Journal of Visualized Experiments

Modeling age-associated neurodegenerative diseases in Caenorhabditis elegans

--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE61169R4
Full Title:	Modeling age-associated neurodegenerative diseases in Caenorhabditis elegans
Section/Category:	JoVE Biology
Keywords:	Ageing; A-synuclein; Cell Death; Ionstasis; Necrosis; neurodegeneration; Poly-glutamate; Proteostasis
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)

TITLE:

Modeling Age-Associated Neurodegenerative Diseases in *Caenorhabditis elegans*

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KEYWORDS:
Ageing, A-synuclein, Cell death, Ionstasis, Necrosis, Neurodegeneration, Poly-glutamate, Proteostasis

SUMMARY:

Here, we introduce and describe widely accessible methodologies utilizing some versatile nematode models, including hyperactivated ion channel-induced necrosis and protein aggregate-induced neurotoxicity, to monitor and dissect the cellular and molecular underpinnings of age-associated neurodegenerative diseases.

ABSTRACT:

Battling human neurodegenerative pathologies and managing their pervasive socioeconomic impact is becoming a global priority. Notwithstanding their detrimental effects on the human life quality and the healthcare system, the majority of human neurodegenerative disorders still remain incurable and non-preventable. Therefore, the development of novel therapeutic interventions against such maladies is becoming a pressing urgency. Age-associated deterioration of neuronal circuits and function is evolutionarily conserved in organisms as diverse as the lowly worm *Caenorhabditis elegans* and humans, signifying similarities in the underlying cellular and molecular mechanisms. *C. elegans* is a highly malleable genetic model, which offers a well-characterized nervous system, body transparency and a diverse repertoire of genetic and imaging techniques to assess neuronal activity and quality control during ageing. Here, we introduce and describe methodologies utilizing some versatile nematode models, including hyperactivated ion channel-induced necrosis (e.g., *deg-3(d)* and *mec-4(d)*) and protein aggregate (e.g., α-syunclein and poly-glutamate)-induced neurotoxicity, to monitor and dissect the cellular and molecular underpinnings of age-related neuronal breakdown. A combination of these animal neurodegeneration models, together with genetic and pharmacological screens for cell death modulators will lead to an unprecedented understanding of age-related breakdown of neuronal function and will provide critical insights with broad relevance to human health and quality of life.

INTRODUCTION

Over the last two decades, *C. elegans* has been widely used as a model organism to investigate the molecular mechanisms of necrotic cell death. *C. elegans* offers an

exceptionally well-characterized and mapped nervous system, transparent body structure and a diverse repertoire of genetic and imaging methods to monitor in vivo cellular function and survival throughout ageing. Thus, several *C. elegans* genetic models of neurodegeneration have been already developed to assess neuronal viability. In particular, well-described and used nematode models include the hyperactive ion channel-induced necrosis[1-3] and cell death triggered by increased protein aggregation[4-10] and heat stroke[11,12], among others.

Short-term exposure to sub-lethal temperatures conferred resistance against necrotic cell death, triggered by a subsequent heat stress both in nematodes and mammalian neurons[11]. Interestingly, daily preconditioning of nematodes at a mild elevated temperature protects against neurodegeneration, which is inflicted by diverse stimuli, such as ionic imbalance (e.g., *mec-4(u231)* and/or *deg-3(u662)*) and protein aggregation (e.g., α-synuclein and polyQ40)[11,13].

Here, we describe versatile methodologies using *C. elegans* to monitor and evaluate age-dependent neurodegeneration in well-established models of human diseases, such as excitotoxicity-triggered cell death, Parkinson's and Huntington's disease. Moreover, we underline the neuroprotective role of heat preconditioning in several models of neurodegeneration. A combination of these techniques together with genetic and/or pharmacological screens will result in the identification and characterization of novel cell death modulators, with potential therapeutic interest.

PROTOCOL:

1. Necrotic cell death-induced by hyperactive ion channels

NOTE: Gain-of-function mutations in the gene family of degenerins, including *mec-4* and *deg-3* among others, results in the generation of hyperactive ion channels triggering necrotic cell death of six touch receptor neurons required for mechanosensation in worms[3]. Necrosis induced by the aberrant stimulation of degenerins displays several mechanistic and morphological similarities to excitotoxicity in mammals. The maintenance of energy metabolism and calcium homeostasis has a crucial role on neuronal survival during necrosis[11]. The following strains can be used to monitor necrotic cell death triggered by hyperactive ion channels, *mec-4(u231)X* and *deg-3(u662)V*.

