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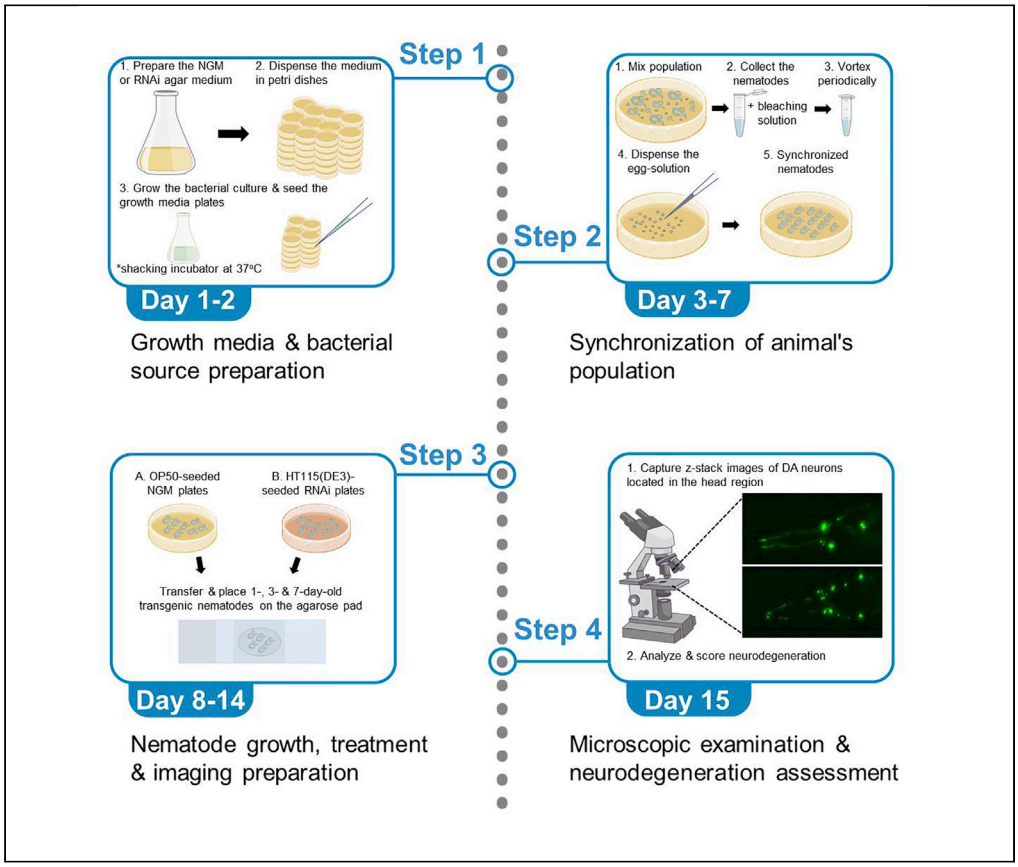
Protocol

Assessment of dopaminergic neuron degeneration in a *C. elegans* model of Parkinson's disease



Transgenic *Caenorhabditis elegans* that expresses the full-length wild-type human α-synuclein in dopaminergic neurons provides a well-established Parkinson's disease (PD) nematode model. Here, we present a detailed protocol to monitor and dissect the molecular underpinnings of age-associated neurodegeneration using this PD nematode model. This protocol includes preparation of nematode growth media and bacterial food sources, as well as procedures for nematode growth, synchronization, and treatment. We then describe procedures to assess dopaminergic neuronal death *in vivo* using fluorescence imaging.

Konstantinos Palikaras, Tanima SenGupta, Hilde Nilsen, Nektarios Tavernarakis

palikarask@med.uoa.gr (K.P.)
hilde.nilsen@medisin.uio.no (H.N.)
tavernarakis@imbb.forth.gr (N.T.)

Highlights

A Parkinson's disease nematode model to study α-synuclein-mediated neurotoxicity

Comprehensive approach for scoring cell death of dopaminergic neurons in *C. elegans*

Genetic tools to investigate the tissue specific effects on neurodegeneration

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Assessment of dopaminergic neuron degeneration in a *C. elegans* model of Parkinson's disease

Konstantinos Palikaras,[1,6,*] Tanima SenGupta,[2,3,6] Hilde Nilsen,[2,3,*] and Nektarios Tavernarakis[4,5,7,*]

[1]Department of Physiology, School of Medicine, National and Kapodistrian University of Athens, 11527 Athens, Greece

[2]Department of Clinical Molecular Biology, University of Oslo, Oslo, Norway

[3]Department of Clinical Molecular Biology, Akershus University Hospital, Lørenskog, Norway

[4]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Heraklion, 71300 Crete, Greece

[5]Department of Basic Sciences, Faculty of Medicine, University of Crete, Heraklion, 71300 Crete, Greece

[6]Technical contact

[7]Lead contact

*Correspondence: palikarask@med.uoa.gr (K.P.), hilde.nilsen@medisin.uio.no (H.N.), tavernarakis@imbb.forth.gr (N.T.)
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SUMMARY

Transgenic *Caenorhabditis elegans* that expresses the full-length wild-type human α-synuclein in dopaminergic neurons provides a well-established Parkinson's disease (PD) nematode model. Here, we present a detailed protocol to monitor and dissect the molecular underpinnings of age-associated neurodegeneration using this PD nematode model. This protocol includes preparation of nematode growth media and bacterial food sources, as well as procedures for nematode growth, synchronization, and treatment. We then describe procedures to assess dopaminergic neuronal death *in vivo* using fluorescence imaging. For complete details on the use and execution of this protocol, please refer to SenGupta et al. (2021).

