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TITLE:

Quantitative Approaches for Scoring In vivo Neuronal Aggregate and Organelle Extrusion in Large Exopher Vesicles in *C. elegans*

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SUMMARY:
This protocol describes approaches for detection and quantitation of large aggregate and/or organelle extrusions (~4 µm) produced by *C. elegans* cells in the form of membrane-bound exophers. We describe strains, growth conditions, scoring criteria, timing, and microscopy considerations needed to facilitate dissection of this debris expulsion mechanism.

ABSTRACT:
Toxicity of misfolded proteins and mitochondrial dysfunction are pivotal factors that promote age-associated functional neuronal decline and neurodegenerative disease across species. Although these neurotoxic challenges have long been considered to be cell-intrinsic, considerable evidence now supports that misfolded human disease proteins originating in one neuron can appear in neighboring cells, a phenomenon proposed to promote pathology spread in human neurodegenerative disease.

C. elegans adult neurons that express aggregating proteins can extrude large (~4 µm) membrane-surrounded vesicles that can include the aggregated protein, mitochondria, and lysosomes. These large vesicles are called “exophers” and are distinct from exosomes (which are about 100x smaller and have different biogenesis). Throwing out cellular debris in exophers may occur by a conserved mechanism that constitutes a fundamental, but formerly unrecognized, branch of neuronal proteostasis and mitochondrial quality control, relevant to processes by which aggregates spread in human neurodegenerative diseases.

While exophers have been mostly studied in animals that express high copy transgenic mCherry within touch neurons, these protocols are equally useful in the study of exophergenesis using fluorescently tagged organelles or other proteins of interest in various classes of neurons.

Described here are the physical features of *C. elegans* exophers, strategies for their detection, identification criteria, optimal timing for quantitation, and animal growth protocols that control for stresses that can modulate exopher production levels. Together, details of protocols outlined here should serve to establish a standard for quantitative analysis of exophers across laboratories. This document seeks to serve as a resource in the field for laboratories seeking to elaborate molecular mechanisms by which exophers are produced and by which exophers are reacted to by neighboring and distant cells.

INTRODUCTION

The neurotoxic challenges of aggregates and dysfunctional mitochondria have long been considered to be cell-intrinsic, but more recently it has become clear that misfolded human disease proteins originating in one neuron can also spread to neighboring cells, promoting pathology[1]. Likewise, mammalian mitochondria can be sent out of the cell of their original production for transcellular degradation[2] or for rescue of mitochondrial populations in challenged neighboring cells[3]. Vesicles of various sizes have generally been observed to transfer cellular materials to neighboring cells or to fluid surroundings[4]. Some extruded vesicles approach the size of the average neuronal soma (average touch neuron soma ~ 6 µm) and can accommodate large aggregates and organelles.

A striking example of large vesicle extrusion that can carry protein aggregates and organelles occurs in *C. elegans* touch receptor neurons that express a high copy number reporter construct encoding a noxious aggregation-prone, degradation-resistant mCherry[5]. Extrusions from the touch neurons, called exophers, are ~4 µm average diameter, selectively include mCherry or other aggregates, and are delivered directly into the neighboring hypodermis, which normally surrounds the touch receptor neurons. The hypodermis attempts lysosome-based degradation, but some non-digestible contents such as mCherry aggregates can be re-extruded by the hypodermis into the fluid-filled pseudocoelom of the animal, from which the mCherry can be taken up by remote scavenger cells called coelomocytes for long term storage (**Figure 1**, **Figure 2**)[5].

The large extruded exopher vesicles leave the cell surrounded by touch receptor plasma membrane and can contain aggregated human disease proteins, mitochondria, and lysosomes. The process of exopher production appears to involve sorting of potentially toxic species (for example an aggregation-prone expressed mCherry is segregated from soluble, inoffensive proteins like GFP that remains mostly in the neuronal soma). In this way, directed expulsion of the threatening entities is accomplished by the neuron[5]. A proteostasis challenge, such as stress induced by *hsf-1* genetic disruption, autophagy knockdown, MG132-mediated proteasome inhibition, or transgenic expression of human disease proteins such as Huntington's disease-associated expanded polyglutamine Q128 or Alzheimer's disease-implicated fragment $A\beta_{1-42}$, can increase the numbers of neurons that produce exophers[5].

As exophers have only recently been documented, what is known of their biology merits description. Exophers were discovered in, and are the most well studied in, *C. elegans* touch receptor neurons. There are six *C. elegans* mechanosensory touch neurons that have cell bodies distributed around the body (**Figure 3A**) and are called microtubule cells because their ultrastructure features distinctive 15 protofilament microtubules. The touch receptor neurons are the anterior AVM (anterior ventral microtubule neuron), ALMR, and ALML (anterior lateral microtubule neurons right and left), the more central PVM (posterior ventral microtubule neuron), and the posterior PLMR and PLML (posterior lateral microtubule neurons right and left) in the tail. Interestingly, the six touch receptor neurons produce exophers at different rates, despite expressing the same offensive transgene (**Figure 3C**). Of the six mechanosensory touch receptor neurons, the ALMR neuron undergoes exophergenesis more often than the other touch neurons. Quantitation of exopher numbers from touch neurons is thus usually established by focusing upon the ALMR.

Exophergenesis is a dynamic process that typically begins with swelling of the neuronal cytoplasm (**Figure 1A-B**). Cellular contents, organelles, or protein aggregates are collected to one side of the neuronal soma, most commonly toward the posterior end of the ALMR neuron (away from the projecting neurite), forming a pre-exopher domain (PED) (**Figure 1B**). The early protrusion is observed as the PED begins to project outwards, forming a recognizable protruded bud. The late bud is defined when the widest diameter of the pre-exopher domain is approximately ⅓ larger than the diameter of the constriction of the soma-exopher neck (**Figure 1C**). Exophers can be ejected in nearly any direction from the soma, but most exophers exit posteriorly from the cell body and remain in approximately the same focal plane as the originating soma.

The exopher can move away from the originating soma as the neck of the bud narrows into a thin filament. Exophers can remain attached to the soma via this filament (**Figure 1D, arrow**) and can later become detached. Cellular contents such as calcium, aggregates, and mitochondria can be transferred via this filament into the attached exopher[5], although the bulk of extruded material is put into the exopher compartment by the massive budding event. Exophers are considered mature when there is no visible connecting tube or thin filament and the exopher is fully separated from the sending soma (**Figure 1E**)**.**

Exophers produced by *C. elegans* touch neurons immediately encounter the hypodermis, the tissue that surrounds the touch neuron. Most commonly, the exopher vesicle appears to travel within the hypodermis posteriorly towards the tail, and can be fairly distant from the soma before exopher contents appear targeted for degradation (for example, the distance can be ~100 µm away from the soma (**Figure 1F**)). The fluorescent exopher vesicle breaks up into many smaller vesicles within the hypodermis, taking on an appearance referred to as “starry night” (**Figure 1G** and **Figure 2**). In the “starry night” stage, punctate fluorescent material can be observed scattered across the hypodermal syncytium into many smaller points of fluorescence as compared to the original solitary exopher. Starry night can look punctate under low magnification and with higher magnification, can look punctate and/or networked within the hypodermis. The fluorescent signal of the starry night is typically dimmer than the exopher and the neuronally

expressed fluorescence (**Figure 2B-C**). The dispersal of mCherry into many punctate vesicles is thought to involve phagosome maturation and fusion with the endosomal/lysosomal network of the hypodermal cell. Some exopher materials are likely degraded in the hypodermal lysosomal network, but residual species that are resistant to degradation (such as mCherry aggregates) are thrown out of the hypodermis into the pseudocoelom, a fluid compartment that can contain cellular debris. The fluorescent material is later taken in by remote scavenger cells called coelomocytes (**Figure 2C**), which can concentrate, store, and again attempt degradation of mCherry.

The phenomenon of aggregate extrusion and transfer appears conserved across phyla, having been reported in genetic models such as *C. elegans*[5-7] and *D. melanogaster*[8,9] as well as in multiple mammalian models. Exopher-like extrusions have been reported for mammalian cells[10], an observation suggesting that conserved mechanisms might underlie aggregate and organelle expulsion. Exopher production may thus be a conserved mechanism of cellular debris management that constitutes a fundamental, but formerly unrecognized, branch of neuronal proteostasis and mitochondrial quality control, which, when imbalanced, might actively contribute to neurodegenerative disease. Identification of the molecules involved in debris discrimination and sorting, transport to a distinct subcellular locale, extrusion, formation/scission of the tubular connection linking the soma and late exopher, and recognition of the large extruded vesicle for remote degradation by a neighboring cell remain for future work. Studies in nematode and fly models will be critically important to defining mechanisms of aggregate and organelle collection and transfer, utilizing unbiased genetic approaches and powerful cell biological tools offered by these models to identify participating molecules in physiological context.

Critical first steps in deciphering mechanisms operative in exopher biology involve defining protocols for reproducible in vivo exopher quantification. The *C. elegans* model offers a particular advantage for such efforts since the body is transparent and exophers can be readily observed when they contain fluorescently tagged proteins or organelles. Exophers have been reported to be generated by *C. elegans* dopaminergic neurons PDE and CEP, ASE and ASER sensory neurons, and dye-filling amphid neurons[5]. Because exophers produced by touch receptor neurons are best characterized, the focus here is on the use of touch neurons for exopher analysis. However the basic approach can be applied to measure exopher production from any cell. Protocols to detect and quantitate exophers produced by *C. elegans* touch receptor neurons that transgenically express an mCherry protein are outlined, with an emphasis on cargoes that can be monitored and temporal constraints in scoring. This article defines approaches toward in vivo exopher identification, and the quantitation of environmental and genetic conditions that modulate exopher production. Protocols emphasize critical attention to constant non-stress conditions for the determination of baseline exopher production and for comparisons across genotypes.

PROTOCOL:

1. **Strains useful for exopher detection**

1.1. Select a strain that expresses fluorescent cargoes within the neurons of *C.* elegans to readily visualize exophers.

