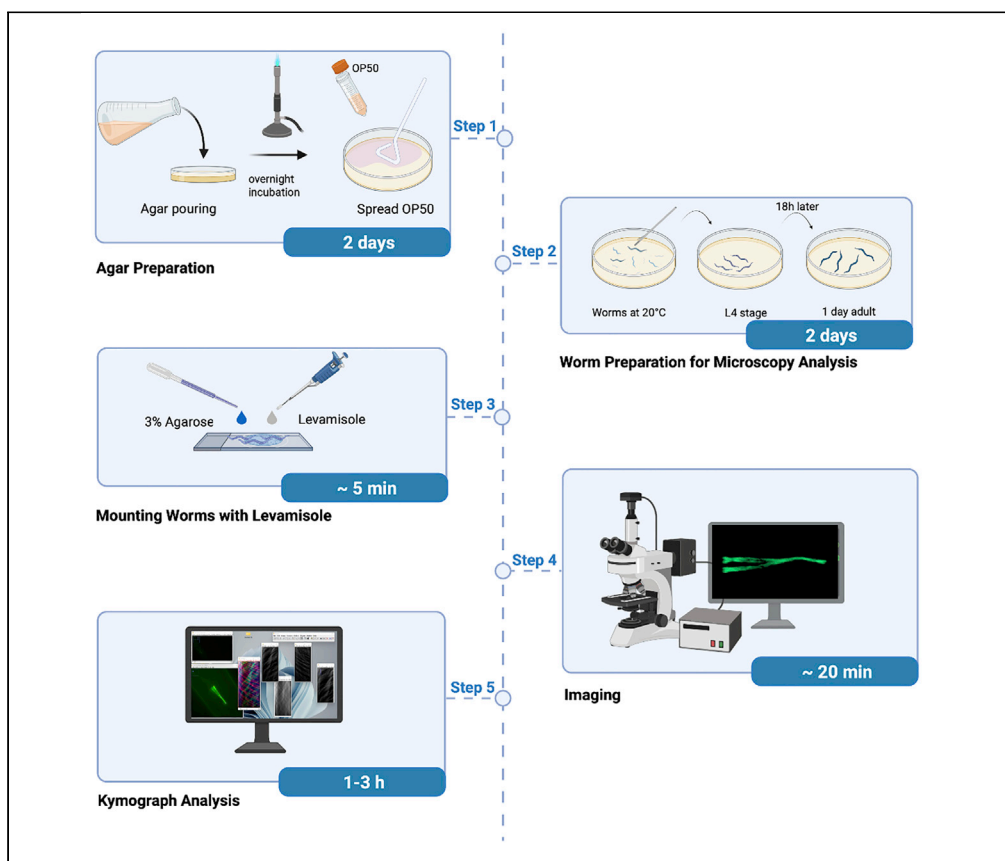## Protocol

Protocol for determining the average speed and frequency of kinesin and dynein-driven intraflagellar transport (IFT) in *C. elegans*

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Highlights

In vivo time-lapse videos followed by kymograph analysis

Kymograph-based analysis can reveal the speed and frequency of IFT

C. elegans immobilization enables collecting high-quality time-lapse movies

Quantify the IFT speed and frequency by synchronizing animals

Here, we present a protocol to image a fluorescent-labeled intraflagellar transport (IFT) component in *Caenorhabditis elegans* with fluorescence microscopy, including steps of sample preparations, *in vivo* live-cell imaging, and post-microscopy analysis with kymographs. This protocol breaks down all processes into three categories: (1) pre-imaging preparations, (2) preparations for the time of image acquisition, and (3) post-imaging analyses. The protocol can be applied to determine the speed and frequency of moving particles.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Protocol for determining the average speed and frequency of kinesin and dynein-driven intraflagellar transport (IFT) in *C. elegans*

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SUMMARY

Here, we present a protocol to image a fluorescent-labeled intraflagellar transport (IFT) component in *Caenorhabditis elegans* with fluorescence microscopy, including steps of sample preparations, *in vivo* live-cell imaging, and post-microscopy analysis with kymographs. This protocol breaks down all processes into three categories: (1) pre-imaging preparations, (2) preparations for the time of image acquisition, and (3) post-imaging analyses. The protocol can be applied to determine the speed and frequency of moving particles.
For complete details on the use and execution of this protocol, please refer to Cevik et al. (2021).

BEFORE YOU BEGIN

A kymograph-based protocol can be employed for determining the frequency and velocity of kinesin motor-driven intraflagellar transport (IFT) in cilia, an antenna-like projection from the cell surface. Using a kymograph-based protocol, we demonstrated that IFT frequency is drastically reduced in specific mutant backgrounds, notably *wdr-31; elmd-1; rpi-2,* in *C. elegans* (Cevik et al., 2021).

The present protocol will describe each step that may be utilized to calculate the frequency and speed of kinesin motor-driven motilities. Firstly, obtain *C. elegans* expressing fluorescence tagged IFT and prepare them for in vivo time-lapse analysis, download and install ImageJ and KymographDirect and KymographClear for subsequent analysis.

Preparing the Nematode Growth Media (NGM)

⊙ **Timing: 1 day**

The NGM plate preparation is the general procedure for all *C. elegans* labs, and NGMs are seeded with *E. coli* OP50 bacteria. *E. coli* OP50 bacteria is a derivative of the *Escherichia coli* strain and a key food source for *C. elegans* in laboratories. The commonly used growth condition of *C. elegans* is at 20 degree Celsius (°C), additionally, some temperature-sensitive strains incubate at 15°C. Thus, the NGM preparation protocol for 1 liter (L) will be provided below, and the protocol can be scaled to the volume required.

A glass bottle (2 L, autoclaved), stir bars (autoclaved), a magnetic stirrer, a digital weighing scale, a water bath, a graduated cylinder, a spoon (autoclaved), 10 μL- 1 milliliter (mL)

micropipettes, and micropipette tips (autoclaved) will be required in the NGM medium preparation procedures.

