CHAPTER

1 Assessing polyglutamine tract aggregation in the nematode *Caenorhabditis elegans*

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Chapter outline

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Abstract

Proteome integrity is a prerequisite for cellular functionality and organismal viability. Its compromise is considered an inherent part of the aging process and has been associated with the onset of age-related, neurodegenerative pathologies. Although the molecular underpinnings of protein homeostasis (proteostasis) have been extensively studied, several aspects of its regulation remain elusive. The nematode *Caenorhabditis elegans* has emerged as a versatile, heterologous model organism to study the dynamics of aggregation-prone human proteins *in vivo*. Here, we describe an experimental pipeline for the analysis of polyglutamine (polyQ) tract aggregation, as a measure of the state of proteostasis, during aging.

Abbreviations

APS	ammonium persulfate
DRPLA	dentatorubral-pallidoluysian atrophy
DTT	dithiothreitol
HD	Huntington's disease
LB	Luria-Bertani
NGM	nematode growth media
PN	proteostasis network
polyQ	polyglutamine
Q	glutamine
ROI	region of interest
RT	room temperature
SBMA	spinal and bulbar muscular atrophy
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TBS-T	tris-buffered saline supplemented with Tween-20
TEMED	tetramethylethylenediamine
WLB	worm lysis buffer

1 Introduction

Proteome integrity is constantly undermined by extrinsic stressful insults, as well as endogenous by-products of cellular metabolism. A significant fraction of the proteome across the evolutionary spectrum is dedicated to proteostasis surveillance

(Muller et al., 2020), underlining the paramount importance of maintaining a healthy protein repertoire for preserving cellular, tissue, and organismal homeostasis. The proteostasis network (PN), comprised of an elaborate array of chaperones and proteolytic degradation machineries coordinated by transcriptional stress response pathways, safeguards proteome integrity (Higuchi-Sanabria et al., 2018; Hipp, Kasturi, & Hartl, 2019). Of particular note, the competence of the PN declines with aging, contributing to the manifestation of late-onset proteinopathies (David et al., 2010; Lopez-Otin et al., 2013). Hence, decelerating PN decline has emerged as a promising strategy to relieve the elderly of the deleterious consequences of proteotoxicity.

The pathogenic expansion of the CAG trinucleotide, coding for glutamine (Q), underlies the manifestation of nine neurodegenerative disorders, including Huntington's disease (HD), spinal and bulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA) and six autosomal dominant forms of spinocerebellar ataxia (SCA1, 2, 3, 6, 7, and 17). All polyglutamine (polyQ) disorders are characterized by similar clinical and pathological outcomes, albeit the affected genes do not share genetic or functional homology (Lieberman, Shakkottai, & Albin, 2019). Mutant proteins harboring elongated polyQ tracts become unstable, undergoing conformational changes that render them prone to aggregation. The transition from native monomers to intermediate oligomers and eventually insoluble aggregates has been described *in vitro* and confirmed *in vivo* (Minakawa & Nagai, 2021). Aggregation-prone proteins implicated in polyQ disorders are widely expressed throughout the body, but degeneration mainly occurs in specific brain regions. Accumulating evidence suggests that peripheral cells, such as cardiomyocytes, skeletal muscles, gastrointestinal cells and hepatocytes, may also accumulate polyQ aggregates, thus contributing to pathology (Huang et al., 2019; Sathasivam et al., 1999; Singh & Agrawal, 2021).

A multitude of studies using invertebrate and vertebrate animal models have indicated that overexpression of an extended polyQ tract is sufficient to induce neuronal degeneration *in vivo* (*C. elegans*: (Morley et al., 2002), *Drosophila*: (Marsh et al., 2000), zebrafish: (Miller et al., 2005) and mice: (Ordway et al., 1997)). Animal models are instrumental for deciphering the regulatory mechanisms that govern polyQ homeostasis on the organismal level and testing potential therapeutic strategies to alleviate protein aggregation. During the last two decades, polyQ tracts of different lengths have been successfully expressed in various *C. elegans* tissues (Brignull et al., 2006; Gidalevitz et al., 2006; Morley et al., 2002; Moronetti Mazzeo et al., 2012; Sinnige et al., 2021). With particular interest for this review, Moronetti Mazzeo and colleagues generated a transgenic strain where the expression of a fluorescently-tagged Q44 stretch is driven by the intestine-specific promoter *vha-6* ($p_{vha\text{-}6}$Q44::YFP) (Moronetti Mazzeo et al., 2012). Intestinal Q44::YFP was reported to aggregate during aging or upon administration of osmotic stress (Moronetti Mazzeo et al., 2012). Another report also documented a role of macroautophagy, a central self-degradation cellular process, in preserving Q44::YFP homeostasis (Kumsta et al., 2017).

