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Live Animal Imaging and Cell Sorting Methods for Investigating Neurodegeneration in a C. elegans Excitotoxic Necrosis Model

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TITLE:

Live Animal Imaging and Cell Sorting Methods for Investigating Neurodegeneration in a *C. elegans* Excitotoxic Necrosis Model

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KEYWORDS:

Excitotoxicity, glutamate, neurodegeneration, regulated necrosis, neuroprotection, *C. elegans*, transcription, mitochondria, microscopy, FACS

SUMMARY:

In a *C. elegans* excitotoxicity model, this protocol employs in vivo imaging to analyze the regulation of necrotic neurodegeneration, the effect of genes encoding candidate mediators, and involvement of mitochondria. Cell dissociation and sorting is used to specifically obtain at-risk neurons for cell-specific transcriptomic analysis of neurodegeneration and neuroprotection mechanisms.

ABSTRACT:

Excitotoxic necrosis is a leading form of neurodegeneration. This process of regulated necrosis is triggered by the synaptic accumulation of the neurotransmitter glutamate, and the excessive stimulation of its postsynaptic receptors. However, information on the subsequent molecular events that culminate in the distinct neuronal swelling morphology of this type of neurodegeneration is lacking. Other aspects, such as changes in specific subcellular compartments, or the basis for the differential cellular vulnerability of distinct neuronal subtypes, remain under-explored. Furthermore, a range of factors that come into play in studies that use in vitro or ex vivo preparations might modify and distort the natural progression of this form of neurodegeneration. It is therefore important to study excitotoxic necrosis in live animals by monitoring the effects of interventions that regulate the extent of neuronal necrosis in the genetically amenable and transparent model system of the nematode *Caenorhabditis elegans*. This protocol describes methods of studying excitotoxic necrosis in *C. elegans* neurons,

combining optical, genetic, and molecular analysis. To induce excitotoxic conditions in *C. elegans*, a knockout of a glutamate transporter gene (*glt-3*) is combined with a neuronal sensitizing genetic background (*nuls5* [*Pglr-1::GαS(Q227L)*]) to produce glutamate receptor hyperstimulation and neurodegeneration. Nomarski differential interference contrast (DIC), fluorescent, and confocal microscopy in live animals are methods used to quantify neurodegeneration, follow subcellular localization of fluorescently labeled proteins, and quantify mitochondrial morphology in the degenerating neurons. Neuronal Fluorescence Activated Cell Sorting (FACS) is used to distinctly sort at-risk neurons for cell-type specific transcriptomic analysis of neurodegeneration. A combination of live imaging and FACS methods as well as the benefits of the *C. elegans* model organism allow researchers to leverage this system to obtain reproducible data with a large sample size. Insights from these assays could translate to novel targets for therapeutic intervention in neurodegenerative diseases.

INTRODUCTION:

Excitotoxicity is the leading cause of neuronal death in brain ischemia and a contributing factor in multiple neurodegenerative diseases[1-9]. Disruption of oxygenated blood flow to the brain (e.g., due to a blood clot) results in the malfunction of glutamate transporters, leading to accumulation of glutamate in the synapse. This excess of glutamate over-activates post-synaptic Glutamate Receptors (GluRs) leading to an excessive (catalytic, non-stoichiometric) influx of Ca^{2+} into neurons (**Figure 1A**). This detrimental influx leads to progressive postsynaptic neurodegeneration that morphologically and mechanistically ranges from apoptosis to regulated necrosis[10-12]. Although they were based on successful interventions in animal models, multiple clinical trials of GluR antagonists that sought to block Ca^{2+} entry and promote cell viability have failed in the clinical setting[13-16]. A likely critical contributor to these failures is the fact that (in contrast to the animal models) treatment in the clinical setting is administered hours after stroke onset, causing the intervention to block late-acting neuroprotective mechanisms, while failing to interrupt degenerative signaling downstream of GluRs[14,16,17]. An alternative approach, which is based on thrombolysis, can only be administered within a severely restricted time window, leaving many patients (who suffer stroke at home with poorly identifiable time of onset) unable to benefit from it[17]. These setbacks emphasize the need to focus excitotoxicity research on the study of events occurring after GluR hyper-stimulation and differentiate subsequent degenerative cascades from concurrent neuroprotective processes. This approach can help prevent cell damage and identify efficient drug targets that can be administered later after damage onset.

One approach to identify subsequent events in excitotoxicity is to study the cell-death signaling mechanisms downstream of GluR hyperstimulation, such as those leading to mitochondrial collapse. Drastic malfunction of mitochondrial physiology and dynamics is a hallmark of neurodegeneration, as seen in excitotoxicity[18-20]. While all cells depend on mitochondrial function and availability for survival, activity, and cellular maintenance, neurons are particularly dependent on mitochondrial energy production to support signal transmission and propagation. Specifically, neurons spend ~50% of their signaling-related energy consumption to restore resting membrane potential following the activation of postsynaptic receptors/channels[21], with high dependence on oxygen and glucose. The reduced availability of glucose and oxygen observed in stroke leads to serious mitochondrial alterations, causing further reduction in ATP

production[19,22-24]. However, studies to identify the sequence of events that lead to mitochondrial collapse produced controversial results and lacked consensus. Analyzing mitochondrial morphology can help understand these events leading to mitochondrial pathology since it is a good indicator of neuronal health[25-29]. Filamentous mitochondria are representative of a healthy neuron, whereas fragmented mitochondria reveal substantial neuronal damage that could lead to cell death. Analyzing mitochondrial morphology in live animals under different genetic conditions can help focus on specific genes and pathways involved in mitochondrial-dependent neurodegeneration in excitotoxicity.

Another approach to identifying subsequent events that might regulate the extent of excitotoxic neurodegeneration is to study the transcriptional neuroprotective mechanisms that mitigate some of the effects of excitotoxicity[14,16]. However, the lack of specificity of key neuroprotective transcription factors and the divergence of experimental setups impede the success of efforts to clearly identify core neuroprotective programs (especially in regulated necrosis).

Therefore, both the study of downstream death signaling pathways and the study of transcriptional neuroprotection in excitotoxicity encountered great difficulties and disagreements on observed outcomes. Much of this controversy is likely to arise from the use of ex vivo or in vitro models of excitotoxicity, and the variability introduced by the specificity of different experimental setups. It is therefore highly beneficial to focus on identifying core mechanisms that are highly conserved, and study them in vivo. The simple model system of the nematode *C. elegans* offers a particularly effective option, due to the potent combination of particularly powerful and diversified research tools, the conservation of core cell-death pathways, and the rich information on the structure and connectivity of its nervous system[30-33]. Indeed, the seminal work of the Driscoll lab on the genetic analysis of necrotic neurodegeneration in mechanosensory neurons is an excellent demonstration of the power of this approach[34]. Importantly for the analysis of excitotoxicity, the conservation of signaling pathways in the nematode includes all major components of glutamatergic neurotransmission[35,36].

The nematode excitotoxicity model builds on these seminal studies, allowing the researcher to study biochemical processes akin to those that occur in stroke and other neurodegenerative diseases affected by glutamate-dependent neurotoxicity. To induce excitotoxic conditions in *C. elegans* this experimental approach uses an excitotoxicity strain that is the combination of a knockout of a glutamate transporter gene (*glt-3*) and neuronal sensitizing genetic background (*nuls5* [*Pglr-1::GαS(Q227L)*]) to produce GluR hyperstimulation and neurodegeneration[37]. This excitotoxicity strain exposes 30 specific (*glr-1* –expressing) neurons that are postsynaptic to glutamatergic connections to excitotoxic neurodegeneration. Of these 30 at-risk neurons, individual neurons go through necrosis as the animal progresses through development (with mixed stochasticity and partial preference towards certain specific neurons[38]), while at the same time cell corpses are also being gradually removed. In combination with the accessibility of many mutant strains, this approach allows the study of multiple pathways that affect neurodegeneration and neuroprotection. These approaches have already been used to analyze some of the downstream death signaling cascades[39] and transcriptional regulators of excitotoxic

neurodegeneration in excitotoxicity[38,40]. Like other cases of necrotic neurodegeneration in the worm[41], the nematode's excitotoxic neurodegeneration does not involve classic apoptosis[40].

This methods paper describes the basic system to induce, quantify, and manipulate excitotoxic necrotic neurodegeneration in *C. elegans.* Furthermore, it outlines two main protocols that are currently in use to streamline studies of specific aspects of nematode excitotoxicity. By using fluorescent reporters and live in vivo imaging the researcher can study mitochondrial involvement and dynamics in the nematode model of excitotoxic neurodegeneration. To determine the effect of specific neuroprotective transcription factors, the investigator can use cell type-specific expression of fluorescent markers, dissociation of animals into single cells, and FACS to isolate specific neurons that are at risk of necrosis from excitotoxicity. These cell-type specific isolated neurons can then be used for RNA sequencing in strains that harbor mutations in key transcription factors. Put together, these methods can allow researchers to tease out the molecular underpinnings of excitotoxic neurodegeneration and neuroprotection in vivo with great clarity and precision.