1. First, prepare the following solutions for NGM: 1 M KPO_4 buffer (pH 6.0, autoclaved); 5 mg/mL cholesterol in ethanol (filtered), 20 mg/mL nystatin (filtered), 1 M $MgSO_4$ (autoclaved), and 1 M $CaCl_2$ (filtered). Please refer to the tables in the materials and equipment section for the NGM preparation, since they provide a complete list of solutions and media preparations.
2. Weigh the chemical reagents (NaCl, Bacto Peptone, and Agar) in the amounts indicated in the below table for 1 L NGM preparation, then pour 1 L ddH_2O into an autoclaved glass bottle, add a stir bar, and autoclave.
3. When the NGM autoclave is almost completed, turn on the magnetic stirrer and set the temperature to 50°C. Next place the NGM containing bottle on the magnetic stirrer and leave it there until it cools to around 50°C.
4. Use micropipette filter tips to add ready-to-use solutions in the following amounts into the autoclaved hand-safe NGM bottle: 1 mL of 20 mg/mL nystatin, 1 mL from 5 mg/mL cholesterol, 1 mL from 1 M $MgSO_4$ (filtered $MgSO_4$), 25 mL from 1 M KPO_4 (pH 6.0), and 1 mL from 1 M $CaCl_2$ (filtered $CaCl_2$). Place the bottle back on the magnetic stirrer and mix for 10 min at 450 rpm at 50°C, allowing each to be homogeneously mixed.
5. The solutions should be poured near a flame in a sterile cabinet. Next, pour the NGM into a sterile Petri dish (size: 60 mm × 15 mm) around 10 mL for each plate. A peristaltic pump or automatic media/agar plate pouring system can be used to pour plates.

   Optional: Turn on ultraviolet (UV) light in the biosafety cabinet to sterilize inside before starting spreading plates.

6. Leave the poured plates in the cabinet at 20°C for 16 h.

   Note: Do not move freshly poured agar plates until it is completely solidified since it will cause uneven agar surface.

Culturing *Escherichia coli* bacteria OP50

⊙ **Timing: 1 day**

7. Fill a glass bottle with 1 L of ddH_2O after adding 25 g of LB (Lysogeny Broth), then autoclave it.
8. OP50 strains of *E. coli* bacteria are grown in 100 mL of liquid LB at 250 rpm at 37°C for 16 h.
9. Sterilize the interior of the biosafety cabinet with aseptic procedures and turn on the Bunsen burner. Drop approximately 70 μL of *E. coli* OP50 liquid culture to NGM plates and spread it by using a glass bacterial cell spreader near the flame.

   Note: Seeding NGM with LB-grown *E. coli* OP50 should be performed under sterile conditions. When adding *E. coli* OP50, use a micropipette filter tip and change it every 10–15 plates. To avoid contamination, immerse the glass bacterial cell spreader in 100% ethanol before lighting it on fire. Allow the ethanol to completely burn off or it may kill the germs as it spreads.

   ⚠ CRITICAL: *E. coli* OP50 spread NGM plates should be monitored in the next few days to ensure that no new bacterial contaminations have occurred. Contaminated plates should not be used and must be discarded. We recommend, for every month, isolating and cultivating a new single *E. coli* OP50 bacteria colony in LB under sterile conditions from a carefully streaked agar plate.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli OP50	*Caenorhabditis* Genetics Center (CGC)	N/A
Chemicals, peptides, and recombinant proteins		
Cholesterol	Carl Roth	8866.1 - 100 gr
Nystatin	RPI (Research Products International)	N82020-10.0
Immersion Oil	Sigma-Aldrich	56822 - 50 mL
Levamisole Hydrochloride	ChemCruz, Santa Cruz Biotechnology, Inc.	sc-205730, 5 g
Agarose	Prona Agarose Biomax	D00216PR
LB Broth, Miller Formulation	VWR Life Science	J106 - 1 kg
K_2HPO_4	Merck	105101.1000 - 1 kg
KH_2PO_4	Merck	104873.1000 - 1 kg
$CaCl_2$	CARLO ERBA Reagents	10043-52-4
$MgSO_4.7H_2O$	CARLO ERBA Reagents	10034-99-8
Sodium chloride (NaCl)	ISOLAB	969.033.1000 - 1 kg
Bacto™ Peptone	BD Bioscience™	211677 - 500 g
Agar-Agar, Kobe I	Carl Roth	5210.2 - 1 kg
Experimental models: Organisms/strains		
C. elegans: Strain GOU2362: ift-74(cas499[ift-74::gfp) II.	*Caenorhabditis* Genetics Center (CGC) (Yi et al., 2017)	WB Strain: GOU2362; WormBase: WBStrain00007886
wdr-31(tm10423)II;elmd-1(syb630) III; rpi-2(ok1863)IV. [GOU2362, ift-74 (cas499 [ift-74::gfp)) II.]	(Cevik et al., 2021)	N/A
Deposited data		
The codes used for creating Figure 1C	This paper	Protocol-for-determining-the-average-speed-and-frequency-of-kinesin-and-dynein-driven-IFT, [https://github.com/thekaplanlab/Protocol-for-determining-the-average-speed-and-frequency-of-kinesin-and-dynein-driven-IFT]
The TIFF movies used for speed and frequency analysis	This paper	Zenodo, [https://doi.org/10.5281/zenodo.6568214]
Software and algorithms		
Carl Zeiss microscope	Carl Zeiss	Axio Vert.A1
Upright fluorescence microscope	Leica	LEICA DM6 B
Electron Multiplying Charge-Coupled Devices (EMCCDs) Camera	Andor	Andor iXon (897) Ultra EMCCD (Camera)
Camera and Microscope Software	Andor	Andor iQ 3.6.2 Software
Stereo Microscope	Carl Zeiss	Stemi 508
ImageJ	(Schindelin et al., 2012)	https://imagej.nih.gov/ij/download.html - Version 1.53o
KymographDirect	(Mangeol et al., 2016)	https://sites.google.com/site/kymographanalysis - Version 2.1
KymographClear	(Mangeol et al., 2016)	https://sites.google.com/site/kymographanalysis - Version 2.0
Workstation	HP ProDisplay P232	HP Z640 Workstation, (RAM): 160.0 GB, Processor: Intel(R) Xeon(R) CPU E5-2620 v3 2.40 GHz, 64-bit Operating System, Display resolution - 1920 × 1080 pixels
Oasis™ 160 Cooling System	Andor	N/A
R Core Team	N/A	https://www.r-project.org/
Other		
Petri dishes (sterile) - 60*15 mm	FiratMed	8870000046