NOTE: **Table 1** lists strains that have been used to visualize exophers produced in touch receptor neurons[5,11,12]. In principle, any cell or neuronal type can be tested for exopher production by using a cell or tissue specific promoter to drive expression of a fluorescent protein that aggregates or is otherwise selected for extrusion.

1.2. Alternatively, use a dye-filling assay to visualize exophers in the amphid head neurons, which are open to the environment and amenable to backfilling[5,13].

2. Growth media

2.1. Prepare standard nematode growth media (NGM) to culture strains according to standard methods[14,15].

NOTE: FuDR, used commonly to block progeny production, and lack of food dramatically effect exopher production. Keep the population continuously fed (avoid even short periods of bacterial food exhaustion) and maintain animals at a constant temperature.

3. Animal husbandry critical for consistent exopher production

3.1. Raise animals on consistent media and with consistent bacterial food sources. Animals *must not* run out of bacterial food, even for short periods of time since food limitation can dramatically change exopher production levels.

3.2. Keep media recipes and preparation uniform throughout a study.

NOTE: Changing media can affect basal levels of exopher production. Agar batches can influence baseline exopher levels, so when supply lots change, make a note of the date. Throw out stock plates after two weeks to ensure healthy bacterial food and to prevent dried agar, which causes changes in agar osmolarity that influence exopher levels.

3.3. For basal conditions, keep animals at a constant temperature of 20 °C. Rearing animals at variable temperatures (even acute changes in temperature) can cause variations in the timing of maximal exopher production.

NOTE: Temperature variability is not limited to culture conditions. Temperatures variations during experiments or at the lab bench can be impactful. For example, temperatures within a microscope room should not differ dramatically from the culture incubator or lab bench.

3.4. Do not use pharmacological anti-fertility interventions because fertilized eggs are critical for early adult production of exophers.

NOTE: Use of Fluoro-deoxyuridine (FuDR)[16] or C22[17], must be avoided. When performing life-span or old-aged animal experiments, age-synchronized populations should be maintained by physically removing adults from their smaller progeny by picking them onto fresh plates spread with bacteria rather than using common pharmacological anti-fertility interventions.

3.5. Do not use contaminated cultures and reinitiate experiments in the event of biological compromise of the population or the plate. Bacterial or fungal contamination can induce stresses and metabolic changes in animals and must be absent from experimental populations.

3.5.1. To maximize reproducible results, maintain cultures for at least two healthy, well-fed, contamination-free generations at 20 °C before experimentation to avoid potential environmentally-induced epigenetic changes.

4. Age synchronization for exopher scoring by bleaching, sucrose flotation, or L4 larvae picking

4.1. Keep experimental populations the same biological age, as exopher detection patterns vary with adult age and comparison of animals of mixed age populations can confound results.

NOTE: Generally, peak exopher production for *C. elegans* mechanosensory ALMR neurons occurs on adult day 2-3 (**Figure 3D**)**,** as measured from days post L4 stage. Thus adult day 1 is 24 hours after the L4 larval stage that is distinguished by "white crescent" vulva morphology (**Figure 5E**).

4.2. Prepare synchronized egg populations by bleaching gravid adults.

4.2.1. Collect gravid adults filled with eggs by washing animals growing on an NGM plate. To wash, flood the plate with 1 mL of M9 buffer, pipet up and down to collect liquid with suspended animals and pipet into a 1.5 mL microcentrifuge tube. Pellet animals by gravitational settling or gentle centrifugation with a mini centrifuge and remove the supernatant.

4.2.2. Add 150 µL of 5M NaOH and 150 µL of 6% sodium hypochlorite (bleach) in 1 mL in H_2O and mix by inversion for approximately 5 minutes.

NOTE: Fresh bleaching solution ensures that the animal cuticle can be disrupted for egg-harvest. Progress in disrupting the cuticle can be monitored under a dissecting microscope; adults should break and release eggs at the point that bleaching should be stopped.

4.2.3. Gently centrifuge with a minicentrifuge tube for 20 s and remove the supernatant. Add 1 mL of M9 buffer and centrifuge again, leaving about 100 µL on top of the pellet.

4.2.4. Repeat steps 4.2.3 twice to remove traces of bleach solution.

4.2.5. Resuspend the eggs in the remaining volume and transfer to a fresh seeded NGM plate. Adults will be lysed, but many viable eggs should be in the preparation.

4.3. Prepare synchronized populations by timed egg-lay.

4.3.1. Pick 20 gravid adults to a seeded NGM plate using standard transfer protocols[14].

4.3.2. Allow animals to freely crawl and lay eggs for 1.5 h (mutant strains with low brood sizes may require the introduction of more adult animals).

4.3.3. Remove all adult animals from the plate by picking, leaving the synchronized egg population behind. Check plates a few hours later to verify that no viable adults have been missed during adult removal.

4.4. Prepare synchronized egg populations by sucrose flotation selection of eggs.

4.4.1. Collect animals and eggs from five NGM plates upon which gravid animals have been laying eggs for at least 24 hours by flooding plates with M9 solution with 0.1% detergent (such as Tween 20 or Triton X-100) and collecting into a 15 mL tube. Pellet adults by gentle centrifugation at room temperature (2,000 x *g* for 30 s).

4.4.2. Remove supernatant and wash animals in 15 mL of fresh M9 three times, discarding the supernatant after each wash, being sure to keep the pellet enriched in animals and eggs.

4.4.3. Retain 2 mL of supernatant and resuspend the pellet. Add 2 mL of 60% weight by volume sucrose.

4.4.4. Centrifuge at 2000 x *g* for 5 min. The solution will now display an upper phase highly enriched in eggs.

4.4.5. Transfer about 2.5 mL of the upper phase to a new 15 mL tube and add 10 mL of M9.

4.4.6. Mix by inversion for 1 min, and then centrifuge 2000 x *g* for 1 min.

4.4.7. Remove the supernatant and wash the egg-enriched pellet in M9. 10-15 µL of the egg-pellet can be distributed to a fresh OP50-seeded NGM plate.

NOTE: This method prepares a great number of eggs; do not allow collected animals to run out of OP50 *E. coli* food.

4.5. Prepare synchronized populations by picking animals at the L4 stage of development.

4.5.1. Grow animals on seeded NGM plates as described above.

NOTE: *C. elegans* develop in four discrete stages. At 20 °C, a newly laid egg takes about 9 hours to hatch (**Figure 5A**)**.** Post hatch, an animal passes through larval stage 1 (L1) to larval stage 4

(L4), with each stage lasting 8-12 hours between each molt (**Figure 5A-F**)**.** Therefore, a plate prepared by inoculating with eggs should have many L4 animals to pick about 40 hours after eggs are introduced.

4.5.2. Identify L4-staged animals by locating the white half-moon crescent shape of the developing vulva (**Figure 5E**)**.**

NOTE: animals at the L4 stage are uniform in size and in body pigmentation. Pick animals with the white crescent to a fresh growth plate for examination of staged animals. The following day (~ 24 hours later) should be counted as adult day 1.

4.5.3. Score a population of animals daily from adult days 1-4.

NOTE: Exophers are typically scored at day 2 of adulthood, which is the peak exopher production under basal conditions. However, because the exophergenesis peak and timing may be shifted by environmental or genetic changes that are being studied, it is advised to score a population of adult animals daily over four days to generate the most comprehensive picture (**Figure 3D**).

5. Detection of exophers using a fluorescent microscope

5.1. Observe exophers using a high-magnification pseudo-stereo dissecting microscope that is outfitted for fluorescence microscopy.

5.2. Immobilize animals on NGM plates by pipetting 100-200 µL of 10-100 mM levamisole/tetramisole solution onto the NGM agar plate surface. After 2-4 minutes, the animals become paralyzed and can be observed directly on the agar plate. Animals can recover from paralysis after they are picked to a levamisole-free plate.

NOTE: Immobilization treatments are not absolutely required, such that with a trained eye, neuronal identification and exopher presence can be scored by visually following crawling animals under the microscope on the plate when determining whether or not an exopher has been produced.

5.3. Observe fluorescent neurons using a total magnification of 100x to accomplish dissecting microscope detection of exophers.

NOTE: Scoring exopher events using dissection microscopy allows for the observation of large numbers of animals with relative ease directly on the agar plates on which they are reared.

5.4. Live imaging and mounting reporter strains for exopher studies using confocal microscopy

5.4.1. Use a confocal microscope to live-image intracellular dynamics and characteristics of exophergenesis.

NOTE: Live imaging is an advantageous approach for observing subtle details of exopher production because exopher production is a dynamic process.

5.4.2. Restrict animal movement for high resolution live imaging using convenient methods, including utilization of levamisole or tetramisole at 10-100 mM or the application of hydrogel polystyrene microbeads (with diameters of 15 µm, 30 µm or 40 µm)[18].

5.5. Slide preparation for compound and confocal microscopy

5.5.1. Mount 20-50 animals into an immobilizing agent on a microscope slide. Reusable ringed cytology slides painted with 13 mm diameter raised-rings are useful for mounting.

5.5.2. Pick live animals into 5-20 µL of a paralytic such as 10-100 mM levamisole or tetramisole within the painted circle or on the agar pad.

5.5.3. Wait 4 minutes for paralysis, and then cover the slide with a coverslip (recommend No. 1½ (0.16 – 0.19 mm) or No. 2 (0.17 - 0.25 mm).

5.6. Mounting small numbers of animals

5.6.1. Do not crush mounted animals; when observing only a few (less than 20) animals per slide, there is a risk of crushing some of the animals due to unequal pressure of the coverslip. This risk can be minimized by using a low percentage agarose pad for mounting.

5.6.2. Make a 2-4% agarose pad slide, and then add 2-15 µL of paralytic solution to the pad. Keep in mind levamisole and tetramisole diffuse into the pad, decreasing their effective concentration.

5.6.3. Mount by picking animals into a 2-15 µL drop of paralytic solution or microbeads resting on the agar pad. Place the coverslip on top and check that the animals are intact[18].

5.7. Agar pad preparation

5.7.1. To prepare 2% agar pads, heat 2% agarose in M9 solution and microwave until the agarose is in a homogeneous and molten state.

