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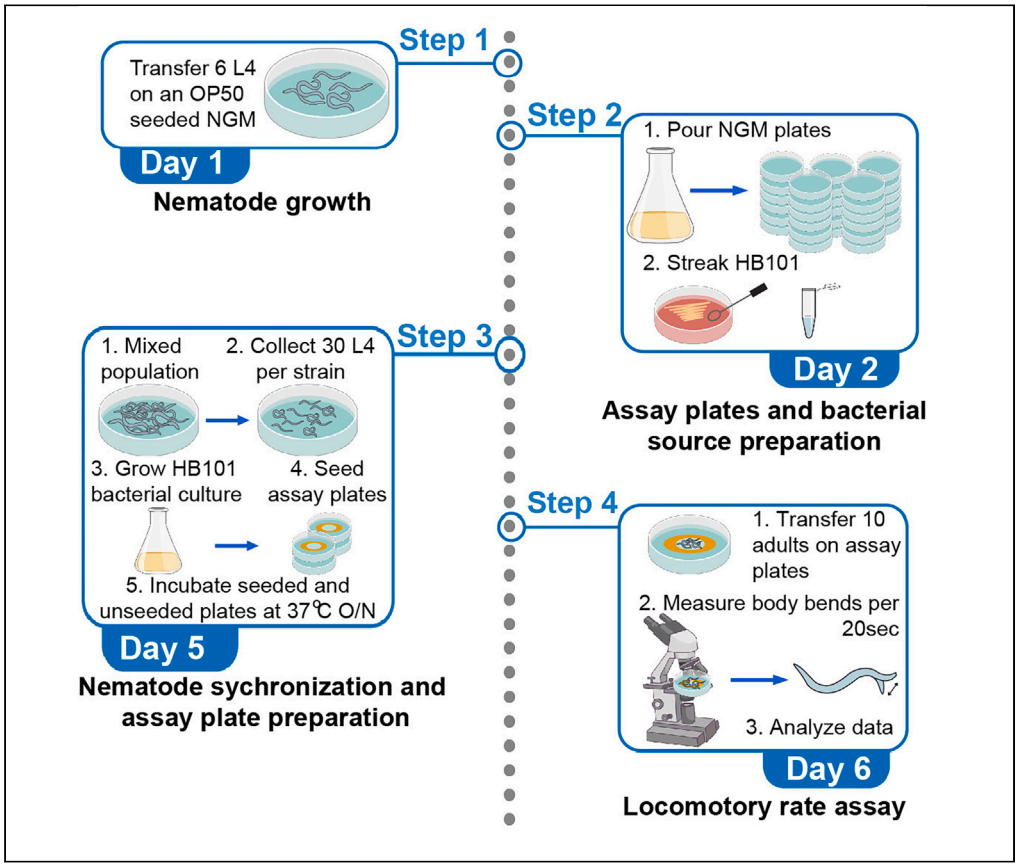
Assessing locomotory rate in response to food for the identification of neuronal and muscular defects in *C. elegans*



C. elegans is a bacteria-eating soil-dwelling nematode. Typical cultivation of laboratory-reared populations occurs on bacteria-covered solid media, where they move along with sinusoidal undulations. Nematodes decelerate when they encounter food. Dopaminergic and serotonergic neurotransmission regulate this behavior. Here, we describe the procedure for determining food-dependent locomotion rate of fed and fasting nematodes. We detail steps for assay plate preparation, *C. elegans* synchronization, and assessment of locomotion. The behaviors we describe provide information regarding the animal's physiological neuronal and muscular function.

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

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Highlights

C. elegans locomotory rate conveys information on neuronal and muscle functionality

Locomotory rate assays to measure animal movement in body bends per time interval

Techniques for assessing basal and enhanced slowing responses

Quantitative analysis of behavioral outputs

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Assessing locomotory rate in response to food for the identification of neuronal and muscular defects in *C. elegans*

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SUMMARY

C. elegans **is a bacteria-eating soil-dwelling nematode. Typical cultivation of laboratory-reared populations occurs on bacteria-covered solid media, where they move along with sinusoidal undulations. Nematodes decelerate when they encounter food. Dopaminergic and serotonergic neurotransmission regulate this behavior. Here, we describe the procedure for determining food-dependent locomotion rate of fed and fasting nematodes. We detail steps for assay plate preparation,** ***C. elegans*** **synchronization, and assessment of locomotion. The behaviors we describe provide information regarding the animal's physiological neuronal and muscular function.**

For complete details on the use and execution of this protocol, please refer to Petratou et al. (2023)[1] and Sawin et al. (2000).[2]

BEFORE YOU BEGIN

C. elegans exhibits a well-defined locomotion pattern of sinusoidal undulations[3,4] when crawling on a solid surface or swimming in a liquid medium. Discrepancies in locomotion wavelength, amplitude, and frequency are symptomatic of abnormalities in certain types of neuronal or muscle cells. In this protocol, we quantitate the locomotory rate of worms as the number of body bends per time unit (20 s). These assays have been previously developed and used in numerous publications.[1,2,5–7] Food presence decelerates the animal and therefore it reduces the number of body bends per time unit, a behavior referred to as basal slowing response (BSR). The dopaminergic system regulates basal slowing response, therefore changes in BSR provide information on dopaminergic neurotransmission. Fasting for 30 min before the experiment induces a pronounced deceleration known as enhanced slowing response (ESR). The serotonergic neurotransmission[2] regulates ESR. Finally, measurements acquired exclusively from seeded plates, provide information on neuronal and muscle functionality and may be utilized for screening tests.[5] In this protocol, we outline the three alternative experimental procedures mentioned above and provide an experimental example employing wild-type Bristol N2 animals and the mutant strains *dop-3(vs106)* and *tph-1(mg280)* as control strains defective in dopaminergic and serotonergic signaling, respectively. Employment of more strains proportionally increases preparation time for each step.