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cover glass - (24 × 24 mm field; 0.13–0.17 thinness)	ISOLAB	075.00.004
Microscope slide	ISOLAB	I.075.05.003

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MATERIALS AND EQUIPMENT

20 mg/mL Nystatin

Reagent	Final concentration	Amount
Nystatin	n/a	20 mg
Ethanol	70%	1 mL
Total	20 mg/mL	1 mL

Filter it with 0.22 μm pore size filter, aliquot into sterile Eppendorf tubes, and stock it at −20°C for several months.

5 mg/mL Cholesterol

Reagent	Final concentration	Amount
Cholesterol	n/a	5 mg
Ethanol	n/a	1 mL
Total	5 mg/mL	50 mL

Filter it with 0.22 μm pore size filter, and stock it at +4°C for several months.

1 M KPO_4 at pH 6.0

Reagent	Final concentration	Amount
KH_2PO_4	n/a	108.3 g
K_2HPO_4	n/a	35.6 g
ddH_2O	n/a	1 L
	1 M	1 L

Autoclave, adjust with pH meter, and stock it at 20°C for several months.

$MgSO_4$

Reagent	Final concentration	Amount
$MgSO_4$	n/a	120.266 g
ddH_2O	n/a	1 L
Total	1 M	1 L

Autoclave and filter it with 0.22 μm pore size filter. Keep it at 20°C for several months.

1 M $CaCl_2$

Reagent	Final concentration	Amount
$CaCl_2$	n/a	110.98 g
ddH_2O	n/a	1 L
Total	1 M	1 L

Autoclave and filter it with 0.22 μm pore size filter. Keep it at 20°C for several months.

NGM

Reagent	Final concentration	Amount
NGM part 1: Prepare agar mixture		
NaCl	n/a	3 g
Bacto Peptone	n/a	2.5 g

(Continued on next page)

Continued		
Reagent	Final concentration	Amount
Agar	n/a	17 g
ddH$_2$O	n/a	975 mL
Total	n/a	1000 mL
Autoclave and cool to 50°C while stirring.		
NGM Part 2: Add Required Chemicals to Buffer (from stocks above)		
20 mg/mL Nystatin	n/a	1 mL
5 mg/mL Cholesterol	n/a	1 mL
1 M MgSO$_4$	n/a	1 mL
1 M KPO$_4$	n/a	25 mL
1 M CaCl$_2$	n/a	1 mL
Total	n/a	1000 mL
Stir for 10 min at 450 rpm at 50°C, then pour into Petri dishes.		

⏸ **Pause point:** NGM plates can be stored up to 4 months at +4°C.

Lysogeny Broth (LB)

Reagent	Final concentration	Amount
LB (lysogeny broth)	n/a	25 g
ddH$_2$O	n/a	1 L
Total	n/a	1 L
Autoclave and store at +4°C.		

⏸ **Pause point:** LB can be stored at +4°C for several months.

M9 Buffer

Reagent	Final concentration	Amount
KH$_2$PO$_4$	n/a	3 g
Na$_2$PO$_4$.7H$_2$O	n/a	5.8 g
NaCl	n/a	5 g
MgSO$_4$.7H$_2$O	n/a	0.25 g
ddH$_2$O	n/a	1 L
Total	n/a	1 L
Autoclave and store it at 20°C.		

⏸ **Pause point:** M9 buffer can be stored at room temperature for several months.

3% Agarose

Reagent	Final concentration	Amount
Agarose	n/a	3 g
ddH$_2$O	n/a	100 mL
Total	3%	100 mL
Heat it in the microwave to melt the agarose, and place the 3% agarose-containing bottle in a 65°C water bath.		

⏸ **Pause point:** 3% agarose can be stored at 65°C up to 7 days.

Levamisole		
Reagent	Final concentration	Amount
Levamisole Hydrochloride	n/a	0.05 g
M9 Buffer	n/a	500 μL
Total	500 mM	500 μL

Mix and stock it at −20°C. Dilute it from stock solution to 10 mM in M9 buffer for analysis.

⏸ **Pause point:** Stocked levamisole can be stored at –20°C for several months.

