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Caenorhabditis elegans as a Model System for Discovering Bioactive Compounds against Polyglutamine-mediated Neurotoxicity

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TITLE:

Caenorhabditis elegans as a Model System for Discovering Bioactive Compounds against Polyglutamine-mediated Neurotoxicity

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SUMMARY:

Here, we present a protocol to assess the neuroprotective activities of test compounds in *Caenorhabditis elegans*, including polyglutamine aggregation, neuronal death, and chemoavoidance behavior, as well as an exemplary integration of multiple phenotypes.

ABSTRACT:

Age-related misfolding and aggregation of pathogenic proteins are responsible for several neurodegenerative diseases. For example, Huntington's disease (HD) is principally driven by a CAG nucleotide repeat that encodes an expanded glutamine tract in huntingtin protein. Thus, the inhibition of polyglutamine (polyQ) aggregation and, in particular, aggregation-associated neurotoxicity is a useful strategy for the prevention of HD and other polyQ-associated conditions. This paper introduces generalized experimental protocols to assess the neuroprotective capacity of test compounds against HD using established polyQ transgenic *Caenorhabditis elegans* models. The AM141 strain is chosen for the polyQ aggregation assay as an age-associated phenotype of discrete fluorescent aggregates can be easily observed in its body wall at the adult stage due to muscle-specific expression of polyQ::YFP fusion proteins. In contrast, the HA759 model with strong expression of polyQ-expanded tracts in ASH neurons

is used to examine neuronal death and chemoavoidance behavior. To comprehensively evaluate the neuroprotective capacity of target compounds, the above test results are ultimately presented as a radar chart with profiling of multiple phenotypes in a manner of direct comparison and direct viewing.

Video Link here

INTRODUCTION:

Progressive neurodegeneration in HD involves pathogenic mutant huntingtin with an abnormal stretch of polyQ encoded by CAG trinucleotide repeats[1–3]. Mutant huntingtin proteins with more than 37 glutamine repeats are prone to aggregate and accumulate in the brains of HD patients and animal models[4,5], which ultimately leads to neurodegeneration[6]. Despite the lack of clarity on the roles of polyQ aggregates in disease pathology[5], the inhibition of polyQ aggregation and its associated toxicity is a useful therapeutic strategy for HD and other polyQ diseases[4,7,8].

Due to the conservation in neuronal signaling pathways and easy-to-construct transgenic disease models, *Caenorhabditis elegans* has been widely used as a major model organism for the investigation of neurological disorders[9–12]. For example, transgenic *C. elegans* models expressing aggregation-prone polyQ expansions can objectively mimic HD-like features such as selective neuronal cell loss, cytoplasmic aggregate formation, and behavioral defects[13]. Investigation of the potential effects of test samples to reverse these phenotypes in established polyQ nematode models has led to the identification of a variety of promising therapeutic candidates, e.g., polysaccharides[7,14,15], oligosaccharides[16], natural small molecules[17,18], and herbal extracts and formulas[19,20].

Described here are two main polyQ *C. elegans* models and relevant protocols for potential applications as exemplified by the study on astragalan, a polysaccharide isolated from *Astragalus membranaceus*[7]. For the polyQ aggregation assay in *C. elegans*, the model used is the transgenic strain AM141, which shows fluorescent puncta dispersed in its body wall muscle when reaching adulthood due to the expression of the Q40::YFP fusion protein, a polyQ tract of 40 residues (polyQ40) fused to yellow fluorescent protein (YFP)[21,22]. The strain HA759 was used to examine neuronal survival and chemoavoidance behavior as it expresses both green fluorescent protein (GFP) and Htn-Q150 (a human huntingtin-derived polyQ tract of 150 residues) strongly in ASH neurons but weakly in other neurons, resulting in progressive neurodegeneration and ASH cell death[7,13]. A comprehensive summary of the neuroprotective potential of therapeutic candidates is provided by integrating results from different assays.

PROTOCOL:

NOTE: See **Table 1** for the recipes of solutions used in this protocol.

1. Preparation of materials for the *Caenorhabditis elegans* assay

1.1. Maintenance of *C. elegans* strains

1.1.1. Obtain *C. elegans* (AM141 and HA759) and *Escherichia coli* (OP50 and NA22) strains (see the **Table of Materials**).

1.1.2. Maintain the nematodes on the nematode growth media (NGM) plate seeded with *E. coli* OP50 at 20 °C for AM141[21] or 15 °C for HA759[23].

1.2. Preparation of *E. coli* OP50 bacterial culture

1.2.1. Pick a single colony of *E. coli* OP50 from a Luria-Bertani (LB) streak plate and inoculate it into 50 mL of liquid LB culture.

1.2.2. Incubate the OP50 bacteria in a shaker at 37 °C and 200 rpm until an optical density of ~0.5 at 570 nm (OD_{570}).

1.2.3. Store the OP50 bacterial culture at 4 °C and use it within two weeks.

1.3. Preparation of NGM plates with OP50 bacteria

1.3.1. Add 20 g of agar, 2.5 g of peptone, 3.0 g of NaCl, and 975 mL of deionized water to a 1 L autoclavable bottle. Autoclave at 121 °C for 30 min.

1.3.2. Place the liquid NGM agar bottle on the bench to cool to ~60 °C, and then add the following sterile stock solutions: 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 1 mL of 5 mg/mL cholesterol, and 25 mL of 1 M potassium phosphate (pH 6.0).

1.3.3. Pour 20 mL of NGM into a sterile 90 mm Petri dish, and leave the plates on the bench to cool and solidify. Keep the plates upside down and allow them to dry on the bench at room temperature for 2 days.

1.3.4. Dispense 200 µL of the OP50 bacterial culture onto each NGM plate and spread evenly with a sterile glass coating rod. Close the lids and incubate the plates overnight at 37 °C.

1.3.5. Store the NGM plates seeded with OP50 in a plastic box with a cover at room temperature and use within two weeks.

1.4. Preparation of age-synchronized *C. elegans* population

1.4.1. Collect gravid adult nematodes into a sterile 1.5 mL microcentrifuge tube and centrifuge at 1000 × *g* for 1 min. Wash three times and resuspend the nematodes in M9 buffer.

1.4.2. Add an equal volume of bleach solution and agitate gently for 3–5 min. Monitor the bleaching every 15 s under a dissecting microscope.

NOTE: The bleach solution must be prepared freshly before use by mixing equal volumes of 10% NaOCl and 1 M NaOH (**Table 1**)[20].

1.4.3. Once most of the nematodes are broken, stop the digestion by diluting with M9 buffer. Quickly centrifuge to remove the supernatant and resuspend the pellet in M9 buffer to repeat the washing three times.

1.4.4. Resuspend the pellet in S medium. Let the nematode residues settle down by gravity for 2–3 min while the eggs remain in the supernatant.

1.4.5. Aspirate the supernatant into a new sterile microcentrifuge tube. Collect the eggs by centrifugation at 1000 × *g* for 1 min.