5.7.2. To achieve an agar pad of sufficient quality, alternate mixing and microwaving at low power for less than 20 seconds. Avoid the inclusion of air bubbles within the pad by placing boiling agar on a heating block and allowing the bubbles to rise to the surface.

5.7.3. Use a Pasteur pipette to draw agar from deep within the molten solution below the risen bubbles.

5.7.4. Prepare two taped slides and place on either side of a clean glass microscope slide upon

a flat surface. To make the taped slides place two 5 cm strips of laboratory tape upon each slide (**Figure 6A**)**.**

5.7.5. Using a Pasteur pipet, place a single drop of agar upon the clean microscope slide sandwiched between the taped slides (**Figure 6B**)**.**

5.7.6. Carefully and quickly, cover the drop of molten agar with a fourth clean slide by placing in across the taped slides (**Figure 6Cc**).

NOTE: The slide should gently press the molten agar into a flattened circle about 0.4 mm thick (the thickness of the tape) (**Figure 6D**). The agar should quickly cool.

5.7.7. Remove the top slide by sliding it off (**Figure 6E**)**.** Agar pads dry quickly and are best used within minutes. Once the top slide is removed, use the gel pad immediately for mounting animals. Avoid using pads with air bubbles.

5.7.8. Store agar pads up to 30 minutes encased between the two glass slides. Dried agar causes animals to clump together and desiccate. Mount animals within 2-15 µL of paralytic solution or microbeads and cover with coverslip; screen the slide within 20 minutes of paralysis and mounting (**Figure 6**).

NOTE: Because stress conditions can alter exopher rates, avoid paralytics that can induce oxidative stress (ex: sodium azide) when screening for exophers.

5.8. Detection of exophers using a spinning-disk confocal microscopy

5.8.1. Observe cell biological features such as organelles and other contents with 1.4 numerical aperture objectives at 63x and 100x.

5.8.2. Use software capable of stage-control and image acquisition utilizing the multidimensional acquisition. Microscopes and image processing software should also be suitable for imaging and data collection as these steps involve standard imaging approaches.

6. **Identifying touch neurons and scoring for exophers with mounted animals**

6.1. Mount paralyzed adult animal (**Figure 6**).

6.2. Identify the desired Z-plane. Use low magnification bright-field (10-40x) to identify the suitable Z-plane of the animal, taking note of the positioning of the animal, head-tail orientation, and location of the vulva - which are landmarks for later neuronal and exopher identification (**Figure 3A & Figure 5E**)**.**

6.3. Focus on the fluorescence signal of the chosen reporter. Staying in the same Z-plane, switch to widefield fluorescence viewing at 10-40x for the chosen cytosolic reporter.

NOTE: In this example fluorescent expression is driven by the *mec-4* mechanosensory touch neuron-specific promoter. High copy arrays, and different fluorophores have variability in expression and therefore variable fluorescent intensity. Adjust if needed.

6.4. Scroll within the Z axis to observe the depth of the animal and fluorescent expression in the focal plane. While doing so, confirm the head-tail orientation; the head/pharynx will have the fluorescent nerve ring and in this case, the tail will contain 1-2 visible PLM soma (**Figure 3A**).

6.5. Identify touch neurons

6.5.1. Identify whether the animal is mounted on the left or the right side (**Figure 3A**).

NOTE: Considering the 3-dimensionality of the animal, the best imaging resolution is accomplished on the side closest to the optics.

6.5.2. Identify the soma and neuronal processes. The soma should be the most obvious (brightest), marked by a round cell body at the end of the process.

6.5.3. Identify the soma (ALM, ALMR, AVM) by observing - start at the head to identify the nerve ring and lateral neuronal processes.

6.5.4. At 10-40x magnification, slowly scroll through the Z-axis to identify the plane of the attached process.

6.5.5. Once the process is identified, follow it laterally in the posterior direction towards the vulva, where the soma will be apparent. Once the most in-focus neuronal soma is found, it can be identified by using other neuronal landmarks as follows:

6.5.6. Use the **AVM,** a nearby ventral neuron, to help assign animal orientation. If the AVM neuron is in the same plane as the ALM then the animal is resting upon its side and the neuron outside that plane is the ALMR . If the AVM neuron is not in the same plane as the ALM in question, the closest touch neuron to the focal plane is ALML.

6.5.7. Identify the **PVM** neuron, another ventral touch neuron located near the tail, to indicate whether the anterior touch neuron is in the same plane. If so, the touch neuron observed is ALML.

6.5.8. Get a sense of the position of other soma bodies, near the area of interest (fluorescent neurons located on either side of the soma), and in all Z-planes, even if it is not possible to get the deepest neuron set in clear focus.

NOTE: The identification of all touch neuron somas is important because out-of-focus soma can be mistaken for exophers.

7. Identifying and scoring for exophers

7.1. Once a touch neuron is found, inspect it for large protrusions (exopher domains) large enough to be considered a bud exopher, (reaching *at least* 1/5th the size of the originating soma) (Figure 1C).

NOTE: The average exopher measures around 2-8 µm in diameter, while the average soma of a (ZB4065 *bzIs166*[P*mec-4*::mCherry1]) animal measures 6-10 µm in day 2 adults (**Figure 7B**).

7.2. If no bud or exopher domain is observed, inspect the neuronal soma for an attached thin filament emanating from the soma. Attached exophers tend to be located closer to the originating soma and in a similar Z-plane.

NOTE: Exophers do not always remain attached to the soma. The detection of an attached filament is a definitive indication that the object is an exopher.

7.3. To identify an unattached exopher, look for the contents of an exopher. Exophers can concentrate expelled fluorescent proteins and are therefore often brighter than the soma.

NOTE: The contents of exophers are heterogeneous and variable. Cellular organelles such as lysosomes and mitochondria can also be extruded within exophers (**Figure 4C-E**).

7.4. Look for unattached exophers in different focal planes than the plane in which the originating soma was found. Although exophers have been seen to protrude from the ALM soma in any direction, it is typical that exophers protrude away from the soma, in a posterior direction from the neuronal process.

7.5. Check for large, spherical objects that are not positioned and identified as neuronal somas. Exophers can be irregularly shaped but are typically spherical structures. Exophers become degraded over time, so older exophers tend to have a more irregular shape.

NOTE: Mature or older exophers are distinguished from the dispersed “starry night” stage via the brighter fluorescence intensity of exophers and their spherical shape.

7.6. Investigate the “starry night” phenotype as evidence of earlier exophergenesis. Exophers progress into a “starry night” stage as the exopher breaks up into smaller vesicles and the surrounding hypodermis attempts to degrade the exopher contents (**Figure 1G, 2B, 3B & 7A**).

NOTE: The starry night stage is marked by fragmented and scattered (sometimes networked) fluorescent debris that has lost structural integrity and displays a dim fluorescence in comparison to the touch neurons and exopher structures.

7.7. Look for instances of “multiple exopher events”. Exophers are usually produced as a

singular occurrence (1 exopher emanating from 1 soma) but under some circumstances more than one exopher can be released from a single soma (**Figure 7D**).

NOTE: Mature exophers can degrade into multiple vesicles as they are degraded in the hypodermis. Distinguishing whether each exopher was generated by an independent exophergenesis event or whether one original exopher split to create an additional vesicle can only be determined by time-lapse observation.

7.8. Keep in mind that **not all morphological abnormalities mature into exophers**.

7.8.1. Do not score a distended soma as an exopher. An extended or pointed soma can be observed on occasion (especially with age or under stress), but an extension without a clear constriction site is not scored as an exopher.

7.8.2. Reject small resolved buds that do not attain 1/5th the size of the soma in exopher event quantification.

7.8.3. Do not count neurite outgrowths as exophers. Mature neurites can extend dramatically with age (usually in the opposite direction of the neuronal process) and fluorescent protein can migrate into the distal end of such structures[19].

NOTE: These neurite outgrowths are not exophers as they have a distinct developmental pattern over days and weeks, do not form buds, and do not detach (**Figure 7E**)**.**

7.9. Identify fluorescent entities that are *not* exophers.

NOTE: It is important to get an idea of this background to ensure correct identification of the extruded fluorescent entity vs. autofluorescence.

7.9.1. **Transgenic fluorescent expression vs autofluorescence.** Do not mistake autofluorescence for transgenic expression. The true exopher signal will not be in the intestine or gut (DIC confirmation can be used to identify these tissues) and exopher signal will be significantly brighter than background autofluorescence.

NOTE: Autofluorescence is caused by gut granule intestinal fluorescent pigmentation and accumulates with age. It is heterogenous, especially among different wavelengths.

7.9.2. **Signal from embryos.** Do not mistake embryo signal for exophergenesis. Confirm suspicions of embryo signal by switching from fluorescence to brightfield illumination and checking for associations of signal with eggs in the uterus.

7.9.3. **Out of plane or nearby soma bodies.** Avoid mistaking an out of plane soma for an exopher by identifying all nearby soma bodies, even out-of-focus somas at the start of the observation.

NOTE: If scoring for exophers from ALMR, identify and account for the location of AVM and ALMR somas. More details on soma body identification are described in (**Figure 3A**).

8. **Scoring and statistics**

8.1. Score exophers as binary (yes, there is an exopher/no, there is not an exopher).

8.2. Consider exopher detection as an 'exopher-event" for a given neuron. An exopher event can constitute observation of a single exopher near a soma or multiple exophers.

NOTE: To quantify numbers of individual exophergenesis events use time-lapse observation

8.3. Count exopher events per a particular identified cell because different cells do not produce exophers at the same rate (see for example **Figure 3C**). ALMR neurons produce the most baseline exophers in the strains described herein and thus often this is the cell selected for exopher quantification from touch receptor neurons.

8.4. For statistics, in general, conduct at least 3 biological trials, of *at least 30* animals scored per trial with the corresponding number of observations required for analysis of the disruption.

8.4.1. For multiple trials involving one or two mutants/treatments compared to control, the Cochran-Mantel-Haenszel test is appropriate to determine *p* values.

8.4.2. For trials involving more than two mutants of treatments compared to control, it is also appropriate to use a binary logistic regression analysis to evaluate significance for any number of categorical predictors.