STEP-BY-STEP METHOD DETAILS

Pre-imaging preparations

To generate reproducible results, especially *in vivo* live transport assay in *C. elegans*, the pre-imaging preparations are as critical as subsequent steps, including microscopy imaging and post-imaging analysis. For example, the age of the worm affects the frequency of kinesin and dynein-driven IFT motility along cilia in *C. elegans* (Zhang et al., 2021). Furthermore, because unfavorable
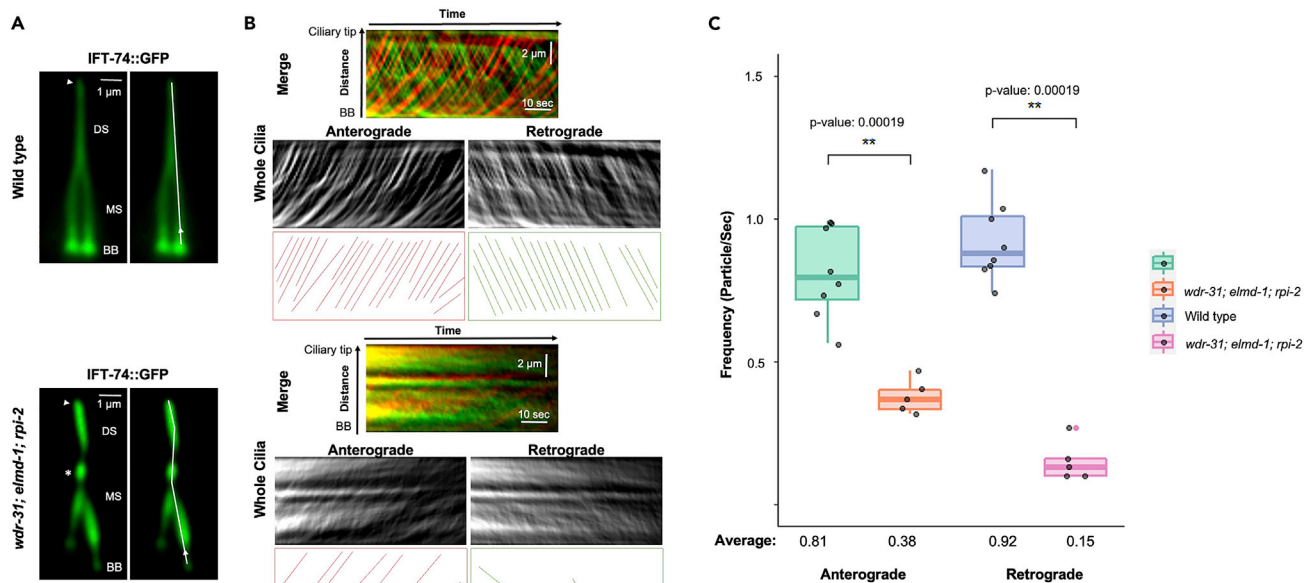

Figure 1. Kymograph results for wild-type and *wdr-31; elmd-1; rpi-2* triple mutant

(A) Fluorescence microscopy of GFP labeled endogenous IFT-74 (human IFT74), an IFT-B component, in the phasmid sensory neuron cilia (PHA/PHB cilia) of wild type *and wdr-31(tm10423)II;elmd-1(syb630) III; rpi-2(ok1863)IV* triple mutant. IFT-74 (green) marks entire cilium including basal body (BB), middle segment (MS), and distal segment (DS). A white arrow indicates the ciliary tip, whereas an arrow mark indicates the direction for making the kymograph. The PHA/PHB cilia localization of IFT-74 in wild type is shown on the top panel, whereas the IFT-74 cilia staining, including accumulations in the middle of the cilia, is displayed on the bottom panel in *wdr-31; elmd-1; rpi-2*. The asterisks (*) denotes accumulations within cilia. Scale Bar: 2 μm.

(B) The kymographs shown were created using ImageJ on the KymographClear2.0 from time-lapse videos of IFT-74::GFP in wild type and *wdr-31; elmd-1; rpi-2*. Red and green lines on merged kymographs represent anterograde and retrograde particles, respectively. Anterograde and retrograde kymographs together with representative drawings are displayed under merged kymographs. The X-axis represents distance (μm), while the Y-axis represents time (30 s).

(C) The average IFT particle frequency of IFT-74::GFP in wild type (WT) and *wdr-31; elmd-1; rpi-2* triple mutants are depicted in the plot. The number of traveling particles on each kymograph were found using the KymographDirect for wild type, and the IFT frequency was determined by dividing the total number of traveling particles on a kymograph the by the duration. The average IFT frequencies in both directions for wild type and *wdr-31; elmd-1; rpi-2* were determined and plotted. While the average IFT anterograde frequency of wild-type is 0.81 particles/s, it was lowered to 0.38 particles/s in the triple mutant. In wild type and *wdr-31; elmd-1; rpi-2*, the average IFT retrograde frequency was 0.92 particles/s and 0.15 particles/s, respectively. Kruskal-Wallis test was used to assess statistical significance, and two asterisks (*) signify statistical significance for both directions between wild-type and *wdr-31; elmd-1; rpi-2* triple mutants. P values are indicated in plot (R Core Team, 2020).

environmental conditions, including starvation and contamination (mold, fungi, etc.) may impact the health of *C. elegans*, they likely influence a variety of cellular processes, especially IFT. Consistent with this logic, starvation increases the speed of anterograde IFT (from the base of cilia to the tip of cilia) in *C. elegans* (Kimura et al., 2018). We recommend having contamination-free, well-fed, and synchronized young worms (1 and 2 days old) for the microscopy analysis. For reproducible results, cultivating healthy *C. elegans* in a sterile Petri dish with contamination-free NGM (Nematode Growth Media) spread with OP50 strain at well-controlled temperatures (20°C) is critical.

Preparation of next steps for *in vivo* live microscopy analysis

⏱ Timing: 1 day

1. Culture worms for at least one generation without starving and any contaminations at 20°C.
2. The day before microscopy analysis, transfer 100 L4s (fourth-stage larvae, the last stage of larvae before becoming adults) into a new plate, and the microscopy analysis can be done on a 1-day adult.
3. Prepare 3% of agarose in a 250 mL bottle and keep it at 65°C in the water bath.

   ⚠ CRITICAL: We do not advise allowing agarose to re-solidify since it necessitates re-melting with the microwave, which changes the percentage of agarose in each microwave heating.

   Note: We do not recommend using the freshly prepared 3% of agarose on the day of preparation, wait for 1 day, because when making an agarose pad in the subsequent days, agarose pads become less brittle, giving better agarose pads. You can use the 3% of agarose up to 7 days.