PROTOCOL:

1. Strains used to Investigate Excitotoxic Neurodegeneration & Neuroprotection

1.1. Use the nematode excitotoxicity strain ZB1102 as the reference point for standard excitotoxic neurodegeneration.

NOTE: Glutamate-dependent excitotoxicity in *C. elegans* is produced in strain ZB1102 by combining a knockout (*ko)* of a glutamate transporter with a transgene that sensitizes neurons in these animals to neurotoxicity and is expressed in a subset of neurons postsynaptic to glutamatergic connections[37]. This genetic combination is referred to as the nematode excitotoxicity strain, and it is freely available from the *Caenorhabditis* Genetics Center (CGC).

1.2. To study the effect of genes encoding candidate regulators of excitotoxic necrosis, combine a mutation in such genes (e.g., *dapk-1*, or *crh-1*) with the nematode excitotoxicity strain.

1.3. Conduct genetic crosses according to standard *C. elegans* methods[30], as outlined in wormbook.org[42,43].

1.4. Since the molecular basis of the mutation is typically documented, follow the cross progeny by genotyping the specific locus using PCR. Differentiate WT vs mutant by fragment size (for deletions) or sequencing (for point mutations).

NOTE: **Table 1** outlines strains that were used to study distinct pathways of neurodegeneration and neuroprotection, specifically for this protocol.

1.5. Derive all critical strains by two independent lines and test them separately to confirm the validity of the observed phenotype.

2. Growth Media and Animal Husbandry

2.1. Grow worms at 16-25 °C on either standard NGM plates[30,37,42] or MYOB plates[38,44] seeded with OP50 *E.coli* as per standard methods.

NOTE: MYOB plates give identical results as NGM plates but are slightly easier to prepare.

2.2. Maintain the experimental strains consistently well fed.

NOTE: Starvation can affect neurodegeneration and reduce the number of dying head neurons. If worms are poorly fed or starved, plate them onto fresh plates and wait a few generations before continuing experiments. Transgenerational effect of starvation will wane after a few generations.

3. Quantification of degenerating head neurons by Nomarski differential interference contrast (DIC) and scoring

3.1. Use the nematode excitotoxicity strain (ZB1102: *glt-3(bz34) IV; nuIs5 V*) as the experimental control for quantification, representing normal excitotoxicity levels. The protocol does not follow the same animal through different developmental stages. Instead, it gives a snapshot of a mixed-stage population of animals, so that in total one collects information from different animals to represent all developmental stages.

NOTE: The *nuIs5* transgene [*Pglr-1::GαS(Q227L). Pglr-1::GFP*][45] (which expresses an activated Gαs and GFP in neurons postsynaptic to glutamatergic connections) produces a background GluR-independent necrotic neurodegeneration level of ~1 dying head neuron/animal (at any given time during development). The addition of *glt-3 (ko)* increases necrotic neurodegeneration of the postsynaptic *nuIs5*-expressing neurons in a GluR-dependent manner, going up to 4-5 dying head neurons/animal in the third larval stage (L3)[37].

3.2. For the test strains, use animals from a recently completed genetic cross, or thaw strains of interest from -80 °C frozen stocks prepared from fresh crosses. Wait a few generations (≥4) before scoring a phenotype and neurodegeneration.

3.3. Cut and remove a small piece of agar from a plate of a mixed-stage population of well-fed animals, and mount it onto a coverslip by flipping the agar chunk over, so that the animals that were crawling on the surface of the agar now face the coverslip.

NOTE: Animals can still move, but are now somewhat restrained and can be observed without anesthetic. Such a chunk can be used for ~1 hour before replacing it with a fresh one.

3.4. Using an inverted DIC scope with x10 ocular and either x40 or x63 objective, scan animals at random by sliding the coverslip manually. While other imaging steps described below can be

conducted on either inverted or upright microscopes, the examination of worms in the agar chunk requires an inverted scope.

3.5. Identify the developmental stage of each animals by the shape of its uterus (refer to uterus diagrams in wormbook.org).

3.6. For each animal, record its developmental stage and the number of dying (i.e., vacuolated) neurons. Count and record the total number of dying neurons in the head, the number of dying neurons in the retrovesicular ganglion (which gives easy identification of specific cells), and the number of dying neurons in the tail (which is unaffected by glutamate and serves as an internal control, confirming that the sensitizing transgene *nuIs5* is fully active).

3.7. Record this data in a table where animals from a given strain are grouped by categories of developmental stage.

3.8. In each session, collect data at random from worms of several developmental stages; perform several scoring sessions across multiple days to collect enough data for statistical analysis. Record neurodegeneration levels at multiple stages and construct a bar graph similar to **Figure 1C**.

NOTE: This method will allow one to determine if a certain treatment or mutation raises/decreases neurodegeneration levels at all stages (shifting the distribution up/down) or shifts the peak in neurodegeneration to an earlier or later developmental stage (shifting the distribution left/right).

3.9. Perform data collection while blinded to the identity of the genotype. Confirm in two independent isolates of the genetic line (to minimize the danger of unintended effects of other/unexpected genetic differences between isolates), and pool data for analysis.

3.10. In each strain, calculate the average and SEM of the number of degenerating neurons in the head (including the retrovesicular ganglion) in each developmental stage (**Figure 1D**). Perform a similar analysis of dying retrovesicular ganglion –only and tail neurons, as necessary.

4. Identification of specific degenerating head neurons

4.1. Mount individual (or small groups of-) animals on an agar pad[46], and paralyze the worm with tetramisole (see below, section 5).

4.2. With a combined fluorescence and DIC scope (upright or inverted) locate specific vacuolated neurons.

4.3. Determine the specific neuron identity of the vacuolated neuron by tracking its GFP labeled processes and comparing the location of the cell body and shape of the processes to those of the neurons known to expresses *glr-1* using WormAtlas[47].

NOTE: Alternatively, identification can be assisted by new methods to quickly identify neurons by multi-color labeling that are coming soon from the Hobert lab[48].

5. Live imaging of neuronal mitochondrial morphology by fluorescent microscopy of reporter strains

5.1. For imaging of mitochondrial changes in degenerating postsynaptic neurons (which are labeled with cytoplasmic GFP in the original excitotoxicity strain) examine mito-mCherry fluorescence.

5.2. Cross the test excitotoxicity strain with strains that label mitochondria of postsynaptic neurons with red fluorescence (by expressing a fusion between the fluorescent protein and the N-terminal of TOM-20 under *glr-1* promoter; for details on constructs and strains, see[49]). Animals can now be imaged using a regular epifluorescence microscope (upright or inverted) or a confocal microscope.

5.3. To paralyze the worm without affecting neuronal survival, pipette 5 µL of 10 mM tetramisole onto a freshly prepared agarose pad. See Arnold et al.[46] for detailed protocol on pad preparation.

5.4. Place animals onto the center of the tetramisole drop and mount with a coverslip.

5.5. Seal the sides of the coverslip with nail polish and allow the nail polish to dry.

5.6. Within 20 minutes of tetramisole treatment and using a scope with DIC and fluorescence imaging, locate worms using a 20x objective lens.

5.7. Once the head of the worm is located (or neurons of interest) move to a 100x oil objective and focus on the fluorescence labeling of the mitochondria in the soma.

5.8. Capture Z-stack images using DIC, GFP, and TxRed filter settings.

6. Neuronal mitochondria morphology scoring & quantification

6.1. Analyze mitochondrial morphology either during live imaging of the worm or after image acquisition using ImageJ or other imaging software.

6.2. For easy identification and repeated analysis of specific neurons, identify the three neurons in the retrovesicular ganglion, RIGL/R and AVG (in some cases only two are present due to cell corpse clearance in excitotoxicity).

6.3. Categorize mitochondria into three main groups: filamentous, intermediate, and fragmented[50-52].

NOTE: Filamentous mitochondria appear as continuous thin structures in the soma of neurons, usually surrounding the nucleus. Intermediate mitochondria appear as a combination of at least one apparent filamentous network, albeit with breaks, and some fragmentation in the soma. Fragmented mitochondria exhibit complete breaks in the mitochondrial network, have swollen appearance, and are scattered throughout the soma (**Figure 2A**).

6.4. For each worm, score the percent of neurons with filamentous, intermediate, or fragmented mitochondria for a total of at least 30 worms.

6.5. Perform statistical analysis using one-way ANOVA followed by post hoc Tukey's test for each mitochondrial morphology between strains[53].

7. Buffer and reagent preparation for worm dissociation for FACS of neurons at risk of neurodegeneration

7.1. Prepare M9 buffer as follows: 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, H_2O to 1 L. Sterilize by autoclaving. Add 1 mL of filter-sterilized 1 M $MgSO_4$. Store at room temperature.

7.2. Prepare Egg buffer as follows: 118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$; autoclave to sterilize. Add 2 M of HEPES pH 7.3 stock solution (previously filtered with a 0.2 μm bottle top filter) to a final concentration of 25 mM. Adjust pH to 7.3 with 1 N NaOH (no more than 10 mL). Use osmometer to ensure the final osmolarity is between 335 -345 mOsm. Filter sterilize egg buffer with 0.2 μm bottle top filters. Store in 4 °C.