REPRESENTATIVE RESULTS

Multiple fluorescent reporters can be used to measure exophers. Touch neuron exophers are readily visualized in vivo via fluorescent tagging of proteins that may be selected for extrusion, by labelling of organelles that can be extruded, or by tagging cell membranes. **Table 1** identifies touch neuron expressed fluorescent reporters that have been used to monitor exophers, with representative examples included in **Figure 4**. Cargoes that are known to be extruded in exophers include a fusion of the N-terminal domain of human huntingtin to expanded polyglutamine (Q128) (**Figure 4B**), lysosomes that are GFP-tagged with lysosomal associated membrane protein (LMP-1) (**Figure 4C**), and mitochondria tagged with matrix-localized GFP (**Figure 4D**). Cytoplasmic GFP is not strongly expelled and is preferentially retained in the soma[5], although GFP can weakly visualize exophers (**Figure 4A**). When GFP is fused to proteins that are expelled, this tag can be used to visualize exophers. An important point is that by tagging different proteins, a large range of questions on the expulsion of specific cargoes and organelles, as well as on the proteins and membranes that make up exophers, can be addressed.

A pseudo-stereomicroscope setup is an effective tool for viewing exophers in animals upon agar plates. This setup is a hybrid of compound and stereoscopic technology that includes high

numerical aperture optics on each magnification, pseudo-stereo technology (discrete objectives over a stereoscopic base), and a zoom operating switch for viewing at magnifications intermediate to installed objectives. A microscope such as this should be equipped with 10x eyepieces and objectives powerful enough for observing neuronal morphology and exopher production for high-throughput scoring (2x objective used for scanning/picking, 10x objective used for identifying and scoring).

While magnification capabilities of standard stereomicroscopes typically have high enough resolution to see the network of touch neurons expressing fluorescent proteins, standard dissecting microscopes are not sufficient for observing subcellular details of exophers like the tubular connections of soma to exopher. Such observations necessitate confocal microscopy (see the **Table of Materials** for equipment details).

Exopher quantitation studies require strict controls to eliminate experimental stresses. The attentive maintenance of consistent growth conditions is required for reproducible exopher production. More specifically, exopher production is stress-responsive such that consistent feeding, constant temperature, and contamination-free growth across generations are critical for reproducibility. Under basal growth conditions with high neuronal expression of mCherry, exopher production is relatively low (5-15% of ALMRs produce exophers) but some stresses, including osmotic and oxidative stress, can increase exopher rates. While mCherry expression can be thought of as stress, a corollary of the stress-sensitivity of exopher levels is that, if properly controlled, experimental stress introduction can be a strategy to more easily induce and observe exophergenesis.

Timing and anticipated exopher production levels. Exophers are virtually absent during larval development. The period of peak exopher production in young adult life appears to be highly restricted to during adult days 1-4, most commonly being evident at adult day 2 or 3. Because the peak can shift ahead or back a little, the most complete evaluation of an exopher production profile is to score multiple trials daily over adult days 1-4. In general, an ALMR produces one major exopher, with the vesicle persisting for at least 24 hours. The exopher can be produced fairly quickly (on the order of minutes at its fastest). Most commonly, only one major exopher is produced per neuron in early adult life, but production of multiple exophers is possible.

In general exopher production by ALMRs expressing mCherry under basal conditions ranges from 5-15% of ALMRs examined within the optimal timeframe of adult day 2-3 (**Figure 3D**). Proteostasis crises[5], as well as exposure to other stresses can modulate exopher level. Stress or genetic perturbations can increase exopher production to detection rates as high as 90% of ALMR neurons producing exopher extrusions.

Feeding-based RNAi for testing roles of specific genes in exophergenesis. The nematode *C. elegans* is commonly subjected to RNAi knock down by feeding animals transformed *E. coli* strain HT115 that express a double stranded RNA (dsRNA) targeting a gene of interest[20]. HT115 bacteria can be used when scoring for exophers in feeding RNAi[5]. While transcripts in most tissues can be targeted by RNAi using this technique, neurons are more refractory. Sensitivity to RNAi can be

calibrated using animals that express the transgenic dsRNA transporter SID-1 under a neuron-specific promoter. In this way neuronal tissue can be sensitized to RNAi[21].

Tissue-specific knockdown of a gene of interest can be accomplished by expressing a component of endogenous RNAi metabolism within a mutant that is deficient in that component. For example: the Argonaute protein RDE-1 can be expressed specifically in the neurons of *rde-1* mutant animals to achieve knockdown of a gene of interest **only** in neurons when animals are exposed to an RNAi intervention targeting that gene.

Using standard nematode RNAi protocols[20,22], exposure of the parents at the L4 stage to the RNAi and allowing their progeny to develop consuming transformed HT115 bacteria until adulthood generates the strong genetic knock-down but be attentive to potential developmental delays induced by RNAi as experimental animals may grow differently than an empty vector control. It is important to always include the empty vector control for negative control comparison. HT115 bacteria can be used when scoring for exophers in feeding RNAi experiments does not significantly change baseline exopher production from the standard OP50 *E. coli* strain. However, note that some genes are effective at changing exophergenesis rates even during shorter periods of RNAi exposure[5]. If targeting of certain genes leads to developmental failure, avoid exposing animals to lifelong knockdown, animals can simply be picked at the L4 stage onto RNAi plates for exposure from L4 to adult D2 or D3.

Tables and Figure Legends

Table 1. Strains that have been used for visualization of touch neurons, touch neuron-exophers, and exopher contents.

Figure 1: Stages of Exophergenesis. The process of making and ejecting an exopher is called 'exopher-genesis'. The dynamic process of exopher formation can take several minutes to several hours. Depicted are examples of soma and exopher morphology at specific steps during the dynamic exophergenesis process in a high-exopher producing strain, ZB4065 *bzIs166*[P_{mec-4}mCherry]. All images are of day 2 adult ALM neurons taken with a 100x objective. (**A**) Normal soma. Adult mechanosensory touch neuron ALM transgenically expressing P_{mec-4}mCherry. The soma morphology depicted is typical of young adult neurons in this strain, with mCherry concentrations in the cytoplasm. (**B**) Early bud phase. The first observable step of exophergenesis involves polarization of selected cytoplasmic material to the edge of the soma membrane. This step is often accompanied by an expansion or swelling of the soma. In the case of the touch neurons, the pre-exopher domain (PED) extends into the surrounding hypodermis (not visible here). Note the greater concentration of mCherry material into the early bud domain. (**C**) Late bud phase. Upon further cellular polarization and an expansion of the pre-exopher domain, a constriction between the soma and exopher (arrow) becomes evident. This event signals the transition to the late bud phase. Although in the late bud stage the cell exhibits a clear constriction site and separate soma and exopher domains, it is not yet pinched off completely from the soma; the budding exopher may be attached by a thick stalk (arrow). The budding domain is considered an early exopher when the diameter of the exopher domain in question is

roughly ⅓ larger than the diameter of the construction site/stalk. (**D**) Early-exopher phase. Early exophers can be attached by a stalk from the departing soma—the diameter of this connection can thin as the exopher moves away from the soma. Cytoplasmic material can be transferred from the soma to the exopher via this tube, although most material is loaded during the process of budding out. Exophers can detach from the soma as depicted in (**E**), separated exophers are considered mature exophers (**F**). The mature exopher can transit through the surrounding hypodermal tissue, moving away from the departing soma. (**G**) Breakdown of the mCherry-labelled exopher into smaller vesicles within the hypodermis results in a scattered punctate appearance of the mCherry material, most likely as it enters the hypodermal endolysosomal network. The dispersed punctate signal is called the "starry night" phase. Degradation of some exopher contents is likely accomplished by hypodermal lysosomes, but some material is not fully degraded and is often re-extruded by the hypodermis into the pseudocoelom. The post-exophergenesis mCherry transit is described in more detail in **Figure 2.**

Figure 2: mCherry extruded from touch neurons in exophers engages the surrounding hypodermal lysosomal network but is eventually extruded into the pseudocoelom where coelomocytes can store/degrade the mCherry. (**A**) Cartoon summary of how mCherry extruded in exophers transits the body after expulsion by neurons. During exophergenesis selected cellular contents such as mCherry become localized and bud off from the sending neuronal soma in an independent vesicle surrounded by the neuronal and hypodermal plasma membranes. Since the touch neurons are embedded in the hypodermal tissue, as the exopher domain buds outwards it moves further into the hypodermis. The exopher can transit the hypodermis, and after hours to days, exopher contents can fragment within the endolysosomal network of the hypodermis. The mCherry can appear as scattered puncta throughout the hypodermis, a stage called "starry night". After a few days, some of the mCherry debris can pass out of the hypodermis into the surrounding pseudocoelom, where scavenger cells called coelomocytes can get access to, and take up, mCherry debris that can be stored. (**B**) Example of the appearance of the starry night mCherry vesicles. Image of an ALM soma tagged with mCherry with large exopher fragments and starry night vesicles. Strain is ZB4065 *bzIs166*[P_{mec-4}mCherry]. (**C**) Example of mCherry concentration in distant coelomocytes. Sideview of an adult animal day 10 of strain ZB4065 *bzIs166*[P_{mec-4}mCherry] showing mCherry concentrated in coelomocytes (arrows). Some starry night vesicles are also evident. In general coelomocyte concentration becomes evident after about adult day 5 of life. (**D**) Cartoon reproduction of (**B**), with touch neurons and processes outlined in red, as are brightest exopher fragments; scattered small vesicles of different Z-depths are shown in lighter pink. (**E**) Cartoon version of image of (**C**), showing neuronal process in red, debris in pink and coelomocytes in green.