BEFORE YOU BEGIN

C. elegans strains and culture conditions

We followed standard procedures for nematode maintenance (Stiernagle, 2006). *C. elegans* strains were grown on nematode growth medium (NGM) plates seeded with the non-pathogenic *Escherichia coli* OP50-1 and HT115(DE3) bacteria. Animals were cultured at 20°C. The following strains were used to evaluate dopaminergic neuronal loss: BY273: *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$α-synuclein^{wt}], RB877: *nth-1(ok724)III*, IR2355: *nth-1(ok724)III*; *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$α-synuclein^{wt}]. To investigate the tissue specific effects on neurodegeneration, we used the following transgenic animals: dopaminergic neuron-specific RNAi UA196: *sid-1(pk3321); baIn11*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$α-synuclein^{wt}]; *baIn33*[$p_{dat\text{-}1}$SID-1; $p_{myo\text{-}2}$mCherry], pan-neuronal RNAi IR2531: *sid-1(pk3321)V*; *uIs69*[$p_{unc\text{-}119}$SID-1; $p_{myo\text{-}2}$mCherry]V; *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$α-synuclein^{wt}], hypodermis-specific RNAi IR2945: *rde-1(ne219)V*; *kzIs9*[$p_{lin\text{-}26}$RDE-1; $p_{lin\text{-}26}$NLS::GFP; *rol-6(su1006)*]; *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$α-synuclein^{wt}], intestine-specific RNAi IR2947: *rde-1(ne219)V*; *kbIs7*[$p_{nhx\text{-}2}$RDE-1; *rol-6(su1006)*]; *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$α-synuclein^{wt}].

Preparation of NGM plates

Timing: 2–3 days

1. Weigh and mix 3 g of NaCl, 2.5 g bacto-peptone, 0.2 g streptomycin and 17 g agar in a 1 L glass bottle.

Alternatives: To avoid any heat-inactivation of streptomycin, it can be added after autoclaving, when the media is cooled down. A conical flask can be used instead of the bottle.

Note: The streptomycin-resistant OP50-1 *E. coli* strain is used. For the preparation of the RNAi agar plates do not add streptomycin. The HT115(DE3) *E. coli* stain is not resistant to streptomycin.

2. Add 900 mL distilled water.
3. Place a magnetic stir bar into the bottle and close its cap. Autoclave for 30 min.
4. Place the autoclaved bottle on the stirrer and air-cool it to 55°C–60°C.
5. Add 1 mL $MgSO_4$ (1 M stock solution), 1 mL cholesterol (5 mg/mL stock solution), 1 mL $CaCl_2$ (1 M stock solution), 1 mL nystatin (10 mg/mL stock solution), 25 mL KPO_4 (1 M stock solution).

Note: Nystatin is an anti-fungal agent. Add 500 μL ampicillin (10 mg/mL stock solution) in the medium for RNAi agar plates.

6. Add distilled sterile water up to 1 L.
7. Use a peristaltic pump and dispense the NGM medium into petri dishes. Add 10 mL of NGM medium per petri dish (60 × 15 mm diameter).

Alternatives: A liquid pipette can be used instead of a peristaltic pump.

8. Leave the NGM plates to solidify.
9. Place the NGM plates at room temperature (22°C–25°C) for a day before use.
10. Upside-down the NGM plates to avoid moisture condensation on the lid and store them for up to 3 weeks at 4°C.

⚠ CRITICAL: Longer storage of NGM plates could affect the salt concentration due to excessive moisture evaporation. Therefore, the same batch of NGM plates should be used for the entire set of experiments.

Bacterial food source

Timing: 2–3 days

OP50-seeded NGM plates

11. Streak *E. coli* OP50 bacteria onto LB agar plate.
12. Incubate the plates at 37°C overnight (~18 h).
13. Pick a single OP50 bacterial colony from an LB agar plate by using a sterilized toothpick (or inoculation needle) and place it in a flask containing 50 mL LB medium and incubate it in a shaking incubator at 37°C for 8 h.
14. Place 200 μL of OP50 culture on NGM plates.
15. Swirl and let the plates dry at room temperature overnight.

HT115(DE3)-seeded RNAi agar plates

16. Streak HT115(DE3) *E. coli* strains expressing the empty vector pL4440 (control) and the pL4440 containing the sequence of *nth-1* gene (*nth-1*) onto LB agar plates containing 100 μg/mL ampicillin and 10 μg/mL tetracycline.

Note: HT115(D3) *E. coli* strain is tetracycline resistant due to *rnc14::Tn10* allele. The RNAi vector pL4440 confers resistance to ampicillin. Thus, RNAi agar plates should contain both ampicillin and tetracycline.

17. Incubate the plates at 37°C overnight (~18 h).

18. Pick a bacterial colony of HT115(DE3) bacteria from each condition (control and *nth-1*) and place them in separate bacteriological culture tubes containing 5 mL LB medium, 5 μL ampicillin (stock solution 100 mg/mL) and 5 μL tetracycline (stock solution 10 mg/mL).
19. Incubate the tubes in a shaking incubator at 37°C overnight (~18 h).
20. Prepare different bacteriological tubes for each condition, and add 5 mL LB medium and 5 μL ampicillin (stock solution 100 mg/mL).
21. Add 350 μL (70 μL per 1 mL LB/ampicillin) of each overnight culture into separate bacteriological culture tubes containing 5 mL LB medium and 5 μL ampicillin (stock solution 100 mg/mL).

Note: Nematodes physiology is affected by high-dose of tetracycline (Vangheel et al., 2014). Thus, tetracycline concentration should be reduced during the preparation of RNAi bacterial cultures.

22. Incubate the cultures in a shaking incubator at 37°C for 4 h until OD_{600} will be 0.5–0.8.
23. Add 1–2 mM IPTG (20 mM stock solution) in each culture and proceed directly with plates seeding.

Alternatives: IPTG can be added in the medium of RNAi agar plate after autoclaving. Fresh IPTG-containing RNAi plates should be prepared every 2 weeks because IPTG efficiency declines over time.

24. Place 200 μL of each culture (control and *nth-1*) on RNAi agar plates.
25. Swirl and let the plates dry at room temperature overnight.

Synchronizing *C. elegans* populations

Timing: 8 days

26. Transfer 10 L4 nematode larvae of each strain in separate OP50-seeded NGM plates. Prepare two plates per genotype/condition.
27. Incubate and let the nematodes to develop and grow at 20°C.
28. After 4–5 days, the plates contain mixed population with the presence of plenty gravid adult worms. Wash the plates with 2 mL M9 buffer and collect the animals in sterile 1.5 mL tubes.
29. Let the animals to settle down by gravity for 1 min and remove the liquid.
30. Add 500 μL freshly made bleaching solution and mix the samples.
31. Vortex the solution for 20 s. Repeat vortexing every minute until the worms are completely dissolved.

⚠ CRITICAL: Do not keep the nematodes for more than 5 min in the bleaching solution, as it might affect viability of embryos.