4. For immobilization of the worms for in vivo live microscopy analysis, 10 mM is working concentration for levamisole, and prepare 10 mM levamisole (an anesthetizing reagent) for 100 μL from a 500 mM stock solution by diluting in M9 buffer.

   ⚠ CRITICAL: There are several anesthetics that can be utilized, including sodium azide, levamisole, tricaine, and 1-Phenoxy-2-propanol. Because of its effect on ATP, sodium azide has an effect on IFT motility and is not recommended for the IFT motility experiment.

   ⚠ CRITICAL: Imaging living *C. elegans* is always challenging and requires a lot of effort as *C. elegans* tend to display movements during live imaging. Levamisole alone will not effectively immobilize the worms, and in our experience, there will be zero movements in 5 of the 25 videos collected for in vivo live microscopy analysis when only anesthetic levamisole was used, thus increasing the overall effort of microscopy analysis. A microfluidic device or polystyrene beads, or a higher concentration of agar pads are alternative ways of immobilizing worms (Kim et al., 2013; Clark et al., 2021). They can be used in conjunction with levamisole.

Preparations for the time of image acquisition

⏱ Timing: 4–5 h

Before beginning image acquisition, ensure that the imaging room's temperature is steady (21°C–22°C) and not fluctuating. Check worms on the day of microscopy imaging to make sure that worms are healthy. We employ a variety of parameters to check the health of worms: 1) They are moving in the plate. 2) There is no bacterial or fungal contamination in the plate. 3) We also make sure they have adequate food on their plate.

5. Begin by turning on the computer and fluorescent microscope (in our example, a Leica DM6 B upright fluorescence microscope), then the camera (Andor iXon Ultra 897 EMCCD Camera), and last the controlling software (Andor iQ 3.6.2 Software). It is worth noting that inverted scope can also be employed here. Find fluorescence signal on the slide with a 20× objective lens, then apply a small amount of immersion oil before rotating a 100× objective lens onto the slide to begin taking a live time-lapse movie (HCX PL APO 100×, 1.40 oil microscope objective).

⚠ CRITICAL: To execute the software and subsequent image analysis, a high-performance computer system is required. However, image processing should be performed on a separate computer so that the computer connected to the microscope is not occupied. A high-quality microscope is necessary, but without high-quality cameras and suitable software, it is not possible to acquire high-quality time-lapse recordings for further analysis. Before purchasing a microscopy complete system for live time-lapse movie analysis, we recommend trying the microscope, camera, and software together.

6. A running protocol must first be set up to take a live time-lapse movie. We therefore spent time finding a suitable exposure time and the gain setting on a fluorescence microscope for an IFT component (IFT-74::GFP; human IFT74) in *C. elegans*. We set the exposure time to 300.0 ms and the gain to 7. We next set up a running protocol of 333.0 ms per second (3 frames per second (s)) with a total of 100 frames on the Andor iQ 3.6.2 Software.

⚠ CRITICAL: The longer camera exposure time produces a sharper image with higher pixel intensity while also photobleaching i.e., the reduction of the sample's fluorescence signal during image capture. The sample will be exposed to light 3 times per second because the protocol is designed to take 3 images per second, resulting in a decrease in the fluorescent signal. A live time-lapse movie, on the other hand, necessitates a particular amount of time, during which light is gathered from the chosen object. The main issue is that the beginning part of the time-lapse movie may have brighter pictures, but the brightness of the images decreases in the later part of the time-lapse movie, which may damage the overall quality of the time-lapse movie. For better quality of time-lapse movies, we recommend reducing the length of the time-lapse movie with a longer exposure time.

⚠ CRITICAL: Because of the nature of endogenously tagged fluorescent proteins, their expression levels might vary and some may be inadequate to be detected by the microscope. Playing with the exposure time and gain will not assist any more until the fluorescent expression is detectable by the microscope system. Make sure to start with a strain containing fluorescent-tagged protein that is well expressed.

7. Use Pasteur pipette to take a little amount of 3% (0.3 mL) agarose (stored at 65°C in the water bath). Drop the agarose liquid onto the 18 mm × 18 mm area of the microscope slide, then place the second slide over the agarose to create a smooth and thin layer. Gently press and wait 1 min for the agarose to solidify. Slowly separate slides from each other without disrupting the agarose pad (Methods video S1: Slide preparation, related to steps 1–4).

⚠ CRITICAL: Agarose pads must be freshly made at the time of image capturing, and once prepared, they must be utilized within 1 minute.

8. Add 2 μL of 10 mM levamisole over the agarose pad under a stereomicroscope (ZEISS Stemi 508), and then transfer 10–15 1-day adult worms using a platinum wire pick onto agar pads. Put cover slide above the worms and gently press. After mounting, we immediately transfer the slide to the fluorescent compound scope for subsequent microscopy analysis (Methods video S1: Worm transformation, related to steps 5–9).

⚠ CRITICAL: Do not harm the worm during transferring; for example, the worm's skin may be injured as a result of the pick's tip, resulting in subsequent bursting while transferring, hence it may affect the result of the IFT assay.

9. Place the slide under the microscope, dab a drop of immersion oil on the cover slide, and look for a worm tail to film. Focus on the GFP signal in the cilia of two phasmid sensory neurons (PHA and PHB) in the tail of worms and start running the protocol that was already set up (Methods video S1: Focusing fluorescent signal, related to steps 12 and 13).

⚠ CRITICAL: When considering the movement of worm parts and the presence of ciliated cells, recording a worm from the tail is less problematic than filming a worm from the head.

⚠ CRITICAL: Putting mouse pointer over the cilia or a part of the cilia can help to understand movement during the recording.

⚠ CRITICAL: We recommend finishing the time-lapse movie within 30 minutes after mounting the slide. In our experience, we notice that after some time, the speed of IFT particles slows down, which might result in inconsistent results.

10. Save all time-lapse series of images in TIFF format taken from the image list section to distinct files for later use with kymograph, speed, and frequency analysis.