Figure 3: Mechanosensory touch neurons produce exophers at different levels with a precise temporal profile. (A) (Top) Cartoon depiction of mechanosensory touch neurons in spatial relation to key anatomical landmarks of *C. elegans* include the pumping pharynx and the neuron-dense nerve ring at the head of the animal, the vulva at the mid body, and the tapered tail. (Bottom) Fluorescently labeled touch neurons expressing GFP as viewed from the top and left side (images adapted from WormAtlas). The red box depicts the area where ALM exophers are typically located. (**B**) High magnification view of the mid body region at which ALM-derived

exophers are produced in a strain expressing [P*mec-4*mCherry]. AVM and ALMR neuron are depicted, and shown is an ALMR exopher along with mCherry starry night debris. ALMR neurons most readily produce exophers. (**C**) ALMR mechanosensory touch neurons more readily produce exophers compared to other touch neurons in hermaphrodites under basal conditions. Mechanosensory touch neuron exopher production on adult day 2, as scored for individual touch receptor neurons is indicated. Strain: ZB4065 *bzIs166*[P*mec-4*mCherry], N>150, error bars are SEM. (**D**) ALMR touch neurons produce more exophers during days 2 and 3 of adulthood compared with the adolescent L4 stage or with animals in advanced age. Strain: ZB4065 *bzIs166*[P*mec-4*mCherry], N>150, error bars are SEM.

Figure 4: Examples of some fluorescent reporters that tag exopher contents. A straightforward way to observe exophers is by creating transgenic animals that express fluorophores from neuronal promoters. The fluorophores allow for visualization of the exopher and transgenic expression induces aggregation and/or proteostress that increases exophergenesis. Exophers produced by amphid neurons can also be observed under native conditions, using dye filling for visualization. Shown are examples of common strains that can be used to observe exophers, (E) exopher, (S) soma. (**A**) Soma and exopher from an ALM of an adult of strain SK4005 *zdIs5*[P*mec-4*GFP],100x objective used for photography, scale bar 3µm. In this strain, exophers that include soluble GFP are measured, but exopher production occurs infrequently. Fusing GFP to proteins that can be preferentially extruded in exophers in other studies confirms that GFP fusions can be detected in mature exophers**. (B**) ALM soma and exopher of an adult of strain ZB4065 *bzIs166*[P*mec-4*mCherry], which expresses mCherry and induces touch neuron exopher production. 100x objective used for photography, scale bar 5 µm. (**C**) ALM soma and exopher of an adult of strain ZB4067 *bzIs167*[P*mec-4*mitogfp P*mec-4*mCherry4]; *igIs1*[P*mec-7*YFP P*mec-3*htt57Q128::cfp *lin-15*+]; selective blue channel used for image of htt57Q128::CFP. The exopher contains htt57Q128::CFP aggregates (arrows), that appear more concentrated in the exopher than in the soma. 40x objective used for photography, scale bar 5µm. (**D-E**) Exophers can contain organelles and organelle-specific tagging with fluorescent proteins enables monitoring of organelle extrusion. (**D**) Lysosomal membrane tag LMP-1::GFP outlines the soma and exopher membrane and tags plasma membranes weakly (plasma membrane localization is a trafficking step on the way to lysosomal targeting) and labels lysosomal organelles strongly. Shown is an adult ALM soma co-expressing P*mec-4*mCherry and the P*mec-7*LMP-1::GFP that localizes to membranes and lysosomes. The soma has an attached exopher with other smaller extrusions likely to be exopher fragments (arrows). GFP positive structures are included in the soma and are present in the large exopher, mostly co-localized with mCherry (yellow)**,** strain: ZB4509 *bzIs166[*P*mec-4*mCherry]*; bzIs168*[P*mec-7*LMP-1::GFP]. 100x objective used for photography, scale bar 5 µm. **E**) A mitochondrial GFP marker can be used to identify mitochondria in soma and exophers. Shown is an adult ALM soma expressing P*mec-4*mCherry and mito::ROGFP, which localizes to the mitochondrial matrix. mito::ROGFP expressed alone, without the mCherry, can also readily be used to identify neurons and score for exophers that include mitochondria. Strain: ZB4528 *bzIs166*[P*mec-4*mCherry]; *zhsEx17* [P*mec-4*mitoLS::ROGFP]. 100x objective used for photography; scale bar 5µm.

Figure 5: Developmental cycle of *C. elegans* and L4 identification. (**A**) At 20 °C an egg takes approximately 9 hours to hatch once laid by the mother. (**B**) A newly hatched animal is in larval stage 1 (L1) and molts into an L2 larva after 12 hours. (**C**) Animals remain in the L2 and the (**D**) L3 larval stages for about 8 hours each. (**E**) Adolescent animals are considered the fourth larval stage (L4) and are marked by a conspicuous developing vulva that appears as a white crescent near the mid body. The presence of this while crescent enables easy identification and picking of L4 staged animals to establish synchronized cultures that later facilitate scoring for exophers. Animals remain in the L4 stage for about 10 hours before their final molt into gravid adults, **F**) identified by developing eggs, visible spermatheca, and the initiation of egg-laying.

Figure 6: Preparation of microscope slide agar pad. (**A**) Prepare two slides with a single strip of laboratory tape placed lengthwise across the top. Place a non-taped microscope slide in between as pictured. **B**) Place a drop of molten agarose on top of the slide. (**C**) Place a clean slide gently on top of the drop, pressing the agarose into a deflated circle pad. (**D**) Remove the taped slides, which act to accomplish an even flattening of the agar that is needed to create an even pad. (**E**) Remove the top slide once the agarose pad has dried. (**F**) Pipette a paralytic solution (levamisole or tetramisole) on top of the agar pad. (**G**) Pick appropriately staged animals into the paralytic. (**H**) Gently cover the animals with a coverslip and ensure animals are alive.

Figure 7: Characters of exophers and exopher identification criteria. (**A**) General criteria that identify an exopher. (**B**) Diameter comparisons between the sending soma and the extruded exopher, measured in µm. Adult ALM somas, N=35, strain: ZB4065 *bzIs166*[$P_{mec\text{-}4}$mCherry] - 6.53 µm average size of soma and 3.83 µm average size of exopher. (**C**) Defining criteria for differentiating between an exopher domain and a budding exopher. (**D**) Most commonly, individual neurons make one large exopher, which later splits or fragments as the hypodermis attempts to degrade its contents. Still, multiple exophers may be observed next to one touch neuron that might derive from either multiple exopher events from one neuron or alternatively, exophers can also bud or fragment themselves. The origin of multiple exopher-like entities can only be determined using time lapse microscopy. Top depicts an ALMR touch neuron soma with a single distant exopher. Bottom depicts an ALMR touch neuron soma with multiple exopher-like extrusions. (**E**) Common morphological features in adult ALM touch neuron somas that may be mistaken for exopher events. **Left top** - A distended ALM soma, with no clear exopher domain or constriction site. **Left middle** - Neurons can have small extracellular protrusions that may be analogous to exophers, but do not meet size requirement criteria to be considered an exopher. **Left bottom –** With age, touch neurons can develop outgrowths along their minor neurite. Often mCherry material can be collected at the tip of the neurite outgrowth. This is not scored as an exopher if the collected mCherry does not meet exopher-to-soma size requirements. **Right** depicts adult ALM neurons that have defining criteria for an exopher domain or an exopher. **Right top** - ALM soma that has a prominent exopher domain that selectively includes mCherry cytosol and mCherry tagged aggregates. The exopher domain constriction site is marked by arrows and meets the size criteria (at least ⅕ the size of the soma). The largest diameter of the exopher domain is almost ⅕ bigger than the diameter of the constriction site, meeting criteria for an exopher event. **Right middle** - ALM soma that has a prominent budding exopher that meets the size criteria. There is a clear constriction site. **Right bottom -** ALM soma that has an attached

mCherry-filled exopher that meets exopher size requirements. The exopher is attached by a thin connecting filament. All images are from strain **ZB4065** *bzIs166*[P_{mec-4}mCherry].

DISCUSSION

The characterization of the in vivo molecular mechanisms of aggregate and organelle elimination in the form of large exophers is in its infancy. Questions as to the designation of cargoes for expulsion, the polarized collection of these cargoes within the cell, the regulation of the decision to generate exophers, the machinery that mediates extrusions, and the interaction of exophers with the degradative machinery in a neighboring cell all remain to be addressed. Furthermore, the in vivo visualization of tubular connections that can pass biological materials that include calcium, aggregates, and mitochondria is interesting and understudied biology in its own right. Questions of why certain cells are more prone to exopher production than others also are unresolved, but can begin to be genetically dissected with the approaches outlined in this protocol.

Described in detail in this protocol are the approaches to achieving reproducible scoring of exopher production, with attention to distinguishing exophers from nearby cell somas, timing of analyses to capture peak of exopher production, and strict control of growth conditions to eliminate unintended stresses that can modulate exopher levels. Both distinction of the large early exopher, or the "starry night' dispersion in the surrounding hypodermis can be quantitated as evidence of exopher production. That being said, neurons expressing mCherry under basal conditions are most often associated with 5-15% of neurons of a specific type producing an exopher. Controlled introduction of stress conditions could be applied to increase exopher production to detection as high as 90% of neurons producing extrusions, particularly useful for genetic or pharmacological screens for modifiers.

In human neurodegenerative disease, large aggregates can transfer from diseased neurons into neighboring cells to promote pathology spread. The exopher mechanism might transpire via a conserved mechanism used for aggregate extrusion across phyla. Defining the in vivo molecules that either enhance the efficiency of this process (considered more effective proteostasis control) or block it might be harnessed to influence design of novel strategies for combating multiple neurodegenerative diseases. As such, the protocol described here could be used for classical genetic mutagenesis screens, genome-wide RNAi screens that systematically knock down genes to identify enhancers and suppressors, or for drug intervention studies that identify candidate pharmacological modifiers of this process. The approach is straightforward, although somewhat laborious. Exophers are so large they can be viewed with a high-magnification dissecting microscope. Still, *C. elegans* neurons are relatively small and looking at their organelles or their membranes require higher power confocal images and is a slow process. Options for higher throughput could involve high content imaging approaches in multi-well plate format.

The application of a standardized approach to exopher scoring should underlie a concerted genetic dissection of the process by which neurons can organize and eliminate cellular debris.