Here, we thoroughly describe an experimental procedure for assessing protein aggregation of the Q44::YFP transgene in *C. elegans*. Specifically, we report an

in vivo imaging method to visualize and quantify Q44::YFP foci number and size during aging. Additionally, we analyze a biochemical protocol based on sodium dodecyl sulfate (SDS) protein solubility and Western blot analysis to estimate the insoluble vs. soluble Q44::YFP fraction. We anticipate that this protocol, appropriately modified or adjusted, can be leveraged for monitoring the dynamics of various aggregation-prone proteins, implicated in human pathology, in *C. elegans*.

2 Materials

2.1 Disposables

1. Microscope slides ($25.4 \times 76.2 \times 1$ mm)
2. Microscope coverslips (18×18 mm)
3. Fingernail polish for sealing the coverslips
4. Standard platinum wire pick: for transferring the worms to NGM plates
5. Eyelash hair attached to a toothpick: for transferring the worms on agarose pads
6. Petri plates (60×15 mm) for the maintenance of *C. elegans* nematodes
7. Petri plates (92×16 mm) for bacterial growth
8. Agarose pads: Add 0.5 g agarose into 25 mL distilled water (H_2O). Heat the solution until the agarose is completely dissolved. While the agarose is still hot, leave a droplet (approximately 1 cm in diameter) in the middle of a microscope slide. Immediately place a second slide on the top of it, and press it until the agarose droplet is flattened to a bigger circle. Wait for about 2 min and carefully remove the second slide, trying not to disturb the agarose droplet. The agarose pad is ready for use in about 5 min.
9. Micropipettes (1–20, 20–200, 200–1000 μL) and tips
10. 1.5 mL microcentrifuge tubes
11. 50 mL plastic conical tubes
12. Glass conical flasks for bacterial cultures
13. 96-well microplates with flat bottom for protein quantification
14. Filter paper (e.g., Whatman® Filter paper)
15. Nitrocellulose membrane (e.g., Amersham Protran Western blotting membrane 0.2 μm)
16. 0.5 mm zirconium oxide beads (e.g., Next Advance)

2.2 Equipment

1. Stereomicroscope (e.g., Nikon SMZ 745)
2. Fluorescent microscope (e.g., Zeiss AxioImager Z2 epifluorescence microscope)
3. Standard tabletop centrifuge
4. Refrigerated centrifuge
5. Microplate absorbance reader (e.g., Spark® Multimode Microplate Reader, Tecan) for protein quantification
6. Tissue homogenizer (e.g., Bullet Blender, Next Advance)

7. Power supply, vertical electrophoresis system and blotting systems (e.g., Mini-PROTEAN® Tetra Vertical Electrophoresis Cell, 4-gel, for 1.0 mm thick handcast gels, with Mini Trans-Blot® Module, Bio-Rad)
8. Laboratory shaker or rocker
9. Chemiluminescence developing system (e.g., Chemidoc Imaging System, Bio-Rad)

2.3 Nematode strains

1. N2: *C. elegans* wild isolate
2. OG412: *drIs20* [$p_{vha\text{-}6}$Q44::YFP + *rol-6(su1006)* + pBluescript II]

2.4 Nematode food

OP50: Uracil auxotroph *E. coli* bacteria. For liquid OP50 bacterial culture, inoculate a single OP50 colony (grown overnight on an LB agar plate) into 50 mL of sterile liquid LB medium. Incubate for 4–5 h in a shaking incubator at 37 °C. Seed 200 µL of the OP50 culture in the middle of freshly prepared NGM plates, and let them grow overnight at room temperature (RT) (for approximately 16 h).