32. Spin down the samples for 30 s at 2,000 g using a table-top centrifuge.

Alternatives: Centrifuge the samples for 1 min at 180 g.

33. Discard the supernatant and keep the pellet.

⚠ CRITICAL: Remove carefully the supernatant without disturbing the egg-pellet.

34. Wash the egg-pellet with 1 mL of sterile M9 buffer.
35. Spin down the samples for 30 s at 2,000 g using a table-top centrifuge.

Alternatives: Centrifuge the samples for 1 min at 180 g.

36. Discard the supernatant and keep the pellet.
37. Repeat twice steps 34–36.
38. Add 200 μL of sterile M9 buffer and solubilize the pellet.
39. Dispense the egg solution to OP50-seeded NGM plates.

Note: If the gene of interest has not been knocked down using RNAi before, examine whether its knockdown could affect animals' development to avoid any severe developmental arrest prior to the experiments. Then, eggs can be placed on HT115(DE3)-seeded RNAi agar plates enhancing the silencing of the gene of interest.

40. Incubate the plates at 20°C.
41. After 3 days, the plates are full of L4 stage nematodes.

Note: Several mutations or RNAi treatments might interfere with normal *C. elegans* development. Thus, any developmental delay or arrest should be taken into consideration for nematodes synchronization prior to any experiment, when animals of different genetic backgrounds are used for neurodegeneration assessment.

Preparation of *C. elegans* strains

Timing: 1–7 days

42. Transfer 20–30 L4 larvae per OP50-seeded NGM plate or HT115(DE3)-seeded RNAi agar plate.
43. Incubate the plates at 20°C.
44. Transfer the nematodes to freshly seeded NGM or RNAi agar plates every two days and incubate them at 20°C.
45. After the respective days, use 1, 3 and 7-day-old transgenic nematodes for microscopic examination and monitor dopaminergic neuron survival.

Alternatives: To avoid worm picking use a 40 μm cell-strainer to separate adults from L1-L2 larvae. Wash the plate with M9 buffer and pass the solution through a 40 μm cell-strainer. L1-L2 larvae will pass through the filter, and adults will remain. Turn the cell-strainer to opposite direction and wash the remained adults with 500 μL of sterile M9 buffer. Carefully collect the adults directly in a freshly seeded NGM plate. This approach has to be done daily; otherwise, the progeny will grow and will be retained by the cell-strainer alongside the adults.

Note: Use at least three separate plates containing transgenic worms for each experimental condition.

⚠ CRITICAL: Non-starved and well-fed animals should be used. Avoid overcrowding that could lead to starvation, which influences organism physiology.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
OP50 *E. coli*	Caenorhabditis Genetics Center	OP50-1
HT115(DE3) *E. coli*	Caenorhabditis Genetics Center	HT115(DE3)
pL4440 in HT115(D3) *E. coli*	Tavernarakis lab	Tavernarakis lab #6
nth-1 in pL4440 in HT115(DE3) *E. coli*	Tavernarakis lab	Tavernarakis lab #2328

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
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 ($CaCl_2$ $2H_2O$)	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
Ethanol absolute	Sigma-Aldrich	Cat# 1070174000
Streptomycin	Sigma-Aldrich	Cat# S6501
Nystatin	Sigma-Aldrich	Cat# N3503
Tetracycline hydrochloride	PanReac AppliChem	Cat# A2228. 0025
Ampicillin sodium salt	PanReac AppliChem	Cat# A0839.0100
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	Cat# I5502
Yeast extract	Sigma-Aldrich	Cat# Y0875
Tryptone	Sigma-Aldrich	Cat# T7293
AgaPure™ Agarose	Canvax	Cat# AG006
Sodium hypochlorite (NaOCl) solution	EMD Millipore	Cat# 105614
Levamisole hydrochloride	Sigma-Aldrich	Cat# PHR1798
Experimental models: Organisms/strains		
C. elegans: *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$a-synuclein^{wt}]	R. Blakely Lab	BY273
C. elegans: N2; *Is*[$p_{dat\text{-}1}$GFP]	Tavernarakis / Nilsen lab	IR2514
C. elegans: *nth-1(ok724)III*	Caenorhabditis Genetics Center	RB877
C. elegans: *nth-1(ok724)III*; *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$a-synuclein^{wt}]	Tavernarakis / Nilsen lab	IR2355
C. elegans: *sid-1(pk3321)*; *baln11*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$a-synuclein^{wt}]; *baln33*[$p_{dat\text{-}1}$SID-1; $p_{myo\text{-}2}$mCherry]	Caldwell Lab	UA196
C. elegans: *sid-1(pk3321)V*; *uIs69*[$p_{unc\text{-}119}$SID-1; $p_{myo\text{-}2}$mCherry]V; *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$a-synuclein^{wt}]	Tavernarakis lab	IR2531
C. elegans: *rde-1(ne219)V*; *kzIs9*[$p_{lin\text{-}26}$RDE-1; $p_{lin\text{-}26}$NLS::GFP; *rol-6(su1006)*]; *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$a-synuclein^{wt}]	Tavernarakis lab	IR2945
C. elegans: *rde-1(ne219)V*; *kbIs7*[$p_{nhx\text{-}2}$RDE-1; *rol-6(su1006)*]; *Is*[$p_{dat\text{-}1}$GFP; $p_{dat\text{-}1}$a-synuclein^{wt}]	Tavernarakis lab	IR2947
Recombinant DNA		
pL4440 (control or empty vector)	Fire lab	Addgene Plasmid #1654
nth-1 in pL4440	Tavernarakis lab	Tavernarakis lab #2328
Software and algorithms		
Zen	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen-lite.html
EVOS FL AUTO 2 software	Thermo Fisher Scientific	https://www.thermofisher.com/gr/en/home/technical-resources/software-downloads/evos-fl-auto2-imaging-system-software-download.html
GraphPad Prism software package	GraphPad Software Inc., San Diego, USA	https://www.graphpad.com/scientific-software/prism/
Other		
Incubators for stable temperature (20 & 37°C)	BIOBASE	BJPX – B80II
Nikon dissecting stereomicroscope	Nikon	SZM645
Zeiss epifluorescence stereomicroscope	Zeiss	Zeiss SteReo Lumar V12
EVOS cell imaging systems	Thermo Fisher scientific	EVOS FL Auto 2
Zeiss confocal microscope	Zeiss	Zeiss LSM 710
Microscope slides 75 × 25 × 1	Marienfeld-Superior	Cat# 1000612
Microscope cover glass 18 × 18	Marienfeld-Superior	Cat# 0101030
Petri plates, 60 × 15 mm	Sigma-Aldrich	Cat# P5481