1.4.6. Discard ~80% of the supernatant and transfer the eggs into a sterile flask containing 20 mL of S medium. Place the flask in a shaker and incubate the eggs overnight without food at 120 rpm to obtain synchronized L1 nematodes.

2. PolyQ aggregation assay

2.1. Preparation of nematodes for the polyQ aggregation assay

2.1.1. Transfer 300–500 synchronized L1 larvae of AM141 to each well of a 48-well plate with 500 µL of S medium containing OP50 (OD_{570} of 0.7–0.8) and 5 mg/mL of astragalan, typically one well per treatment for one time point.

2.1.2. Seal the plate with parafilm and incubate at 20 °C and 120 rpm for 24, 48, 72, and 96 h.

2.1.3. Harvest the nematodes in a sterile 1.5 mL microcentrifuge tube and wash with M9 buffer >3 times by centrifugation (1000 × *g* for 1 min) to remove the remaining OP50. Resuspend the AM141 nematodes in M9 buffer, and keep them ready for image acquisition.

2.2. Acquisition of fluorescent images and data analysis

NOTE: Data are automatically analyzed with this high-content imaging system. If an automated imaging device is not available, a conventional method to prepare an agarose pad for image acquisition can be used to achieve similar performance by using a common fluorescent microscope[7,15,17] (see steps 3.2 and 3.3).

2.2.1. Transfer 10–15 nematodes into each well in a 384-well plate (final volume 80 µL per well). Set 10 replicate wells for each treatment.

2.2.2. Add 10 µL of 200 mM sodium azide to each well to paralyze the nematodes and allow them to settle down to the bottom (5–10 min).

2.2.3. Place the plate in a high-content imaging system to acquire fluorescent images (see the **Table of Materials** for device and software information).

2.2.4. Open the image acquisition software and set up the following parameters.

2.2.4.1. Open the **Plate Acquisition Setup** window and create a new experiment set and name.

2.2.4.2. Select **Magnification** as **2x**, **Camera binning** as **1**, and **Plate type** as **384-well plate**.

2.2.4.3. Set the wells and sites (single site) to be visited.

2.2.4.4. Select the fluorescein isothiocyanate (**FITC) filter** and enable image-based focusing options.

2.2.4.5. Set the **Exposure** as **300 ms**.

NOTE: The above settings can be tested and adjusted to optimize the imaging parameters.

2.2.4.6. Save **Image acquisition** settings and click on the **Acquire Plate** button to run.

2.2.5. Analyze the image data by using the image analysis software.

2.2.5.1. Open the **Review Plate Data** window and select the **Test plate** for image analysis.

2.2.5.2. Double-click on a test well to display its image.

2.2.5.3. Select the **Count Nuclei** as the analysis method and click on the **Configure Settings** button to bring out a window for the following settings.

2.2.5.4. Define the source image from the FITC channel and select the **Standard Algorithm**.

2.2.5.5. Set the image analysis parameters as follows: approximate minimum width = 10 µm (= 2 pixels); approximate maximum width = 50 µm (= 12 pixels); intensity above local background = 1,000–2,000 graylevels.

2.2.5.6. Test the current settings to optimize the method of analysis.

2.2.5.7. Save the settings and run the analysis on all the wells.

NOTE: It takes ~20 min to finish analysis for one 384-well plate.

2.2.5.8. Export the **Total Nuclei** as the total number of Q40::YFP aggregates in each well.

2.2.6. Count the number of nematodes in each well.

2.2.7. Calculate the average number of Q40::YFP aggregates per nematode in each group, and apply a nonlinear curve fit to the data from each time point.

2.2.8. Calculate the inhibition index using eq (**1**) below:

Inhibition index = $(Ncontrol - Nsample) / Ncontrol$ (**1**)

Where $N_{control}$ and N_{sample} are the average number of Q40::YFP aggregates in the control and the treatment groups, respectively.

3. PolyQ-mediated neurotoxicity assays

3.1. Treatment of *C. elegans* with test samples

3.1.1. To prepare nematodes for the polyQ neurotoxicity assay, transfer 300–500 synchronized L1 larvae of HA759 to each well of a 48-well plate with 500 µL of S medium containing OP50 (OD_{570} of 0.7–0.8) and 5 mg/mL of astragalan, typically three replicate wells for each treatment.

3.1.2. Seal the plate with parafilm and incubate at 15 °C and 120 rpm for 3 days[23].

3.1.3. Collect the nematodes by centrifugation and wash 3–5 times with M9 buffer. Resuspend the nematodes in M9 buffer for use in neuronal survival and avoidance assays.

3.2. Preparation of agarose pad

3.2.1. Add 2 g of agarose to 100 mL of M9 buffer (2%, w/v) and heat the agarose solution in a microwave to near-boiling.

3.2.2. Dispense 0.5 mL of melted agarose onto the center of a 1 mm thick microscopy glass slide placed between two pieces of 2 mm thick glass plates. Cover with another slide vertically. Once the agarose cools down and is solidified, gently remove the top slide.

3.3. ASH neuronal survival assay

3.3.1. Add a drop of 20 mM sodium azide onto the agarose pad. Transfer 15–20 HA759 nematodes into the drop to immobilize them.

3.3.2. Place a coverslip gently over the nematodes. Keep the slide under a fluorescence microscope fitted with a digital camera. Select a 40x objective lens and FITC filter to detect GFP-positive ASH neurons in the head region of the nematodes.

3.3.3. Select more than 50 nematodes in each group randomly to count the number of nematodes with GFP-labeled bilateral ASH neurons in their head region[7]. Calculate the survival rate of ASH neurons using eq (**2**) below.

Neuronal survival (%) = $Nsurvival \,/\, Ntotal \times 100\%$ (**2**)

Where $N_{survival}$ and N_{total} are the number of nematodes with GFP-positive ASH neurons and the total number of tested nematodes in each group, respectively.

3.4. Osmotic avoidance assay

3.4.1. Divide a food-free NGM plate (9 cm) into normal (N) and trap (T) zones by an 8 M glycerol (~30 µL) line in the middle. Spread a 200 mM sodium azide (~20 µL) line at ~1 cm away from the glycerol line to paralyze the nematodes crossing through the glycerol barrier into Zone T.

3.4.2. Transfer ~200 nematodes each onto Zone N of three replicate plates for each group. Add a drop of 1% butanedione (~2 µL) onto Zone T (1 cm from the plate edge) to attract the nematodes. Cover the lid of the Petri dish immediately, and incubate at 23 °C for 90 min.

3.4.3. Score the number of nematodes on the N and T zones under a microscope. Calculate the avoidance index using eq (**3**)[20].

Avoidance index = $N \,/\, (T + N)$ (**3**)

Where N and T are the number of nematodes in N and T zones, respectively. Data are presented as means ± standard deviation (SD) of three replicates, representative of >3 independent experiments.

3.4.4. Perform an unpaired, two-tailed *t*-test to compare the data from the astragalan and control groups.

4. Creating a radar chart

4.1. Open the graphing software and import data from different assays into a new sheet. Input the A(X) column as the titles of the radial axes, A(Y) column as the data from the control group, and B(Y) column as the data from the treatment group.