Institutional permissions

Experimentation with the nematode *C. elegans* does not require Institutional Animal Care and Use Committee (IACUC) permission.

C. elegans strains maintenance and synchronization

Timing: 5 days

We follow standard nematode maintenance procedures.[8] Maintain animals on OP50-seeded plates (6 cm) at 20°C.

1. Place 6 hermaphrodites at the L4 stage on an OP50-seeded plate with a flame-sterilized worm picker, consisting of a platinum wire flattened at the tip and attached on a glass Pasteur pipette. Allow the hermaphrodites to grow and lay eggs for four days. Prepare 1 plate for each strain and incubate them at 20°C (troubleshooting 1).
2. On day four, use the worm picker to select L4 stage animals from the mixed population. Transfer ~40 L4 stage hermaphrodite animals from each strain on a new OP50-seeded NGM plate to obtain synchronous animals for the assay. The L4 larvae will develop into Day 1 adults within ~18 h at 20°C, and at this age they will be used in the locomotion assay.

⚠ CRITICAL: Avoid using starved animals since starvation affects the locomotory rate.

Alternatives: Another way to obtain Day one adult animals would be, at step one, to allow ~10 adult hermaphrodite animals to lay eggs on OP50-seeded plates for ~6 h and then remove them with the worm picker. The adults should be on days 1–4 of adulthood, during which period they lay most of their eggs. Incubate them at 20°C for three days until they reach day 1 of adulthood. Another technique for nematode synchronization is bleaching, which is also used for stock decontamination. In this case, a 5% sodium hypochlorite solution (bleaching solution) is used to dissolve the nematode bodies but not their eggshell-protected eggs. For this procedure, apply 2 mL M9 on a plate with mixed population of the desired genotype. Collect approximately 1.5 mL M9 with floating animals from the plate into a sterile 1.5 mL tube. Allow the animals to sink with gravity and remove the supernatant. Add 1.3 mL M9, allow the animals to sink with gravity, or spin them down with a microcentrifuge and remove the supernatant to remove bacteria and wash the animals. Repeat twice the washing step. After the second wash, remove the M9 and add 1.3 mL bleaching solution. Vortex for 6 min for all the animals to dissolve and the eggs to remain. Avoid incubating with bleaching solution for more than 6 min because it reduces the embryo's viability. After bleaching, centrifuge the eggs for 30 sec at full speed (15.000 *g*), remove the supernatant, and wash with 1.3 mL M9. Repeat washing twice more by increasing the centrifugation time by 30 sec each time. Dissolve the eggs in 20–50 μL M9 and spread them on an OP50-seeded NGM plate. Incubate them at 20°C for three days to reach day one of adulthood.[8,9] Using this alternative a big number of animals are obtained and in some cases eggs should be divided to two NGM to avoid starvation.

Preparation of nematode growth medium (NGM) plates

Timing: 2.5 h

This step describes how to prepare NGM plates for nematode maintenance and assay plates, following the standard procedure previously reported.[8,10,11]

3. In a 1 L Erlenmeyer flask, weigh 3 *g* NaCl, 2.5 *g* bacto-peptone, 0.2 *g* streptomycin, and 17 *g* agar.
4. Fill the flask with 500 mL distilled water, add a magnetic stir bar, and cover its mouth with aluminum foil.

Note: Before autoclaving add only 500 mL of water, to avoid overflow of the medium. Afterwards, add sterilized water, as indicated in step 7, up to 1 mL.

5. Autoclave for 30 min (min).
6. Allow to cool at 55°C while stirring.

⚠ CRITICAL: Cool the medium using a water bath (~10 min) or simply air-cooling (~50 min) to 60°C so that you can touch the flask for a while without feeling any discomfort.

7. Aseptically add 1 mL $MgSO_4$ (1 M stock solution), 1 mL $CaCl_2$ (1 M stock solution), 1 mL cholesterol (5 mg/mL stock solution), 1 mL nystatin (10 mg/mL stock solution), 25 mL KPO_4 pH 6 (1 M stock solution), and sterilized distilled water up to 1 L. Mix well by stirring.
8. Dispense the medium into 6 cm petri plates with a peristaltic pump. Pour 7 mL of the medium per plate.
9. Allow it to solidify ~12 h at ~22°C and store at 4°C for up to one month.

⚠ CRITICAL: Store NGM plates upside down to avoid moisture condensation on the lid. Avoid storing NGM for longer because evaporation raises salt concentration. The salt concentration is vital for the animal's physiology and influences a variety of behavioral responses. Use the same batch of NGM plates for each experimental set. Follow sterile procedures while preparing NGM to avoid contamination.

Seeding of plates for nematode maintenance

Timing: 2–3 days

The following steps describe the preparation of OP50 bacterial culture, utilized as a food source for *C. elegans* maintenance.

10. Streak OP50 bacteria on a 9 cm LB plate with a sterile pipette tip and incubate at 37°C for ~12 h.
11. Pick a single colony with a sterile tip and inoculate 50 mL LB. Allow the bacteria to grow by shaking, at 37°C for ~6 h (h), until OP50 OD_{600} = 3 × 10^8 – 1 × 10^9 cfu/mL (OD_{600} 0.9–1.8).
12. Apply 200 μL of OP50 at the center of a 6 cm petri plate. Spread the culture in a circle using the bottom of a fat vial, but not too close to the plate's edges.

⚠ CRITICAL: Avoid growing the bacterial lawn too close to the plate's walls, or the nematodes will crawl out of the medium and dry on the plate wall.

13. Allow the bacterial lawn to grow for ~12 h at 25°C.

⚠ CRITICAL: Maintain aseptic conditions throughout the preparation with a flame.