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Petri plates, 92 × 16 mm	Sigma-Aldrich	Cat# P5481
Cell Strainer 40 μm,	pluriSelect	43-57040-50

MATERIALS AND EQUIPMENT

NGM medium		
Reagent	Amount	Final concentration
NaCl	3 g	50 mM
Bacto-peptone	2.5 g	2.5 mg/mL
streptomycin	0.2 g	0.2 mg/mL
Agar	17 g	17 mg/mL
ddH_2O	900 mL	–
Total	[a]**Add up to 1 L**	–

[a]Autoclave 900 mL NGM medium and cool it to 55°C–60°C, and add 1 mL $MgSO_4$ (1 M stock solution; final concentration: 1 mM), 1 mL cholesterol (5 mg/mL stock solution; final concentration: 5 μg/mL), 1 mL 1 mL $CaCl_2$ (1 M stock solution; final concentration: 1 mM), 1 mL nystatin (10 mg/mL stock solution; final concentration: 10 μg/mL), 25 mL KPO_4 (1 M stock solution; final concentration: 25 mM). Fill with sterilized ddH_2O up to 1 L.

M9 buffer		
Reagent	Amount	Final concentration
KH_2PO_4	3 g	3 mg/mL
Na_2HPO_4	6 g	6 mg/mL
NaCl	5 g	5 mg/mL
ddH_2O	up to 1 L	–
Total	**1 L**	–

Note: Autoclave M9 buffer, and add 1 mL $MgSO_4$ (1 M stock solution) to 1 L M9 buffer so that the final concentration of $MgSO_4$ is 1 mM. Store M9 buffer for up to 2 months at 4°C.

1 M KPO_4 buffer		
Reagent	Amount	Final concentration
KH_2PO_4	102.2 g	0.75 M
K_2HPO_4	57.06 g	0.32 M
ddH_2O	1 L	–
Total	**1 L**	–

Note: Autoclave and store KPO_4 buffer (pH:6) at room temperature (RT). Store KPO_4 buffer for up to 2 months at RT.

Bleaching solution		
Reagent	Amount	Final concentration
NaOH (5N)	1 mL	0.5 N
5% Sodium hypochlorite (NaOCl) solution	2 mL	25%
ddH_2O	7 mL	–
Total	**10 mL**	–

Store bleaching solution for a week at room temperature.

Nystatin stock solution		
Reagent	Final concentration	Amount
Nystatin	10 mg/mL	0.5 g
Ethanol	70%	35 mL
ddH_2O	N/A	15 mL
Total	**N/A**	**50 mL**
Store nystatin stock solution for up to 5 months at 4°C.		

Ampicillin stock solution		
Reagent	Final concentration	Amount
Ampicillin sodium salt	10 mg/mL	1 g
ddH_2O	N/A	10 mL
Total	**N/A**	**10 mL**
Store ampicillin stock solution for up to 6 months at −20°C		

Tetracycline stock solution		
Reagent	Final concentration	Amount
Tetracycline hydrochloride	10 mg/mL	0.5 g
Ethanol	70%	35 mL
ddH_2O	N/A	15 mL
Total	**N/A**	**50 mL**
Store tetracycline stock solution for up to 3 months at −20°C.		

Levamisole solution		
Reagent	Final concentration	Amount
Levamisole hydrochloride	0.5 M	1.2 g
ddH_2O	N/A	10 mL
Total	**N/A**	**10 mL**
Store levamisole stock solution for up to 5 months at 4°C.		

M9/levamisole solution		
Reagent	Final concentration	Amount
Levamisole (0.5 M)	20 mM	400 µL
M9 buffer	N/A	15 mL
Total	**N/A**	**15 mL**
Store M9/levamisole stock solution for up to 2 weeks at 4°C.		

LB liquid medium		
Reagent	Amount	Final concentration
NaCl	5 g	5 mg/mL
Yeast extract	5 g	5 mg/mL
Tryptone	10 g	10 mg/mL
ddH_2O	up to 1 L	–
Total	**1 L**	–
Autoclave and store the LB medium for up to 3 weeks at room temperature.		

LB agar plates		
Reagent	Amount	Final concentration
NaCl	5 g	5 mg/mL
Yeast extract	5 g	5 mg/mL
Tryptone	10 g	10 mg/mL
Agar	15 g	15 mg/mL
ddH_2O	up to 1 L	–
Total	**1 L**	–

Autoclave the LB agar medium. Air-cool the medium to 55°C–60°C. Pour 18 mL LB agar medium per petri dish (92 × 16 mm). Store the LB agar plates for up to 3 weeks at 4°C.

Note: Prepare 100 μg/mL ampicillin and/or 10 μg/mL tetracycline LB agar plates: Add 170 μL ampicillin (100 mg/mL stock solution) and/or 17 μL tetracycline (10 mg/mL stock solution) on LB agar plates and spread the plates by using a sterilized glass spreader.

STEP-BY-STEP METHOD DETAILS

Agarose pad preparation

Timing: 15 min

This step describes how to prepare 2% agarose pads combined with M9/levamisole buffer that will be used for mounting the nematodes. Several methods (e.g., agarose pads, polystyrene nanoparticles, microfluidic chips and anesthetics) have been developed and utilized to immobilize and mount nematodes for long- or short-term imaging (Dong et al., 2018; Kim et al., 2013; Mondal et al., 2016; Mondal and Koushika, 2014). The use of agarose pads is the most common methods because it is a simple, low-cost and versatile technique.

1. Weigh 0.5 g of agarose in a 50 mL glass beaker.
2. Add 25 mL of M9 buffer.
3. Place and heat the mixture in a microwave until the agarose will be dissolved.
4. Stir the mixture periodically and keep it warm on a heating plate.
5. Place an empty microscope slide on the bench.
6. Add a drop of 30 μL of agarose solution (2% final concentration) in the middle of the slide.
7. Take a second microscope slide and place it on the top of the agarose drop and press down gently to flatten it.
8. After 30 s remove carefully the top microscope slide.

