil” objective in the “Magnification” setting in the “Device Control” toolbar to store the calibration for pixel size with your images. Save your settings so that you can reload them next time you image the same molecular marker.
3. Click “Full Chip” and “Show Live” to see continuous video from the camera. You may select a region of the image with the rectangle tool in the “Region Tools” toolbar, and then select “Use Active Region” rather than “Full Chip” in the “Acquire”

panel, in order to capture streaming video from a small region. Alternatively, you can acquire using “Full Chip” and simply magnify the image later (e.g., to zoom in to the CEM cilium).

4. Open the “Stream Acquisition” panel from the “Acquire” menu, and set the number of frames you are going to capture—for 100 ms exposure, 100 frames will take 10 s. We select “Stream to Ram” in the “Acquisition Mode” and “Acquire images at frame rate” in the “Camera Parameters” tab. We do not select “Display preview image during acquisition” in the “Camera Parameters” tab because this may slow down acquisition in Metamorph.
5. When you are ready to acquire a video stream, turn on fluorescence excitation, quickly fine-tune focus, and press the “Acquire” button in the “Stream Acquisition” panel.
6. After the acquisition, the new image stack of your video will appear. Check for movement of GFP puncta by dragging the bar on the top of the stack or by pressing “play.” You should see movement of GFP puncta in the image stream without significant image processing. If you do not see moving particles using a marker known to move in cilia, either photobleaching or improper acquisition settings are typically to blame (*see Note 8*). However, some fluorescently marked molecules may not undergo active transport in cilia. For example, we did not detect moving particles for the TRP channel PKD-2::GFP in CEM cilia [17] or the inversin homolog NPHP-2::GFP in phasmid cilia [18]. To determine if your protein is stationary or mobile, you may need to use another imaging technique such as FRAP (fluorescence recovery after photobleaching [19]).

3.6 Analysis of Kymographs

Because scoring by tracing lines on kymographs includes an element of subjectivity, we typically acquire many streaming videos and then have a lab mate blind the genotypes of the videos before scoring. It is essential to score for particle movement in CEM cilia of several different animals for each genotype to ensure that you account for individual variation or those due to focus issues (*see Note 9*).

1. Load your streaming video stack or multidimensional TIFF back into Metamorph, and select “Kymograph” from the “Stack” menu to open the “Kymograph” panel.
2. Click “Calibrate distance” under the “Measure” menu. In the “Calibrate Distances” panel, click on the option that indicates calibration for 100X objective. Click “Apply to all open images” (*see Note 10*).
3. Using the “Multisegment Line tool” from the “Region Tools” toolbar, carefully draw a line along the cilium or dendrite in the region where you want to measure particle velocity (Fig. 3). When drawing the line, always start proximally, and extend the