4.2. Select the required data and click on **Plot** | **Specialized: Radar** button in the toolbar menu to generate a radar chart.

4.3. Double-click on the radial axis and adjust the **Scale**, **Tick**, and **Tick labels** of each axis as needed.

4.4. Click on **File** in the menu and select **Export Graphs** to save the image as ***.tiff**.

NOTE: The software websites in the **Table of Materials** provide detailed help documents and video tutorials on creating radar charts.

REPRESENTATIVE RESULTS:

The transgenic polyQ strain AM141 strongly expresses Q40::YFP fusion proteins in its body wall muscle cells[7,21]. As shown in **Figure 1A**, the discrete aggregate phenotype of this strain can be identified by the automated imaging and analysis protocol described in this article. The amount of Q40::YFP aggregates in AM141 nematodes increased significantly after 48 h from the L1 stage. However, this tendency to increase was inhibited by astragalan treatment (**Figure 1B**), demonstrating the protective potential of astragalan against polyQ aggregation. Typically either 72 h or 96 h can be conveniently used as the time points to count Q40::YFP aggregates to evaluate the anti-aggregation effect of test samples. In this protocol, the fluorescent aggregates of AM141 nematode were captured by an automated imaging system, although fluorescence microscopes can also be used for this purpose[7].

The *C. elegans* strain HA759 expresses both the GFP marker and Htn-Q150 in its sensory ASH neurons, leading to progressive loss and dysfunction of these neurons[11,13]. The nematodes were mounted on 2% agarose pads to determine the ASH neuronal viability (**Figure 2A**) and visualized microscopically to detect the ASH neurons. A loss of GFP fluorescence in bilateral ASH neurons in the head regions of nematodes indicates the ASH neuronal death (**Figure 2B**). The survival rate of ASH neurons in HA759 nematodes is <40% in the control group after incubation at 15 °C for 3 days[7,20], indicating polyQ-mediated neurotoxicity. Hence, this ASH neuronal survival assay can be used to visually evaluate the effects of test compounds on *C. elegans* neurons, e.g., the neuroprotective effect of astragalan but not *Poria* glycan (**Figure 2C**).

As behavior dysfunction is a major clinical symptom in polyQ diseases, the chemosensory avoidance assay (**Figure 3A**) using large numbers of HA759 nematodes is designed as a simplified test to examine the effect of test samples on the functional loss of ASH neurons mediated by polyQ aggregation. As shown in **Figure 3B,** the avoidance index of HA759 nematodes in the untreated control group was ~0.5, similar to what was reported previously[14]. Interestingly, the avoidance index increased to >0.6 in the nematodes treated with astragalan at 15 °C for 3 days (**Figure 3B**), demonstrating a neuroprotective effect of the polysaccharide against behavioral impairments.

To evaluate the overall neuroprotective capacity of test compounds, the data from the above individual assays can be integrated and presented as a radar chart for multiple phenotypes, making it a unifying feature of polyQ phenotypes suitable for direct comparison and direct

viewing. As shown in **Figure 4**, the area of the triangle in the astragalan treatment group is greater than that of the control group, indicating the anti-polyQ effects of the polysaccharide.

FIGURE AND TABLE LEGENDS:

Figure 1: Effect of astragalan on polyQ40 aggregation. (**A**) Representative images of AM141 nematodes after treatment with or without astragalan for 96 h at 20 °C. The fluorescent images (left panels) were acquired from 384-well plates using a high-content imaging and analysis system, and the Q40::YFP aggregates (right panels) were automatically identified by the system. Insets are the magnified views of nematode images showing the polyQ aggregates. Scale bars = 500 µm. (**B**) Quantification of Q40::YFP aggregates. The number of Q40::YFP aggregates in AM141 nematodes was monitored using the high-content imaging system every 24 h for 4 days after treatment with or without astragalan at 20 °C. Approximately 100–150 nematodes in each group were scored for aggregates at each time point. The results are shown as fitted curves based on the average number of aggregates per nematode. Abbreviations: polyQ = polyglutamine; YFP = yellow fluorescent protein; FITC = fluorescein isothiocyanate.

Figure 2: Effect of astragalan on Htn-Q150-mediated ASH neuronal death. (**A**) Schematic diagram of agarose slide preparation. (**B**) Representative micrographs of HA759 nematodes with ASH neuronal survival and death using 400x magnification. Scale bars = 20 µm. The HA759 nematodes were photographed using a fluorescence microscope after treatment with or without astragalan at 15 °C for 3 days from L1. (**C**) Protective effect of astragalan against polyQ-mediated ASH neuronal death. *Poria* glycan, a polysaccharide from *Poria cocos*, was used as a control. Data are presented as means ± SD of three replicates, representative of more than three independent experiments. Statistical analysis was performed using an unpaired, two-tailed *t*-test to compare data of the control group with those of the astragalan and *Poria* glycan groups. *$p < 0.05$; ns = no significant. Abbreviation: GFP = green fluorescent protein.

Figure 3: Effect of astragalan on Htn-Q150-mediated behavioral dysfunction. (**A**) Schematic diagram of the avoidance assay plate. (**B**) Representative results of avoidance assay. Avoidance index was defined as the ratio of nematodes in the N zone to the total number of nematodes on the plate. The results are presented as mean ± SD of three replicates, representative of three independent experiments. Statistical analysis of avoidance index was performed using an unpaired, two-tailed *t*-test. **$p < 0.01$.

Figure 4: Overall neuroprotective capacity of astragalan. Data from three different assays are imported into OriginPro software to a create radar chart, which is presented to profile the general effect of astragalan on multiple phenotypes mediated by polyQ.

DISCUSSION:

As polyQ aggregation and proteotoxicity are important features of polyQ disorders, such as Huntington's disease[13], we recommend the use of multiple models and methods to comprehensively evaluate the neuroprotective capacity of test compounds, including the

polyQ aggregation assay in the AM141 strain, the ASH neuronal survival assay in the HA759 strain, and the chemosensory avoidance assay in the HA759 strain. The protocols presented here have been used to evaluate the neuroprotective capacities of test samples against polyQ toxicity, including inhibitory effects on both polyQ aggregation and associated neurotoxicity[7,14,15,16,17,19,20], demonstrating their potential in drug discovery for HD and other polyQ diseases.

An automated imaging and analysis system is introduced for the detection and counting of polyQ aggregates in the polyQ aggregation assay. This method has the advantages of being high-throughput and time-efficient and results in significantly reduced subjective errors in the laborious counting process. For an entire 384-well plate, it only takes <1 h to finish image acquisition and analysis. However, the conventional microscopic imaging method has also shown similar performance in this laboratory without using the automated imaging device[7].

A total of 100–150 nematodes per treatment are recommended in a typical Q40::YFP aggregation assay for each time point, which can be performed in replicate wells containing 10–15 nematodes each. However, it should be noted that L1 larvae may be more sensitive to some treatments or higher concentrations. Therefore, higher doses of test compounds might inhibit their growth, leading to false-positive results due to slow growth and, thus, delayed polyQ aggregation. Usually, a food clearance assay can be performed to address this concern and ensure the appropriate concentration range of test compounds[23].