Note: Several agarose pads can be prepared (Ramachandran et al., 2015; Rieckher and Tavernarakis, 2017; Walston and Hardin, 2010; Wang et al., 2021). Leave the top microscope slide as a cover to eliminate the evaporation and preserve the agarose pads humidity longer (~ 30 min).

9. Proceed with the sample preparation.

Mounting nematodes

Timing: 5–10 min

This step describes the mounting process of transgenic nematodes on the agarose pads before the image acquisition.

10. Add 10 μL 20 mM M9/levamisole buffer on the agarose pad.

Note: Levamisole is an agonist of cholinergic receptors and influences directly neuronal function and physiology (Culetto et al., 2004; Fleming et al., 1997; Kim et al., 2001; Podbilewicz

and Gruenbaum, 2006). Thus, lower concentration of levamisole (e.g., 1 or 5 mM) can be used, especially for experiments that require extended periods of live-cell imaging.

11. Use an eyelash, which is glued on a toothpick or platinum wire worm picker, to pick and transfer the transgenic nematodes into the M9/levamisole droplet. Transfer 15–20 animals per drop.

Note: Transfer carefully the transgenic animals one by one into the droplet to avoid nematodes injury or even death. Old nematodes are more sensitive to mechanical forces.

Alternatives: A worm pick can be used instead of eyelash to transfer the transgenic animals into the droplet.

12. Place gently a coverslip on the top of the transgenic nematodes. Troubleshooting 1.
13. Use nail polish and seal the coverslip on the agarose pad.

⚠ CRITICAL: Sealing of the coverslips is required for the maintenance of humidity during the imaging process. Sealing is highly recommended for long-term imaging experiments.

14. Proceed to microscopic examination of the specimens.

Image acquisition

Timing: 1–2 h

15. Use an epifluorescence (e.g., EVOS FL AUTO 2) or a confocal (e.g., Zeiss LSM 710) microscope combined with a camera.
16. Place the prepared slides under the microscope.
17. Properly focus and detect single transgenic nematodes co-expressing green fluorescent protein (GFP) and α-syn in dopaminergic neurons.

Note: The hermaphroditic nematode *C. elegans* has 8 dopaminergic neurons, 6 (CEPs and ADEs) in the head region and 2 (PDEs) in the middle body (Figure 1A). The male nematodes display additional 6 dopaminergic neuronal cells, which are located in the tail (Sulston et al., 1975).

18. Capture z-stack images of the head and the middle body region by using 20× objective lens. Troubleshooting 2.

Note: EVOS FL AUTO 2 software and Zeiss ZEN (black edition) were used for image acquisition.

⚠ CRITICAL: Use the same imaging settings (e.g., lens, magnifiers, filter exposure time, laser intensity, gain etc.) during the entire microscopic examination for all the condition that will be used for analysis.

19. Save the acquired images.
20. Process the acquired z-stack images with the Zeiss ZEN software (black version), to obtain the maximum intensity projection (Figure 2).
21. Proceed to the analysis of the acquired images.

Scoring degeneration of dopaminergic neurons

Timing: 1 h

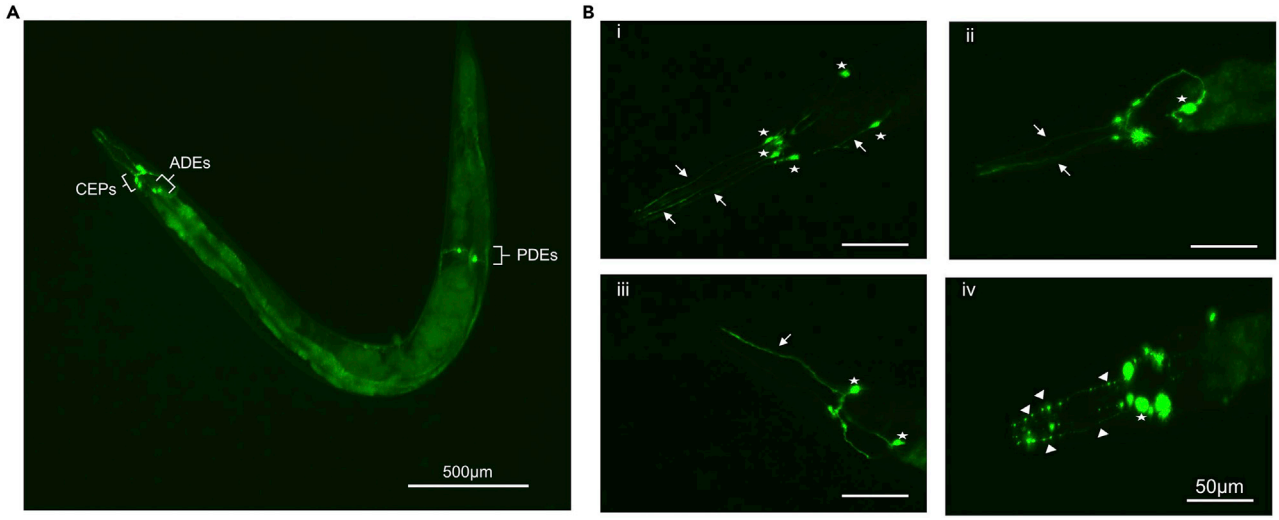

Figure 1. Dopaminergic neuronal circuit in *C. elegans*

(A) Dopaminergic neuronal circuit consists of 8 neurons the *C. elegans* hermaphrodite. Transgenic nematodes expression cytosolic GFP under the *dat-1* promoter display two pairs of CEPs and a pair of ADE neurons in the anterior part and a pair of PDE neurons in the posterior part of the nematode body. (B) CEPs and ADEs neurons form a well-structure network in the head region (i). Dopaminergic neuronal circuit is gradually deteriorated with age in transgenic animals expressing α-synuclein. CEPs and ADEs neurons present dendritic or outgrowths loss (ii), entire loss of their cell bodies (iii), axonal and some blebbing (iv). Remnants of neuronal cell bodies (asterisks), intact neuronal processes (arrows) and axonal beading (arrowheads) are depicted. Scale bars, 500 μm and 50 μm.