2.5 Reagents

2.5.1 Reagents for bacterial cultures

1. Luria-Bertani (LB) liquid medium: For the preparation of 1 L liquid LB medium, dissolve 10 g sodium chloride (NaCl), 10 g Tryptone and 5 g yeast extract in 900 mL distilled H_2O. Stir the mix until it is completely dissolved. Adjust the pH to 7.0 with sodium hydroxide (NaOH), and bring the volume to 1 L with H_2O. Sterilize by autoclaving.
2. LB solid medium: For the preparation of 1 L solid LB, mix the ingredients of the aforementioned recipe for liquid LB with 15 g agar in 700 mL distilled H_2O. The powder will not dissolve completely, as agar is only dissolved after autoclaving. Adjust the pH to 7.0 with NaOH and sterilize by autoclaving. Bring the volume to 1 L with sterile H_2O and when the medium is cooled enough (approximately 50 °C), dispense it to petri plates [approximately 18 mL per plate (92 × 16 mm)].

2.5.2 Reagents for nematode growth and maintenance

1. Nematode Growth Media (NGM): To prepare 1 L of NGM, dissolve 3 g NaCl, 2.5 g bactopeptone, 17 g agar and 0.2 g streptomycin in 800 mL distilled H_2O. Sterilize by autoclaving. After the solution has cooled down (approximately 50 °C), add 1 mL of 1 M calcium chloride ($CaCl_2$), 1 mL of 1 M magnesium sulfate ($MgSO_4$), 25 mL of 1 M potassium phosphate (KPO_4), 1 mL of cholesterol (5 mg/mL in 100% ethanol) and 1 mL Nystatin (10 mg/mL in 70% ethanol). All the solutions, except for cholesterol and Nystatin, need to be sterilized by autoclaving. Bring the volume to 1 L with sterile H_2O, and after 5-10 min of stirring, dispense the solution to petri plates (7 mL per 60 × 15 mm plate).

2. M9 Minimal Medium Buffer: Dissolve 3 g monopotassium phosphate (KH_2PO_4), 6 g disodium phosphate (Na_2HPO_4) and 5 g NaCl in 800 mL distilled H_2O. Sterilize by autoclaving. After the solution has cooled down, add 1 mL of sterile 1 M magnesium sulfate ($MgSO_4$), and bring the volume to 1 L with distilled water.
3. Bleaching solution for synchronizing nematode population: To prepare 10 mL of bleaching solution, add 1 mL of 5 N NaOH and 2 mL of 5% sodium hypochlorite (NaOCl) in 7 mL distilled H_2O.
4. Tetramisole for anesthetizing the nematodes: To prepare 100 mM stock tetramisole solution, dissolve 0.24 g tetramisole hydrochloride powder in 10 mL M9 buffer. Keep it as a stock solution at 4 °C. For the working solution, dilute the stock in M9 at a final concentration of 20 mM and keep it at RT.

2.5.3 Reagents for the biochemical detection of polyQ aggregates

1. Worm Lysis Buffer (WLB): To prepare 10 mL WLB mix 200 µL of 1 M Tris-Cl pH 7.4 stock solution (20 mM final concentration), 200 µL of 1 M NaCl stock solution (20 mM final concentration) and 10 µL of 1 M $MgCl_2$ stock solution (1 mM final concentration). Bring the volume to 10 mL with distilled H_2O and keep it at RT.
2. Urea Lysis Buffer: To prepare 1 mL Urea Lysis Buffer, add 0.48 g Urea (8 M final concentration), 200 µL 10% (*w*/*v*) SDS buffer (2% (*w*/*v*) final concentration), 25 µL of 1 M Tris-Cl pH 8.0 stock solution (50 mM final concentration) and 25 µL of 1 M dithiothreitol (DTT) stock solution (50 mM final concentration). Bring the volume to 1 mL with distilled H_2O and keep it at RT to avoid SDS precipitation. Add the DTT immediately before use.
3. Protease inhibitors (e.g., cOmplete™, Mini Protease Inhibitor Cocktail, Roche).
4. 6× sample buffer for SDS PAGE protein sample preparation: To prepare 10 mL solution, mix 3.75 mL of 1 M Tris-Cl pH 6.8 stock solution (375 mM final concentration), 1.2 g SDS (12% (*w*/*v*) final concentration), 6 mL glycerol (60% (*v*/*v*) final concentration), 6 mg bromophenol blue (0.06% (*w*/*v*) final concentration) and 120 µL β-mercaptoethanol (12% (*v*/*v*) final concentration). Warm the solution at 40 °C to avoid the formation of SDS precipitates. Bring the final volume to 10 mL with distilled H_2O. Aliquote per 1 mL and store at 20 °C. Avoid freeze-thaw cycles. Once thawed, a vial is stable for several weeks at RT.
5. 10% (*w*/*v*) SDS: Dissolve 5 g SDS in 50 mL distilled H_2O. Store at RT.
6. Acrylamide-Bisacrylamide 37.5:1 mix (e.g., Acrylamide 4 K solution 30% (*w*/*v*), AppliChem).
7. 10% (*w*/*v*) APS: Dissolve 5 g ammonium persulfate (APS) in 50 mL distilled H_2O. Aliquote and store at −20 °C.
8. Tetramethylethylenediamine (TEMED).
9. 10% SDS polyacrylamide resolving gel: In a plastic conical tube mix 1.5 mL of 1.5 M Tris-Cl pH 8.8 stock solution, 2 mL 30% Acrylamide-Bisacrylamide mix, 2.5 mL distilled H_2O, 60 µL 10%(*w*/*v*) SDS. Vortex briefly. Add 60 µL 10%