Note: OP50 is an easily growing bacterial culture. Incubation of 200 μL OP50 on an NGM at 22°C for ~12 h gives nice bacterial lawns for a wide range of OD_{600} of the liquid culture.

Seeding of assay plates

Timing: 2–3 days

The steps below demonstrate how to prepare an HB101 bacterial culture and seed NGM plates for monitoring the locomotory rate (troubleshooting 2).

14. Streak HB101 bacteria on a 9 cm LB plate and incubate it at 37°C for ~12 h.
15. Pick a single colony with a sterile pipette tip and inoculate it in 50 mL liquid LB, shaking, at 37°C for ~7 h, until HB101 OD_{600} = 4 × 10^8 – 6 × 10^8 cfu/mL (OD_{600} 1.3–1.6).

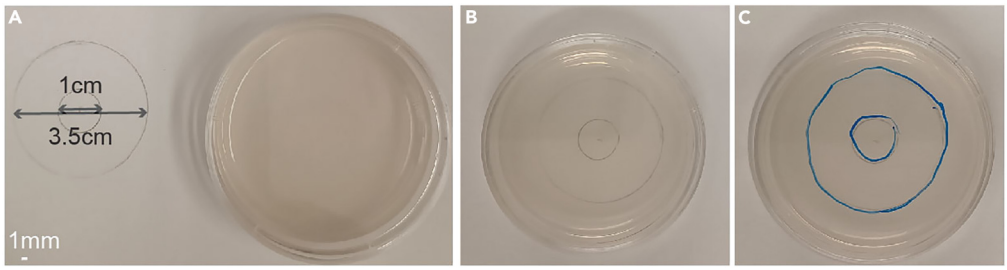

Figure 1. Use a drawn ring as a guide for spreading HB101 bacteria

Draw the ring on a piece of paper (A) and use it as a guide (B) to copy it at the bottom of the assay plates (C). Scale bar, 1 mm.

16. On a paper sheet, draw a ring with an inner diameter of ~1 cm and an outer of ~3.5 cm. Use it as a guide to draw the ring with a marker at the bottom of the assay plates (Figure 1).
17. Spread 100 μL HB101 with the bottom of a glass test tube in a ring-shaped manner, leaving the center free of bacteria.

⚠ CRITICAL: Generate bacterial lawns of similar thickness for all biological replicates, as the thickness of the lawn impacts *C. elegans* locomotory rate. Create thin bacterial lawns to limit deceleration due to mechanical hindrance at its thick peripheral area (Figure 2).

18. Prepare one plate for each strain and incubate them 15–17 h at 37°C along with the same number of unseeded NGM plates, all from the same batch.

⚠ CRITICAL: Always seed the assay plates the day before the assay.

Note: HB101 liquid bacterial culture when spread on an NGM plate does not grow easily to a bacterial lawn, compared to OP50, and requires an increased OD_{600} and incubation ~12 h at 37°C in order to grow a thinner bacterial lawn.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli: OP50 strain	Jonathan Ewbank lab; Caenorhabditis Genetics Center (CGC)	WB-STRAIN: WBStrain00041971; WormBase ID: WBStrain00041971
Escherichia coli: HB101 strain: *E. coli* [supE44 hsdS20(rB-mB-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1]	Caenorhabditis Genetics Center (CGC)	WB-STRAIN: WBStrain00041075; WormBase ID: WBStrain00041075
Chemicals, peptides, and recombinant proteins		
Agar	Sigma-Aldrich	Cat# 05040
Bacto-peptone	BD, Bacto	Cat# 211677
Sodium chloride (NaCl)	EMD Millipore	Cat# 106404
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich	Cat# M7506
Cholesterol	SERVA Electrophoresis	Cat# 17101.01
Calcium chloride dehydrate ($CaCl_2$ * 2 H_2O)	Sigma-Aldrich	Cat# C5090
di-Potassium hydrogen phosphate (K_2HPO_4)	EMD Millipore	Cat# 137010
Potassium dihydrogen phosphate (KH_2PO_4)	EMD Millipore	Cat# 104873
di-Sodium hydrogen phosphate (Na_2HPO_4)	EMD Millipore	Cat# 106586
Streptomycin	Sigma-Aldrich	Cat# S6501

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nystatin	Sigma-Aldrich	Cat# N3503
Yeast extract	Sigma-Aldrich	Cat# Y0875
Tryptone	Sigma-Aldrich	Cat# T7293
Sodium hydroxide (NaOH)	Sigma-Aldrich	Cat# 106498
Sodium hypochlorite (NaOCl)	Sigma-Aldrich	Cat# 1056142500
Ethanol absolute	VWR BDH Chemicals	CAS# 64-17-5
Experimental models: Organisms/strains		
C. elegans: Strain wild type (N2) Bristol isolate	*Caenorhabditis* Genetics Center	WormBase ID: WBStrain00000001
C. elegans: Strain LX703: *dop-3(vs106)*	*Caenorhabditis* Genetics Center	WormBase ID: WBStrain00026374
C. elegans: Strain MT15434: *tph-1(mg280)*	*Caenorhabditis* Genetics Center	WormBase ID: WBstrain00027519
Software and algorithms		
GraphPad Prism 8.0.2	GraphPad Software Inc., San Diego, USA	https://www.graphpad.com/
Microsoft Excel	Microsoft	https://www.microsoft.com/
Other		
Incubators for stable temperature (20°C & 37°C)	BIOBASE	BJPX – B80II
Shaking incubator with stable temperature (37°C)	Sigma-Aldrich	Eppendorf New Brunswick, Excella 24 Inc Shaker, EPM1352-0014
Dissecting stereomicroscope	Nikon	SZM745
Petri dishes (60 *15 mm)	Sigma-Aldrich	Cat# P5237
Petri dishes (92 *16 mm)	Fisher Scientific	Cat# NC9565080
Peristaltic pump	Wheaton Science Products	WHE-374302
1.5 mL microcentrifuge tubes	Sigma-Aldrich	Cat# Z606340