22. Open the acquired images by using ZEN software.
23. Examine the transgenic nematodes and evaluate dopaminergic neuronal loss by the following complementary methods:
    a. Measure average fluorescence pixel intensity from dopaminergic neurons (CEPs, ADEs, PDEs) expressing GFP under the dopaminergic neuron specific promoter using the Zeiss ZEN software (blue version).
    b. Open an acquired image with ZEN Blue software, click graphics, select draw spine contour, mark the GFP positive neuronal cell body, and note the mean intensity value.

Note: Create similar shape or size of region of interests (ROIs) to improve and increase the measurements' accuracy.

    c. Follow this step to score the intensity for each CEP, ADE and PDE neurons (Figure 3).

Alternatives: Image analysis software, such as FIJI (https://imagej.net/software/fiji/) or Qupath (https://qupath.github.io), could be alternatively utilized to quantify the average fluorescence pixel intensity from dopaminergic neurons.

⚠ CRITICAL: Use transgenic animals (IR2514) expressing only the cytosolic GFP under the *dat-1* promoter to examine whether its activity is affected by the respective treatments or genetic backgrounds.

    d. Score neurodegeneration by monitoring specific morphological features of dopaminergic neurons in the head region. *C. elegans* contains 3 pairs of head dopaminergic neurons, which form well-structured neuronal processes (Figure 1Bi). Nematodes expressing α-synuclein in dopaminergic neurons display age-dependent degeneration that is characterized by dendritic or outgrowths loss (Figure 1Bii), entire loss of neuronal cell bodies (Figure 1Biii), axonal and some blebbing (Figure 1Biv). Count the neuronal cells bodies with wild type morphology to signify the

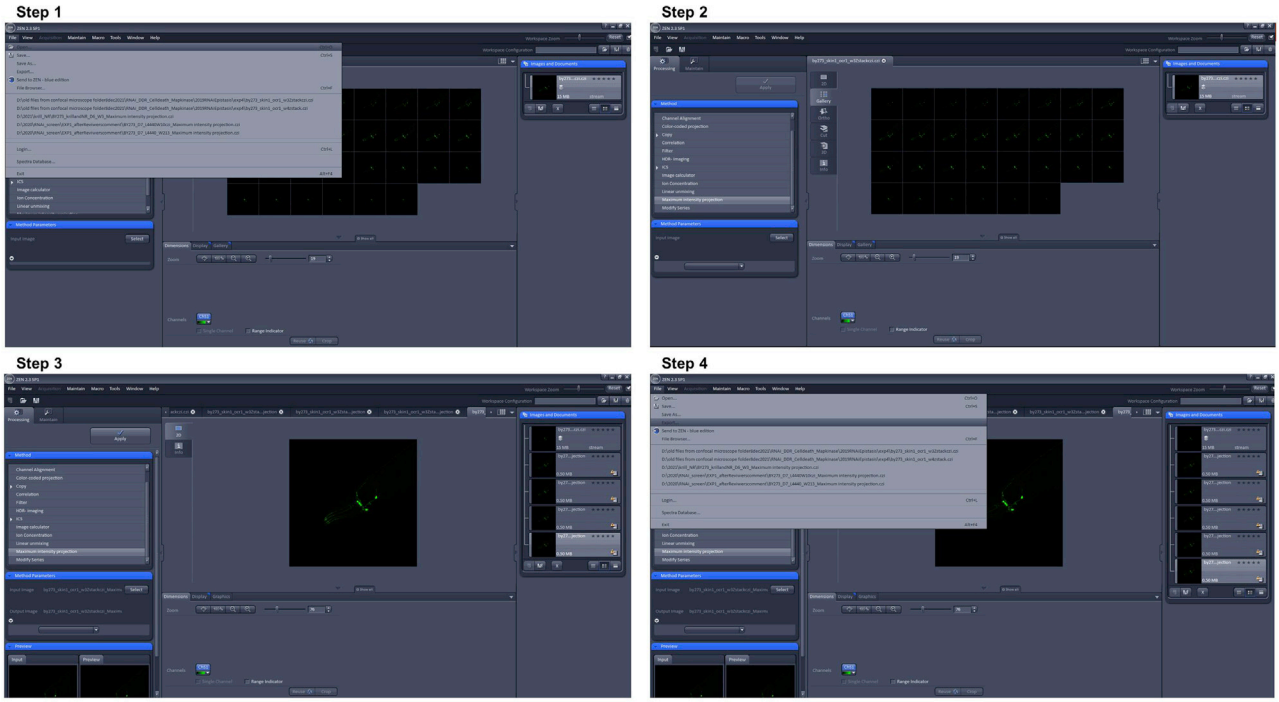

Figure 2. Maximum intensity projection by using the Zeiss ZEN software

Step 1. Open an acquired Z-stack image (.czi file) with the Zeiss ZEN software (black version; https://www.zeiss.com/microscopy); Step 2. In the processing tab under Method section select "Maximum intensity projection"; Step 3. Select the Z-stack image and press select button followed by apply button to create the Maximum intensity projection image; Step 4. Export the maximum projection intensity image to your respective drive.

survival of dopaminergic neurons in the total number of imaged animals. Use 3 different plates with 15–20 animals per strain/condition in each experimental set up. Troubleshooting 3, 4, and 5.

Note: The assessment of the morphological alterations in dopaminergic neurons is a subjective method. Thus, (1) positive controls should always be included in each experimental set up and (2) each experiment must be conducted in a double-blind manner.

24. Import and analyze the data by using a statistical software package (e.g., GraphPad Prism).

Note: Repeat each assay at least three times.

EXPECTED OUTCOMES

Transgenic animals expressing the human α-synuclein together with the cytosolic GFP in dopaminergic neurons are a well-characterized and established PD nematode model (Cooper and Van Raamsdonk, 2018). The accumulation of α-synuclein aggregates promotes the gradual degeneration of dopaminergic neurons with age (Figures 4A and 4B).

Recently, the current protocol was used to demonstrate an intricate link between the base excision repair (BER) pathway efficiency and PD pathophysiology (SenGupta et al., 2021). The BER deficient *nth-1(ok724)* mutants display enhanced neuroprotection against α-synuclein during ageing (Figures 4A and 4B). To examine whether NTH-1 DNA glycosylase acts in a cell-autonomous manner and regulates neuronal viability, PD nematodes were subjected to RNAi against *nth-1* in specific tissues. Interestingly, pan-neuronal or dopaminergic neuron-specific knock down of NTH-1 is sufficient to facilitate neuronal survival (Figures 4C and 4D), whereas hypodermal or intestinal RNAi against *nth-1* do not provide any neuroprotective effect (Figures 4E and 4F).