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DISCLOSURES:

None

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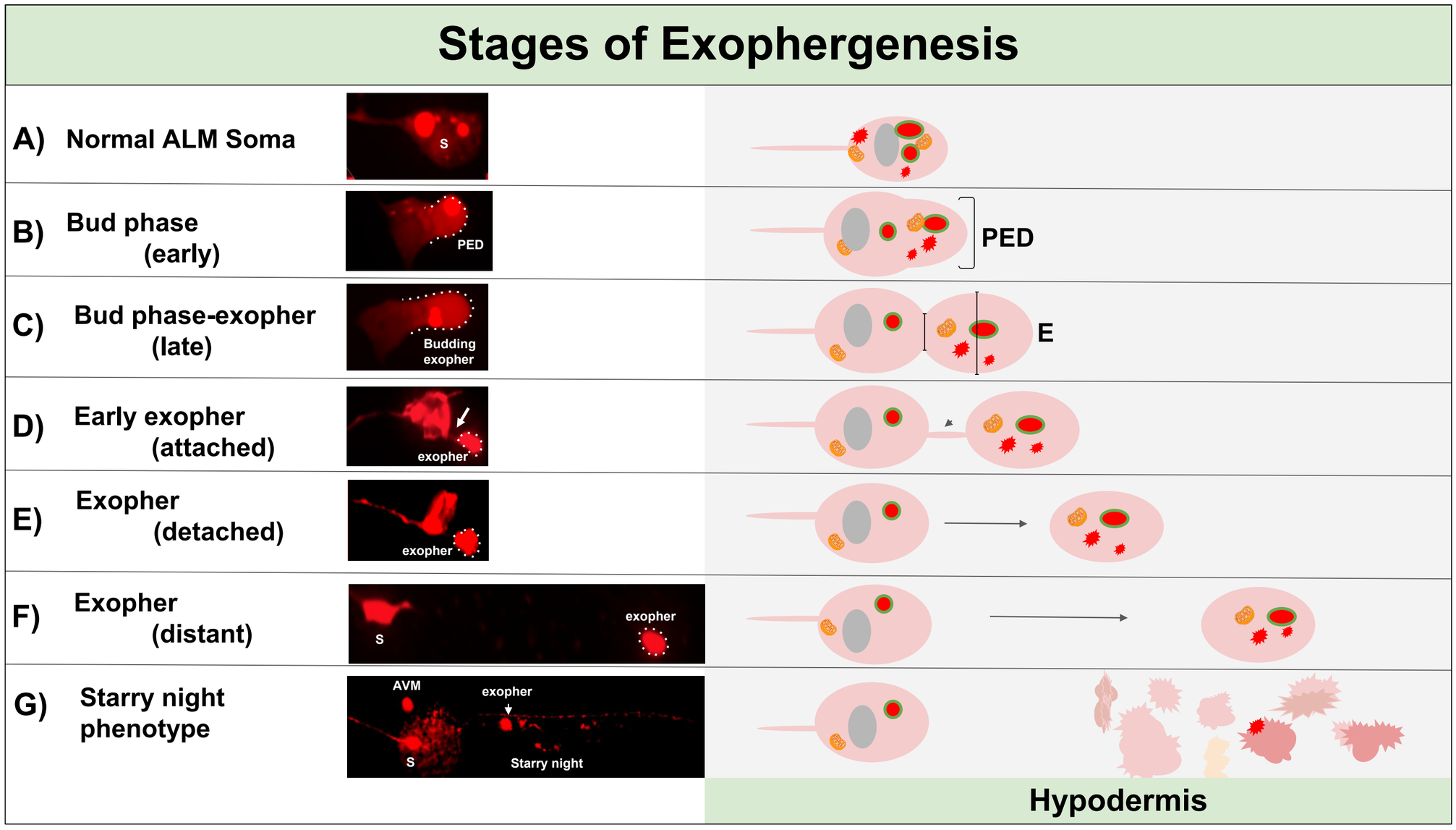


Figure 1

Figure 2

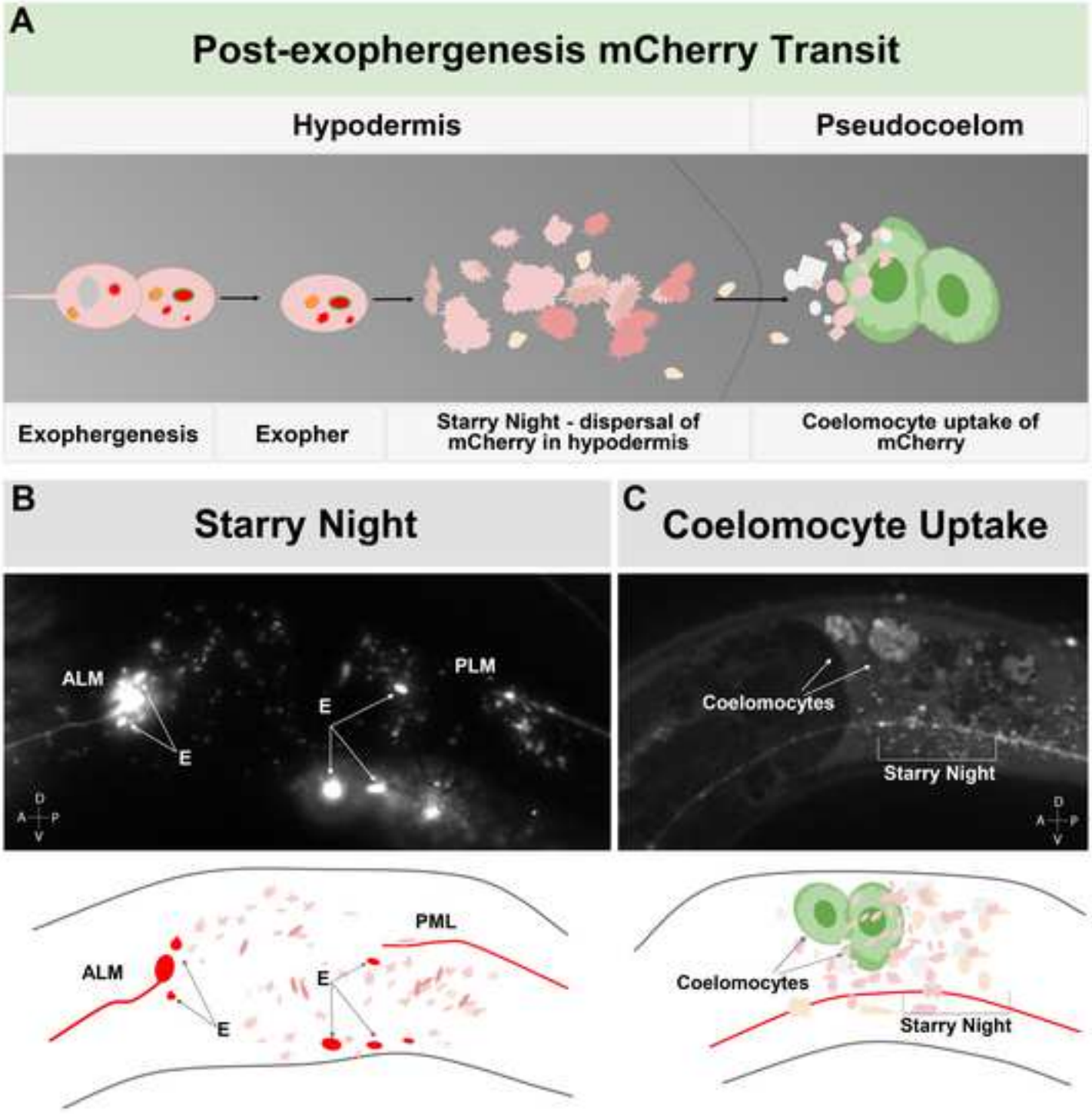
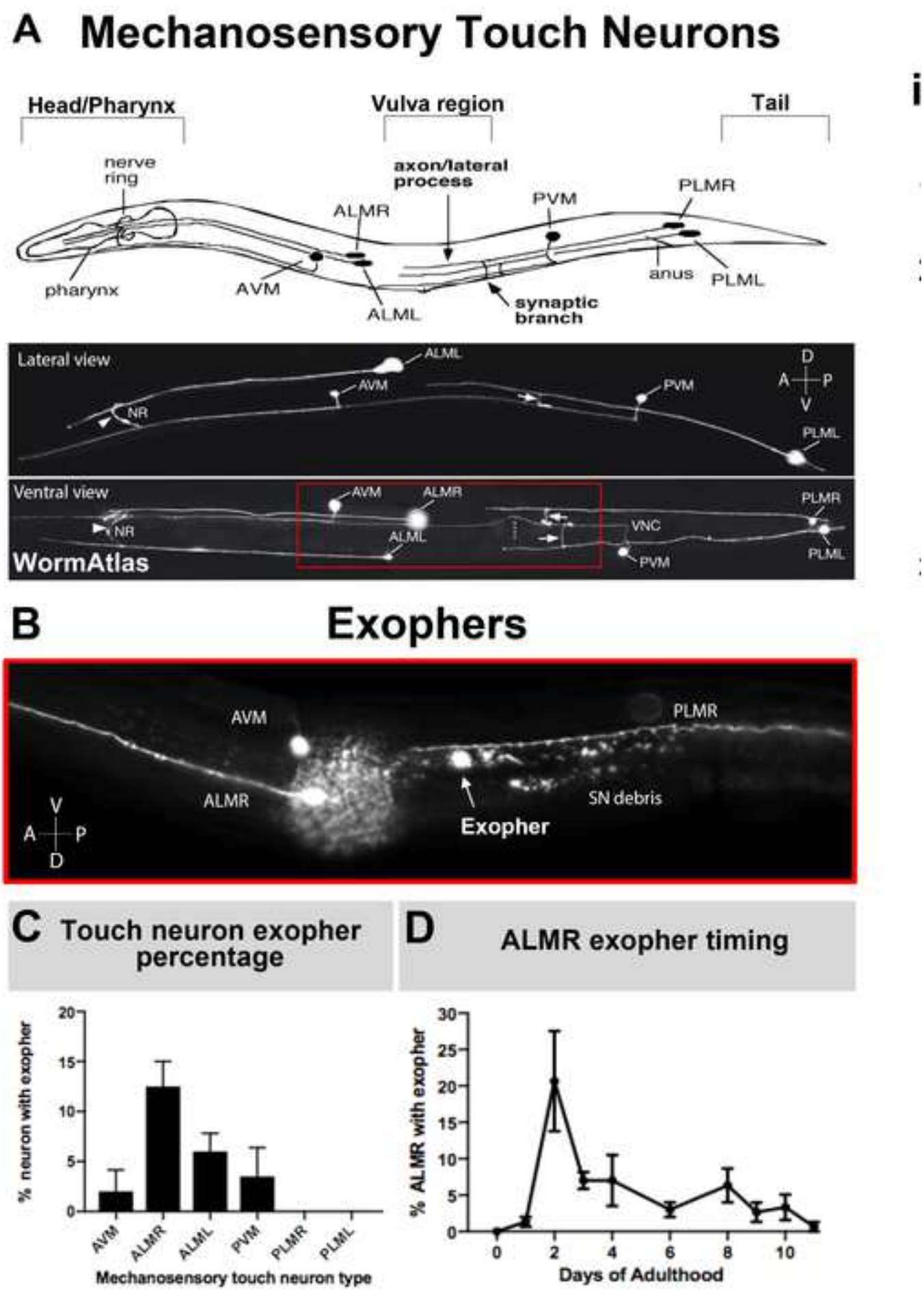