MATERIALS AND EQUIPMENT

NGM medium		
Reagent	Final concentration	Amount
NaCl	50 mM	3 *g*
Bacto-peptone	2.5 mg/mL	2.5 *g*
Streptomycin	0.2 mg/mL	0.2 *g*
Agar	17 mg/mL	17 *g*
$MgSO_4$	1 mM	1 mL
$CaCl_2$	1 mM	1 mL
Cholesterol	5 mg/mL	1 mL
Nystatin	10 mg/mL	1 mL
KPO_4	25 mM	25 mL
ddH_2O	-	Up to 1 L
Total	-	**1 L**

Note: Mix NaCl, bacto-peptone, streptomycin, and agar in 400–500 mL ddH_2O and autoclave. Cool to 55°C–60°C and add sterilized $MgSO_4$ (stock solution 1 M), sterilized $CaCl_2$ (stock solution 1 M), cholesterol (stock solution 5 mg/mL), nystatin (stock solution 10 mg/mL), sterilized KPO_4 (stock solution 1 M) and sterilized ddH_2O up to 1 L. While still hot pour 7 mL per 6 cm petri plate using a sterilized peristaltic pump.

M9 buffer		
Reagent	Final concentration	Amount
KH_2PO_4	3 mg/mL	3 *g*
Na_2HPO_4	6 mg/mL	6 *g*

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
NaCl	5 mg/mL	5 *g*
$MgSO_4$	1 mM	1 mL
ddH_2O	-	Up to 1 L
Total	-	**1 L**

Note: Mix all reagents (apart from $MgSO_4$), add ddH_2O up to 1 L, and autoclave. Then, add $MgSO_4$. Store at 4°C for up to 2 months.

1 M KPO_4 buffer		
Reagent	Final concentration	Amount
K_2HPO_4	0.32 M	57.6 *g*
KH_2PO_4	0.75 M	102.2 *g*
ddH_2O	-	1 L
Total	-	**1 L**

Note: Autoclave and store at 22°C for up to 2 months.

- **1 M $MgSO_4$ stock solution:** add 24.65 *g* $MgSO_4$(*7 H_2O) and ddH_2O up to 100 mL.

  Note: Autoclave and store at 22°C for up to 2 months.

- **1 M $CaCl_2$ stock solution:** add 14.7 *g* $CaCl_2$ (*2H_2O) and ddH_2O up to 100 mL.

  Note: Autoclave and store at 22°C for up to 2 months.

- **10 mg/mL Nystatin stock solution:** add 0.5 *g* Nystatin in 50 mL 70% Ethanol.

  Note: Store at 4°C for up to 2 months.

- **5 mg/mL Cholesterol stock solution:** add 1 *g* cholesterol in 200 mL 100% Ethanol.

  Note: Stir ~12 h to dissolve. Store at 4°C for up to 5 months.

Bleaching solution		
Reagent	Final concentration	Amount
NaOH (5 N)	0.5 N	1 mL
5% sodium hypochlorite (NaOCl) solution	1%	2 mL
ddH_2O	-	7 mL
Total	-	**10 mL**

Note: Always prepare fresh bleaching solution.

LB liquid medium		
Reagent	Final concentration	Amount
NaCl	5 mg/mL	5 *g*
Yeast extract	5 mg/mL	5 *g*

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
Tryptone	10 mg/mL	10 *g*
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: Autoclave, dispense in 50 mL per 250 mL flask, and store at 22°C for up to 3 weeks.

LB agar plates: Prepare the recipe as in LB liquid.

- Add 15 *g* agar to a final concentration of 15 mg/mL.
- Autoclave and air-cool to 55°C–60°C.
- Pour 17 mL medium per 9 cm petri plate with a sterilized peristaltic pump.

Note: Store at 4°C for up to one month.

STEP-BY-STEP METHOD DETAILS

Preparation of materials and animals on the day of the assay

Timing: 40 min

This section describes how to prepare assay plates and the nematode strains before the experiment.

1. Remove the assay plates from the 37°C and incubate them at 22°C for at least 30 min.

   Note: To obtain uniform cooling of all dishes to 22°C, place them on the bench in a single layer and side by side.

   ⚠ CRITICAL: Temperature affects the animal's locomotion. Use plates cooled at 22°C at all times.

2. Label the plates with the designated genotype. Each genotype requires one HB101-seeded and one unseeded NGM plate.
3. Place three 20 µL M9 solution droplets on a petri plate lid.
4. With a hair-pick (eyelash glued on a toothpick), collect 15 one-day adult animals in the first droplet of M9 and allow them to wash for 30 sec.
5. Transfer the animals in the second droplet with the hair-pick and let them settle for 30 sec.

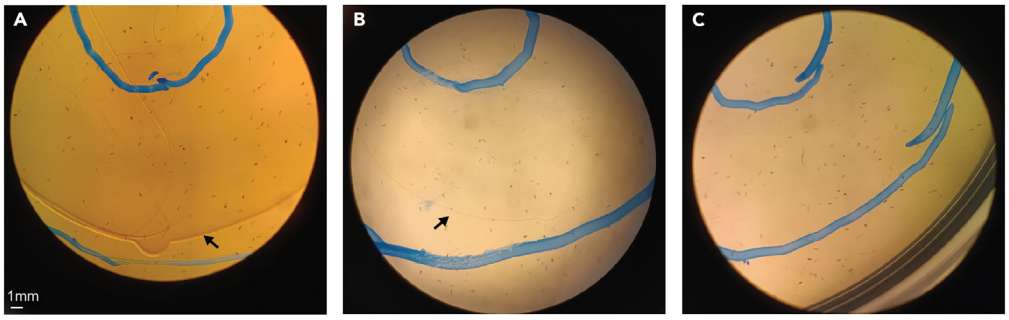

Figure 2. Bacterial lawns of varying thicknesses created by HB101 on assay plates
Generate a thin bacterial lawn on the assay plates as shown in (B) and avoid overly thick (A) or too thin lawns (C). (A, B) Arrows indicate the thick lawn periphery. In (C), the lawn is hard to observe because it is too thin. Scale bar, 1 mm.