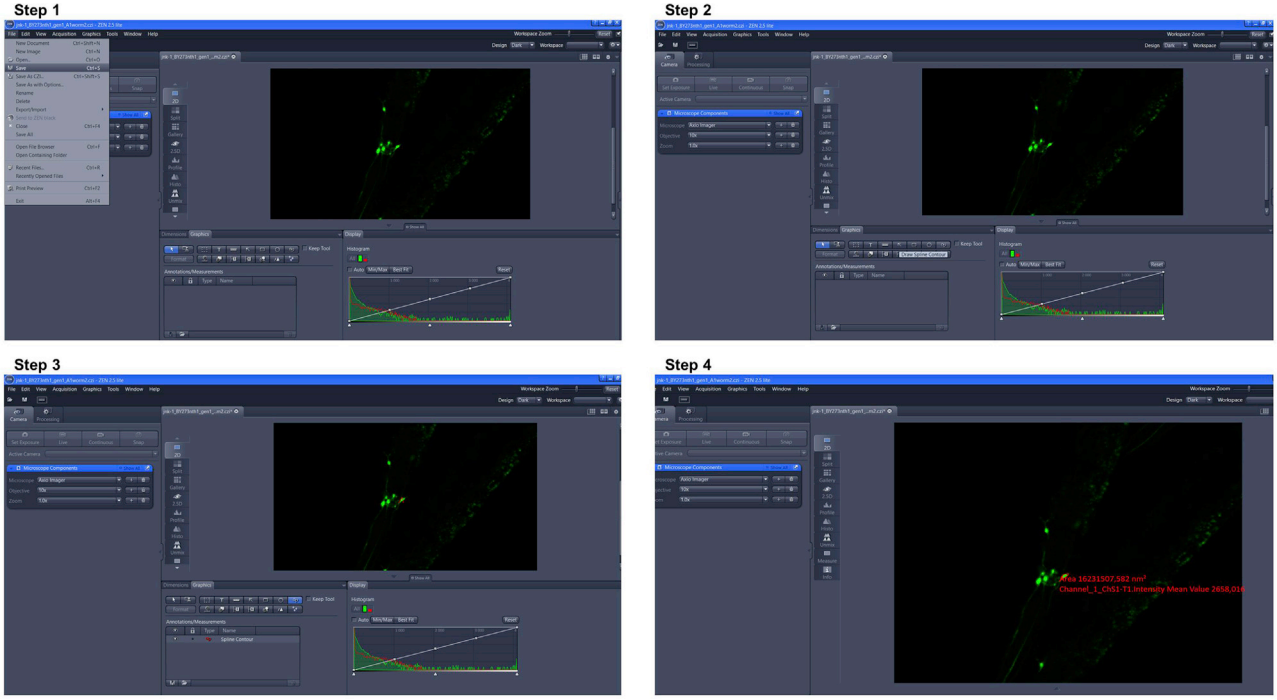

Figure 3. Measure average fluorescence pixel intensity by using the Zeiss ZEN software

Step 1. Open an acquired maximum intensity projection image with the Zeiss ZEN software (blue version; https://www.zeiss.com/microscopy); Step 2. Click the graphics tab and select "draw spine contour"; Step 3. Draw the contour around CEPs or ADEs neurons expressing cytosolic GFP; Step 4. The pen symbol marks the end of contour and provides area and intensity mean values, which can be noted in excel file for further analysis.

LIMITATIONS

The neurodegeneration assessment assay described here in *C. elegans* PD model is quite robust with high reproducibility. However, live cell imaging of *C. elegans* PD neurons can be challenging for the first time. Therefore, it is advisable to train the eye by following the neurons in a few settings. As the scoring is subjective, (1) the use of well-known inducers or inhibitors of α-synuclein-induced neurodegeneration and (2) blinded repeats are the optimum solution to avoid observer bias (Cooper and Van Raamsdonk, 2018; Maulik et al., 2017; Offenburger et al., 2018).

During slide preparation, coverslip must be placed gently to avoid air bubbles and rupturing of animals. Imaging should be performed as quickly as possible after slide preparation to avoid dehydration of the worms, which can significantly reduce the quality of images. In addition, worms might not survive long-term imaging. Thus, time must be calibrated and planned well.

TROUBLESHOOTING

Problem 1

Excessive rupture of transgenic nematode bodies is happening upon the placement of the coverslip (step 12).

Potential solution

To avoid excessive rapture of nematodes, increase either the volume of M9/levamisole drop or the number of the animals in the drop.

Problem 2

Low image quality (e.g., blurry image) due to (A) increased fluorescence intensity during the acquisition process or (B) extensive bubble formation (step 18).