⚠ CRITICAL: Discard the time-lapse series of images where worms move. Alternatively, the ImageJ plugin for drift correction can be used to help correct movements in time-lapse movies. The following plugin can be downloaded for manual drift correction: https://imagej.net/plugins/manual-drift-correction#:~:text=Manual%20Drift%20Correction%20plugin%20allows,by%20interpolating%20between%20key%20landmarks.

⚠ CRITICAL: The image file size for each time-lapse TIFF file of 100 frames is 200.0 megabytes (MB). Make sure you have enough storage for all microscope analyses, and that you keep the original files. The main challenge, however, is the long-term storage of each time-lapse image. The external hard drives are easy to fail. Cloud storage is another option for long-term data storage, although it needs ongoing subscriptions.

Post-imaging analyses

⊙ Timing: 1–3 h

11. For generating Kymographs and speed analysis, we use two software: ImageJ, a freely available image analysis software, and KymographDirect and KymographClear, automated packages for kymograph analysis, from https://imagej.nih.gov/ij/ and https://sites.google.com/site/kymographanalysis, respectively (Mangeol et al., 2016; Schindelin et al., 2012). Download and install both of them.

12. Once KymographDirect and KymographClear related files are downloaded from the website mentioned above, copy and paste the "KymographClear 2.0a.ijm", "KymographDirect2.1.aliases", and "KymographDirect2.1.ini" to the "toolsets" file in the "macro" file in the ImageJ file. Open ImageJ and, install "KymographClear 2.0a.ijm" using the install button on Plugins. Additional buttons will appear on ImageJ which allow users to run KymographDirect, and KymographClear on ImageJ. The user must install the kymograph generation-related plugins to the ImageJ panel each time opens.

Note: KymographDirect and KymographClear website provides step-by-step instructions about how to install and use these tools: https://sites.google.com/site/kymographanalysis.

13. Copy and paste your TIFF files into their dedicated new files created for each TIFF at a particular location on your computer.

> ⚠ CRITICAL: Dedicated files for each time-lapse TIFF will help avoid confusion. Kymographs are created automatically from each TIFF file during TIFF file analysis; thus, no kymographs will be mixed up when they have their dedicated files.

Determining the frequency of anterograde and retrograde IFT

> ⏱ Timing: 1–2 days

14. Open a single TIFF file with the Kymograph Open Sequence plugin (KymographClear plugin) (yellow file figure in the panel) previously installed on ImageJ. Choose the first of two options: "open a sequence and compute average image" or "open a sequence and compute max intensity image". After you upload the TIFF file, the "Sequence options" tab will appear. In the "Sequence options" tab, type 1 in the section of "number of images", then click OK. The time-lapse image will appear and zoom on cilia (Methods video S2: Opening files with ImageJ, related to steps 1 and 2).

> *Note:* Fiji is an ImageJ2-based image analysis software option, however, subsequent analysis needs kymographs created using KymographDirect and KymographClear, both of which are incompatible with Fiji.

> ⚠ CRITICAL: ImageJ and Fiji have alternative kymograph generation plugins, but they require manual drawing of lines in order to produce the kymograph. With manual drawing, two lab members separately did an internal study using the same movies and determined IFT speeds, and their findings were slightly different. The discrepancy was most likely caused by differences in kymograph drawing lines (the slope of line drawings differed). Furthermore, we compared manual kymograph analysis findings to those obtained by KymographDirect and KymographClear using the same videos and discovered that the average IFT speed of manual kymograph analysis was slower than that of KymographDirect and KymographClear.

Methods video S2: Time-lapse microscopy images of yeast cells, related to step 1.

15. Using ImageJ's panel, draw a segmented line above the cilia from the base to the end (entire cilia). The line delineates the area where the kymograph will be built and measurements (IFT speed and frequency) will be taken (Methods video S2: Determining cilia parts, related to step 3).
16. Next, in the KymographDirect toolbar, select "Kymograph generation action tool" and set the line width to 5 to produce kymographs for anterograde and retrograde motilities. Kymographs (Anterograde, retrograde, and merged) and speed-related files are automatically saved in the same folder as the TIFF file (Methods video S2: Kymograph generation, related to step 4).
17. Launch the KymographDirect tool and upload kymograph from the file. In the KymographDirect tool, the subpanel is located on the left panel, and the kymograph is in the middle. Anterograde particles appear on a kymograph when the "forward" option is selected, whereas retrograde particles appear when the "backward" option is selected. The quantity of anterograde and retrograde particles may be observed in the subpanel's "Select a line" option (Methods video S2: Counting particles, related to step 6). After noting the number of anterograde and retrograde particles in the time-lapse videos examined, you can proceed to the next time-lapse movies.

⚠ CRITICAL: Intensity threshold and brightness & contrast settings help to reduce background noise during the counting process if you want to check lines as manually. Playing with the threshold on the subpanel will change the number of particles.

18. Create an excel file to save all particle numbers with the experiment name and directions for further analysis (Methods video S2: Counting particles, related to step 6).
19. To calculate the average frequency of anterograde or retrograde transport events for each strain, use at least 10 kymograph findings.

Determining the average speed of anterograde and retrograde IFT

⏱ Timing: 1–2 days

In *C. elegans,* ciliary cargos and signaling molecules are distributed across cilia by kinesin and dynein driven IFT complex. Anterograde IFT, mediated by the kinesin motor, refers to transport happenings from the ciliary base to the ciliary tip, whereas retrograde IFT, mediated by cytoplasmic dynein, refers to transport events that occur from the ciliary tip back to the ciliary base. *C. elegans* channel cilia have a middle segment (~4 μm long, microtubule doublets) and a distal segment (~2.5 μm long, microtubule singlets). In the middle segment of channel cilia, Kinesin II and OSM-3 (human KIF17) motors move at a slower rate (the mean velocity of 0.7 μm/s) carrying IFT components, IFT cargos, and signaling molecules, and all of these cargos are handed over to OSM-3, which travels at a quicker rate (the mean velocity of 1.2 μm/s). The retrograde transport is achieved by cytoplasmic dynein at a velocity of 1.10 μm/s. In the following protocol, we will go over kymograph-based IFT speed measurements using the KymographDirect tool.