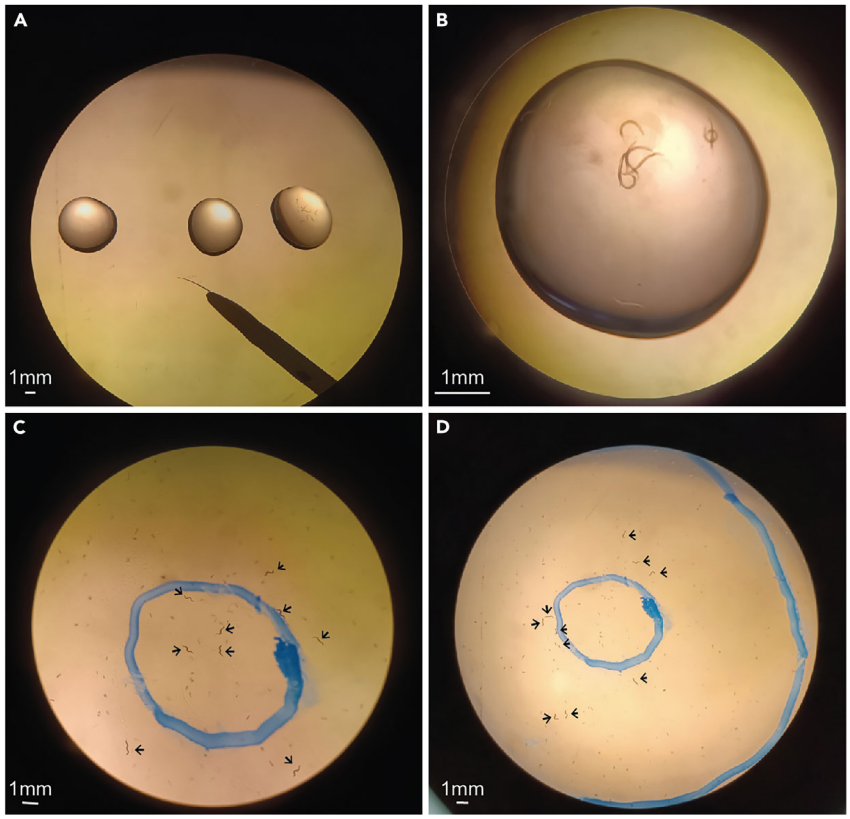

Figure 3. Nematode washing in M9 drops before transferring to the assay plate

Wash nematodes clean from bacteria in three 20 μL M9 drops (A and B) prior to the assay. Following washing, place the animals with the eyelash at the center of the assay plate (C). After 3 min, all nematodes already move onto the bacterial lawn and are ready for counting (D). Arrows point to the nematodes on the assay plate. Scale bars are indicated on each image and correspond to 1mm.

6. Then transfer them to the third droplet with the hair-pick and wash them for ~30 sec until they are bacteria-free.

⚠ CRITICAL: Wash the nematodes thoroughly to remove all bacteria, as they constitute mechanical and gustatory stimuli for *C. elegans* and affect its locomotory rate (Figure 3).

Note: Use the hair-pick preferably for transferring a small number of nematodes to or from liquid drops, because it is thinner, easier to handle and transfers less bacteria with worms than the platinum worm-picker.

Locomotion assay I: Basal slowing response

Timing: 1.5 h

The locomotion rate of ten to fifteen animals, expressed as body bends per 20 s, is measured on HB101 seeded and unseeded NGM plate.

7. Transfer the animals with the hair-pick to the unseeded region at the center of the assay plate (Figure 3).
   a. Start with either the unseeded or the HB101-seeded NGM.

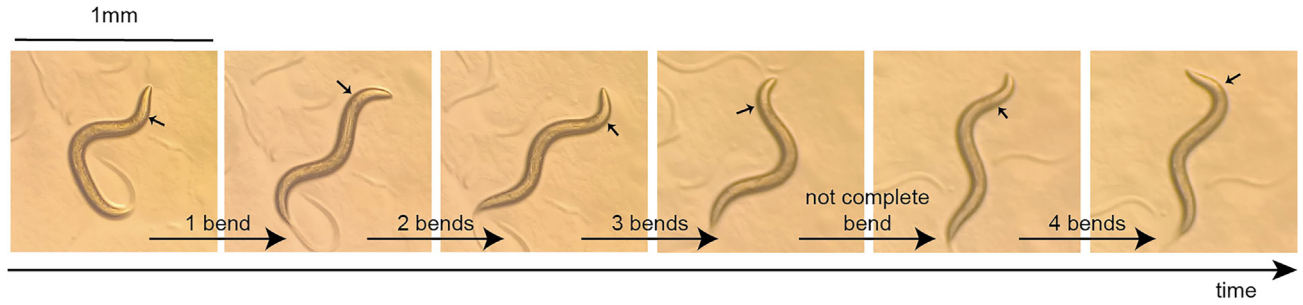

Figure 4. Monitoring nematode's body bends

Sequential illustrations of an animal's movement. The images present an animal performing four body bends through time. Arrows indicate the region behind the pharynx where the body bends complete. Scale bar, 1 mm.