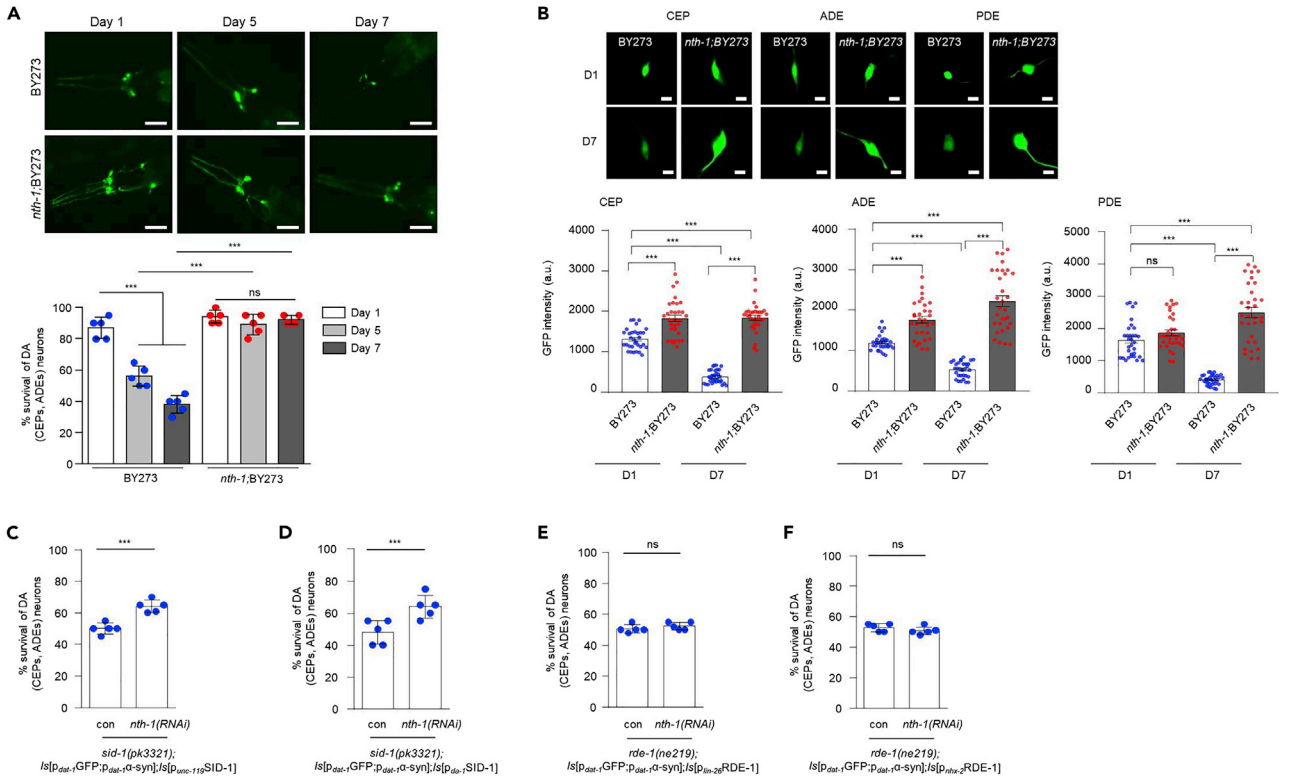

Figure 4. Neurodegeneration assessment in PD nematode model

(A–F) Transgenic animals co-expressing the human α-synuclein protein and cytoplasmic GFP in dopaminergic neurons display age-dependent degeneration signified by (A) altered cellular morphology and (B) decreased GFP intensity (n = 35 nematodes per condition; ns $p>0.05$, ***$p<0.001$; one-way ANOVA followed by Bonferroni's multiple comparison test). Scale bars, 50 μm and 5 μm. Pan-neuronal (C) and dopaminergic neuron (D) specific depletion of NTH-1 protect against α-synuclein-mediated toxicity, whereas knocking down of *nth-1* in hypodermis (E) and intestine (F) does not promote the survival of dopaminergic neurons (n= 5 biological replicates, 40 animals per condition; ***$p<0.001$; unpaired *t*-test). Error bars denote SEM.

Potential solution

- The presence of the residual bacteria during the worm picking and transferring into the M9/levamisole droplet should be reduced. Therefore, the use of the eyelash is recommended to decrease the bacterial load in the specimen. Alternatively, worms could also be picked to an NGM (or RNAi) plate with no bacterial lawn & left to move around for ~30 min prior to transfer with the eyelash if residual bacteria persist.
- Place gently and slowly the coverslip on the top of the sample. Oblique-angle lowering down is recommended to avoid bubble formation.

Problem 3

Although 1-day-old control transgenic animals co-expressing GFP and α-synuclein under the *dat-1* promoter do not display dopaminergic neuronal loss, excessive degeneration can be observed in young transgenic nematodes (step 23).

Potential solution

- Reduce the scan and imaging time. Long-term imaging process could induce photodamage and neuronal death. In case of long-term or time-lapse imaging process, 10% agarose pads combined with polystyrene nanoparticles could be alternatively used (Kim et al., 2013; Rieckher et al., 2018).
- Remove dead and censored nematodes from the imaging process. Neuronal morphology can be affected by several genetics and environmental factors, including internal hatching, starvation, temperature fluctuations among others, that influence animals' viability.

- Use freshly prepared agarose pads and M9/levamisole solution to maintain humidity throughout the imaging process. Sample dehydration alters the morphological features of neurons.
- Avoid the use of sodium azide (NaN3) as an anesthetic. Sodium azide could induce necrotic cell death even at low concentration upon long-term exposure (Artal-Sanz et al., 2006; Sato et al., 2008).
- Long-term cultivation of nematodes in the laboratory results in the accumulation of random genomic mutations that could affect animals' physiology. Every three months, thaw and renew *C. elegans* strains to maintain their genetic background.

Problem 4

7-day-old control transgenic animals co-expressing GFP and α-synuclein do not present increased levels of neurodegeneration (step 23).

Potential solution

- Avoid temperature fluctuations and use well-fed animals. Nematodes have to be grown under optimal physiological conditions. Several stress conditions, such as starvation and short-periods of heat shock, are shown to promote neuroprotection (Griffin et al., 2019; Kourtis et al., 2012; Steinkraus et al., 2008).
- Do not cultivate continuously *C. elegans* strains. Regularly thaw new nematodes to avoid genetic drift. Newly thawed nematodes should be cultured for at least three generation before being utilized in any experimental set up.

Problem 5

The *E. coli* OP50 and HT115(D3*)* bacterial strains differentially affect α-synuclein-induced neurodegeneration in transgenic nematodes of the same age and/or genetic background (step 23).

Potential solution

The *E. coli* OP50 and HT115(D3*)* are two distinct bacterial strains, which differ in their nutrient and metabolite composition (Coolon et al., 2009; Gracida and Eckmann, 2013; MacNeil et al., 2013; Pang and Curran, 2014). Therefore, these two distinct bacterial food sources differentially impact *C. elegans* gene expression, cellular responses and physiology (Gracida and Eckmann, 2013; MacNeil et al., 2013; Pang and Curran, 2014; Urrutia et al., 2020; Zhou et al., 2019). To avoid these differential diet effects, RNAi feeding protocol can be performed by using a genetically engineered OP50 strain enabling the silencing of gene of interest (OP50i) (Xiao et al., 2015).

RESOURCE AVAILABILITY

Lead contact

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

Materials availability

All materials are available upon request.

Data and code availability

This study did not generate or analyze any datasets.

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AUTHOR CONTRIBUTIONS

K.P. and T.S. conducted experiments; K.P. and T.S wrote the manuscript; H.N. and N.T wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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