Figure 3



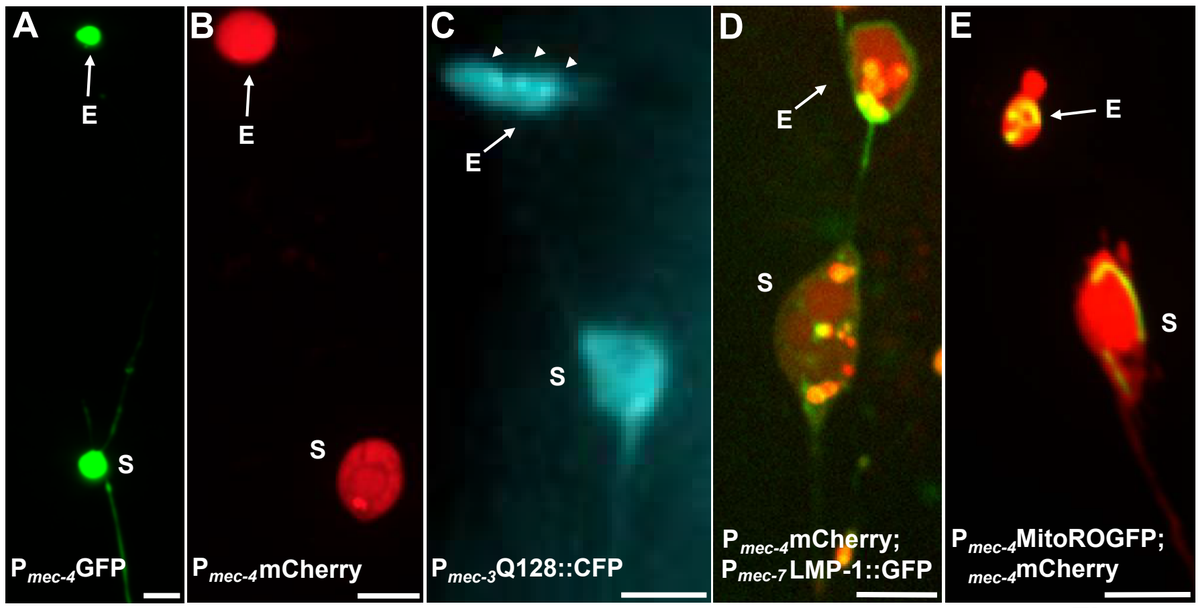


Figure 4

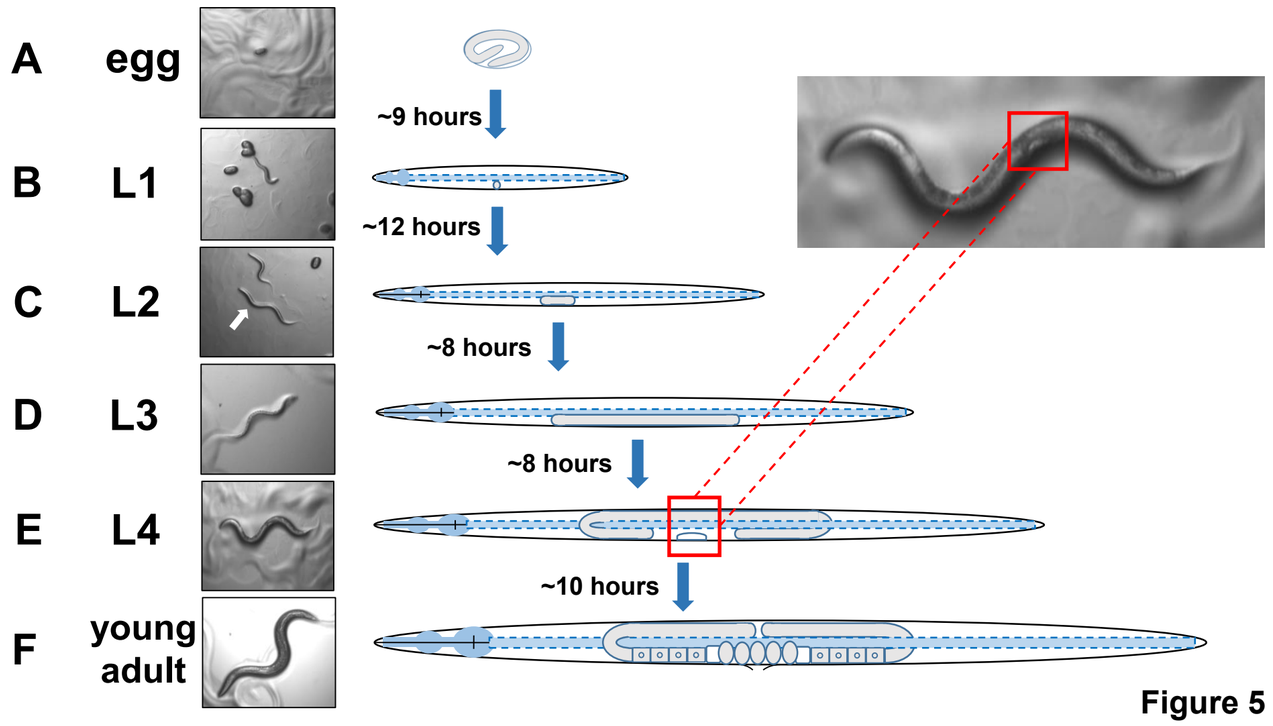


Figure 5

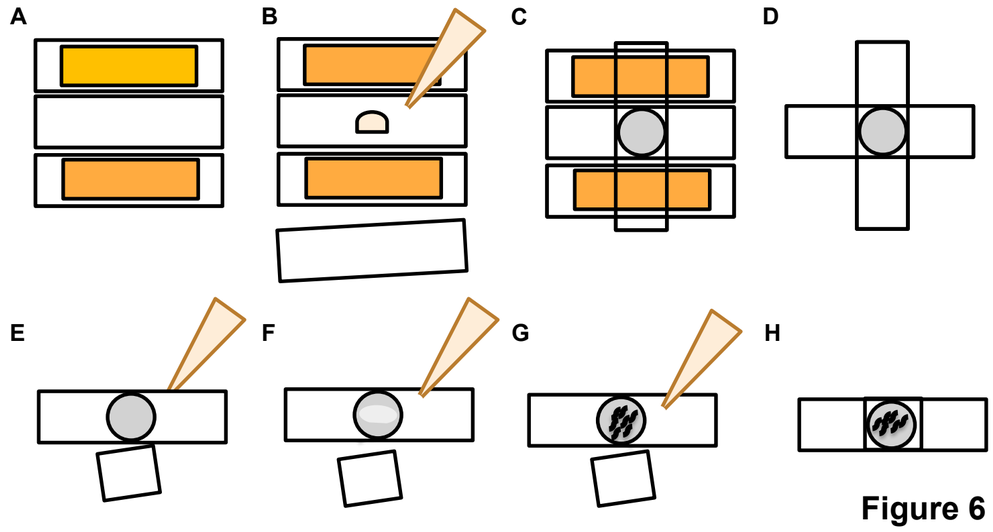
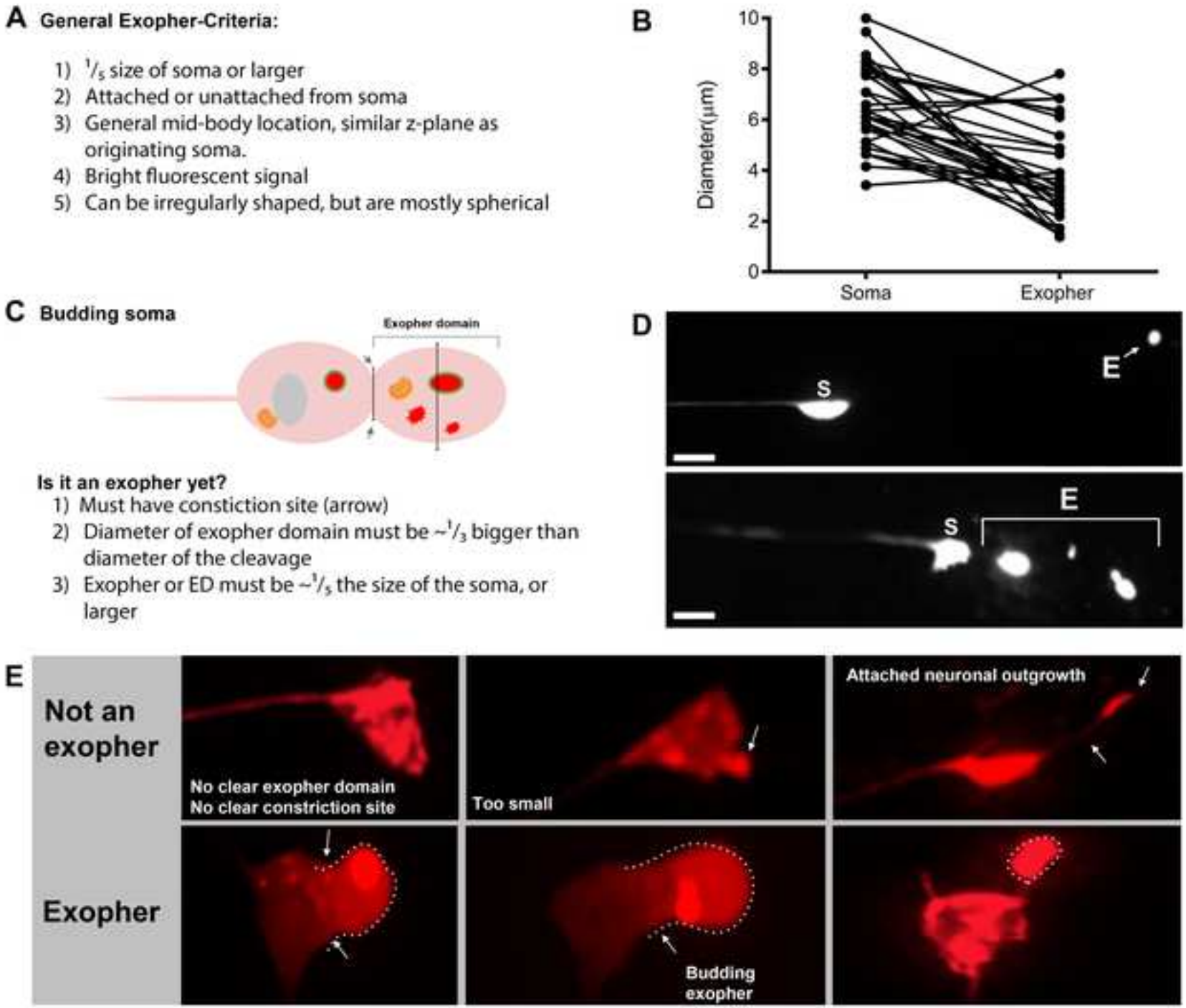