8. Cover the plate with its lid and place it under the stereomicroscope for 3 min to allow the nematodes to recover and adjust.

   Optional: If required, wipe out the moisture from the lid before proceeding.

   Alternatives: Instead of manually transferring nematodes in M9 drops, collect the animals with M9 using a P1000 in a 1.5 mL tube. Allow the animals to settle for ~1 min, remove supernatant, and apply 1 mL clean M9. Repeat this washing step twice and then transfer the worms to the center of the assay plate in ~20–50 µL M9. Remove the excess M9 from the assay plate with a Kimwipe and let the animals recover for 5 min instead of 3 min. In this case, a larger number of animals should be used initially as several of them will be lost during washing steps. Utilizing M9 drops enables the use of fewer animals because they all recover in good shape and none is lost or injured as during pipetting. Furthermore, it takes less time for washing but requires experience in handling the hair-pick.

9. Set a timer for 20 s, monitor the nematodes, and count and record how many times the head of each animal changes direction (body bends) during that period. Measure the body bends per 20 sec for 10 animals (troubleshooting 3 and 4).

   ⚠ CRITICAL: Avoid tapping the plate and keep the lid on during step 9. Mechanical stimuli such as plate tapping or airflow may affect nematodes' motility.

10. Repeat steps 3–9 for the second assay plate (either the seeded or the unseeded).
11. Repeat for each strain with a seeded and an unseeded NGM. The timing indicated applies to two strains. Time increases by 20–30 min for every additional strain.

    ⚠ CRITICAL: Take measurements from different animals and avoid counting the same nematode repeatedly (troubleshooting 3). Record 10–15 measurements from the seeded (troubleshooting 4) and 10–15 measurements from the unseeded plate. Try to preserve the same number of measurements for each strain, as this will facilitate your statistical analysis.

A bend is considered complete when the whole head area, starting from directly behind the pharynx, moves to the opposite direction. Advance one count every time the animal makes a complete bend in the opposite direction (Figure 4). Measure bends during both forward and backward locomotion. When assessing the HB101-seeded plate, do not count animals that dawdle in the thick edge of the bacterial lawn or crawls off food.

Note: Perform the locomotion assay at 20°C–23°C. However, in the case of increased day-to-day temperature fluctuations prefer a 20°C consistent room temperature, to promote reproducibility.

Locomotion assay II: Enhanced slowing response

Timing: 2 h

The locomotion rate of ten to fifteen animals, expressed as body bends per 20 s, is measured on HB101 seeded and unseeded NGM plates after 30 min starvation.

Repeat the same steps as in Locomotion Assay I (Basal slowing response) but with the following modifications.

12. In step 1 (in step-by-step method details), equilibrate at 22°C one more unseeded NGM for each strain.

Note: Equilibration of the unseeded NGM to 22°C is necessary if the NGM plate was not prepared the previous day and was therefore stored at 4°C.

13. In step 2 (in step-by-step method details), label this plate "starvation plate".
14. After step 6 (in step-by-step method details), transfer the animals with the hair-pick to the "starvation plate" and let them starve for 30 min (troubleshooting 5).
15. Proceed with step 7 (in step-by-step method details).

Locomotion assay III: Locomotion rate on a seeded plate

Timing: 2 h

The locomotion rate of ten to fifteen animals, expressed as body bends per 20 s, is measured on HB101-seeded NGM plates.

Repeat the protocol as in Assay I only for the HB101-seeded plate.

Alternatives: In this assay, the OP50 bacterial strain may replace the HB101. However, a thin bacterial lawn remains a prerequisite. Additionally, in step 9, body bends can be recorded for 9 min instead of 20 sec. The 9 min of counting are separated into 3 blocks of 3 min each, with 30 min intervals between them. In this case, divide by 9 the number of body bends counted per animal for the 9 minutes to calculate body bends per minute per animal. Determine the mean body bends per min for each strain by averaging the body bends per min for all 10 nematodes measured for each strain. Calculate the standard deviation for the 10 measurements.

EXPECTED OUTCOMES

In a successful BSR (assay I), we expect wild-type animals to produce 13–17 body bends per 20 sec on the seeded plate (same for assay III), and 18–24 body bends per 20 s on the unseeded plate. In the ESR (assay II), the bends on the unseeded plate remain the same, but they decrease to 3–6 bends on the seeded one.

We demonstrate an experimental example of the basal and the enhanced slowing response assays (Figure 5). The dopaminergic pathway mediates the BSR, whereas the ESR requires the serotonergic pathway. Therefore, we use the *dop-3(vs106)* strain, which is deficient in the D2-like dopamine receptor DOP-3, as control for BSR (Figures 5A and 5B). In the ESR, we utilize the strain *tph-1(mg280)* as a negative