1.1. Maintenance, synchronization and preparation of mutant worms for examination of necrotic cell death

1.1.1. Day 1: Pick L4 larvae of *mec-4(u231)* or *deg-3(u662)* mutant nematodes onto Nematode Growth Media (NGM; **Table 1**) plates seeded with *Escherichia coli* (OP50) using a dissecting stereomicroscope.

1.1.2. Place 10 L4 nematodes per seeded NGM plate and grow them at the standard temperature of 20 °C.

1.1.3. Day 5: Wash the plates with 1 mL of M9 buffer (**Table 1**) and collect the animals in a 1.5 mL tube.

1.1.4. Centrifuge at 10,000 x *g* for 30 s and remove the supernatant.

1.1.5. Add 0.5 mL of bleaching solution (7 mL of H_2O, 1 mL of 5 N NaOH and 2 mL of bleach).

NOTE: Bleaching solution gradually lose its efficiency; hence it should be prepared daily.

1.1.6. Vortex and monitor periodically until the worms have dissolved.

NOTE: Avoid bleaching for periods longer than 5 min because the embryos' viability will be affected.

1.1.7. Centrifuge at 10,000 x *g* for 30 s and remove the supernatant.

1.1.8. Wash twice the pellet (eggs) with 1 mL of M9 buffer.

1.1.9. After washing, resuspend the eggs in 200 µL of M9 buffer and incubate them for 25 min at 34 °C in a water bath.

1.1.10. Maintain a separate group of control sample (eggs) at 20 °C.

1.1.11. Pipette 100 µL of control or heat shock-treated eggs and place them on unseeded NGM plates. Each plate contains at least 100-200 eggs.

1.1.12. Incubated the eggs at 20 °C until hatching.

1.2. Mounting L1 larvae for examination using differential interference contrast (DIC; Nomarsky) microscopy

1.2.1. Prepare 2% agarose pads.

1.2.2. Use 1 mL of M9 buffer to wash the plates and collect L1 larvae nematodes in 1.5 mL tubes.

1.2.3. Centrifuge at 10,000 x *g* for 30 s.

1.2.4. Remove the supernatant and keep the pellet (nematodes).

1.2.5. Add 100 µL of 20 mM M9/levamisole buffer to anesthetize the nematodes.

NOTE: Avoid sodium azide as an anesthetic. Sodium azide triggers mitochondrial damage and oxidative stress leading eventually to cell death induction

1.2.6. Pipette 10 µL of M9/levamisole buffer containing L1 worms and mount them on 2% agarose pads (**Table 1**).

1.2.7. Gently place a coverslip on the top of the sample.

NOTE: Seal the coverslip with nail polish to preserve humidity throughout the process of imaging.

1.2.8. Observe worms using differential interference contrast (DIC; Nomarsky) microscopy.

1.2.9. Score neurodegeneration of the six touch receptor neurons by counting cells with the characteristic vacuolated appearance per nematode.

2. Protein aggregate-induced neurodegeneration

NOTE: The following strains can be used to investigated protein aggregates-induced neurotoxicity: (**A**) overexpression of human α-synuclein in dopaminergic neurons, UA44: *Is*[baIn1; p_{dat-1}α-syn, p_{dat-1}GFP] and (**B**) overexpression of human polyglutamine protein (PolyQ) pan-neuronally, AM101: *rmsIs110*[p_{rgef-1}Q40::YFP][6,10].

2.1. Maintenance, synchronization and preparation of transgenic nematodes for neurodegeneration assay

2.1.1. Use a dissecting stereomicroscope to monitor *C. elegans* development and growth.

2.1.2. Day 1: Synchronize the population of transgenic nematodes by picking and transferring 15-20 L4 larvae of each strain on freshly OP50-seeded NGM plates.

NOTE: Use at least three plates of each strain per condition.

2.1.3. Incubate and let the nematodes to grow at the standard temperature of 20 °C.

2.1.4. Day 2: Perform daily preconditioning for 30 minutes by transferring the plates in an incubator set at 34 °C. Then, return the preconditioned nematodes back at the standard temperature of 20 °C.

NOTE: Different genetic backgrounds might be sensitive to high temperature for long periods exposure.

2.1.5. (**A**) If investigating overexpression of human α-synuclein in dopaminergic neurons, UA44: *Is*[baIn1; p_{dat-1}α-syn, p_{dat-1}GFP]: on Day 9, monitor 7-days-old transgenic nematodes for dopaminergic neuronal cell death.