(*w*/*v*) APS and 6 µL TEMED immediately before casting the gel, as the addition of APS and TEMED will initiate the polymerization of acrylamide.

10. 5% Stacking gel: In a plastic conical tube mix 0.75 mL of 0.5 M Tris-Cl pH 6.8 stock solution, 0.45 mL 30% Acrylamide-Bisacrylamide mix, 1.77 mL distilled H_2O, 30 µL 10% SDS. Vortex briefly. Add 30 µL 10% APS (*w*/*v*) and 3 µL TEMED immediately before casting the gel.
11. 10× Running buffer: For 1 L add 30 g Tris-Base (250 mM final concentration), 144 g glycine (1.92 M final concentration) and 10 g SDS (1%(*w*/*v*) final concentration) in 1 L distilled H_2O. Store at RT and dilute 1:10 in distilled H_2O to prepare the 1× working solution.
12. 1× Transfer buffer: For 2 L dissolve 6 g Tris-Base (25 mM final concentration) and 28.8 g glycine (192 mM final concentration) in 1.6 L distilled H_2O. Stir until both chemicals are dissolved. Then add 400 mL methanol (20% (*v*/v) final concentration) and store at 4 °C until use. Transfer buffer can be reused for at least two times.
13. 10× Tris-Buffered Saline (TBS) pH 7.6: For 1 L add 24 g Tris-base (200 mM final concentration) and 88 g NaCl (1.5 M final concentration) in 900 mL distilled H_2O. Adjust pH to 7.6 with 12 N HCl and bring the volume to 1 L with distilled H_2O.
14. 1× TBS supplemented with 0.1% Tween-20 (TBS-T): To prepare 1 L add 100 mL 10× TBS in 900 mL distilled H_2O. Then use a tip with a cut end to add 1 mL Tween-20. Stir until the detergent is dissolved.
15. Ponceau S solution: To prepare 100 mL solution, dissolve 0.5 g Ponceau S in 5 mL acetic acid. Bring the final volume to 100 mL with distilled H_2O. The solution can be reused multiple times.
16. 5% Blocking buffer: Dissolve 2 g non-fat milk powder in 40 mL 1× TBS-T.
17. Antibodies: Primary antibodies: anti-GFP, anti-α-tubulin and secondary HRP-conjugated antibodies.
18. Chemiluminescence detection kit (e.g., Pierce™ ECL Western blotting Substrate, Thermo Scientific).
19. Protein quantification kit (e.g., Pierce™ BCA Protein Assay Kit, Thermo Scientific).

3 Methods

3.1 Microscopic analysis for monitoring polyQ aggregation

3.1.1 Nematode preparation for imaging in the microscope

1. Transfer five L4-staged nematodes of the OG412 strain in two OP50-seeded NGM plates and incubate them at 20 °C.
2. After about 5 days, the plates will be full of gravid adult hermaphrodites. Wash them with 1.5 mL of M9 buffer and collect the liquid in a sterile 1.5 mL microcentrifuge tube. Allow the animals to settle by gravity for 2 min and discard the liquid. Add 500 µL of freshly-made bleaching solution. Shake by hand or

vortex the solution for a few seconds. Repeat shaking/vortexing every 2 min until the worm bodies are completely dissolved. Do not bleach for more than 5 min, as the resulting eggs may not hatch. Spin the tube in a tabletop centrifuge (~3000 rpm) for 30 s to pellet the eggs. Discard the supernatant and wash the egg pellet with 500 μL sterile M9 buffer. Centrifuge once more using the same conditions and resuspend the egg pellet in 100 μL sterile M9 buffer. Dispense the egg solution to OP50-seeded plates. Allow the animals to grow at 20 °C.