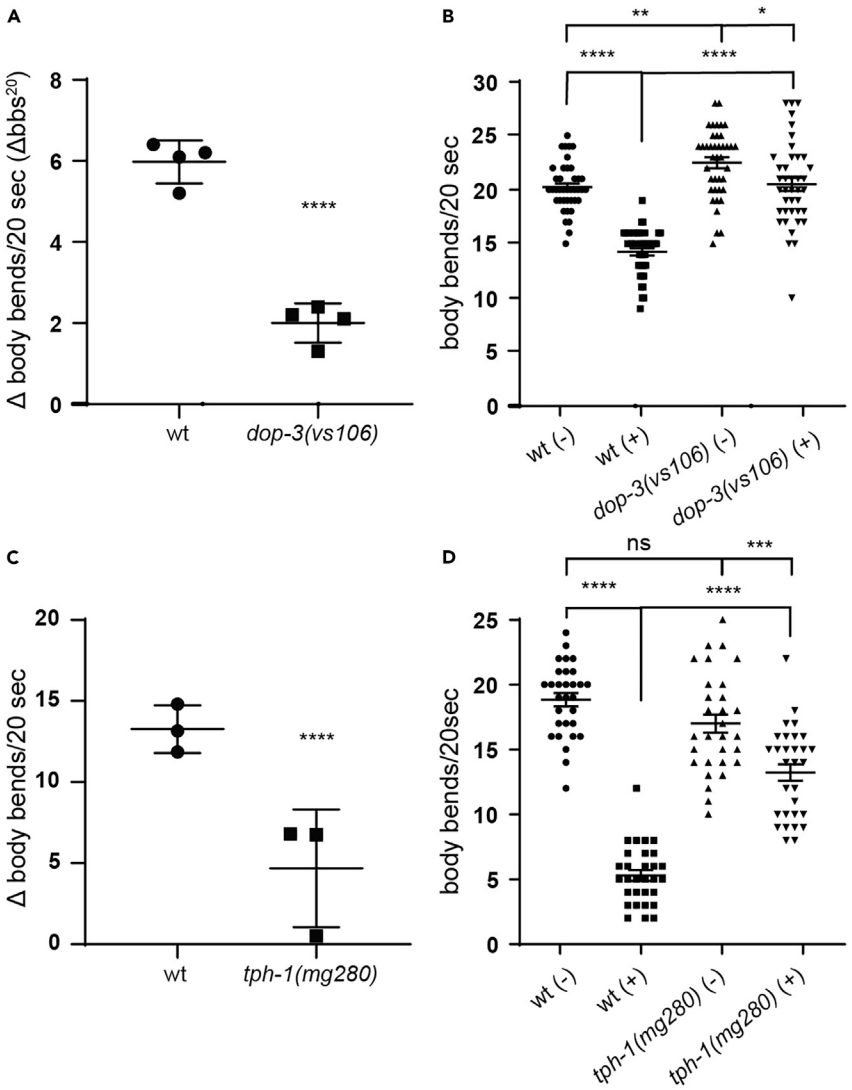

Figure 5. Locomotory rate of control and mutant animals in the basal and enhanced locomotion rate assays

(A and B) Basal slowing response of wt and *dop-3(vs106)* mutant animals. (A) The basal slowing response expressed as Δ body bends per 20 s. The slowing index of the *dop-3(vs106)* mutants is reduced, pointing to faster movement in the presence of food. (B) The BSR expressed as body bends per 20 s. The mutant animals display increased number of body bends per 20 sec on a plate with food, compared to wild type and therefore show a reduced BSR.

(C and D) Enhanced slowing response of wt and *tph-1(mg20)* mutants. (C) The enhanced slowing response expressed as Δ body bends per 20 sec. Mutant *tph-1(mg280)* animals display reduced slowing index compared to wild type. (D) The enhanced slowing response expressed as body bends per 20 sec. The body bends per 20 s of *tph-1(mg280)* mutant animals on a seeded plate are almost the same as on the plate without food. (A, C) Scatter plots, dots represent the ratio Δ body bends/20 sec of independent biological replicates. The ratio Δ body bends/20 sec corresponds to body bends/20 sec on an empty plate minus body bends/20 sec on a plate with food. Error bars represent SD. (B, D) Scatter plots, dots represent the body bends per 20 sec of each animal tested in all biological replicates. Error bars represent SEM. The symbol (-) indicates the absence of HB101 bacterial lawn during the measurement, while the symbol (+) indicates the presence of food. (A-D) We tested 10 animals in every replicate of each BSR experiment. In all experiments, we used one-day-old adult animals. Non-significant (ns) $p = 0.1234$, *$p = 0.0332$, **$p = 0.0021$, ***$p = 0.0002$, ****$p < 0.0001$. Two-way ANOVA. (A, B) wt (-) n = 40, wt (+) n = 40, *dop-3(vs106)* (-) n = 40, *dop-3(vs106)* (+) n = 40, 4 biological replicates. n represents the number of animals. (C and D) wt (-) n = 30, wt (+) n = 30, *tph-1(mg280)* (-) n = 30, *tph-1(mg280)* (+) n = 30, 3 biological replicates. n represents the number of animals.

control, which is a mutant for tryptophan hydroxylase, an enzyme required for serotonin biosynthesis (Figures 5C and 5D). We used the Bristol N2 strain was as wild type. In both cases, the mutant strains demonstrate defective responses compared to control animals. In the BSR wild type animals display a Δ body bends/20 sec (Δbbs^{20}) slowing index (see "quantification and statistical analysis" section) of 5.975 ± 0.5315 (SD), while *dop-3(vs106)* mutants have a slowing index of 2 ± 0.483 (Figure 5A). These index values show that the *dop-3* mutants exhibit a substantially lower slowing response on the seeded plate than the wild type. When we display body bends per 20 sec instead of the slowing index (Figure 5B) we also observe that the *dop-3(vs106)* mutants move faster on the empty plate than the control. Wild type animals perform 20.23 ± 0.3881 (SEM) mean body bends/20 sec on the unseeded plate and 14.25 ± 0.2297 on the seeded, while *dop-3(vs106)* mutants display 22.29 ± 0.3664 on the unseeded plate and 20.50 ± 0.7071 on the seeded. Therefore, *dop-3(vs106)* animals exhibit basal slowing, but to a considerably lesser extent than control animals (Figure 5B).

Concerning the ESR, the N2 strain has a Δbbs^{20} of 13.27 ± 1.478 (SD) and *tph-1(mg280)* has a Δbbs^{20} of 4.683 ± 6.323 (Figure 5C). The mean body bends/20 sec of the wild type on the unseeded plate is 18.83 ± 0.4339 and on the seeded 5.3 ± 0.3387 (SEM), while the mean body bends/20 sec of the *tph-1(mg280)* mutant on the unseeded plate is 17 ± 0.6146 and on the seeded plate is 13.23 ± 0.5922 (Figure 5D). Therefore, we observe that the *tph-1* mutant displays a severely reduced slowing index.