2.1.6. (**B**) If investigating overexpression of human polyglutamine protein (PolyQ) pan-neuronally, AM101: *rmsIs110*[p_{rgef-1}Q40::YFP]: on Day 5, measure neuronal PolyQ aggregates in the head region of 4-days-old transgenic animals expressing Q40::YFP.

2.2. Mounting the samples for microscopic examination

2.2.1. Prepare 2% agarose pads (**Table 1**).

2.2.2. Add 10 µL of 20 mM M9/levamisole buffer drop at the center of the agarose pad.

2.2.3. Pick the respective transgenic nematodes and transfer them in a M9/levamisole drop.

NOTE: Place 20-30 nematodes per drop.

2.2.4. Place gently a coverslip on the top of the sample.

NOTE: Seal the coverslip with nail polish to preserve humidity throughout the process of imaging.

2.2.5. Proceed to microscopic examination of the respective samples.

2.3. Acquisition process and data analysis of transgenic nematodes co-expressing α-synuclein and cytosolic GFP in dopaminergic neurons

2.3.1. Use an epifluorescence microscope combined with a camera (e.g., EVOS FL Auto 2).

2.3.2. Detect and capture z-stack images of the head region at 20x magnification.

2.3.3. Save and collect the maximum intensity projection images.

2.3.4. Proceed to the analysis of the acquired images.

2.3.5. Examine the transgenic worms for neurodegeneration by scoring the following cellular characteristics, (i) loss of fluorescence from neurons expressing GFP under the promoter of *dat-1*, (ii) neurons showing soma and/or axonal blebbing, outgrowths or dendritic loss.

2.3.6. Import and analyze the data by using a software package (e.g., Excel).

3. Acquisition process and data analysis of transgenic nematodes expressing pan-neuronally PolyQ40 fused with YFP

3.1. Use an epifluorescence microscope combined with a camera (e.g., EVOS FL Auto 2).

3.2. Detect and capture z-stack images of the head region at 20x magnification.

3.3. Save and collect the maximum intensity projection images.

3.4. Proceed to the analysis of the acquired images by using Fiji software.

3.5. Open images with Fiji program[14].

3.6. Select **Split Channels** command via the **Image** and **Color** drop-down menu.

NOTE: Keep the green channel image.

3.7. Use the **freehand selection** tool to manually set the fluorescent region of interest (ROI; e.g., head).

3.8. Add the respective ROI in **ROI Manager** via **Analyze** and **Tools** drop-down menu.

3.9. Subtract the background to 50% by selecting **Process** and **Subtract Background**.

3.10. Set up and apply threshold values via the menu command **Image** | **Adjust** | **Threshold**. Keep and set the same threshold values throughout image analysis of the entire experiment.

3.11. Select the respective ROI from the **ROI Manager**.

3.12. Analyze the number of protein aggregates by using the menu command **Analyze** and **Analyze Particles**.

3.13. Repeat steps 3.5-3.12 for each acquired image.

3.14. Copy the display values from the separate Results window.

3.15. Paste/Import and analyze the results by using a software package.

4. Report statistical analysis

4.1. Use at least 30 nematodes for each experimental condition. Perform three biological replicates.

4.2. Use statistical analysis software to either conduct student *t*-test (comparison between two groups) or ANOVA (comparison among multiple groups) for statistical analysis with $p < 0.05$ as significant.

REPRESENTATIVE RESULTS:

Necrotic cell death-induced by hyperactive ion channels

Using the procedures presented here, *mec-4(u231)* and *deg-3u662)* mutant embryos were either incubated for 25 min at 34 °C or kept at the standard temperature of 20 °C. Upon hatching, the number of neuronal cell corpses was determined at the L1 larval stage of both groups. Necrotic cell death is diminished in nematodes that hatched from heat shock preconditioned eggs (**Figure 1A-1B**).

Protein aggregate-induced neurodegeneration

Transgenic nematodes overexpressing (A) human α-synuclein and cytoplasmic GFP in dopaminergic neurons and (B) human polyglutamine protein (PolyQ) fused with YFP pan-neuronally, were exposed daily for 30 min at 34 °C. Heat shock preconditioning promotes neuroprotection against α-synuclein-induced cell death in 7-day-old adult hermaphrodites (**Figure 2A**) and decreases Q40::YFP protein aggregates in the head region of 4-day-old adults (**Figure 2B** and **Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1. Hyperactive ion channel-induced necrosis. (**A**) Representative DIC microscopy image of *mec-4(u231)* L1 larva, with arrowheads indicating characteristic necrotic vacuoles. At early stage of neurodegeneration swollen nuclei are displayed within cells. Image was acquired by using a 40x objection lens. Scale bar, 20 μm. (**B**) Number of neuronal cell corpses at the L1 larval stage of development, per 100 animals carrying the neurotoxic *mec-4(u231)* or *deg-3(u662)* alleles. Necrotic cell death is suppressed in L1 larvae hatched from preconditioned eggs compared to untreated counterparts (n= 100 animals per genotype and assay; Data represent mean ±S.E.M., ****P*< 0.001 for untreated versus preconditioned; *t*-test)