3. After about 3.5 days, the plates will be full of young day 1 adult animals. Use at least 25–30 worms for microscopic observation (day 1 sample). Transfer adequate number of animals to new OP50-seeded plates (25–30 animals per plate) and incubate them at 20 °C. The following days use 25–30 animals for microscopic observation (day 3, 4, 5, 6, 7, 8, 10 samples). Ensure to transfer the animals to new OP50-seeded plates when needed to prevent bacteria depletion.
4. Place a 5 μL drop of 20 mM tetramisole in the middle of a freshly made agarose pad.
5. Transfer worms to the droplet using an eyelash hair attached to a toothpick.
6. Cover with a coverslip and seal with fingernail polish, to prevent animals drying out during the imaging process.
7. Observe the worms under a fluorescent microscope at 10× magnification and acquire images of whole animal bodies. Ignore animals that seem to be damaged (mainly due to mishandling during preparation). Ensure that all images are acquired by using the same settings (i.e., exposure time, brightness, contrast) and include a scale bar.

3.1.2 Image analysis for quantification and characterization of polyQ aggregates using the FIJI software

1. Open the first image in FIJI and with the "Straight" tool draw a line of the same length with the scale bar. From the "Analyze" menu select "Set scale." In the dialog box enter the "Known distance" based on the scale bar and select the appropriate unit of length. Select the "Global" function, in order to automatically apply the calibration to the rest of the images.
2. In the opened image select the whole animal body by using a selection tool and add the selected Region of Interest (ROI) in the ROI Manager (Edit→Selection→Add to Manager). Repeat for all the animals in the image.
3. When all ROIs are added in the ROI manager, from the "Image" menu select "Color" and "Split channels." Keep only the image of the green channel.
4. If necessary, from the "Process" menu select "Subtract background" and in the opened dialog box set the appropriate "Rolling ball radius" to remove the background noise.
5. From the "Image" menu select "Adjust" and "Threshold." In the opened dialog box set the second bar at the maximum level (255), adjust the upper bar at a level where all the puncta are visible (we usually set it at 70, but it depends on the image acquisition settings) and press "Apply."

6. For each ROI run the "Analyze particles" command from the "Analyze" menu. In the opened dialog box, in the "Show" menu select "Outlines" and ensure that you have ticked "Display results," "Summarize" and "Exclude on edges."
7. Repeat steps 2–7 for all images. Save the "Results" data, containing information regarding the puncta size and the "Summary" data, containing information regarding particle number per worm.
8. Perform the statistical analysis using Microsoft Excel or Graphpad Prism softwares.

Once image processing parameters (i.e., subtract background setting and threshold values) are standardized to accurately select puncta, a macro may be used to automate the analysis of Q44::YFP particles in selected ROIs. Using the macro shown in Fig. 1, we analyze the puncta number and size in images containing multiple worms. For the automated analysis, calibrate the scale as described in step 1 and modify the macro command in order to appropriately select the rolling ball radius and threshold values (macro lines 11, 14). For each image, add all ROIs of the picture in the ROI manager and run the macro. The macro generates a dialog to select a working space on the computer, for example, the desktop. All subsequent steps are performed automatically and the "Results" data and "Summary" data are produced. Using the aforementioned analysis, we show that the mean number and size of Q44::YFP aggregates significantly increase during aging (Fig. 2A–C).

20220930_macro for particle analysis.ijm

```
1  dir1 = getDirectory("Choose Source Directory ");
2  N=roiManager("count")-1;
3  dir2=dir1+"Image.tif";
4  saveAs("Tiff", dir2);
5  run("Split Channels");
6  selectWindow("Image.tif (red)");
7  close();
8  selectWindow("Image.tif (blue)");
9  close();
10 selectWindow("Image.tif (green)");
11 run("Subtract Background...", "rolling=100");// set your own rolling value
12 setAutoThreshold("Default");
13 run("Threshold...");
14 setThreshold(70, 255); //set your own threshold
15 setOption("BlackBackground", false);
16 run("Convert to Mask");
17 run("Analyze Particles...", "  show=Nothing display exclude summarize");
18 selectWindow("Image.tif (green)");
19 for (i = 0; i < N; i++)
20 {
21 selectWindow("Image.tif (green)");
22 roiManager("Select", i);
23 run("Analyze Particles...", "show=Nothing display exclude summarize");
24 }
25 selectWindow("Image.tif (green)");
26 close();
```

FIG. 1

Overview of the semi-automated analysis for estimating the number and size of Q44::YFP foci.