The HA759 transgenic nematodes used in polyQ neurotoxicity assays coexpress OSM-10::GFP and Htn-Q150, making it possible to unambiguously identify bilateral ASH sensory neurons. Hence, ASH neuron survival is evaluated by the presence or absence of GFP expression; usually, ~40–75% of ASH neurons in the control nematodes are dead[23,24]. Interestingly, the *pqe-1* (polyglutamine enhancer-1) genetic mutant background in the HA759 strain (*pqe-1;Htn-Q150*) accelerates polyQ-mediated toxicity, leading to the death of most ASH neurons within three days, even at 15 °C, and therefore this strain is grown at 15 °C for the neuronal survival assay, as previously reported[23,24].

Functional loss of ASH neurons in HA759 nematodes may occur before the detection of cell death and protein aggregates[13]; therefore, the osmotic avoidance behavior assay is essential for the assessment of polyQ-mediated toxicity. To minimize the potential impact of less active HA759 nematodes at low temperature on behavioral experiments, the avoidance assay plates are incubated in a humidified 23 °C incubator rather than at 15 °C as in the neuronal survival assay using this strain. In addition, it has been reported that Htn-Q150/OSM-10::GFP transgenic nematodes are highly sensitive to nose touch; hence, an alternative detection of ASH neuron function is the nose touch assay[13].