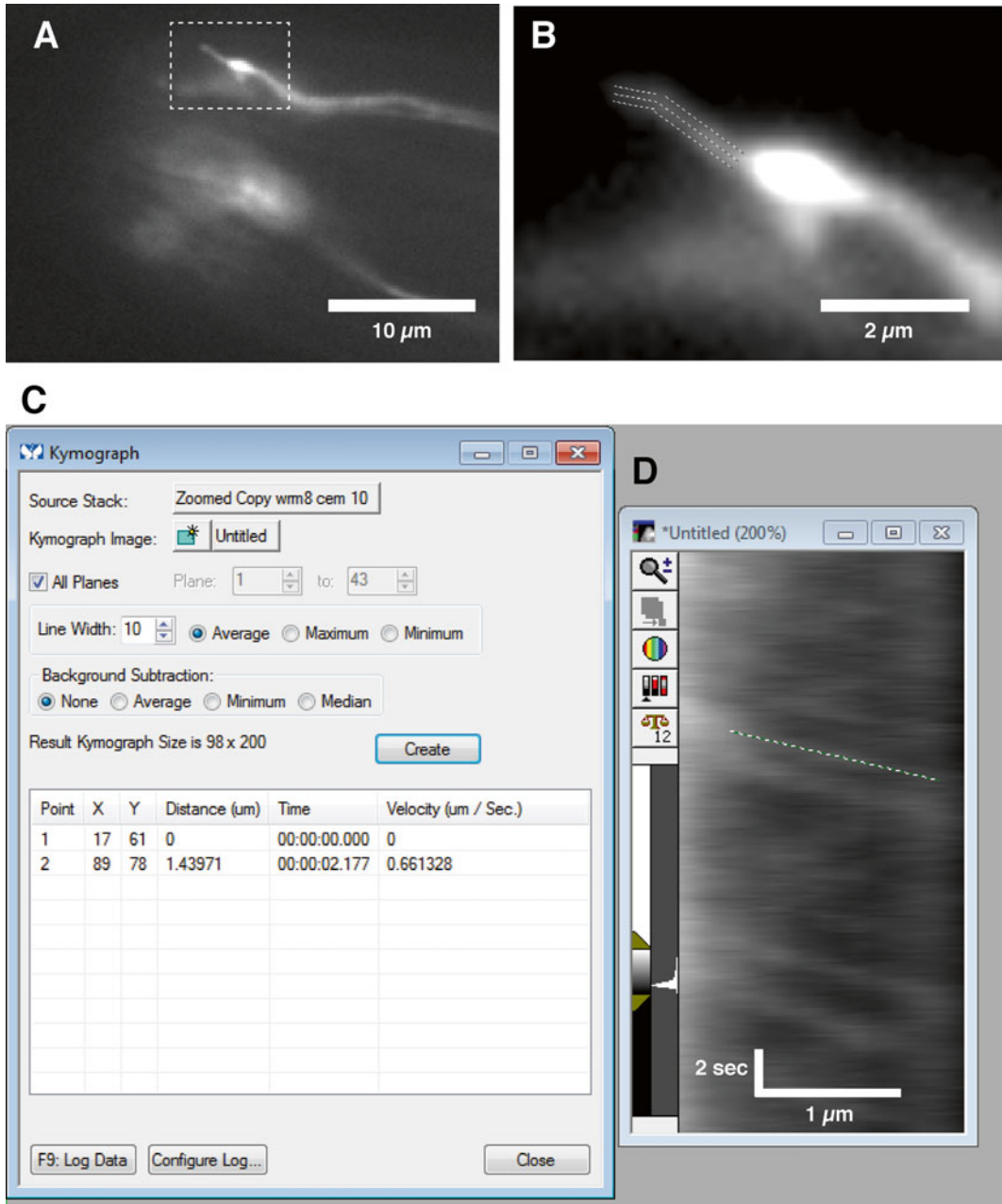


Fig. 3 Analysis of motile particles in streaming images. **(a)** The tip of adult male nose showing OSM-3::GFP fluorescence in a CEM neuron. At the tip of a dendrite, a CEM cilium extends from the bright area at the ciliary base. **(b)** Enlargement of area enclosed by *dashed-line box* in A with a *line* traced down center of the CEM cilium; *outer dashed lines* are set by “Line Width” of 10 pixels, selected in “Kymograph” panel (shown in **c**). This line will be used to generate kymograph. **(c)** “Kymograph” panel with “Line Width” of 10 pixels selected. Clicking “Create” produces the kymograph (shown in **d**). **(d)** Several diagonal lines in the kymograph shown represent motile OSM-3::GFP puncta. The *dotted white line* in the kymograph was traced over a *diagonal line* and automatically analyzed in the kymograph panel (in **c**), showing a velocity of 0.661328 μm/s

line distally so that your kymographs will always have the same orientation and you will be able to distinguish anterograde from retrograde movement.

4. In the “Kymograph” panel (Fig. 3), adjust “Line Width” to include the brightest part of cilium (~5–10 pixels). For “Background subtraction,” selecting “None” will not subtract any background from the image, while “Minimum” will subtract the lowest detected value from each pixel in the stack. Either one usually works well.
5. Click “Create” in the “Kymograph” panel to create a kymograph which plots the pixel values for each pixel in the line you drew for each frame of the streaming video, resulting in rectangle with “time” plotted vertically and “distance” plotted horizontally (Fig. 3). You will (hopefully) see diagonal lines, representing particles that have moved during your streaming video (*see* **Note 11**). You can then use the “Straight-line tool” from the “Region Tools” toolbar to trace the diagonal lines in your kymograph; the slope of the line, dD/dT , is the particle velocity (Fig. 3). Because Metamorph stores the pixel and time dimensions for your video, the velocity will be calculated automatically and appear in the “Kymograph” panel (Fig. 3).
6. The data points can be manually logged, or Metamorph can automatically store them in an Excel or text file. To store measurements automatically, click “Open Log” at the bottom of the “Kymograph” panel and follow instructions. Thereafter, to log each data point, click “F9: Log Data” in the “Kymograph” panel or simply press the F9 key.
7. After scoring kymographs from several animals for each genotype, use Graphpad Prism software to perform one-way ANOVA tests to detect differences between genotypes.