Figure 2. Daily preconditioning at 34 °C confers neuroprotection against α-synuclein and decreases polyQ aggregates in *C. elegans*. (**A**) Survival of anterior dopaminergic neurons (CEPs and ADEs) in untreated and preconditioned nematodes co-expressing cytoplasmic GFP together with the human α-synuclein. Preconditioned nematodes display enhanced neuroprotection compared to untreated. Remnants of neuronal cell bodies (asterisks) and axonal beading (arrowheads) are seen in untreated nematodes (top panel). Both soma (asterisks) and neuronal processes (arrowheads) are preserved upon preconditioning. Images acquired by using a 20x objection lens and depict maximum intensity z-projection. Acquisition details: Bright, 0.15. Scale bar, 20 μm. Worms were scored for neuronal survival of the anterior dopaminergic neurons on the seventh day of adulthood. (**B**) Neuronal polyQ aggregates detected on the fourth day of adulthood in the head region of untreated and preconditioned transgenic nematodes. Preconditioned transgenic worms present less neuronal Q40::YFP aggregates compared to untreated. Representative images of the head region are shown with arrowheads indicating polyQ protein aggregates in neuronal cells. Images acquired by using a 20x objection lens and depict maximum intensity z-projection. Acquisition details: Bright, 0.0175. Scale bar, 20 μm. 30-35 animals were quantified per condition in each of three independent experiments. Data represent mean ±S.E.M., ***P < 0.05, unpaired *t*-test.

Figure 3. Image analysis by using Fiji software. 1. Open an acquired image with Fiji software; **2.** Select “Split channel” command via the “Image” and “Color” drop down menu to convert the image; 3, **4.** Keep “green channel” image and by using the “freehand selection” tool, enwrap the fluorescent region of interest (ROI; e.g., head). Add the respective ROI in “ROI Manager” via “Analyze” and “Tools” drop-down menu; **5, 6.** Subtract the background to 50% by selecting “Process” and “Subtract Background”; **7, 8.** Set up and apply threshold values via the menu command “Image”, “Adjust” and “Threshold”; **9.** Select the respective ROI from the “ROI Manager”; **10-12.** Analyze the number of protein aggregates by using the menu command “Analyze” and “Analyze Particles”.

Table 1: Recommended recipes for reagents used. All the reagents recipes used in the presented protocol are outlined here.

DISCUSSION

Here, we introduce and describe widely accessible methodologies for growth, synchronization and microscopic examination of some versatile *C. elegans* models investigating age-dependent neurodegeneration. Particularly, we assess and dissect

the cellular and molecular underpinnings of age-related neuronal breakdown by using hyperactivated ion channel-induced necrosis and protein aggregate-induced neurotoxicity[1-5,7,9-11].

Although the described procedures for in vivo cell death assessment are straightforward and can be easily performed in any laboratory, there are some critical steps that should be taken into consideration. Caloric restriction and starvation are known to induce multiple stress pathways, such as autophagy, that might interfere with neurodegeneration or protein aggregates accumulation[13,15,16]. Thus, well-fed and non-starved nematodes should be used. Heat shock preconditioning confers neuroprotection against several neurodegenerative stimuli and is used as an established cell death modulator[11,13]. However, some mutants are susceptible to high temperature exposure for long periods. Thus, the appropriate developmental stage, age and duration of heat shock preconditioning should be experimentally determined each time, when animals of different genetic backgrounds, that might be sensitive to high temperatures, are used. A gradual increase of nematode intestinal autofluorescence is observed during aging. Thus, neuronal cell bodies and processes close to the intestinal area should be avoided during the imaging process of AM101 strain. Focus on neuronal cells, which are located in the head and/or tail region to bypass intestine-derived autofluorescence. Use M9 buffer instead of water to generate M9/levamisome buffer and 2% agarose pads. M9 buffer ensure a favorable osmotic environment protecting the nematodes from drying out throughout the microscopic visualization and analysis.