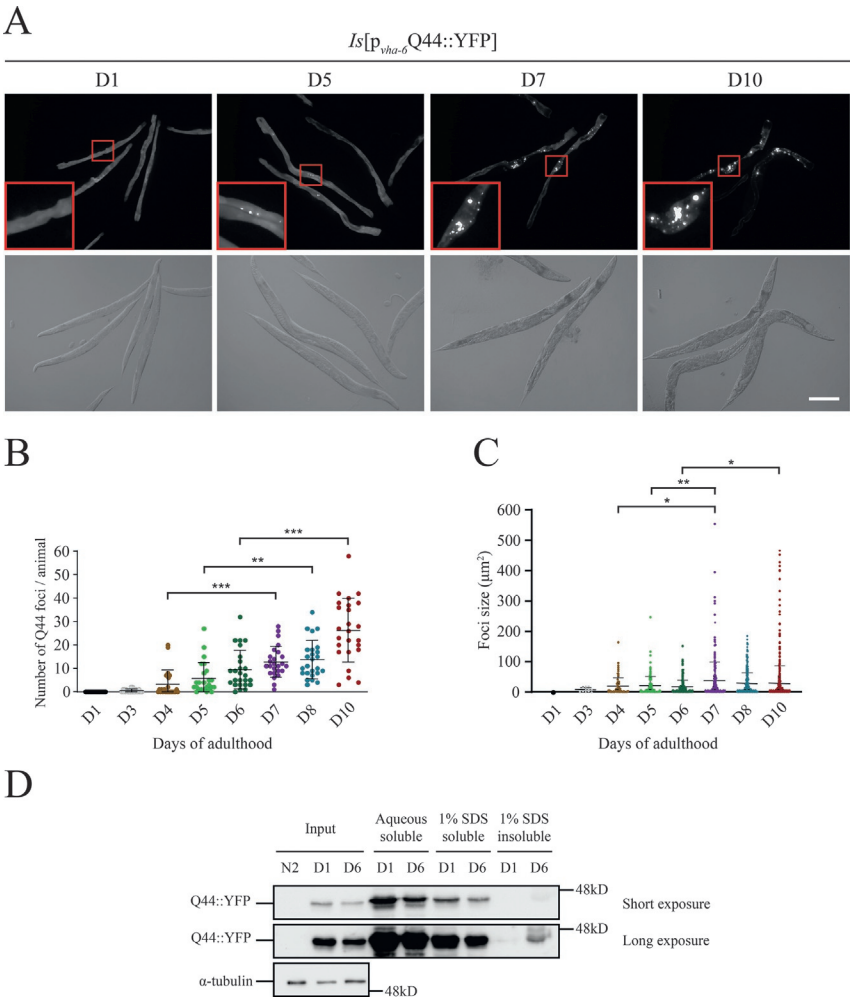

FIG. 2

Microscopic analysis combined with SDS protein solubility assay for monitoring Q44::YFP dynamics during *C. elegans* lifespan. (A) Longitudinal analysis of Q44::YFP aggregation in wild-type animals. Red inboxes highlight representative intestinal regions. Scale bar, 200 μm. (B) Quantification of intestinal Q44::YFP foci number per animal in different days of adulthood ($N = 25$ animals; $^{***}p < 0.001$, $^{**}p < 0.01$; one-way ANOVA; error bars, SD). (C) Quantification of Q44::YFP foci size (μm^2) in different days of adulthood (N = 25 animals; $^{**}p < 0.01$, $^{*}p < 0.05$; one-way ANOVA; error bars, SD). (D) Western blot against Q44::YFP and α-tubulin proteins. 1 μg of N2 and OG412 lysates were used as inputs to validate equal protein content between samples and ensure antibody specificity against the Q44::YFP protein. A prominent band at approximately 40 kD corresponds to the Q44::YFP protein. α-tubulin was used as a loading control.

3.2 Biochemical analysis of polyQ aggregation during aging

3.2.1 Nematode preparation for biochemical analysis of polyQ aggregation

1. Following the instructions described in Section 3.1.1 (steps 1–3), prepare at least 100 day 1 adult animals of the OG412 strain and collect them in a microcentrifuge tube containing 1 mL M9 buffer. Allow the animals to settle by gravity for 2 min and discard the liquid. Wash the worm pellet twice with M9, flash-freeze it in liquid nitrogen and store it at −80 °C. Follow the same procedure to obtain additional time-point samples.
2. In parallel prepare 100 day 1 adult animals of the wild-type (N2) strain following the instructions described above and store the worm pellet in −80 °C. N2 lysate will be used as control sample, to distinguish Q44::YFP specific bands.