NOTE: The salts dissolve easier when using $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ to make respective $MgCl_2$ and $CaCl_2$ stock solutions. You can also make a stock of 10x egg buffer and dilute it when needed in sterile deionized water.

7.3. Prepare SDS-DTT as follows: 20 mM HEPES pH 8.0, 0.25% SDS, 200 mM DTT, 3% sucrose. In a tissue culture hood, sterilize SDS-DTT with 0.2 μm syringe filter. Store 300 μL aliquots in -20 °C covered in foil to protect from light.

7.4. Prepare pronase solution as follows: prepare the day of the dissociation 15 mg/mL pronase in egg buffer. Store on ice.

8. Age Synchronization for neuron specific FACS

8.1. Use animals that combine the excitotoxicity genotype (and other mutations, as desired) with transgenic expression of a strong fluorescent marker that can be readily used for sorting (e.g., FJ1244: *pzIs29 [Pglr-1::NLS::LAC-Z::GFP::glr-1 3'UTR] X*)[54]. Grow animals on two or three 100mm NGM/MYOB plates at 20 °C until the plate is full of predominantly gravid worms.

8.2. Wash worms off the plates using M9 buffer. Transfer worms into 50 mL conical tubes using a glass 10 mL serological pipette.

8.3. Centrifuge at room temperature for 2.5 min at 250x *g* and remove the supernatant.

8.4. Resuspend the worm pellet in 10 mL of bleach solution (2% 10 N NaOH and 5% fresh household bleach in sterile deionized water).

8.5. Place tubes on a shaker horizontally, rock at low speed. Ensure that the worms do not settle to the bottom of the tube. The bleach cracks open the worm's cuticle but does not affect embryos, which are protected by the egg shell.

8.5.1. This bleaching step takes approximately 5 minutes, but this varies. To ensure over-bleaching does not occur, monitor the process by retrieving a sample every minute: Gently pipette 10 µL from each tube on a glass microscope slide and examine the worms using a dissecting microscope.

8.6. Once most of the gravid worms are cracked open but not completely dissolved, stop the bleaching step by filling the conical tubes with egg buffer.

8.7. To retrieve the eggs/embryos, centrifuge the tubes at 250x *g* for 2.5 min, remove the supernatant and wash again with egg buffer.

8.8. Repeat four more washes.

8.9. Resuspend the eggs by gently pipetting the pellet and spread on four 100 mm seeded NGM/MYOB plates. Let embryos hatch and grow at 20 °C for 3 days.

NOTE: The plates should now be full of predominantly gravid worms.

8.10. Repeat the age synchronization by repeating this bleach/age synchronization protocol.

8.11. Pipette the eggs onto eight 100 mm NGM/MYOB plates. Let animal hatch and grow at 20 °C until at the desired larval stage.

9. **Whole worm cell dissociation for FACS**

9.1. Grow synchronized worms to the desired developmental stage. Gently wash off 100 mm plates with M9 buffer and glass serological pipettes and transfer to 50 mL conical tubes.

9.2. Add cold M9 up to 45 mL. Place the tubes on ice for 30 minutes to allow the worms to settle at the bottom by gravity, while any residual bacterial debris from the plates will float in the supernatant.

9.3. Remove the supernatant and wash the worms with fresh M9.

9.4. Repeat the 30 minute gravity settling on ice.

9.5. Remove the supernatant, add M9 up to 45mL, and centrifuge at 250 x *g* for 5 minutes.

9.6. Transfer the pellet to a microcentrifuge tube and add M9 up to 1 mL.

9.7. Spin at 14,000 rpm on a tabletop centrifuge for 1 min and remove supernatant.

9.8. To disrupt the cuticle, resuspend the worm pellet with SDS-DTT using (approximately) twice the volume of the pellet, and incubate with rocking at room temperature for 4 minutes for L2-adult stages.

NOTE: Do not exceed 4 minutes incubation in SDS-DTT because fluorescent protein signal will drastically decrease with longer SDS-DTT treatment. Since the SDS-DTT is light sensitive it should not be more than 3 months old because the solution may have lost potency with time; Dissociations work best with recently -prepared SDS-DTT solution.

9.9. Add 1x egg buffer (pH 7.3, 335-345 mOsm) up to 1 mL to stop the SDS-DTT treatment.

9.10. Spin at 14,000 rpm on a tabletop centrifuge for 1 minute and remove supernatant.

9.11. To further disrupt the cuticle and dissociate the animals' cells, resuspend pellet with room temperature pronase solution, using triple the volume of the pellet.

9.12. Incubate at room temperature for 15-30 min.

9.13. Pipette up and down 40 times every 5 min. with a P-200 or P-1000 pipette, touching the bottom of the tube with the pipette tip.

NOTE: The pressure created by the tip touching the bottom of the tube facilitates worm dissociation. Use a filter tip so the worms do not accidently enter the pipette shaft during rapid pipetting.

9.14. After 15 min, check the progress of the worm dissociation by gently pipetting 5µL on a glass slide and observing the progress under a dissecting microscope. When most (~90%) of the intact worms have burst, the process is complete.

NOTE: Pronase incubation can be extended if many worms remain intact.

9.15. In a cell culture hood, add 1x egg buffer up to 1.5 mL to stop the pronase treatment.

9.16. Transfer to FACS tubes, add 5mL egg buffer, and spin at 800 x *g* for 5 min.

9.17. Remove supernatant and resuspend in 3 mL pf egg buffer.

9.18. Place a 70 µm cell strainer cap on new FACS tubes, pipette cell suspension on strainer, and spin at 800 x *g* for 1 min. Collect the efflux cell suspension.

9.19. Place a 5 µm cell strainer cap on new FACS tubes, pipette cell suspension on strainer, and spin at 800 x *g* for 1 min.

9.20. Add DAPI for a final concentration of 0.5 µg/mL in egg buffer, place on ice with a lid/cover to protect from light.

9.21. Perform FACS as soon as possible. Fluorescent protein signal decreases with time and light exposure, therefore it is important to perform dissociation protocol as quickly as possible and perform FACS immediately after the dissociation is complete.

NOTE: Worm dissociation protocol was adapted from protocols from the Miller lab[55-57], the Murphy lab[58], and the Shaham lab[59].

10. FACS machine operation modifications for identification of *C. elegans* neurons

10.1. Use 4 liters of chilled (4 °C) egg buffer instead of standard sheath fluid when sorting *C. elegans* neurons.

NOTE: The osmolarity of *C. elegans* cells is much higher than mammalian cells and standard sheath fluid will burst the neurons. All of the machine settings and calibrations are done after the egg buffer is added, because the viscosity of the egg buffer is different than that of standard sheath fluid. The sorting technician will run the diagnostic beads through the machine to ensure the lasers are working properly.

10.2. When sorted neurons are to be used in subsequent transcriptomics studies, sort a minimum of 100,000 GFP+ cells directly into 800 µL of Trizol-LS + 10 µL of RNase inhibitor.

10.3. Invert cells in Trizol 15 times to mix.

10.4. Snap freeze in a dry ice/ethanol bath and store at -80 °C until ready to extract RNA from all samples for one RNA sequencing experiment.

NOTE: While most sorted cells are collected directly into Trizol for transcriptome analysis, make sure to retrieve a small sample of GFP+ sorted cells (from each strain) collected into egg buffer, for microscopy validation of the efficiency of sorting.

11. FACS gating strategy

11.1. For identifying GFP positive signal use the 488 nm laser with the 530/30 filter and 502 long pass (LP).

11.2. For identifying DAPI positive and negative cells use the 405 nm laser with the 450/50 filter.

11.3. For identifying auto-fluorescent cells that could be mistaken for GFP-positive cells use the 488 nm laser with the 610/20 filter and 595 LP (personal communication, Stanka Semova, Operations Manager, Flow Cytometry Resource Center, Rockefeller University).

11.4. Perform a standard gating strategy and remove events with a large side scatter area (SSC-A) and small forward scatter area (FSC-A), which are likely to represent clumps of cells and debris.

11.5. Isolate foreword scatter singlets and then side scatter singlets.

11.6. Isolate cells with high GFP signal and low autofluorescence signal.

NOTE: Cells high for autofluorescence and lower in GFP are not true GFP positive cells.

11.7. Threshold for GFP+ gate is determined by comparing GFP- cells (i.e. N2) to GFP+ cells[55,56].

11.8. Remove any dead cells with a live/dead gate, based on the selective permeability of DAPI to dead cells: Threshold for live dead gate is determined by comparing unstained cells to DAPI stained cells.

NOTE: When sorting multiple samples, flush the stream between samples to ensure there is no cross contamination.

12. Microscopy of sorted neurons to validate the efficiency of FACS gating strategy

12.1. Draw a circle on a glass microscope slide with a liquid repellent pen.

12.2. Pipette 10 µL of sorted cells suspension in egg buffer inside the circle. Place a coverslip over the sample and seal with nail polish around the perimeter of the cover slip.

12.3. After the nail polish has dried, check under upright/inverted fluorescent microscope that the sorted cells are indeed primarily GFP + cells.