20. To measure the average speed of the anterograde IFT in the middle segment, draw a segmented line above the cilia from the base to the middle segment (4 μm), and repeat the step 25 (Figures 2A and 2B). In a brief, use the KymographDirect toolbar to generate kymograph for anterograde and retrograde transport events. System automatically calculates the average anterograde IFT and retrograde IFT speeds.
21. Users should hit "Save" on the upper save button on the KymographDirect tool to save files to an existing file called "Results.". The file named "velocity vs. time" files will be used to calculate average anterograde IFT velocities in the middle segment, and we have supplied a walk-through video to help you calculate average IFT speeds (Methods video S2: Speed, related to step 7).
22. To determine the average velocity of the anterograde IFT in the distal segment, draw a 2.5 μm segmented line from the ciliary tip and repeat the step 16 and rest of the steps mentioned above (Figures 2A and 2B). This procedure may be repeated to calculate the average IFT frequency for retrograde, with kymographs obtained to calculate the average IFT frequency.
23. Next, open the "Results" file and choose the "velocity vs. time" text file. Copy-paste all results from this text file to an excel file to find all results for further analysis (Methods video S2: Saving velocity results, related to steps 8 and 9).
24. Average all velocity and time results for the whole lines in the excel file, separately (Methods video S2: Average speed calculation, related to steps 10 and 11).
25. Add more analysis until achieving at least 100 particles for each strain. Then, calculate the average velocity in the overall experiments. We always undertake two independent analyses and then increase the number of IFT particles whenever we observe statistically significant differences in IFT rates between the wild type and mutant strains examined.

Optional: A more visually appealing display of merged kymograph images would aid readers in estimating the number of moving particles. KymographClear2.0 generates a merged
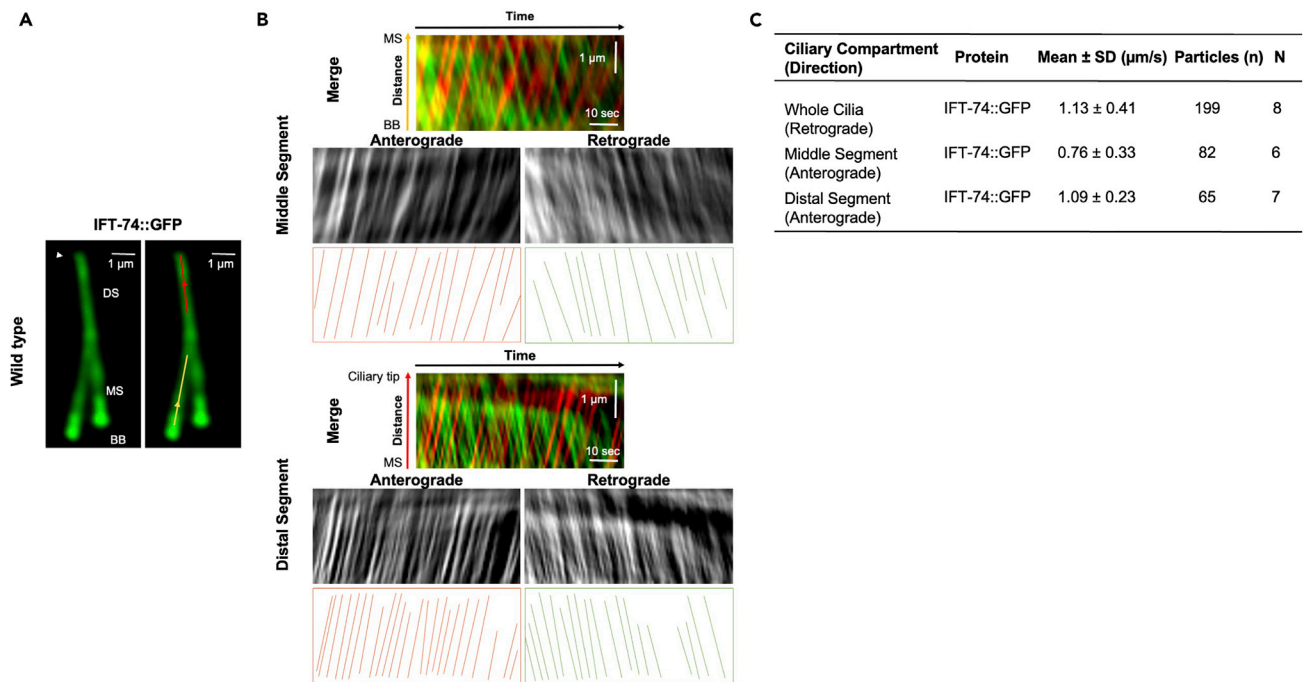
Figure 2. IFT velocity for anterograde and retrograde directions for IFT-74::GFP

(A) The wild type phasmid sensory cilia (PHA and PHB cilia) are shown with GFP tagged endogenous IFT-74. IFT-74 (green) stains the whole cilium, including the basal body (BB), middle segment (MS), and distal segment (DS). A white arrow indicates the tip of the cilia. The red and yellow arrows represent the locations where the MS and DS kymographs were created, respectively. Scale Bar: 1 μm.

(B) The kymographs for the middle and distal segments are created with the ImageJ software's KymographClear2.0 plugin. The red and green lines on the merged kymographs for each kymographs depict particles traveling anterograde and retrograde directions, respectively. Separate anterograde and retrograde kymographs (grayscale) are exhibited under combined kymographs with representative drawing panels of particles. The yellow and red arrow marks on the combined kymographs reflect the kymograph's orientation for MS and DS, respectively. The x-axis denotes distance (m), while the y-axis denotes time (30 s). Scale bar for distance and time is shown on the merged kymographs.