3.2.2 SDS solubility assay

1. Thaw worm pellets on ice (4 °C) and aspirate the supernatant liquid if needed.
2. Estimate the volume of each worm pellet (approximately 20–30 µL) and add an equal volume of 0.5 mm zirconium oxide beads to each microcentrifuge tube.
3. Add at least 2 volumes of WLB supplemented with 1× protease inhibitors (WLB + PI) to each microcentrifuge tube. Due to the small volume of the worm pellets (20–30 µL), we recommend adding 100 µL WLB + PI for efficient homogenization.
4. Perform the homogenization using a tissue homogenizer at 4 °C. For the Bullet Blender (Next Advance) adjust the following settings: Speed→10, Time→3 min
5. Visually inspect the lysates to ensure complete homogenization. If necessary repeat the homogenization process once using the same settings.
6. Leave the beads to settle by gravity and transfer the lysates to new microcentrifuge tubes.
7. Determine protein concentration of each sample by using a protein quantification kit (e.g., Pierce™ BCA Protein Assay Kit, Invitrogen).
8. Dilute all samples with WLB + PI, in order to achieve equal concentration. Keep 1 µg of each sample as input and use an equal amount of each sample (we used 25 µg) to proceed.
9. Centrifuge at 17000 g for 15 min at 4 °C.
10. Without disturbing the pellets, transfer the supernatants to new microcentrifuge tubes, and store them on ice as the "aqueous-soluble protein fractions."
11. Resuspend the pellets in 50 µL WLB + PI supplemented with 1% (*w*/*v*) SDS. Care must be taken to use the exact same volume of buffer in each sample, to ensure the quantitative nature of analysis.
12. Centrifuge at 17000 g for 15 min at RT to avoid SDS precipitation.
13. Transfer the supernatants to new microcentrifuge tubes and store them at RT as the "SDS-soluble protein fractions."
14. Wash the pellets with 100 µL WLB + PI supplemented with 1% (*w*/*v*) SDS.
15. Centrifuge at 17000 g for 15 min at RT. Discard the supernatants.

16. Resuspend the pellets in 25 µL of freshly-made Urea lysis buffer and incubate at RT for 10 min. Care must be taken to use the exact same volume of buffer in each sample, to ensure the quantitative nature of analysis.
17. Dilute Urea-treated samples with 25 µL WLB + PI supplemented with 1% (*w*/*v*) SDS and store them as the "SDS-insoluble protein fractions."
18. Add a suitable volume of 6× sample buffer to all samples in order to achieve a final concentration of 1× and boil the samples at 95 °C for 5 min.

3.2.3 Characterization of Q44::YFP protein solubility during aging by Western blot

1. Assemble the glass plate and spacer in the gel casting apparatus following the manufacturer's guidelines. Prepare 6 mL of 10% SDS-polyacrylamide resolving gel, as described in Section 2.5.3. Add approximately 4.5 mL of the resolving gel and 1 mL of isopropanol on top. Save the remaining quantity of the resolving gel in the conical tube to check for gel polymerization. In parallel prepare the stacking gel solution, without adding the polymerization factors (APS and TEMED). When the resolving gel is polymerized, remove isopropanol, and wash with dH_2O. Add the polymerization factors to the stacking gel solution, and pour it on top of the resolving gel, until the gel plate is filled. Insert the comb to create the wells and allow polymerization to occur.
2. Once the gel is polymerized, remove the comb and place the gel in the electrophoresis tank, according to the manufacturer's instructions. Fill the tank with 1× Running buffer. Use one of the wells to load a pre-stained protein marker, in order to distinguish protein molecular weights. Load the samples and perform the electrophoresis at 70 V for 15 min and then at 100–120 V until the bromophenol blue front reaches the end of the gel. Because there is no loading control for different fractions, it is important to load in the gel the exact same quantity among samples for every fraction, in order to perform a quantitative analysis.
3. Once the electrophoresis is completed proceed to the wet transfer. Assemble the transfer cassette containing the sponges, filter papers, nitrocellulose membrane and gel, according to the manufacturer's instructions and place it into the transfer apparatus filled with ice-cold 1× Transfer buffer including a cooling unit. Perform the transfer at 100 V for 1 h.
4. Once the transfer is completed you may optionally perform Ponceau-S staining of the membrane, to ensure that proteins are properly transferred. To this end, incubate the membrane with Ponceau-S solution for a few minutes in a Tupperware on a shaker. Then wash the membrane using distilled H_2O or TBS-T, until the protein bands are detectable. Perform additional washes to completely de-stain the Ponceau-S stain.
5. Incubate the membrane with 5% Blocking buffer on a shaker for 1 h at RT.
6. Dilute the primary antibody (anti-GFP) in 5% Blocking buffer, according to the data sheet and incubate the membrane with the diluted antibody overnight at 4 °C on a shaker.