NOTE: Perform this check after each sorting session to validate the FACS gating strategy[57].

13. RNA Extraction and RNA quality quantification on a Quality Control automated electrophoresis system

NOTE: All RNA work should be executed with extreme care to avoid contamination with RNase (including careful preparation of reagents, consumables, and best RNase-safe practices).

NOTE: Perform all steps where samples contain phenol and/or chloroform in a fume hood.

13.1. Thaw vials of cells in Trizol at room temperature.

13.2. Using a filter tip P200 pipette tip, pipette up and down several times to homogenize the sample.

13.3. Incubate at room temperature for 5 minutes.

13.4. Add 0.2 mL of chloroform per 1 mL of Trizol reagent used for lysis, then securely cap the tube.

13.5. Invert tubes 15 times, and incubate (at 20–25 °C) for 2–3 minutes.

13.6. Centrifuge the sample for 15 minutes at 12,000 × *g* at 4 °C. The mixture separates into a lower red phenol-chloroform, an interphase, and a colorless upper aqueous phase.

13.7. Exclusively collect the upper aqueous phase containing the RNA and transfer to a new low DNA/RNA bind 1.5 mL tube.

NOTE: In some instances, if the ratio of cells in egg buffer that dropped out of the cell sorter into Trizol is greater than a 1:3 sample:Trizol-LS ratio, the high salt content of the egg buffer can cause the layers to be inverted; if this occurs add more Trizol-LS and repeat the inversion and centrifugation. This can also be avoided if you take note of the increase in sample volume after sorting; if the 1:3 ratio is not maintained, add sufficient trizol before beginning the RNA extraction.

13.8. To further purify the RNA use RNA extraction column chemistry.

13.9. Use the Quality Control automated electrophoresis system to measure the RNA Integrity Number (RIN) and confirm RNA RIN of 8.0 or greater for input into subsequent RNA sequencing experiments.

REPRESENTATIVE RESULTS

Nematode model of excitotoxicity and identification of vacuolated degenerating neurons

Data shown here is reproduced from previous publications[37,38]. To mimic excitotoxic-induced neurodegeneration, a glutamate-transporter gene knockout (*glt-3)* is combined with a neuronal sensitizing transgenic background (*nuIs5* [*Pglr-1::GαS(Q227L);Pglr-1::GFP)*]). The transgenic construct is expressed in neurons expressing GLutamate Receptor 1 (GLR-1, a critical subunit of AMPA receptors) and is tracked via GFP fluorescence expressed in these same neurons. Degenerating neurons are observed using DIC and appear as swollen vacuoles (**Figure 1B**). The number of dying head neurons is significantly increased when the *nuIs5* transgenic construct is

combined with *glt-3 ko*. This number is greatly reduced when all GluRs that contribute to Glu-induced currents in these cells are knocked out, suggesting Glu-dependent neurodegeneration, which is a hallmark of excitotoxicity (**Figure 1C**). **Figure 1D** shows the role of the nematode homolog of the transcription factor CREB in nematode excitotoxicity. Knockout of CREB/CRH-1 significantly increases the number of dying head neurons, demonstrating a conserved role in neuroprotection. **Table 1** shows the various strains we use in addition to our excitotoxic strain to study processes that can contribute to neurodegeneration in our nematode model.

Visualization and analysis of neuronal mitochondria using fluorescent reporters and in vivo imaging

To observe mitochondrial morphology in excitotoxicity, expression of the transgene *odIs122*[49] (a gift from the Rongo Lab), which labels mitochondria in *glr-1* -expressing neurons with mito-mCherry fluorescent protein, was combined with the excitotoxicity strain. Using 100x objective of a scope with DIC and fluorescence imaging, mitochondria in the RIGL/R and AVG neurons of the retrovesicular ganglion were examined and categorized into filamentous, intermediate, or fragmented groups (**Figure 2A**). Degenerating neurons with vacuolated appearance exhibited robust fragmentation of mitochondria (**Figure 2B**).

Fluorescence activated cell sorting of *glr-1* expressing neurons for transcriptomics

Animals from synchronized cultures of control or worms transgenic for *pzIs29* (expressing a nuclear GFP marker under the *glr-1* promoter)[54] were dissociated into cells, and GFP-expressing neurons were collected by FACS. To validate our sorting gates (**Figure 3A & 3B**), we use microscopy to screen for GFP expression in sorted neurons versus an unsorted *C. elegans* sample (**Figure 3C**).

TABLE AND FIGURE LEGENDS

Table 1. Strains that used here to study excitotoxicity and the involvement of mitochondria, and to sort at-risk neurons for transcriptomics analysis.

Figure 1. Nematode Excitotoxicity Model.

A) Schematic of Excitotoxicity Mechanism. In stroke, lack of energy for neurotransmitter clearance causes a buildup of synaptic Glutamate (Glu), over-stimulation of GluRs, excessive Ca^{2+} influx and cell death by regulated necrosis or apoptosis. Cells exposed to non-maximal insult intensity show either delayed death or recovery and survival. This later-stage neuroprotection is largely mediated by TFs that trigger defensive transcriptional programs. **B) Identification of Necrotic Neurons.** Necrotic neurodegeneration is seen in DIC optics as vacuole-looking structures, mostly near the nerve ring of the worm (approximate scale bars of 10µm). Red arrows indicate degenerating cells (only some of which are clearly seen in this focus plane). **C) Quantification of excitotoxic neurodegeneration.** A deletion of *glt-3* combined with the *nuIs5* sensitizing background gives rise to GluR –dependent excitotoxicity, seen by significant increases in levels of neurodegeneration. This extended neurodegeneration is reversed by deletion of all GluRs active in these neurons (reproduced from[37]). **D) The effect of CREB/CRH-1 on excitotoxicity** (Reproduced from[38]). Average number of degenerating head neurons per animal

in different developmental stages, comparing the original excitotoxicity strain (*glt-3;nuIs5*) to a similar strain where CREB/CRH-1 is eliminated (*crh-1;glt-3;nuIs5*). In this and all subsequent bar graphs: Error bars represent SEM of the number of degenerating head neurons per animal. One-way ANOVAs were performed at each life stage. Asterisks represent statistical significance of the difference between the indicated groups, where (when used-) * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$; *** indicates $p \leq 0.001$, n.s. denotes non-significant difference between groups.

Figure 2. Visualization of neuronal mitochondria using fluorescent reporter
A) Categorizing mitochondria based on morphology. Images of at-risk neurons labeled with GFP and mito-mCherry (in strain IMN66: *glt-3(bz34) IV; nuIs5 V; odIs122 X*) using an inverted scope with DIC and fluorescence imaging. Filamentous mitochondria appear as a continuous, circular structure in neuronal soma, while intermediate mitochondria have thin filaments with partial breaks/small amounts of fragmented mitochondria. Fragmented mitochondria have complete breaks with swollen appearance in the neuronal soma. Blue dashed lines indicate the soma of neurons. **B) Mitochondria in a degenerating neuron.** Vacuolated neurons which indicate neurodegeneration have apparent fragmented and swollen mitochondria in their soma. (Scale bar: 5μm)

Figure 3. Fluorescence activated cell sorting of *glr-1* expressing neurons. Forward scatter singlets gate and GFP + gates shown for samples containing **A)** Neurons expressing nuclear GFP under the *glr-1* promoter, or **B)** wild type cells (devoid of any transgenes expressing fluorescent proteins). **C**) Images of FACS sorted GFP+ neurons and unsorted cells used to verify the success of sorting. (Scale bar: 10μm)

DISCUSSION

While the prevalent controversies and failures suggest that excitotoxicity presents an exceptionally hard process to decipher, the analysis of excitotoxicity in the nematode offers a particularly attractive strategy to illuminate conserved neuronal cell death pathways in this critical form of neurodegeneration. The investigator can rely on the rich collection of research tools available in this system, and particularly on the animal's transparency (allowing in vivo analysis) and the large repertoire of viable mutants (available freely from the CGC or prepared in-house by techniques like EMS mutagenesis and CRISPR). Furthermore, research progress benefits from the relative ease of identification of specific neurons that are known to mediate well-described roles in the nematode nervous system. Indeed, one can now identify and quantify degeneration of specific neurons[38] and target them for analysis of mitochondrial damage or transcriptomics. This can allow the identification of cell-type specific processes that confer particular vulnerability or resistance.

The powerful molecular tools available in *C. elegans* research and the genetic tractability of this model organism empower the researcher to dive deeper into the biology of causes of neuronal cell death and protection during excitotoxicity. Moreover, the transparency of the nematode also allows the study of dynamic processes such as mitochondrial morphology changes in an excitotoxicity specific context in live animals. *C. elegans'* short life cycle and ease of maintenance allow generation of large data sets, in specific neurons, and under diverse conditions (e.g., aging).

There are a few points of special practical significance. The use of sodium azide is a common approach to immobilizing *C. elegans* for microscopy[60]. However, sodium azide is an inhibitor of the mitochondrial respiratory chain and causes neurodegeneration[61]. It is therefore critical to avoid sodium azide, and it is best to quantify the effect of excitotoxicity modifiers in non-treated animals by examining animals on an agar chunk with DIC optics. When necessary, use a muscle-paralyzing agent (such as tetramisole[30,62], though neuronal side effects should be considered).