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

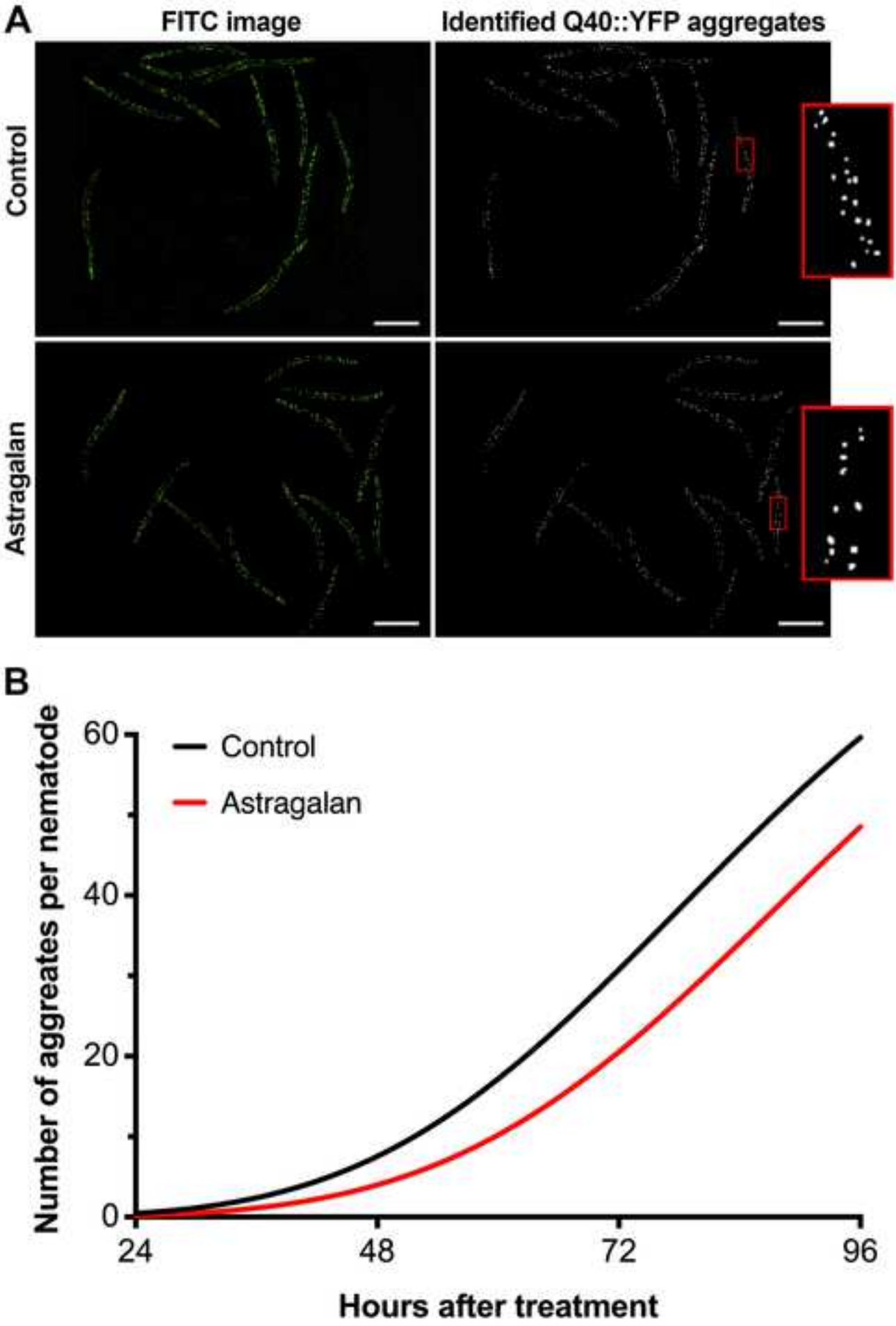


Figure 2

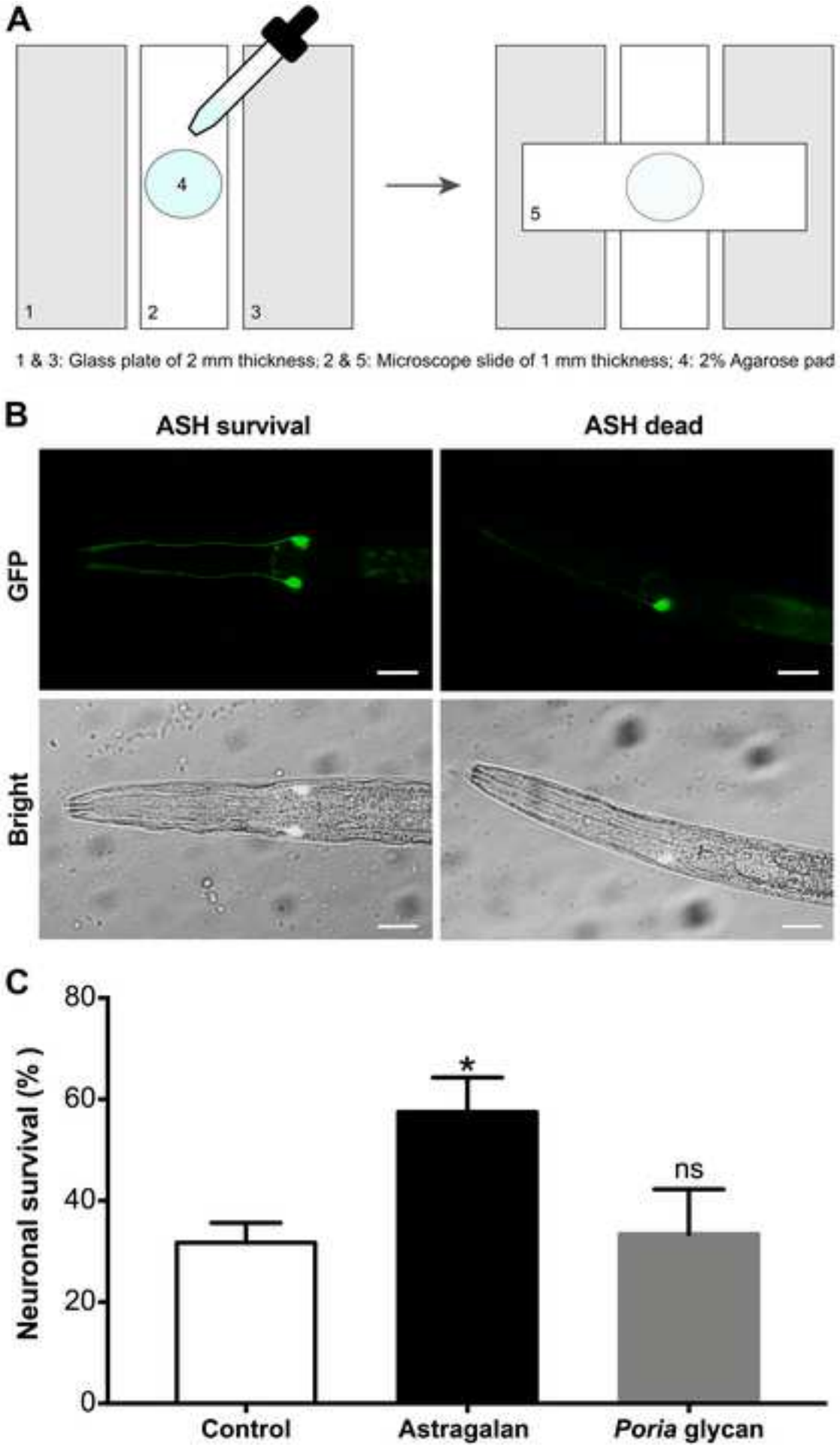
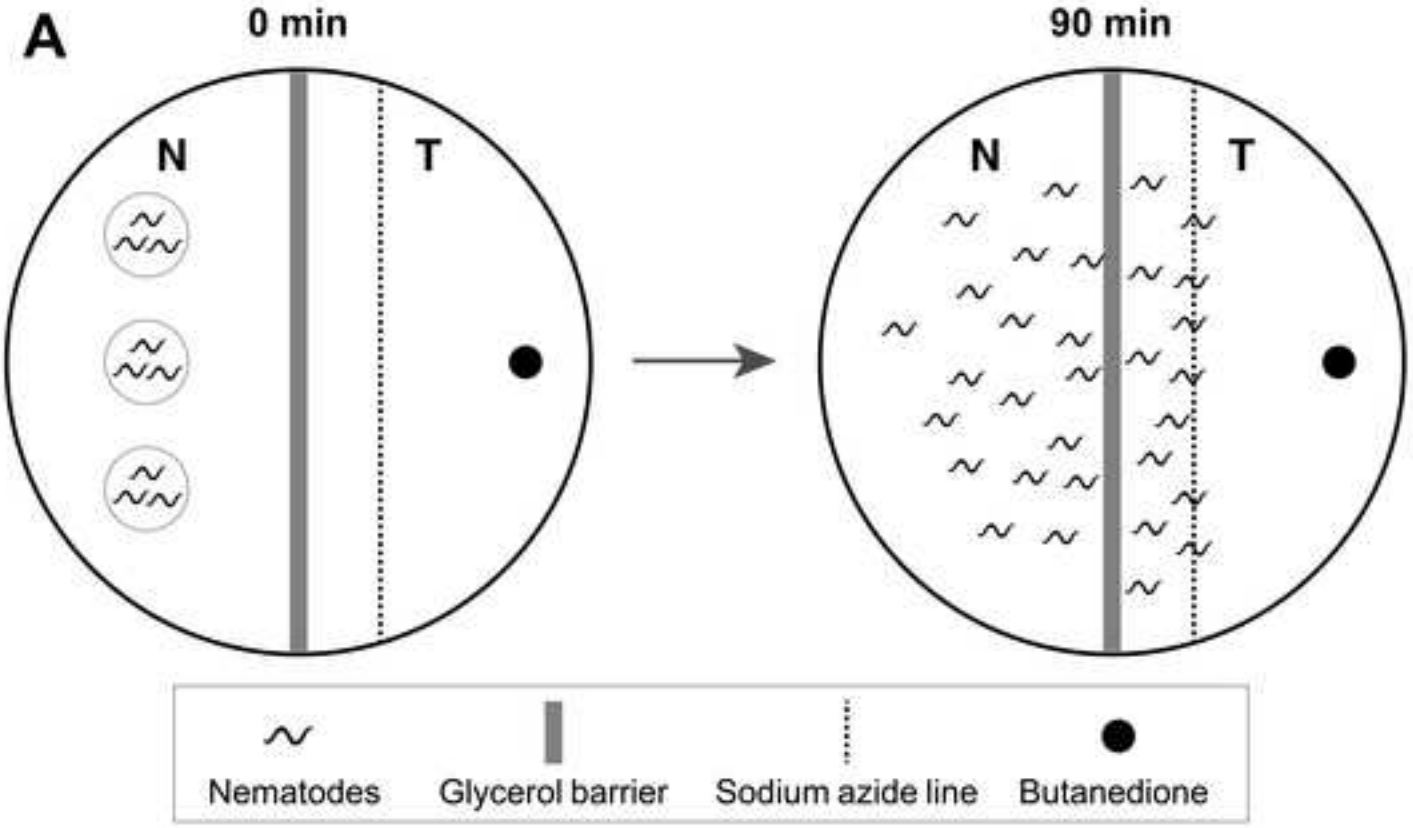