4 Notes

1. We often use the *pha-1(e2123ts)* mutation to easily maintain stable transgenic lines [20]. Animals with this mutation develop normally at the permissive temperature of 15 °C, but fail to develop a normal pharynx at the restrictive temperature of 20–25 °C, and therefore do not survive to adulthood [20]. Because the *myEx685* transgene contains DNA encoding the fluorescent marker (*klp-6p::osm-3::gfp*) as well as the plasmid pBX, which contains a wild-type copy of *pha-1*, the extrachromosomal array is conveniently maintained by growing animals at 20–25 °C. Under these conditions, only animals that have inherited the extrachromosomal array, which rescues the lethality of the *pha-1(e2123ts)* mutation, survive to adulthood [20].

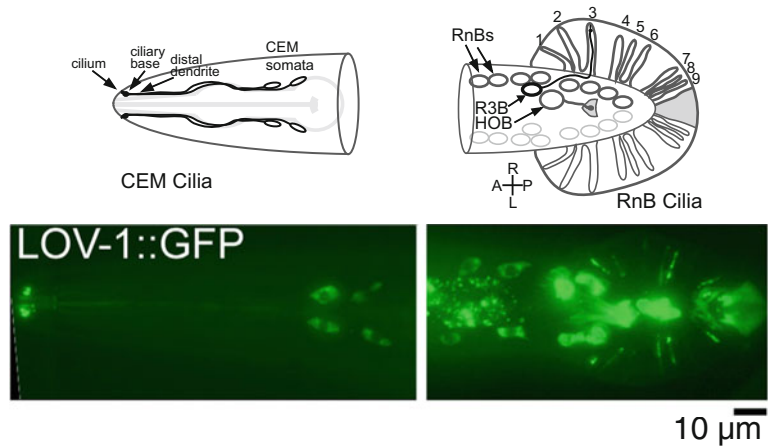


Fig. 4 *C. elegans* male-specific ciliated neurons in diagram (*top*), and in vivo (*bottom*) visualized using a LOV-1::GFP marker. Male-specific neurons with sensory exposed to the environment include the four CEM neurons in the head and the B-type ray neurons (“RnBs”) and the HOB neuron in the tail. These neurons express the tagged polycystin LOV-1::GFP (diagrams reproduced from [15] and pictures reproduced from [27]). This LOV-1::GFP reporter does not move in cilia but is released in ciliary extracellular vesicles [28]

The *myEx685 [klp-6p::osm-3::gfp + pBX]* transgene uses the *klp-6* promoter to drive expression of GFP-tagged kinesin-2 motor OSM-3 in only a subset of ciliated sensory neurons (including the male-specific B-type CEM, RnB, and HOB neurons; Fig. 4). In this example, we describe how to measure OSM-3::GFP motility in male-specific CEM cilia, which coexpress the polycystins LOV-1 and PKD-2 (homologs of mammalian PKD1 and PKD2, respectively [21, 22]).

2. Worms should be raised in incubators to provide a stable temperature for growth. The example transgenic strain PT2098 of genotype *pha-1(e2123ts) III; him-5(e1490) V; myEx685 [klp-6p::osm-3::gfp + pBX]* must be grown at 20–25 °C to maintain the transgene. Typically, animals are raised from egg to adulthood in about 3 days at 20 °C. Exposure to temperatures higher than 25 °C is not healthy for *C. elegans*.
3. To avoid possible variability of data caused by differences in the age and health of the animals, image only healthy young adult males. Take care to avoid transferring hermaphrodites along with your L4 males to eliminate differences in neuronal activity due to sexual experience of hermaphrodites. In this way, each male will have never encountered a potential mate during his adulthood, when his CEM neurons become mature. Therefore, any effects of neuronal stimulation on motor transport will be avoided.