The transgene expressed in *nuls5* animals seems to be subjected to gradual suppression of its expression. As a result, after multiple generations, the effect of the sensitized background begins to wane and reduce baseline neurodegeneration. Therefore, the overall excitotoxicity observed in the worm seems to decline with subsequent generations (e.g., as manifested by the decline in degeneration of tail neurons). To avoid this, outcross the excitotoxic strain to wildtype (N2) worms at least twice using standard protocols, and use freshly outcrossed animals, or frozen stock thereof, for scoring of neurodegeneration. Occasional outcrossing restores overall excitotoxicity and neurodegeneration compared to multi-generational lines.

This protocol describes imaging of neuronal mitochondria in live animals during excitotoxicity. Carefully distinguishing between neurons with fragmented, intermediate, and filamentous mitochondria is critical for determining the qualitative effects of different genes in nematode excitotoxicity. This approach can also be used to discover neuron-specific vulnerability to mitochondrial perturbation.

Isolation of nematode neurons for transcriptome analysis is based on protocols developed in the Kuhn[63,64], Miller[57], Murphy[58], and Shaham[59] labs. This protocol describes sorting of all (30 neurons/animal) at-risk neurons exposed to excitotoxicity, using expression of a bright nuclear GFP in *glr-1* expressing neurons[54]. However, single-cell –specific promoters can also be used to express the fluorescent protein markers, to obtain individual neurons of interest for cell-specific transcriptome analysis (thus circumventing problems of shallow coverage, i.e., small number of reads per gene, seen in some cases with single-cell transcriptome analysis). Sorting large quantities of neurons from excitotoxicity strains animals in the L2 stage seems more successful than when using animals in later stages. This is probably because of the increase fragility of at-risk neurons at the L3 stage, decreasing the representation of at-risk neurons following the sorting process. However, the dissociation and neuron sorting protocols can be used to sort neurons from any *C. elegans* larval stage.

In sum, using the nematode to illuminate key events in excitotoxicity, such as endogenous excitoprotective transcriptional programs and mitochondrial mechanisms, could potentially identify highly conserved core steps in this critical form of neurodegeneration, and might therefore eventually reveal drug targets for late stage stroke intervention.

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The authors have nothing to disclose.

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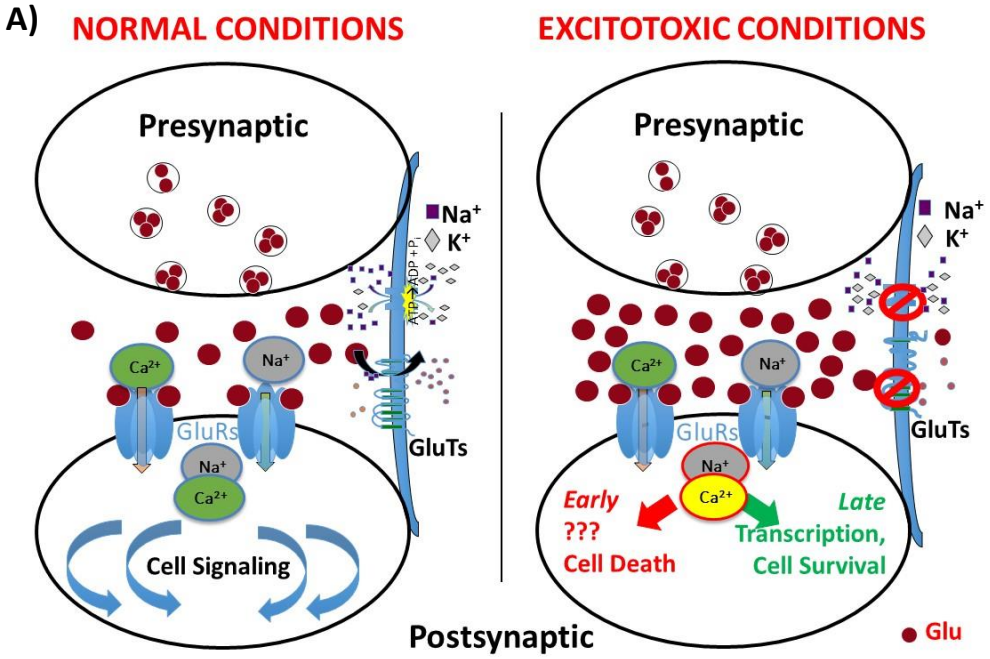