The described methodologies underscore that the combination of nematodes models of neurodegeneration, together with genetic and pharmacological screens for cell death modulators could lead to an unprecedented understanding of age-associated impairment of neuronal circuits and boost the development of novel therapeutic interventions against neurodegenerative disorders promoting human health and quality of life.

ACKNOWLEDGEMENTS

We thank Chaniotakis M. and Kounakis K. for video recording and editing. K.P. is funded by a grant from the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and Technology (GSRT). N.T. is funded by grants from the European Research Council (ERC – GA695190 – MANNA), the European Commission Framework Programmes, and the Greek Ministry of Education.

DISCLOSURES

The authors declare no competing interests.

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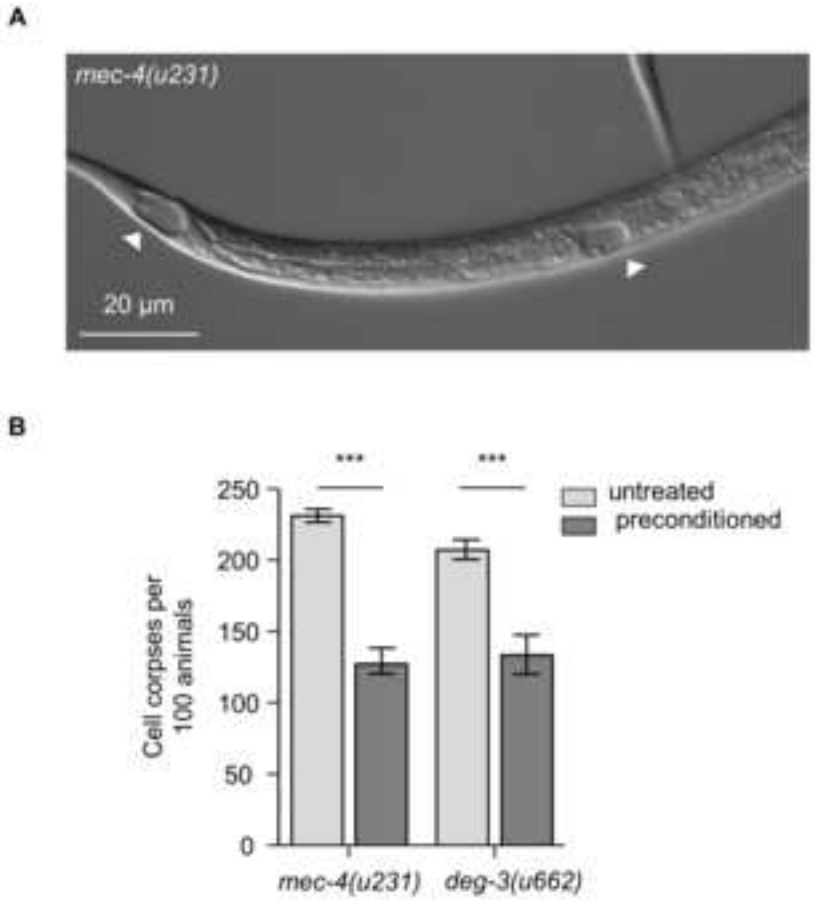