Figure 3



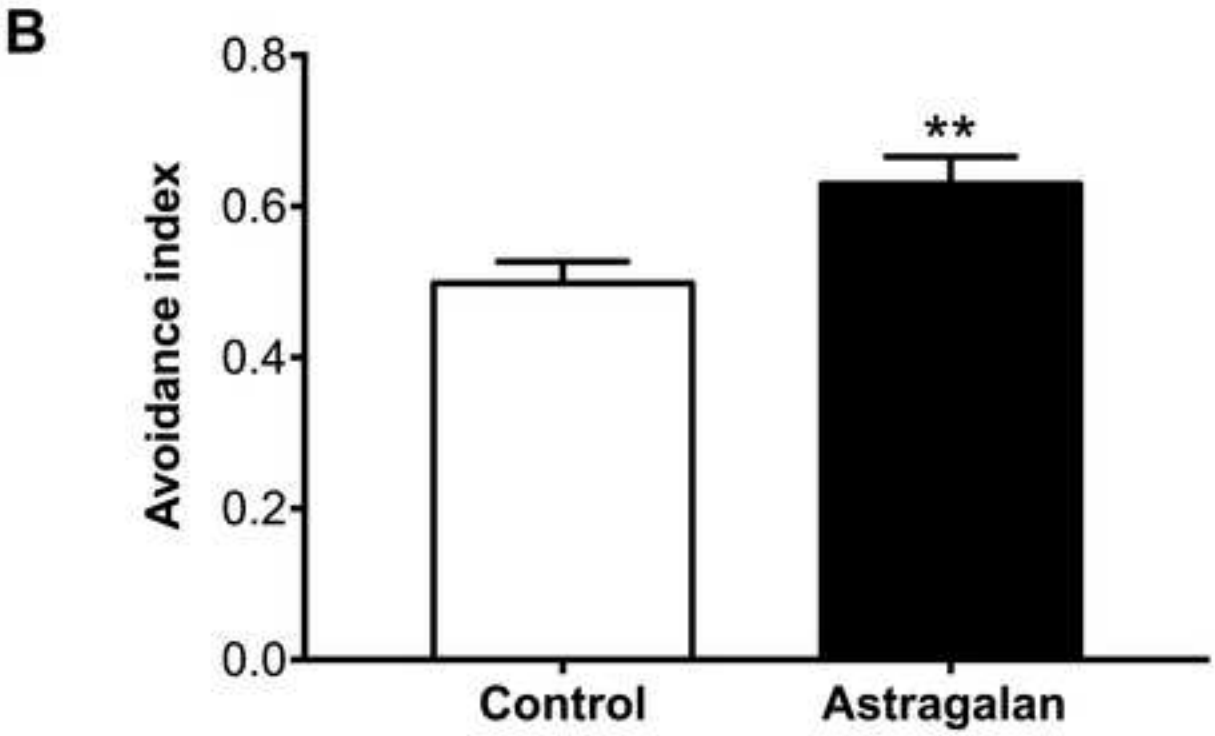


Figure 4

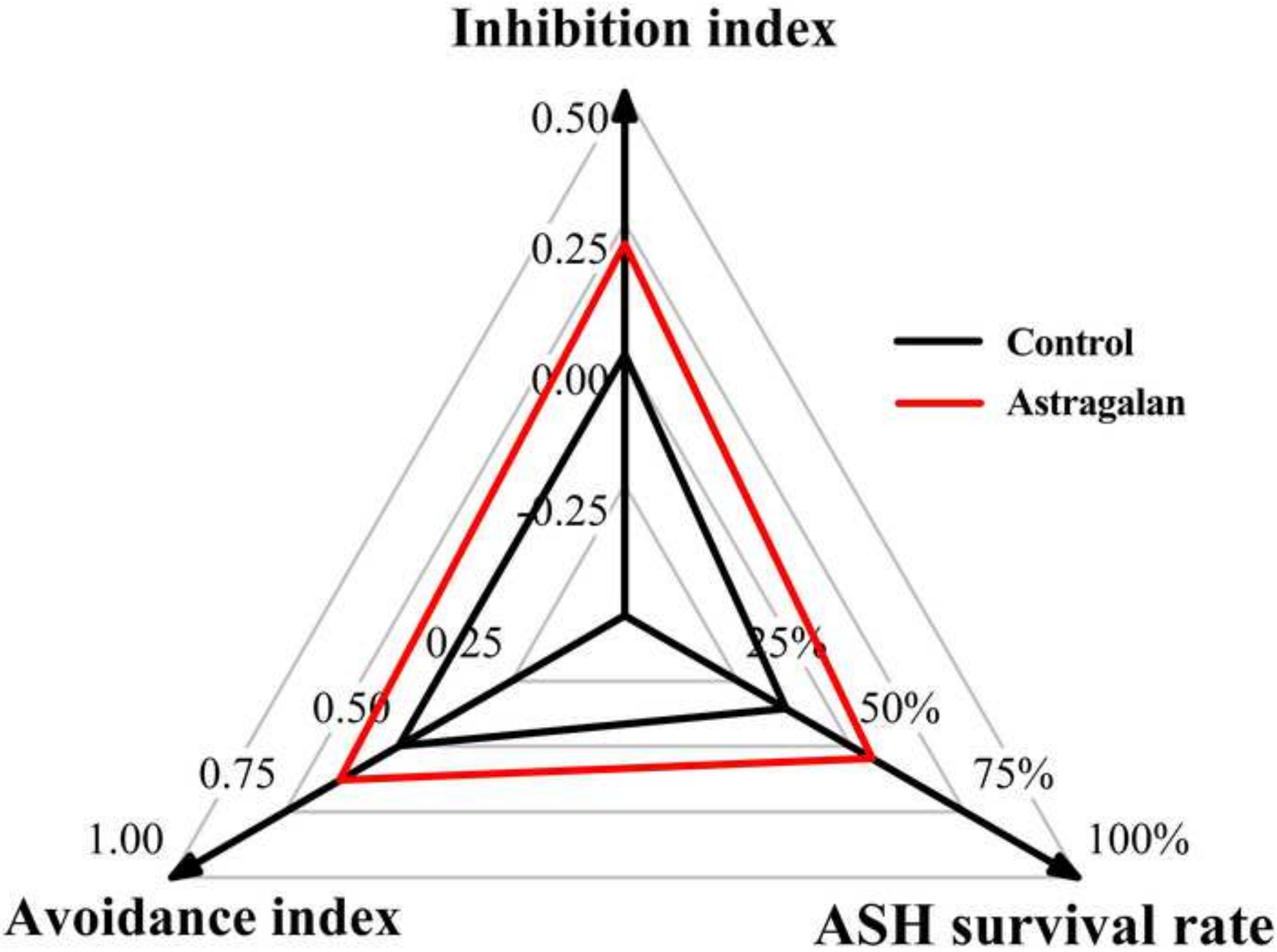


Table 1

Click here to access/download;Table;Table 1 Solution Recipes (1).xlsx

Solution	Preparation instructions	Storage
1 M $CaCl_2$	111 g $CaCl_2$ Add dH_2O to 1 L Autoclave	Store at room temperature (RT)
1 M Potassium phosphate (pH 6.0)	108.3 g KH_2PO_4 35.6 K_2HPO_4 Add dH_2O to 1 L Autoclave	Store at RT
1 M $MgSO_4$	246 g $MgSO_4 \cdot 7H_2O$ Add dH_2O to 1 L Autoclave	Store at RT
Cholesterol in ethanol (5 mg/mL)	0.5 g Cholesterol Add ethanol to 100 mL Do not autoclave!	Store at -20 °C
1 M Potassium citrate (pH 6.0)	Add 10.0 g citric acid monohydrate Add 146.75 g tri-potassium citric acid monohydrate Add dH_2O to 1 L Autoclave	Store at RT
Trace metals solution	0.93 g Disodium EDTA 0.345 g $FeSO_4 \cdot 7H_2O$ 0.1 g $MnCl_2 \cdot 4H_2O$ 0.145 g $ZnSO_4 \cdot 7H_2O$ Add 0.0125 g $CuSO_4 \cdot 5H_2O$ Add dH_2O to 500 mL Autoclave	Store in dark at RT
1 M NaOH	4 g NaOH Add dH_2O to 100 mL	Store at RT
Bleach solution	0.5 mL of 1 M NaOH 0.5 mL of 10% NaOCl	Prepare freshly before use
M9 buffer	5 g NaCl 3 g KH_2PO_4 6 g Na_2HPO_4 Add dH_2O to 1 L Autoclave Add 1 mL of 1 M $MgSO_4$	Store at RT
S basal	5.85 g NaCl 6.0 g KH_2PO_4 1.0 g K_2HPO_4 Add dH_2O to 973 mL Autoclave, and cool to ~60 °C Add 1 mL of 5 mg/mL cholesterol in ethanol	Store at RT
S medium	S basal Add 3 mL of 1 M $CaCl_2$ Add 3 mL of 1 M $MgSO_4$ Add 10 mL of 1 M potassium citrate Add 10 mL of trace metals solution All components have been autoclaved, do not autoclave	Store at RT
5 mg/mL of astragalan	0.1 g astragalan Add 20 mL of S medium Filter through 0.22 µm syringe filter	Store in aliquots at -80 °C
200 mM of sodium azide	1.3 g NaN_3 Add 100 mL of S medium	Store at 4 °C
8 M Glycerol	73.67 g glycerol Add dH_2O to 100 mL	Store at RT
10% Butanedione stock	Mix 10 µL of butanedione with 90 µL of dH_2O	Store at RT
1% Butanedione	Add 10 µL of butanedione stock to 90 µL of dH_2O	Store at RT
1 L of Luria-Bertani (LB) culture	10 g NaCl 10 g Tryptone 5 g Yeast extract Add dH_2O to 1 L	Store at RT

Autoclave

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16 July 2021

Manuscript ID: JoVE63081

Dear Dr. Vidhya Iyer,

Thank you for your email regarding the above manuscript. We are grateful for the critical and helpful reviews made by the editors and reviewers, and have attempted to satisfy the points raised, which are listed as follows.

Sincerely yours,

Zebo Huang Ph.D.
Professor, Institute for Food Nutrition and Human Health

Editorial comments

Comment 1

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have carefully checked the manuscript again and defined all abbreviations.

Comment 2

2. Please provide an email address for each author.

Response: The emails of all authors were provided when the manuscript was submitted to the submission website. They are now also added at the first page of the manuscript (the text in red); if this is not the case, they can be deleted.

Comment 3

3. Please provide a Summary (before the abstract) to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Response: As required, a Summary is added before the Abstract (Lines 19-22).

Comment 4

4. Please revise the following lines to avoid overlap with previously published work: 33-35, 45-49, 66-68, 274-275.

Response: We have modified relevant sentences as required (the text in red: Lines 32-35, 44-47, 63-66, and 290-292).

Comment 5

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: ImageXpress Pico (Molecular Devices, San Jose, CA, USA); MetaXpress software; Mshot MF31-LED fluorescence microscope etc.
Obviously, if you have optimized your protocol with certain instruments/reagents/software, you will need to mention them, but include just the bare minimum information (e.g., name of product) in the manuscript and come up with a generic term to refer to the product (include this in the comments column in the Table of Materials) after the first mention so that you don't keep repeating commercial terms throughout the paper. Comparison of your equipment or software to other commercially available ones is allowed in the discussion, but without unnecessary repetition of these names and only for scientific discussion.