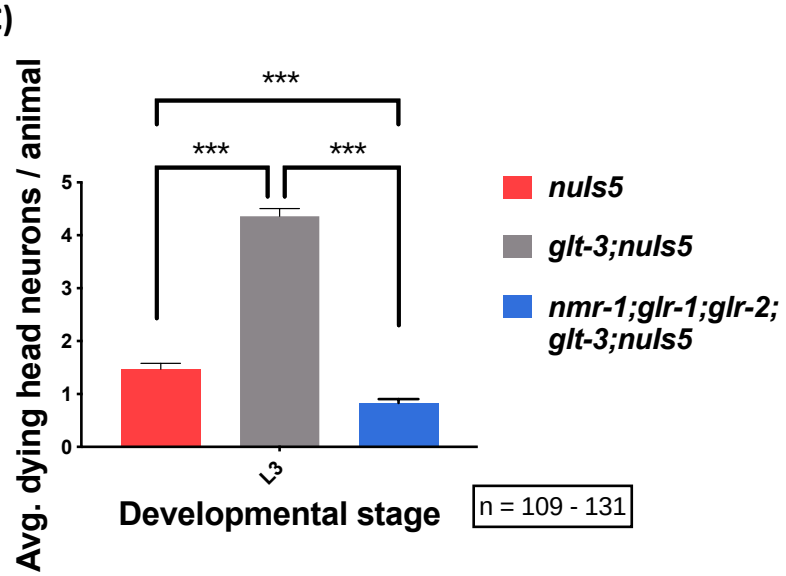

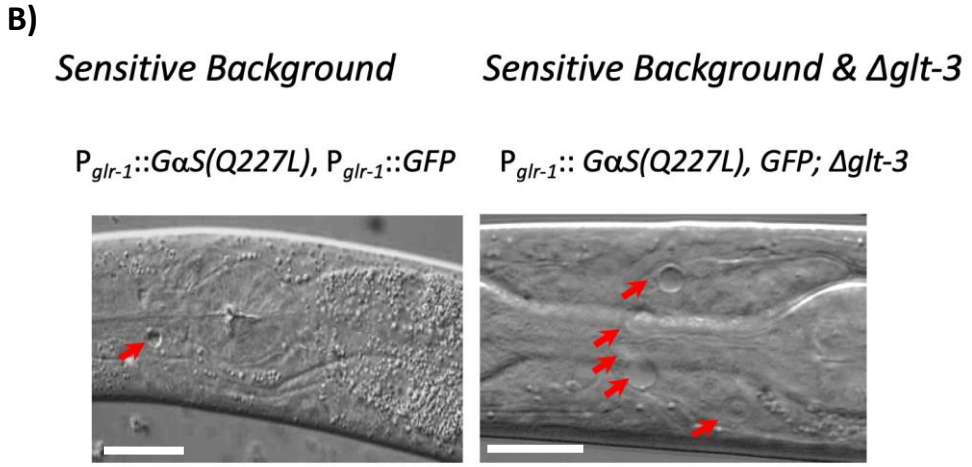

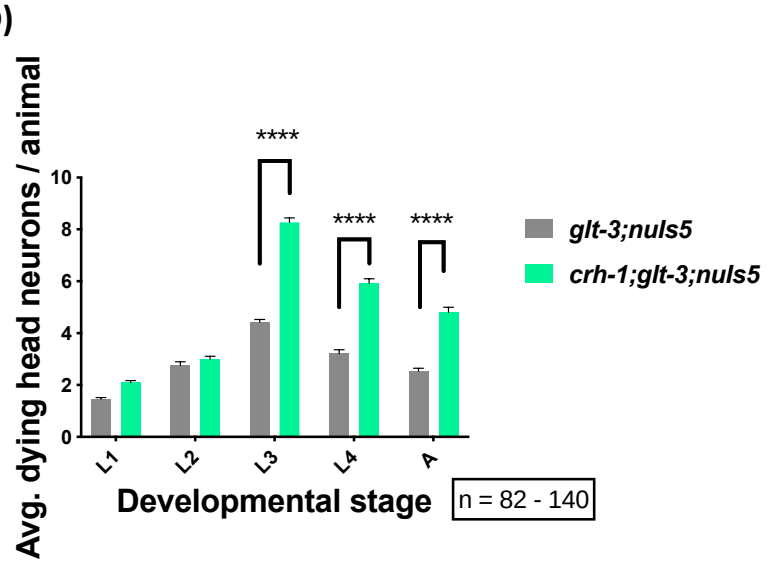

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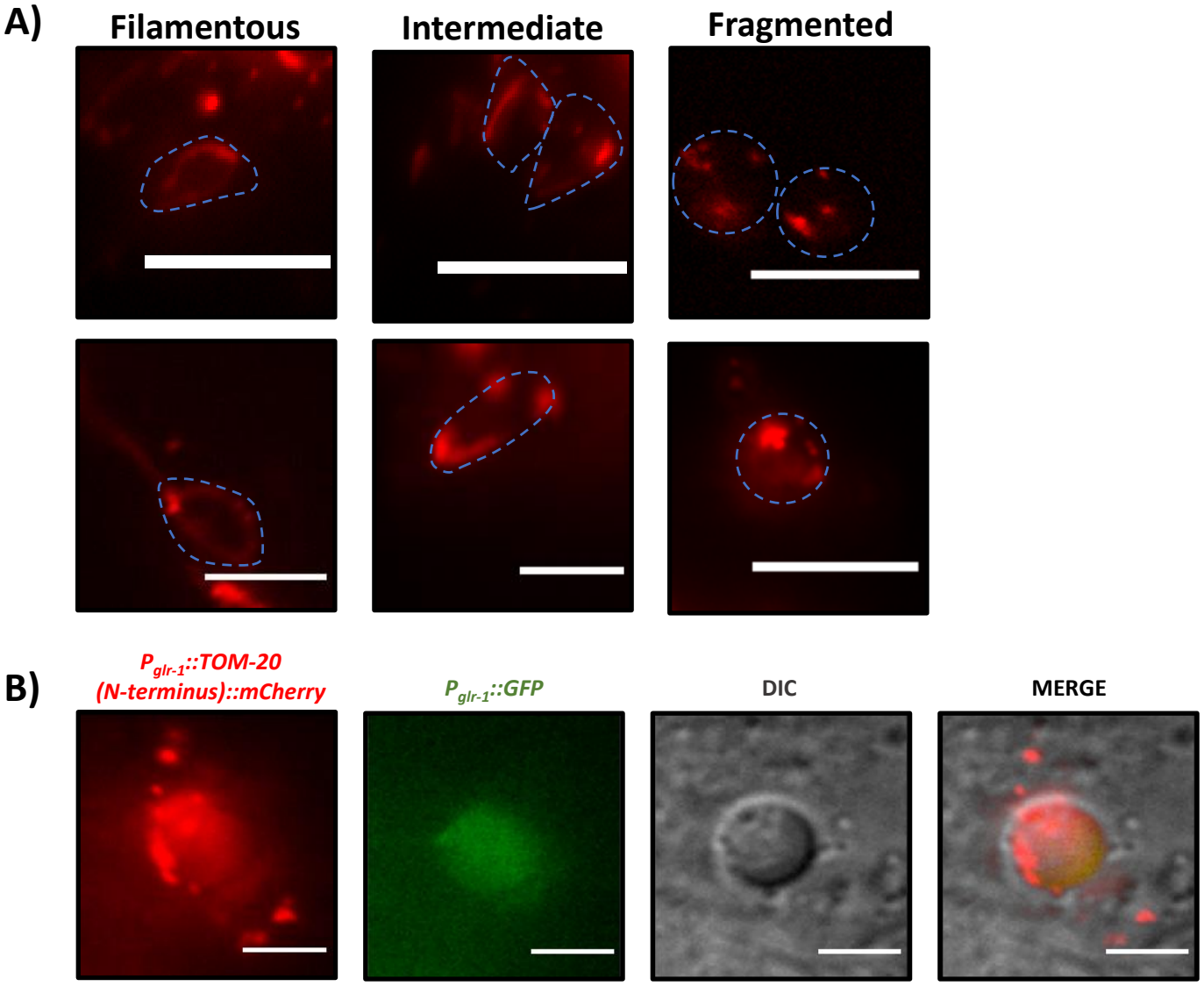

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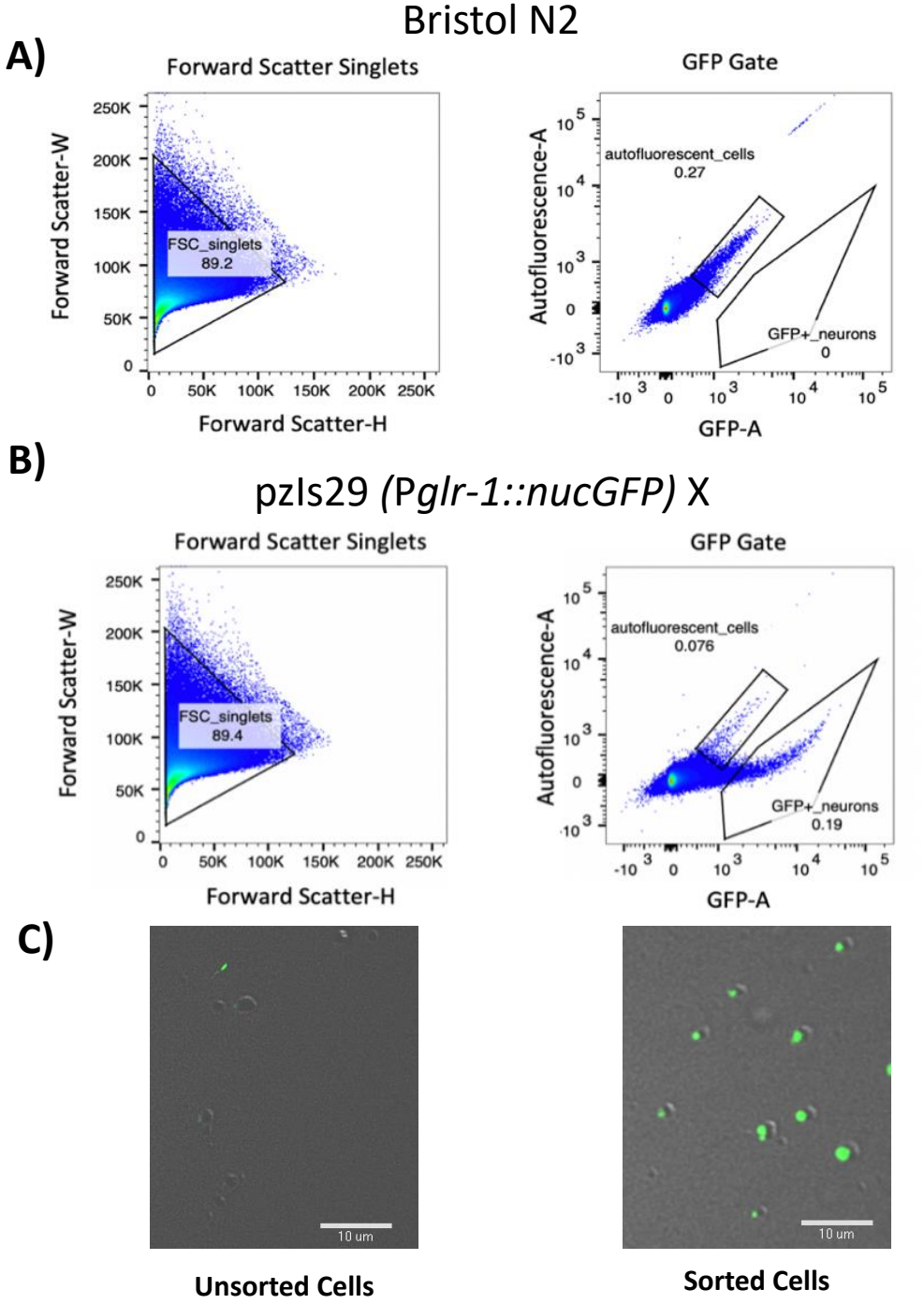

Table

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Strain Name	Genotype	Description	Reference
KP742	*nuIs5 [glr-1::gfp; glr-1::Gαs(Q227L) ; lin-15(+)] V*	Expresses constitutively active G alpha subunit in *glr-1* -expressing neurons. Acts as a neuronal sensitizing background.	45
ZB1102	*glt-3(bz34) IV; nuIs5 V*	Excitotoxicity Strain: Knockout of glutamate transporter 3 (*glt-3)* combined with sensitizing background *nuIs5* to produce excitotoxicity.	37; 38; 39; 40
ZB1336	*nmr-1 (ak4) II; glr-2 (ak10) III, glr-1 (ky176) III; glt-3(bz34) IV; nuIs5 V*	.Combination of the excitotoxicity strain with the knock out of all GluRs mediating ionotropic Glu response in these neurons	37
IMN36	*crh-1 (tz2)III; glt-3(bz34) IV; nuIs5 V*	Combination of the excitotoxicity strain with the knockout of CREB/*crh-1.*	38
	odIs122 [P $_{glr-1}$ ::TOM-20(N-terminus)::mCherry, ttx-3::rfp] X	mCherry fused to the N-terminus of mitochondrial protein TOM-20 expressed in *glr-1* expressing neurons.	49
IMN66	*glt-3(bz34) IV; nuIs5 V; odIs122 X*	Expresses mitochondrial fluorescent transgene *odIs122* in the excitotoxicity strain.	This manuscript
N2	*C. elegans wild type isolate.*	*C. elegans* var Bristol. Wild type *C. elegans* strain used as negative control in FACS experiment.	30
FJ1244	*pzIs29 [Pglr-1:: NLS::LAC-Z::GFP::glr-1 3'UTR] X*	Expression of bright nuclear GFP (nGFP) in *glr-1* -expressing neurons.	54
IMN87	*glt-3(bz34) IV; nuIs5 V; pzIs29 X*	Combination of the excitotoxicity strain with *glr-1::nGFP* expression for FACS sorting of at-risk neurons.	This manuscript