Figure 1
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Figure 2

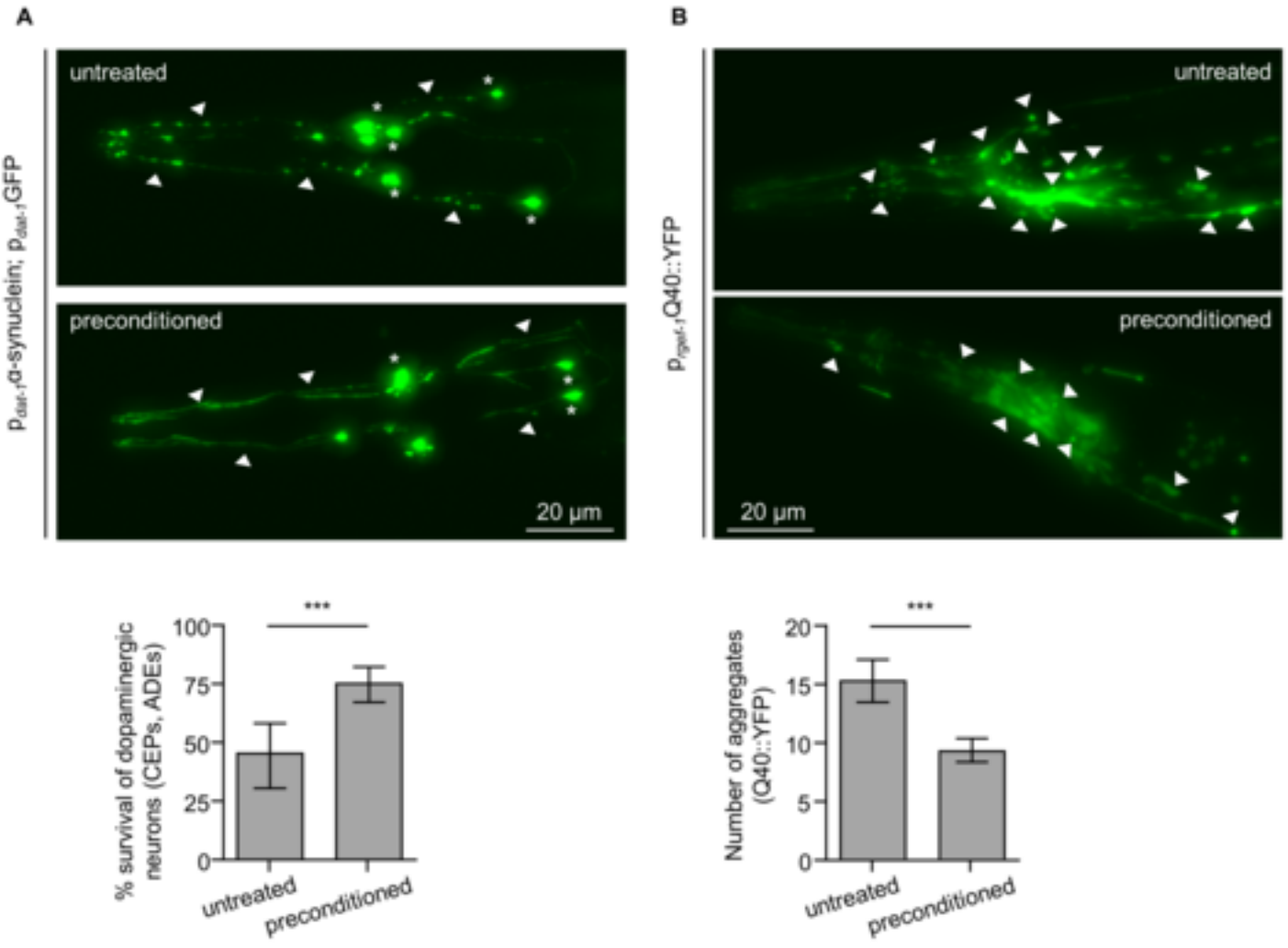

Figure 2
Palikaras and Tavernarakis

Figure 3

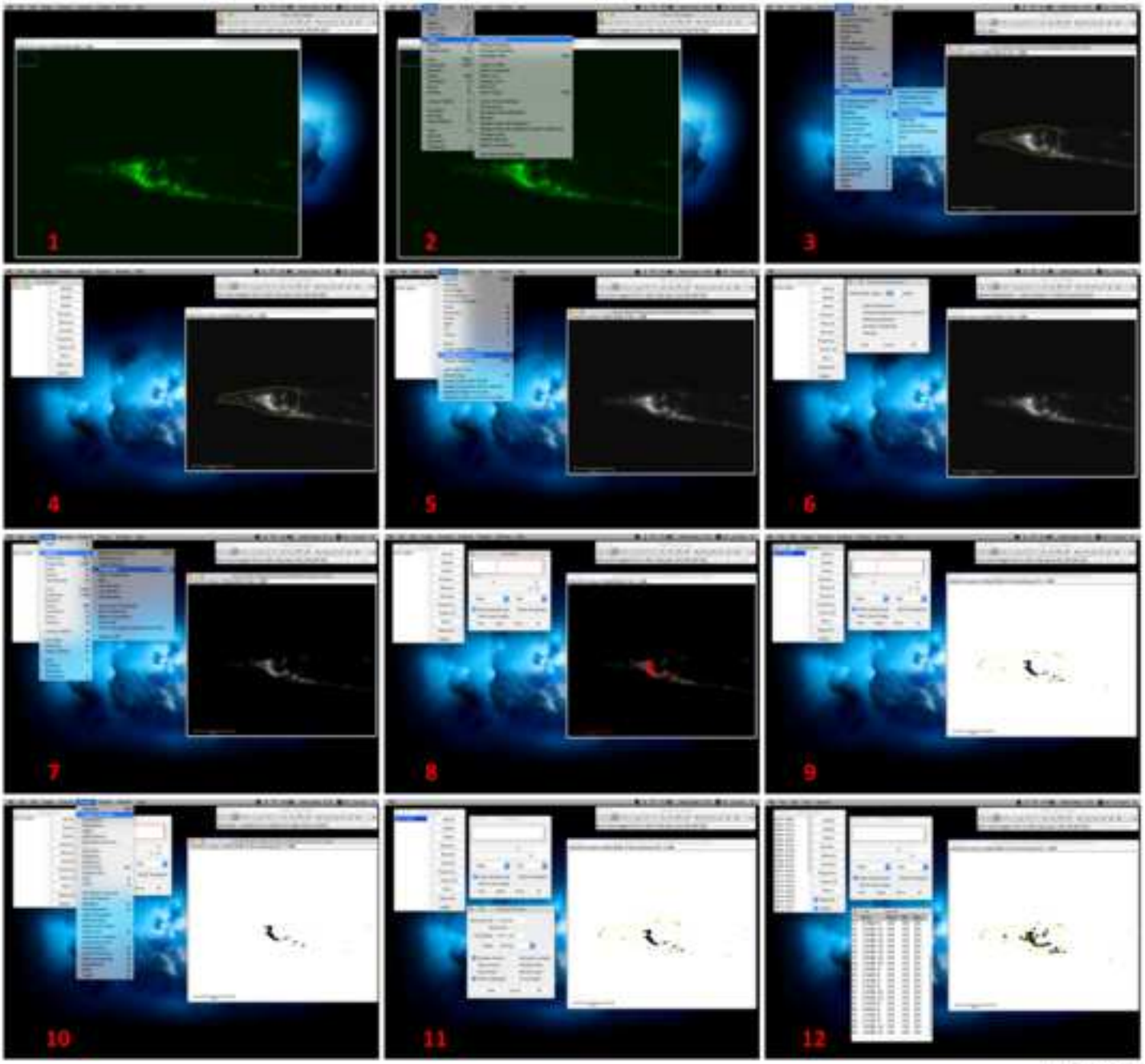

Figure 3
Palikaras and Tavernarakis

Table

Reagent	Recipe
2% agarose pads	1. Weigh 0.5 g of agarose in a cylindrical glass beaker. 2. Add 25 mL of M9 buffer. 3. Heat in a microwave until close to boiling. Take out, stir with a pipette tip and boil again. Repeat until the agarose is dissolved. 4. Place an empty microscope slide on the bench. 5. Put a drop (~ 50 µL) of fresh 2% agarose solution in the middle of the slide. 6. Take a second microscope slide and place it on top of the agarose drop. Gently press down to flatten the drop. 7. Let the agarose harden for 30 seconds and remove gently the top microscope slide. 8. Immediately proceed with the sample preparation, since the agarose pads will start drying within approximately 5 minutes. **Tip:** Leave the top microscope slide as a cover to preserve the humidity longer (~ 1 hour). Thus, several agarose pads can be prepared and used swiftly during the experiments.
M9 buffer	1. Dissolve 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl in 1 L of distilled water and autoclave. 2. Let cool and add 1 mL of 1 M $MgSO_4$ (sterile). 3. Store M9 buffer at 4 °C.
Nematode growth medium (NGM) agar plates	1. Mix 3 g of NaCl, 2.5 g of bactopeptone, 0.2 g of streptomycin, 17 g of agar and add 900 mL of distilled water. Autoclave. 2. Let cool to 55-60 °C. 3. Add 1 mL of cholesterol stock solution, 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 1 mL of nystatin stock solution, 25 mL of sterile 1 M phosphate buffer, pH 6.0, and distilled sterile water up to 1 L. 4. Pipette 10 mL of medium per Petri dish and leave to solidify. 5. Store the plates at 4 °C until used.