Response: Thanks for the detailed instructions. We have removed all commercial language from the text (Lines 142, 144, 153, 195, and 236 in the current version) and added a table of Materials (Table 2) as suggested.

Comment 6

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have examined each step of the protocols and also added references to published materials when necessary, e.g. Lines 81, 110, 137, 180, 199 and 214 (the text in red).

Comment 7

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: We have formatted the manuscript as instructed. Selected text from the protocols Part 2 and Part 3 (lines highlighted in yellow) can be included in the video section.

Comment 8

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Response: We have revised the Discussion section as instructed, including critical steps, potential limitations, methodological significance and future applications. We have also added citations where necessary.

Comment 9

9. Consider calling the current Table of Materials Table 1; it will contain the composition of different media and buffers.

Response: The title of Table 1 is now changed to "Solution Recipes", which contains the composition of media and buffers as in the first version.

Comment 10

10. Please create another table of essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Response: Table 2 ("Materials") is now added to list strains, reagents, equipment and software used.

Reviewer #1

General Comment

Summary:

The authors described protocols to investigate protective effects of bioactive molecules against PolyQ induced toxicity: PolyQ-aggregation assay (Number of PolyQ aggregates in muscle) and PolyQ-mediated neurotoxicity assays (ASH neuronal survival and Chemosensory avoidance assays). The text is well organised and well described, and will be helpful to the scientific community. I recommend the publication after minor revision.

Response: Many thanks!

Comment 1

Major concerns:

The authors should include in Part 1 the preparation of OP50 bacterial culture.

Response: We have added a step for OP50 preparation in Part 1 as suggested (Lines 82-87).

Comment 2

Line 83 (also in line 181): What was the reference used for temperature conditions of HA759 maintenance? Why 15°C instead of 20°C? I did not find this information in GCG.

Response: Thanks for reminding us this important information. The *pqe-1* (polyglutamine enhancer-1) background in HA759 strain (*pqe-1;Htn-Q150*) accelerates polyQ-mediated toxicity, leading to the death of most ASH neurons within three days. Therefore, this strain is grown at 15°C for neuronal survival assay as previously reported (e.g. Varma et al. 2007; Voisine et al. 2007). We have added references to the protocol (Lines 81 and 180 in the current version) and also briefly discussed this point in Discussion (Lines 326-329).

References:

Varma, H., Cheng, R., Voisine, C., Hart, A. C., Stockwell, B. R. Inhibitors of metabolism rescue cell death in Huntington's disease models. *Proceedings of the National Academy of Sciences of the United States of America*. **104** (36), 14525–14530 (2007).

Voisine, C., Varma, H., Walker, N., Bates, E. A., Stockwell, B. R., Hart, A. C. Identification of potential therapeutic drugs for Huntington's disease using *Caenorhabditis elegans*. *PLoS One*. **2** (6), e504 (2007).

Comment 3

Minor concerns:

Line 58: I suggest change "symptoms" to "features" or similar.

Response: We have changed "symptoms" to "features" as suggested (Line 55 in current

version). Thanks!

Comment 4

Line 100: Please spell EP

Response: We have changed “EP” to “Eppendorf” in the manuscript (text in red: Lines 104, 116 and 130).

Comment 5

Line 123: "for the indicated times". Please indicate the times here.

Response: As suggested, we have modified the text to indicate the times (text in red: Lines 128-129).

Comment 6

Line 137: "Add 20 mM of sodium azide in each well". Is it the final concentration of azide? Or, what is the volume that should be added?

Response: A final concentration of about 20 mM will work here. We have modified the text to clarify the confusion (Line 140). Thanks!