Table of Materials

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Name of Material/Equipment	Supplier	Catalog Number
Agar	VWR	AAA10752-0E
Bactopeptone	VWR	90000-264
BD FACSAriaIII	BD	
Bleach	Any household	
$CaCl_2$	VWR	97062-586
$CaCl_2 \cdot 2H_2O$	BioExpress	0556-500G
Cell Strainer, PluriStrainer mini 70um	PluriSelect	43-10070-40
Cell Strainer, PluriStrainer mini 5um	PluriSelect	43-10005-60
Centrifuge - 15-50 mL Sorval benchtop LEGENDX1R TC	Fisher Sci	75618382
Centrifuge - microfuge ; Ependorff 5424	VWR	MP022629891
Chloroform	VWR	97064-680
Cholesterol	Sigma	C8667-25G
DAPI	Fisher Sci	EN62248
Dry ice	United City Ice Cube	
DTT	VWR	97061-340
E. coli OP50	CGC	OP50
Ethanol (100%)	VWR	EM-EX0276-1S
Ethanol (90%)	VWR	BDH1160-4LP
FACS tubes	USA Sci	1450-2810
Filter tips	USA Sci	1126-7810
Glass 10 mL serological pipettes	USA Sci	1071-0810
Heating block	BioExpress	D-2250
Hepes	VWR	97061-824
Immersion Oil - Carl Zeiss Immersol	Fisher Sci	12-624-66A
Isopropanol	VWR	EM-PX1830-4
KCl	VWR	BDH9258-2.5KG
KH_2PO_4	VWR	BDH9268-2.5KG
Low bind 1.5mL tubes	USA Sci	4043-1021
Metamorph Imaging Software	Molecular Devices	
$MgCl_2$	VWR	97063-152
$MgCl_2 \cdot 6H_2O$	BioExpress	0288-500g
$MgSO_4$	VWR	97061-438
Microscope, Confocal, for Fluorescence Imaging	Zeiss	LSM 880
Microscope, Inverted, for Fluorescence Imaging	Zeiss	Axiovert 200 M
Microscope Camera	Q-Imaging	Retiga R1
Microscope Light Source for Fluorescence Imaging	Lumencor	SOLA SE Light Engine
Microscope, Nomarski DIC	Zeiss	Axiovert Observer A1
Microscope, Nomarski DIC	Nikon	Eclipse Ti-S
Na2HPO4	VWR	97061-588
NaCl	VWR	BDH9286-2.5KG
NaOH	VWR	97064-476
Petri dishes, 100mm	Fisher Sci	FB0875712
Petri dishes, 60mm	TriTech	T3308
Pipet Controller	TEquipment	P2002
Pipettor P10 Tips	USA Sci	1110-3000
Pipettor P1000 Tips	USA Sci	1111-2020
Pipettor P200 Tips	USA Sci	1110-1000
Pronase	Sigma	P8811-1G

RNAse away spray	Fisher Sci	7000TS1
RNAse free serological pipettes	USA Sci	1071-0810
RNAse-free 50 mL tubes	USA Sci	5622-7261
RNeasy micro	Qiagen	74004
SDS	VWR	97064-496
Streptomycin sulfate	Sigma	S6501-100G
Sucrose	VWR	AAJ63662-AP
SUPERase·in RNase inhibitor	Fisher Sci	AM2694
Quality Control automated electrophoresis system: Tapestation - H	Agilent	5067-5579
Tapestation - High Sensitivity RNA ScreenTape Ladder	Agilent	5067-5581
Tapestation - High Sensitivity RNA ScreenTape Sample Buffer	Agilent	5067-5580
Tapestation - IKA MS3 vortexer	Agilent/IKA	4674100
Tapestation - IKA vortexer adaptor at 2000 rpm	Agilent/IKA	3428000
Tapestation - Loading tips	Agilent	5067- 5152 or 5067- 5153
Tapestation - Optical Cap 8x Strip	Agilent	401425
Tapestation - Optical Tube 8x Strip	Agilent	401428
Quality Control automated electrophoresis system: TapeStation 22	Agilent	G2964AA
Tetramisole	Sigma	L9756-10G
Tris base	Fisher Sci	BP152-500
Tris hydrochloride	Fisher Sci	BP153-500
Trizol-LS	Fisher Sci	10296-010
Wescor Vapro 5520 Vapor Pressure Osmometer	Fisher Sci	NC0044806
Wheaton Unispense µP Dispenser	VWR	25485-003

Rebuttal Letter

Click here to access/download;Rebuttal Letter;Response to review JoVE61958.docx

Response to review of JoVE submission JoVE61958

Live Animal Imaging and Cell Sorting Methods for Investigating Neurodegeneration in a *C. elegans* Excitotoxic Necrosis Model

Zelda Z Mendelowitz1, Adem Idrizi, and Itzhak Mano

Dear Editor,

We would like to express our sincere thanks to the editors and the reviewers for their close attention and deep evaluation of our manuscript. We addressed all comments, introduced changes to address all editorial notes and nearly all (whenever possible) of the reviewer's comments, as listed below.

We hope that you will now find our manuscript fit for publication in JoVE.

Many thanks

Itzhak Mano, PhD.

CUNY Sch of Med

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done (as best we could)

2. Please revise the title to avoid abbreviations while remaining within the 150-character limit.

Done

3. Please provide scale bars for Figure 1B, 2B, 3C

Done

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Done (as best we could)

6. 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 and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Wheaton Unispense μP Dispenser, BD's FACSAriaIII machine, Zeiss Axiovert

Done

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done

8. 3.2: Are there any special precautions to be taken when mounting agar chunks?

Addressed in the text

9. 4: How is the crossing actually done?

Addressed in the text

10. 5.1: Please provide more details: which constructs are to be used; how do you label the mitochondria? Does the reference contain this information? Please specify.

Addressed in the text

11. 6.1: Please provide details on analysis and figures.

Addressed in the text

12. 6.2 and 6.3: Please indicate what these different types of mitochondria mean for your study outcome and interpretation of results.

Addressed in the text

13. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Done

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Done

16. 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

Done

17. Please write journal names fully in the reference list.

Done

18. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors described live imaging and FACS methods for investigating neurodegeneration using a C. elegans excitotoxic necrosis model. This work provides useful methods for researchers interested in neuronal degeneration. This manuscript is overall well written but needs to provide more details.

Minor Concerns:

1. (2.0 Growth Media) Please describe the purpose of using two different kinds of worm growth media, NGM and MYOB.

Done

2. (5.0 Live imaging of neuronal mitochondria morphology…)(Figure 2A) To observe worm neurons expressing fluorescent protein, why they use inverted microscope instead of upright microscopes? Are there any advantages in this case? Please, describe more.

Addressed in the text

3. (Figure 1A) This schematic of excitotoxicity mechanism is poorly drawn. This figure should be improved.

Done

4. (Figure 1C) In the graph, this mark ("}Nematode Excitotoxicity") seems to be inappropriate.

Addressed

5. (Figure 1D) For the statistical test, the legend and figure do not match. Only *** is observed in the figure, while the figure legend mentioned *,**, ***, and ns.

Addressed

6. (Figure 3A and 3B) It is hard to understand the axis labels on graphs. Please describe more details in both figure legend and the main text.

Addressed

Reviewer #2:
Live Imaging and FACS Methods for Investigating Neurodegeneration in a C. elegans Excitotoxic Necrosis Model
Mendelowitz et al.

Mendelowitz et al. describe how to score glutamate-dependent neuronal deaths in the powerful model system C. elegans and document protocols for sorting-based isolation of at-risk neurons dissociated from whole animals, used for RNA seq. studies. This paper will be of great value to those studying like processes in C. elegans and seems highly suitable for a JoVE publication.

Some revision will address clarity.

In general, I know the authors will have to edit out the use of personal pronouns for JOVE style. There is a lot of that in the paper.

Addressed

Also in genera,l the verb tense use is quite varied-the authors should pick one and stay with it, going through the text to keep tense uniform.

Addressed

Specific comments on grammar, typos:

Line 69 It is believed that much of the difference from animal models is because the intervention was administered hours after stroke onset, blocking late-acting endogenous neuroprotective mechanisms downstream of GluRs 14,16,17 71 .

The "difference from animal models" is not well explained and a bit unclear; possibly better is—clinical failures of protcols suggested by animal work might be because….

Addressed

72 alternative approach, which is based on thrombolysis, can

Addressed

96 Therefore, both the study of downstream death signaling pathways and the study of

transcriptional neuroprotection in excitotoxicity encountered great difficulties and disagreements in outcome.