Name of Material/ Equipment	Company
Agar	Sigma-Aldrich
Agarose	Biozym
AM101: *rmsIs110* [p_{rgef-1} Q40::YFP]	Caenorhabditis Genetics Center (CGC)
Calcium chloride dehydrate (CaCl2.2H2O)	Sigma-Aldrich
Cholesterol	SERVA Electrophoresis
deg-3(u662)V or *deg-3(d)*	Caenorhabditis Genetics Center (CGC)
DIC microscope (Nomarsky)	Zeiss
Dissecting stereomicroscope	Nikon Corporation
Epifluorescence microscope	Thermo Fisher Scientific
Escherichia coli OP50 strain	Caenorhabditis Genetics Center (CGC)
Greiner Petri dishes (60 mm x 15 mm)	Sigma-Aldrich
image analysis software	Fiji
KH_2PO_4	EMD Millipore
K_2HPO_4	EMD Millipore
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich
mec-4(u231)X or *mec-4(d)*	Caenorhabditis Genetics Center (CGC)
Microscope slides (75 mm x 25 mm x 1 mm)	Marienfeld, Lauda-Koenigshofen
Microscope cover glass (18 mm x 18 mm)	Marienfeld, Lauda-Koenigshofen
Microsoft Office 2011 Excel software package	Microsoft Corporation, Redmond, USA
Na_2HPO_4	EMD Millipore
Nematode growth medium (NGM) agar plates	
Nystatin stock solution	Sigma-Aldrich
Peptone	BD, Bacto
Phosphate buffer	
Sodium chloride (NaCl)	EMD Millipore
Standard equipment for preparing agar plates (autoclave, Petri dishes, etc.)	
Standard equipment for maintaining worms (platinum wire pick, incubators, etc.)	
statistical analysis software	GraphPad Software Inc., San Diego, USA
Streptomycin	Sigma-Aldrich
Tetramisole hydrochloride	Sigma-Aldrich
UA44: *Is* [baIn1; p_{dat-1} α-syn, p_{dat-1} GFP]	

Catalog Number	Comments/Description
5040 840,004	
C5080 17101.01	
	Maintain animals at 20 °C Axio Vert A1 SMZ645 EVOS Cell Imaging Systems
P5237	
	https://fiji.sc
137,010	
104,873	
M7506	
	Maintain animals at 20 °C
10 006 12 01 010 30	
106,586	
N3503 211677	
1,064,041,000	
	GraphPad Prism software package
S6501 L9756	
	Upon request: G. Caldwell (University of Alabama, Tuscaloosa AL)

Dear Editor,

We would like to thank you for your time and effort in reviewing our manuscript/video. We considered each of the points in the reviews very carefully and made every possible effort to address them. In doing so, we provide to you the revised manuscript and video.

We believe that with your constructive input, we have been able to resubmit a significantly improved video. A point-by-point response to all the comments follows below (original comments are quoted in **bold**).

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:
1. Please use numbered superscripts to refer to all previous publications.

Corrected

2. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

In the revised manuscript, we have now stated that the authors declare no competing interests.

3. Please move the solution recipes from the Table of Materials to a separate table.

Per the editorial suggestions, we have now moved the recipes in a separate table.

Changes to be made by the Author(s) regarding the video:
1. Please deliver a video at 29.97 fps.

Corrected

2. Please design a title card that fills the frame (there should be no black or gray bars on the sides of the title card), matches the resolution of the video (the title card should not be blurry or blown up, it should be sharp as the video), and is free of errors such as extra pixels (like the one down and to the left of the name Konstantinos).

Corrected

3. 00:03-00:36 The Introduction with Dr. Palikaras speaking does not have synchronized audio and video. This means that the video is playing later than the corresponding audio. This issue should be fixed before publication. Try moving the audio forward in time a bit until the movement of his lips match the audio exactly. Note: This is not a problem in the Conclusion.

Corrected

4. **Editing (Jump Cuts):**
"We have seen some use of ""jump cuts."" These are edit points that feature shots with very similar content transitioned over a single frame, causing the content to "jump" on screen. This style is not appropriate for JoVE videos since it causes confusion and draws attention away from the content. When needing to join very similar shots in succession, use a cross dissolve or fade so the transition between shots is smooth. Here are some of the jump cuts we flagged:
• 01:53
• 02:11
• 03:20
• 03:32
• 03:44"

We have revised the video accordingly.

5. Editing (Extra Shots):
"• 04:34-04:35 There is an extra shot of the incubator here that should be removed.

The extra shot of the incubator has been removed in the revised video.

• 05:41 This seems to be an extra shot of the imaging/measuring step. It can probably be deleted. If you don't have enough time from the shot before, consider making a freeze frame or slowing down the footage so it fits the narrationSome ."

The extra shot of the imaging/measuring step has been removed in the revised video.

6. Graphics:
• 05:53-06:19 Please make the background white (to match the images) so that there are no black margins during this section.

Corrected

7. Please renumber the protocol steps in the video title cards to match the numbering in the written manuscript. I have changed this numbering to fit our publication standard.

We have revised the video accordingly.

8. Please stabilize the protocol section of the video and avoid any speeding up of the footage.

Corrected