7. The following day wash the membrane three times with TBS-T for 10 min on a shaker at RT.
8. Incubate the membrane with the suitable HRP-conjugated secondary antibody, diluted in 5% Blocking buffer according to the datasheet for 1 h at RT on a shaker.
9. Wash the membrane three times with TBS-T.
10. Add the chemiluminescence substrate on the membrane and develop the membrane signal using a Chemiluminescence developing system to detect Q44::YFP.
11. Wash the membrane three times with TBS-T and repeat steps 6–10 for the detection of α-tubulin. By using anti-GFP and anti-α-tubulin primary antibodies generated in different species (e.g., rabbit and mouse), you may use the same nitrocellulose membrane to detect both proteins. To this end, add 2% (*w*/*v*) sodium azide (NaN_3) in the dilution of anti-α-tubulin antibody, which will inhibit the HRP activity of the secondary antibody used for GFP detection. Otherwise, a second gel may be prepared and used to directly detect α-tubulin.

Our biochemical analysis reveals that aged day 6 adult animals show increased levels of SDS-insoluble Q44::YFP compared to young day 1 adults, while the total levels of Q44::YFP protein are not changed (Fig. 2D). Inversely, the levels of aqueous and SDS-soluble Q44::YFP protein decrease in aged nematodes, indicating that Q44::YFP transitions from a soluble to an aggregated state during aging. The enrichment of SDS-insoluble fraction correlates with the elevated number and size of Q44::YFP aggregates during aging (Figs. 2B and C).

4 Concluding remarks

In the current protocol, we describe two complementary approaches to assess Q44::YFP dynamics in *C. elegans*. Fluorescent imaging followed by the semi-automated analysis of foci number and size can serve as an accurate estimate of Q44::YFP aggregation status. When combined with SDS protein solubility assay and subsequent Western blot analysis, it can highlight differences in the ratio of soluble vs. insoluble (aggregated) Q44::YFP. We suggest that a similar protocol, carefully adjusted to the needs of each individual experiment, can be utilized to assess the dynamics of polyQ tracts of variable length expressed in different nematode tissues (muscles, neurons, etc.). Monitoring polyQ aggregation under various experimental conditions can provide ample insight into the molecular mechanisms that underpin proteostasis and unveil therapeutic interventions that can decelerate its decline during aging.

5 Notes

1. Animals should be reared under well-fed conditions for at least three generations before initiating the experiment. Starvation may have a direct impact on proteostasis, thereby affecting Q44::YFP aggregation.

2. Our experience using the OG412 strain has shown that intestine-specific *vha-6* expression is significantly elevated when nematodes are fed with previously UV-crosslinked, dead OP50 bacteria. We can not exclude the possibility that the quality of bacterial food (i.e., different standard bacterial strains used for nematode maintenance) may have a direct impact on Q44::YFP total levels or dynamics. Hence, in every experimental setup including the appropriate controls should always be a priority.
3. The length of the polyQ tract strongly affects its aggregation propensity. Moreover, we have observed tissue-specific differences, even when the length of the polyQ tract is comparable. For example, the AM141 CGC strain expressing Q40::YFP under a muscle-specific promoter exhibits entirely focal fluorescence, even from day 1 of adulthood. This can be attributed to the relative transgene overexpression levels (driven by different tissue-specific promoters), but also to inherent differences in polyQ proteostasis among *C. elegans* tissues.

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Author contributions

A.S, C.P. and N.C. prepared the original draft of the manuscript. A.S., C.P., N.C. and N.T. revised and edited the manuscript. All authors consent to the published version of the manuscript.

Conflicts of interest

The authors have no conflict of interest to declare.

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