Addressed

33 pathways that affect neurodegeneration and neuroprotection. We have already started using these approaches to analyze downstream death signaling cascades

Addressed

In general 1.1 might be confusing to an uninitiated reader. I suggest more precise description of the constructs.

Addressed

140 1.1. Glutamate-dependent excitotoxicity in C. elegans is produced by combining a knockout
141 (ko) of a glutamate transporter gene with
with transgene that sensitizes to neurotoxicity expressed in a particular subset of neurons

We refer to this genetic combination as our excitotoxicity strain (freely available from the Caenorhabditis Genetics Center, CGC). We study the effect of genes encoding candidate regulators of excitotoxic necrosis by combining a mutation in such genes (strains obtained from CGC or produced by us) with our excitotoxicity strain (a bit vague—better if exact names or better descriptions are given) . Table 1 outlines strains that we use to study distinct pathways of neurodegeneration and neuroprotection, specifically for this protocol.

Addressed

169 overnight to dry. (overnight is one word)

Modified

174 psi for at least 45 minutes, allow it to cool let the solution cool and inoculate w

Modified

176 of bacterial culture to dried dry MYOB agar plates and allow them the bacteria to dry/grow

Modified

178 will it be clear what a chunk is?

Addressed

194 Neurodegeneration peaks at in L3 animals, which

Addressed

202 (without anesthetics). Flip the agar chunk so that the animals which that were cra

Addressed

3.3. Using a DIC scope with x10 ocular and either an x40 or x63 objective scan animals at random by 207 sliding the coverslip manually

Addressed

209 Record the number of dying (i.e., vacuolated) head neurons and the developmental stage of each animal in a table where animals from a given strain are grouped by developmental stage

Addressed

211 The number of degenerating neurons in the head, retrovesicular ganglion, and tail per worm is recorded, respectively.

Addressed

214 and further into that paragraph: In each session we collect data at random for several developmental stages.
I think JOVE does not permit personal pronouns?

Addressed

small groups of-) animals on an agar pad 39

Addressed

229 3.5. Calculate averages and SEM of the collective number of degenerating neurons in both the head and retrovesicular ganglion are calculated per animal per developmental stage (Figure

Addressed

252 5.0 Live imaging of neuronal mitochondrial morphology using reporter strains by inverted fluorescent microscopy reads better as:
Live imaging of neuronal mitochondrial morphology by inverted fluorescent microscopy of reporter strains

Addressed

254 sentence has some redundancy; possibly:

Addressed

5.1. For mitochondrial imaging we examine mito-mCherry using strains that label mitochondria of glr-1 -expressing neurons (by expressing a fusion between the fluorescent protein and the N-terminal of TOM-20 under glr-1 promoter 41).

Addressed

266 5.4. Image animals within 20 (add a space) minutes using an inverted scope with DIC and florescence imaging.

Addressed

272 Once the head of the worm is located (or neurons of interest, in this case, we look at the soma of neurons of the 273 retrovesicular ganglion) take overlapping Z-stack images with DIC, GFP, (one sentence)

Addressed

284 them, with few little fragmentation.

Addressed

288 worm, score the number of neurons with filamentous, intermediate, and fragmented mitochondria for a total

Addressed

291 Perform statistical analysis using chi-square test to compare mitochondrial morphology distribution between strains.
It might be useful to explain more here for comparison of 3 parameters?

Addressed

296 . Here we describe neuron sorting of all (30 neurons/animal) a add a space

Addressed

298 However, single-cell -specific promoters can also be used to express the GFP marker used for sorting in individual neurons of interest, for cell-specific transcriptome analysis (thus circumventing problems of shallow coverage seen in some cases with single-cell transcriptome analysis).
The issue here does not read as clearly as it might. Do the authors mean:

However, single-cell-specific promoters can also be used to express the enhanced brightness

GFP marker used for sorting individual neurons of interest, for cell-specific transcriptome analysis (thus circumventing problems of shallow coverage seen in some cases with single-cell transcriptome analysis). (it is not exactly clear what the shallow coverage issue is—dim signal, getting buried among other signals—describe better)

Addressed

345 days. The plates should now be full of predominantly gravid worms and ready to use to harvest synchronized progeny.

Addressed

356 MYOB plates and grown at 20°C until hatched animals are at the desired larval stage.

Addressed

367 9.2 The supernatant is removed and add M9 is added up to 45mL and the tube is centrifuged

Addressed

406 Worm dissociation protocol was adapted from protocols from Miller lab, Murphy lab, & 407 Shaham lab. Useful to have protocol references here.

Addressed

381 DTT treatment: resuspend pellet with SDS-DTT spin at 14,000 rpm on a add space

430 Section 10.3. The cell numbers here are a bit confusing: From one GFP+ sample we sort ~50,000 cells into egg buffer for 430 microscopy validation of sorting.
This seems like a LOT of GFP cells to look at for microscopy validation—are these numbers correct, is there a reason?

Addressed

454 polish around the perimeter of the cover slip. After the nail polish has dried, check under an inverted fluorescent microscope that the sorted cells are GFP + neurons.

Addressed

457 add institution for Dr. Miller

Addressed

509 add institute and full name for Dr. Rongo

Addressed

519 C. elegans in italics

Addressed

531 recover and survive

Addressed

538 reproduced no capital

Addressed

575 rely on
576 animal's

Addressed

Figures 1A possibly could look better of blue glial cell boundary was extended up and down

1B right most panel, red arrow looks like it highlights a viable cell

Addressed

Figure 2—cells might be better visualized with a faint thin dashed line around them?

2B some fluorescence appears outside the cell boundary—is there a cleaner image?

Addressed

Table description section has phrases with and without periods, be consistent

Addressed

ON genotype—check for uniform nomenclature on fusions: odIs122[P glr-1 ::TOM-20(N-terminus)::mCherry, ttx-3::rfp] X
4 pzIs29 [Pglr-1:: NLS::LAC- Z::GFP::glr- 1 3'UTR] X

Addressed

Reviewer #3:
Manuscript Summary:

The present manuscript focuses on methods to quantitatively and effectively analyze excitotoxic necrotic events in the C. elegans neurons. Using their own strain which harbors both a glutamate transporter KO and a sensitizing genetic background by a transgene, authors shows an increased necrosis in developing animals. This probably makes easy to identify novel genetic or external factors which affect neuronal necrosis. Authors also shows other two protocols for the analysis of mitochondria structure and gene expression profile, probably related to the onset of necrosis. The novelty and efficacy of these latter two approaches are not high as authors simply describe the usual procedure for analysis. At the same time, the data from suggested methods are quite hard for readers to conduct similar experiments. Additional figures or explanations to show the benefits of their approach for necrosis study.

Major Concerns:
Authors describe the methods for the observation of mitochondria structure in neuronal cells. Using fluorescent labeled strains, mitochondria are visualized and classified into three categories to evaluate the induction of necrosis. Following to this protocol, it is true that the mitochondria structures can be observed, as authors show in Figure 2. However, is there any way to improve or to accelerate this usual observation?

Addressed , added more details

What is the point to perform this observation in efficacy?

Addressed

Authors should include some helpful points to perform this observation easily but accurately.

Addressed

In addition, the structures of mitochondria are quite dynamic and variable in the cell. The example pictures in Fig. 2A are one example and not easy for me to classify other examples into the three categories. Authors should add several examples and explain how the classification can be performed with efficiently but quantitatively.

Addressed, we added further description

The similar concern is raised against the FACS protocol. What is the important point to follow suggested protocol? Are there any effective points leading a successive experiment for readers? If there, authors should strengthen or highlight such valuable points or options.

Addressed

Minor Concerns:
1. Line 156, authors describe two medias for the culturing of worms. It is helpful to explain the reason or advantage to prepare the MYOB media.

Addressed

2. Line 206, authors explain the method to observe necrosis in living animals on coverslip. I am wondering how long worms can be alive on coverslip. Authors should notice suitable number of worms on a coverslip and the maximum period for healthy condition of animals.

Addressed

3. Line 266, authors explain microscopic equipment to observe mitochondria structures, by epifluorescent or laser confocal microscopy. It is easily speculated that a laser confocal microscopy give us a fine structure of intracellular organelles. Is it possible to promptly classify three structures by using conventional epifluorescent microscopy? If so, why do authors use a laser confocal microscopy? Authors should explain which one is better for easy classification.

Addressed

4. Line 380, authors shows an optional for unsuccessful dissociation. It is not clear that this option will be done before step 9.3, or before 9.4. In either way, do authors mean that the same procedure should be performed by collecting worms and repeat this protocol with addition of option? Please add helpful explanations for readers.

Addressed

5. Line 547, Please indicate what neurons are indicated in Figure 2.

Addressed

6. In Figure 2, please indicate the microscopy acquiring these images. In addition, fragmented mitochondria seem to be located in the nucleus. If this is the case, please describe more detailed patterns of the three classifications.

Addressed

7. In Figure 3, the panel C is not so high quality, especially it is hard to find cells in the image of 'unsorted cells'. Please use clearer image or mark cells in the image.

Addressed