Comment 7

Line 251: Please correct "neurprotective" to "neurOprotective"

Response: We have corrected the mistake (Line 260) and also checked the manuscript for other typo errors. Thanks!

Reviewer #2

General Comment

Manuscript Summary:

This manuscript describes protocols for assessment of neuroprotective capacities of test compounds using transgenic polyQ C. elegans models. The protocols provide detailed assays in terms of polyQ aggregation as well as polyQ-mediated neuronal death and behavioral dysfunction. These details are generally easy to follow and certainly convenient for researchers to screen for anti-polyQ compounds. However, as a method manuscript, I have a few concerns for the authors to address prior to acceptance for publication.

Response: Many thanks! We have attempted to address the concerns as follows.

Comment 1

Major Concerns:

1. Protocol: Part 1, Section 2, Step 5. More details are needed for preparation of OP50 bacterial culture. This is important especially for beginners.

Response: We have added information for preparation of OP50 bacterial culture as suggested (highlighted: Lines 87-92).

Response: We have added a specific step for preparation of OP50 bacterial culture in Part 1 (Lines 82-87) as also suggested by Reviewer 1 (Comment 1). Thanks!

Comment 2

2. Protocol: Part 3, Section 4, Step 6-8. I understand from Step 3, ~200 nematodes are used for each plate, but how many replicate plates usually needed for this chemosensory avoidance assay in each group?

Response: We have added more details to clarify these points (Lines 209 and 216-217).

Comment 3

3. Figure 4: More details are needed for generating the radar chart, e.g. the software used.

Response: To address this point, we have added "Part 4. Creating a radar chart" in the Protocol section (Lines 221-231). The legend of Figure 4 is also modified accordingly (text in red: Lines 295-297). Thanks!

Reviewer #3

General Comment

Manuscript Summary:

C. elegans has proven to be an attractive model organism for prioritizing lead compounds in the early stages of drug development for human diseases. C. elegans possess many characteristics that make it ideal for drug testing including its short lifecycle, small size, ease of culturing in liquid, simple nervous system and plethora of well characterized behavioral assays. In the manuscript entitled "Caenorhabditis elegans as a Model System for Discovering Bioactive Compounds against Polyglutamine-mediated Neurotoxicity", Wang et al. outline multiple protocols to assay the impact of small molecules on polyQ aggregation and polyQ-related neurotoxicity using two different C. elegans models. To assess aggregation, the authors employ a transgenic line expressing 40 glutamines fused to YFP in the body wall muscle (AM141). To evaluate neurotoxicity, the authors use a second transgenic line expressing a

huntingtin protein fragment with 150 glutamines expressed in a subset of neurons (HA759). Using AM141, the authors provide a high through method for counting the number of aggregates in worms treated with the polysaccharide astragalin. Using HA759, the authors describe two protocols for assessing neurotoxicity after treatment with astragalan, one measuring ASH neuronal cell death and a separate protocol for measuring ASH neuron function. This reviewer agrees that protocols for using C. elegans models for drug screening are important for developing treatments for neurodegenerative diseases. However, additional clarification in the steps of the protocols and additional representative datasets are needed before publication.

Response: Many thanks for this general summary! We have modified the text and figures, aiming to clarify the protocols and address the following concerns.

Comment 1

Major Concerns:

1. For the protocol "Effect of astragalan on polyQ40 aggregation", the authors do not indicate whether the data in figure 1A shows the number of aggregates per worm. Please add that detail to the y axis. Also, the authors do not indicate the number of worms that are evaluated for each time point. What is the recommendation for the typical number of worms assayed and measured for the aggregate experiment at each time point? Is it ~500 in control and ~500 with compound?

Response: Thanks for these comments. (1) The data in Figure 1 are indeed the numbers of polyQ aggregates per worm, which is now added in the y axis. (2) The number of worms evaluated for each time point is 100-150 nematodes per treatment, i.e. 10-15 nematodes per well in 10 replicate wells in a 384-well plate (Lines 138-139). This is also the recommendation for a typical independent experiment, i.e. ~10 replicate wells at each time point and >100 nematodes in total for a treatment (text in red in Discussion: Lines 315-317). To clarify, we have modified the text in Protocol (text in red: Lines 124-127) and in Figure 1 legend (text in red: Lines 276-278) in addition to the text in Discussion. (3) To ensure the 10 replicate wells (100-150 nematodes in total) in the 384-well plates of Step 5 (Lines 138-139), the number of nematodes prepared in the 48-well plates of Step 1 (Lines 124-127) is no less than 300. We usually transfer up to 500 nematodes for ease of handling. (4) To better understand the experimental logic and images, we have now swapped panel A and B of Figure 1.

Comment 2

2. In figure 1B, the representative images are not helpful. It is difficult to see any changes in the number of aggregates over time. Please add magnified views of the images as insets within the figures for clarity. In addition, it would be important to include images of the AM141 strain with and without drug treatment. This reviewer is unclear if the representative images are on

AM141 or AM141 treated with the compound. Finally, a statistical analysis should be performed on the dataset.

Response: We have revised this figure as suggested, which is now Figure 1A in the current version as explained in the response to Comment 1. Specifically, images with and without drug treatment, together with insets showing magnified views of the aggregates, are now included. A description of data analysis is added in the figure legend (text in red: Lines 276-278).

Comment 3

3. In figure 2, the authors provide images representing a worm with two intact ASH neurons and an image with one ASH neuron demonstrating neuronal cell death. In addition to these images, the authors should provide a representative set of data quantifying ASH neuronal survival with and without the compound. As the title indicates "Effect of astragalan on Htn-Q150-mediated ASH neuronal death", this reviewer was anticipating a dataset demonstrating the neuroprotective effect of astragalan along with a statistical analysis.

Response: We agree with the reviewer and thus a representative result is now provided in Figure 2 C, which also includes *Poria cocos* glycan as a control in addition to astragalan. A description of data analysis is added in the figure legend (text in red: Lines 283-288).

Comment 4

4. In figure 3, it is unclear if the graph includes data from one osmotic avoidance assay with multiple technical replicates or three independent biological replicates to generate the standard deviation. Please indicate the recommended approach in the "Part 3: PolyQ-mediated neurotoxicity assays 4. Chemosensory avoidance assay" portion of the manuscript. To this point, this reviewer recommends changing the title of this section to "Osmotic avoidance assay"

Response: The results are from three replicate plates, representative of more than three independent experiments. To clarify this point, we have revised the text in Lines 209 and 216-217 (text in red). The title of Section 4 in Part 3 is changed to “Osmotic avoidance assay” (Line 204) as suggested. Thanks!

Comment 5

5. In figure 4, the authors do not provide a protocol or reference for building a radar chart to evaluate compounds. If readers are interested in integrating the three datasets into a radar chart, information on how to generate this presentation format would be helpful.

Response: Thanks for this suggestion. We have added “Part 4. Creating a radar chart” in the Protocol section (Lines 221-231) to address this concern. Relevant information is also provided in the table of Materials (Table 2), which is newly added in the current version.