The third alternative is represented by the measurements of the basal slowing response solely on the seeded NGM.

QUANTIFICATION AND STATISTICAL ANALYSIS

This analysis applies to Assays I and II.

From the collected data (10 observations from the seeded and 10 measurements from the unseeded plate for each strain), calculate the slowing index Δ body bends/20 sec (Δbbs^{20}), as analyzed below, and generate a graph. The slowing index Δbbs^{20} corresponds to the difference between the mean body bends/20 s on the unseeded plate minus the mean on the seeded plate.

1. Collect measurements from 10 animals on the seeded and 10 animals on the unseeded plate.
2. Calculate mean body bends per 20 sec on the seeded (m^s) and the unseeded plate (m^u) for each genotype. You may use software like Microsoft Excel for all calculations.
3. Calculate Δ body bends/20 sec (Δbbs^{20}) according to the following equation:

$$\Delta bbs^{20} = m^u - m^s$$

4. Compute the standard deviation of the mean for each set of ten measurements counted on seeded (SDs) and unseeded plates (SDu) for each strain.
5. Determine the standard deviation for Δ body bends/20 sec for each strain (ΔSD^{20}) according to the following equation.

$$\Delta SD^{20} = \sqrt{(SD_s^2 + SD_u^2)}$$

6. Use software like GraphPad Prism to plot Δ body bends per 20 sec (Δbbs^{20}), together with ΔSD^{20} and the number of measurements (10) of all biological replicates in a grouped table (Figure 5).
7. Analyze the data with Two Way ANOVA using Tukey's test to correct for multiple comparisons. Shapiro-Wilk test may be used to assess normality of data distribution.

Alternatives: Instead of Δ body bends, one may plot body bends per 20 sec from seeded and unseeded plates in a column table and graph. However, the number of columns doubles this way, which may be confusing when testing a large number of genotypes (Figure 5). This alternative applies better to the "Locomotion assay – Alternative III" analysis.

LIMITATIONS

The locomotion of *C. elegans*, similar to most of its behaviors, is influenced by its microenvironment, such as ambient temperature, moisture of plates, food abundance as well as by population maintenance and handling conditions, like population density or starvation of previous generations. Therefore, the locomotion rate assessment assay is sensitive and challenging and necessitates strict quality control. To eliminate data variation always maintain all tested animals under ad libitum conditions for at least three generations. Use fresh NGM plates from the same batch, seeded together with enough amount of OP50 or HB101 bacterial food source. The incubation temperature and moisture should be kept constant avoiding temperature fluctuations. This assay does not apply to animals with the roller phenotype.

TROUBLESHOOTING

Problem 1

Starvation of the animals before the locomotion rate assay (related to step 1, "before you begin" section).

Potential solution

Ensure to use fresh OP50-seeded plates. Apply 200–250 μL freshly prepared OP50 to seed NGM. At the first step of synchronization, transfer no more than 5–6 L4 animals.

Problem 2

HB101 forms a too-thin or too-thick bacterial lawn on the assay plate (related to step 17, "before you begin" section).

Potential solution

Always prepare new HB101 O/D culture coming from a fresh streaking. At this step, it is important to determine the point at which the HB101 culture density yields a thin bacterial lawn.

Problem 3

The body bends/ 20 sec of some animals are measured more than once (Related to step 9, "step-by-step method details" section).

Potential solution

Following data recording from every animal, one may pick the nematode with the worm-picker and burn it. However, this means that, every time a measurement from an animal is obtained, the lid of the plate should be lift and then placed again on the plate following the burning of the animal. During this process, the nematodes are exposed to mechanical stimuli, emerging from moving or accidentally tapping the plate and by airflow (from breathing or air-conditioning). This procedure might increase nematodes' mobility. Working with fewer animals (e.g., 5) would make it easier to monitor them.

Problem 4

During body bend measuring many animals stack and stay almost immobile at the dense periphery of the bacterial lawn (Related to step 9, "step-by-step method details" section).

Potential solution

This is a usual behavior of *C. elegans* that cannot be avoided. To limit this phenomenon, make sure to generate a thin bacterial lawn. Additionally, ensure that the recovery time of the 3–5 min is applied. A possible solution is to use more animals so that there would be enough animals to count

even though there are also many immobile animals. Another alternative would be to wait for some animals until they start moving again.

Problem 5

Some animals are censored during the 30 min starvation due to desiccation on the plate walls (related to step 14, "step-by-step method details" section).

Potential solution

Typically, 15–20 animals are sufficient to ensure that 10 will survive for the experiment. However, you may increase them to 30–40 to avoid having very few nematodes at the end of the starvation step. Alternatively, you may apply 4 M fructose to the plate's periphery. Administer a few drops on the plate's edge and rotate it to create a fructose ring on its perimeter. Add a dye, like Congo Red or Bromophenol Blue, to visualize the ring. Wild-type well-fed animals avoid this osmotic barrier and remain inside the plate, while it does not affect their mobility.[12]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nektarios Tavernarakis (tavernarakis@imbb.forth.gr).

Technical contact

Further information and details about technical specifics concerning the protocol should be directed to and fulfilled by the technical contact, Dionysia Petratou (dipetratou@imbb.forth.gr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any dataset or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, writing – original draft, and visualization, D.P. and N.T.; writing – review and editing, D.P., E.L., and N.T.; investigation, data curation, and visualization, D.P., E.L., and P.F.; methodology, validation, and formal analysis, D.P.; resources, supervision, project administration, and funding acquisition, N.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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