(C) The average anterograde and retrograde velocities of IFT-74::GFP in the middle and distal segments are displayed. The number of particles (n) and worms (N) examined is shown in the table. SD stands for standard deviation.

kymograph in the kymograph file called color-coded direction. This kymograph has anterograde and retrograde directions on a static blue backdrop. To create a visually appealing kymograph during the post-imaging process split the color-coded kymograph using the "Color" option from ImageJ's Image subpanel, and close the blue TIFF file while keeping red and green TIFF files. Arrange the green and red fluorescent signals using brightness/contrast parameters, then merge them using the "Color" option, "Merge Channels." The newly generated kymograph is more aesthetically evident than the color-coded kymograph (Methods video S3).

EXPECTED OUTCOMES

Kinesin and dynein-driven IFT complexes dynamically transport cargos and signaling molecules to and from cilia, and its bidirectional movement in the cilia is near constant in velocity and varies in frequency. In *C. elegans*, Kinesin II and OSM-3 (human KIF17) motors jointly travel to drive IFT components and cargos at an average speed of 0.7 μm/s in the middle segment of cilia (microtubule doublets), and OSM-3/KIF17 takes over IFT components and cargos and transports them at an average speed of 1.2 μm/s in the distal segment of cilia (microtubule singlets), while cytoplasmic dynein transports the IFT components and cargos from the tip to the base at an average speed of 1.10 μm/s.

Using time-lapse movies coupled with kymograph based analysis, we found that the average anterograde (from base to cilia) speed of IFT-74::GFP, an IFT component, in the middle segment of the cilia was 0.76 μm/s, while the speed in the distal part was 1.09 μm/s. The average retrograde speed (from the ciliary tip to the base of cilia) was 1.13 μm/s (Figure 2C). Average speeds in both directions were accordance with average speeds reported. We next determined the average IFT frequencies in both directions for wild type and triple mutant namely *wdr-31(tm10423)II;elmd-1(syb630)III;rpi-2(ok1863)IV*. In our latest work, we documented the nature of mutations in each mutant (Cevik et al., 2021). In summary, wdr-31(tm10423)II has a 160-bp deletion that causes a frameshift, whereas elmd-1(syb630)III and rpi-2(ok1863)IV mutants have 1784-bp and 1143-bp deletions, respectively. When we compared *wdr-31;elmd-1;rpi-2* to wild type, both anterograde and retrograde IFT frequencies in cilia were dramatically reduced (Figures 1A and 1B). It is noteworthy kymographs generated in Figure 1 were utilized to calculate average IFT frequencies and average IFT retrograde speeds.

LIMITATIONS

KymographDirect produces very elegant kymograph with visible lines, and the lines on both anterograde and retrograde kymograph panels generally correlate with the numbers provided by KymographDirect as we performed visual inspection. However, the real issue arises when there are faint lines (in certain mutants) on both anterograde and retrograde kymograph panels, because the system is unable to fully detect weak lines and instead detects non-real lines, resulting in incorrect results. Furthermore, tinkering with the intensity threshold settings does not solve the problem. In such cases, we should not rely on KymographDirect results and instead conduct a visual inspection.

TROUBLESHOOTING

Problem 1

While a longer exposure time results in a sharper image with higher pixel intensity, it also results in a decrease in overall fluorescence signal (photobleaching) of a sample during image capture, resulting in lower image quality (step 15).

Potential solution

To minimize the decline in overall fluorescence signal, reduce the exposure time. Additionally, anti-photobleaching mounting media (for example, commercial mounting media Fluoromount) can be used.

Problem 2

The IFT speed will differ from cilia to cilia or worm to worm, and the IFT speeds of different cilia segments from the same worm will differ significantly. Even though this is to be expected, the IFT speed will be affected by the age and health of the worms, the length of microscopy time, and the temperature of the microscopy rooms, which may result in inconsistent results (pre-imaging preparations).

Potential solution

Use the same stage of well-fed worms (1 day adult) in the analysis.

Ensure the analysis time, which includes mounting and filming, does not exceed 30 min.

Do not use damaged worms for IFT analysis and choose healthy worms.

Make sure the microscopy room temperature does not fluctuate.

Problem 3

Endogenously fluorescent-tagged proteins expression levels might vary and cannot be detected by microscope (step 15).

Potential solution

Begin with strains that have a greater quantity of endogenously fluorescent-tagged proteins (a brighter signal).

Problem 4

The worm may display a slight movement after certain frames in a 100 frames movie (step 19).

Potential solution

Duplicate the time-lapse movie and delete the frames where there is movement in the time-lapse movie. The non-moving portion of the film can be used for further analysis. Do not delete any frames from the midst of the time-lapse movie; instead, delete from the beginning or the end of time-lapse videos.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Oktay I. Kaplan (oktay.kaplan@agu.edu.tr).

Materials availability

Reagents used with this protocol are available from the lead contact upon request.

Data and code availability

Original data have been deposited to Zenodo Data: 10.5281/zenodo.6568214. The codes used to generate the plot and statistics for this study are available at https://github.com/thekaplanlab/Protocol-for-determining-the-average-speed-and-frequency-of-kinesin-and-dynein-driven-IFT.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101498.

AUTHOR CONTRIBUTIONS

Data acquisition, O.C., H.K., S.D.T., S.C., and M.G.T.; analysis, S.C. and M.G.T.; writing – original draft, M.G.T. and O.I.K.; conceptualization, O.I.K.; funding acquisition, O.I.K. and S.C.; supervision, O.I.K. and S.C.; methodology, O.I.K. and S.C.; data curation, O.I.K. and S.C.; project administration, O.I.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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