4. Adult males are more active than hermaphrodites and larvae. Therefore, a lower concentration of levamisole may be used when adapting this method for imaging adult hermaphrodites or larvae. If your worms burst on the slide, you may be inadvertently using levamisole at a higher concentration.
5. If you find you are wasting too much time refocusing the stereodissecting scope between picking up animals off NGM plates and mounting on agar pads, you can use a “booster stage” comprising stacked and taped glass slides to hold slides in the same focal plane as the NGM plates.
6. Take care to minimize photobleaching. Since time-lapse imaging will expose samples to excitation illumination for a significant amount of time, the sample will bleach during acquisition. Note also that you may be bleaching your sample before you even begin imaging. Make sure you turn off fluorescent excitation and use Nomarski optics or simply transmitted white light for all manipulations prior to imaging.
7. If using another fluorescent reporter, adjust the exposure time (typically 100–300 ms), Gain, and/or EM Gain (in the “Special” Tab of the “Acquire” panel), and intensity of the excitation light source until you see an acceptable Live image (see Table 1, e.g., settings). The process of finding the right combination of excitation illumination, microscope, and camera settings is iterative. After determining the best parameters for a particular reporter, use the same parameters for all experiments with this marker.

Table 1
Example molecules tagged for kymography analysis in *C. elegans* CEM male-specific-ciliated sensory neurons

Protein name (<i>C. elegans</i> /mammal)	Class	Location	Exposure/gain settings	Reference
PKD-2/PKD2	Polycystin 2	CEM dendrite CEM cilia	300 ms/5 MHz digitizer (Standard gain) 1×	[17, 26]
OSM-3/KIF17	Homodimeric kinesin-2	CEM cilium	100 ms/5 MHz digitizer (standard gain) 1×	[12, 15]
KAP-1/KAP	Heterotrimeric kinesin-2 subunit	CEM cilium	150 ms/5 MHz digitizer (standard gain) 4×	[12]
KLP-6	Kinesin-3	CEM cilium	150 ms/20 MHz digitizer (standard gain) 1×	[12]
OSM-6/IFT152	IFT B complex	CEM cilium	200 ms/5 MHz digitizer (standard gain) 4×	[15, 23]

Location indicates where motile particles have been analyzed in *C. elegans* male-specific-ciliated sensory neurons. PKD-2::GFP puncta move in CEM dendrites, but active transport was not detectable in CEM cilia [17]

8. Your exposure may be too long or too short to detect movement. Check that the combination of your camera resolution and exposure time is likely to detect movement of the velocity expected of your marker. For example, for our Photometrics Cascade 512B EMCCD camera, the distance calibration for the 100 \times objective is 0.15997 $\mu\text{m}/\text{pixel}$. CEM cilia are approximately 3 μm (or 19 pixels) long. OSM-3::GFP puncta in wild type move at approximately 0.7 $\mu\text{m}/\text{s}$ (or 4.4 pixels/s), so for each frame video acquired with 100 ms exposure time (or approximately ten video frames per second), we would expect to see 0.44 pixel displacement of a moving OSM-3::GFP particle in each frame, and each particle could travel from ciliary base to tip in 4.3 s. In contrast, if the exposure were set to 1000 ms, a given particle might appear to “jump” about one third of the way down the cilium and might not be readily detectable as a single moving particle.
9. Anomalous velocities can result from analyzing video of a cilium that does not reside completely in the plane of focus, causing the cilium to appear shorter than it is in reality. This leads to an underestimate of the velocity.
10. We have found applying “Calibrate Distances” each time before analysis helps to avoid mistakes in the original calibration (i.e., in case you forgot to select the 100X objective in Metamorph when acquiring the stream).
11. Vertical lines indicate stationary puncta. Some stationary puncta are expected. However, if your marker should be moving and is not, several explanations exist. Problems could arise due to overexpression of particular transgenes on the cell or cilium. For example, we have found that some GFP-tagged IFT reporters produce a synthetic ciliogenesis defect depending on the genetic background [23]. Alternatively, problems in transport could arise from poor health of mounted worms, owing to either problems in mounting or culture conditions. For an excellent overview of *C. elegans* biology and methodology, the reader is directed to the Worm Methods section of *Wormbook* (http://wormbook.org/toc_wormmethods.html). Your reporter may be too highly expressed, leading to intense fluorescence obscuring the detail necessary for kymography. Extrachromosomal arrays containing a high copy number of the fluorescent marker genes can lead to excessively high expression or overexpression phenotypes [24]. In this case, you may generate a dimmer reporter by injecting your transgene at a lower concentration and looking at more transgenic lines to select one with appropriate fluorescence expression. In the near future, genomic engineering techniques such as CRISPR-mediated genome editing [25] will be used to GFP-tag the endogenous gene of interest to ensure markers are expressed at native levels.

Acknowledgments

The authors were supported by NJCSCR Grant CSCR15IRG014 (R.O.) and NIH Grants DK059418 and DK074746 (M.B.).

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