Figure 6



Strain name	Genotype
SK4005	*zdIs5* [P_{mec-4} GFP]
ZB4065	*bzIs166* [P_{mec-4} ::mCherry]
ZB4067	*bzIs167* [P_{mec-4} mitogfp P_{mec-4} mCherry4]; *igIs1* [P_{mec-7} YFP P_{mec-3} htt57Q128::cfp *lin-15* +];
ZB4509	*bzIs166[* P_{mec-4} mCherry]*;* *bzIs168* [P_{mec-7} LMP-1::GFP]

ZB4528	*bzIs166* [P*mec-4* mCherry]; *zhsEx17* [P*mec-4* mitoLS::ROGFP]

Description
Cytosolic expression of GFP in touch neurons.
Overexpression of mCherry (*bzIs166*) in touch neurons, produces both cytosolic signal and mCherry aggregates. *bzIs166* is an exopher inducer. mCherry aggregates are predictors of exophergenesis and are preferentially extruded in exophers.
YFP cytosolically labels *mec-7* touch neurons. Co-expressed Q128::CFP aggregates and induces exophers. CFP preferentially silences.
bzIs168 LMP-1::GFP labels plasma membranes and lysosomal membranes. *bzIs168* can be used to identify neuronal membranes, exophers (as they are membrane bound), and lysosomal-membrane structures.

Allele zhsEx17 is a mitochondrially localized reporter that changes its peak excitation wavelength from 405nm (oxidized) to 476nm (reduced) according to the local oxidative environment. It is expressed in the touch neurons and can be used on its own to identify mitochondria in touch neurons and in mito-exophers.

Exopher percentage	Reference
1-8% ALM	Figure 4A, Melentijevic 2017
3-20% ALM (normal conditions). 20-80% ALM (fasting conditions).	Figure 4B, Melentijevic 2017
~25%	Figure 4C, Meletijevic 2017
3-20% ALM	Figure 4D, Melentijevic 2017

3-20% ALM proteo-exopher. % ALM mito-exopher quantitation in progress.	Figure 4E, Melentijevic 2017, Cannon 2008, Ghose 2013

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
95B Scientific CMOS camera	Photometrics Prime		
1,000 µL low retention tips	Sarstedt		
10 mL serological pipette	Appleton Woods	CC214	
10 µL low retention tips	Sarstedt	70.1130.105	
13% sodium hypochlorite	Acros Organics	AC219255000	
15 mL centrifuge tubes	Fisher Scientific	05-539-12	
2 L erlenmeyer flasks	Scientific Laboratory Supplies	FLA4036	
25 mL serological pipette	Appleton Woods	CC216	
300 µL low retention tips	Sarstedt	70.765.105	
50 mL serological pipette	Appleton Woods	CC117	
5-Fluoro-2'-deoxyuridine 98%	Alfa Aesar	L16497.ME	
9 cm sterile Petri dishes	Fisher Scientific	11309283	
absolute ethanol	Vwr	20821.33	
Agar	Sigma Aldrich	A1296	
C. elegans strain wild type	Supplied by CGC	N2	C. elegans strain
calcium chloride dihydrate	Sigma Aldrich	C3881	

cholesterol	Acros	110190250	
dibasic sodium phosphate	Sigma Aldrich	S3264	
E. coli strain OP50	Supplied by CGC	Op50	E coli strain
FBS10 Standard microscope	Meyer Instruments	KSC 410-1-100-1	FBS10 Standard with Plate Base, 100/100 Trinocular Head and Flip zoom
glass pipette 270 mm	Fisherbrand	FB50255	
Heraeus Multifuge X3R	Thermofisher scientific	75004515	
Inoculating Spreaders	Fisher Scientific	11821741	
LB medium capsules	MP biomedicals	3002-031	
LDI – Laser Diode Illuminator	89 North		
levamisole	Sigma Aldrich	16595-80-5	
M4 multipette	Eppendorf	4982000012	
magnesium sulphate	Sigma Aldrich	M7506	
monobasic potassium phosphate	Sigma Aldrich	P0662	
Multitron Standard shaking incubator	Infors HT	INFO28573	
Nalgene 1 L Centrifuge pots	Fisher Scientific	3120-1000	
P10 pipette	Eppendorf Research Plus	3123000020	
P1000 pipette	Eppendorf Research Plus		

P200 pipette	Eppendorf Research Plus	3123000055	
pipeteboy 2	VWR	612-0927	
Polystyrene microbeads	Sigma Aldrich	MFCD00131491	
RC5C plus floor mounted centrifuge	Sorvall	9900884	
Reusable ringed cytology slides	ThermoFisher Scientific	22037242	
SK4005 zdIs5[Pmec-4GFP]	contract Driscoll lab		GFP expressed in touch neurons
sodium chloride	Sigma Aldrich	13422	
Sodium hydroxide	Fisher Chemical	S/4880/53	
Tactrol 2 Autoclave	Priorclave		
Triton-X	Thermofisher scientific	28313	
Tween 20	Sigma Aldrich	9005-64-5	
X-Light V2 Spinning Disk Confocal Unit	CrestOptics		
ZB4065 bzIs166[Pmec-4mCherry]	contract Driscoll lab		mCherry expressed in touch neurons
ZB4067 bzIs167[Pmec-4mitogfp Pmec-4mCherry4]; igIs1[Pmec-7YFP Pmec-3htt57Q128::cfp lin-	contract Driscoll lab		Q128 expressed in touch neurons
ZB4509 bzIs166[Pmec-4mCherry]; bzIs168[Pmec-7LMP-1::GFP]	contract Driscoll lab		mitoROGFP expressed in touch neurons
ZB4528 bzIs166[Pmec-4mCherry]; zhsEx17 [Pmec-4mitoLS::ROGFP]	contract Driscoll lab		autophagy marker expressed in touch neurons
ZEISS Axio Vert.A1	Zeiss		

Editorial Comments:

1)Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Done

2)Protocol Language: The JoVE protocol should be almost entirely composed of numbered short steps (2-3 related actions each) written in the imperative voice/tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief "Note" at the end of the step (please limit notes). Please re-write your ENTIRE protocol section accordingly. Descriptive sections of the protocol can be moved to Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results. Majority of your steps needs re-writing.

Done.

1. Examples NOT in the imperative: 1.1-1.3, 3.1, 3.3, 3.3.1, 3.5, 3.5.2,3.6, etc.

Done.

2. Avoid personal pronoun "your".

Done

3. Ensure that the protocol does not exceed 10 pages; currently it is edging close to 10 pages.

Done.

3)Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:

Done.

1. Several steps appear to be general guidelines, please rewrite your protocol to be more specific. Avoid generic discussions.

Done.

2. 4.4.4, 4.4.7: convert speed to g.

Addressed

4.4.4 Centrifuge at 2000 RCF x g for 5 minutes.

4.4.7 Mix by inversion for 1 minute, then centrifuge 2000 RCF x g for 1 minute.

4)Protocol Numbering: All steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

addressed, There is now a space between each step, all steps are aligned left.

5)Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1. The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.

2. The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3. Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4. Notes cannot be filmed and should be excluded from highlighting.

addressed

6) Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs):

1) modifications and troubleshooting - conditions needed for replication are mentioned.

2) limitations of the technique. "Still, C. elegans neurons are relatively small…"

3) significance with respect to existing methods. "Exophergenesis seems to be conserved across phyla", - Protocol described is useful for genetic dissection, C. elegans is a suitable model to study a human disease.

4) future applications high throughput genetic dissection of aggregate spread mechanisms.

5) critical steps within the protocol - critical steps described are regarding reproducibility, timing, peak, etc.

7) Figures:

1. Add scale bars to all micrographs.

addressed Figure 1 - scale bars not available, addressed in figure legend

2. Avoid excessive text in figures such as in fig 3 and 8. The text should be merged with the figure legend instead.

Reduced text in figure, merged text with figure legend text.

3. Remove the text "Figure #"

addressed

4. Figure 7 should be made into a table and uploaded as an excel file.

Addressed

8) References: Please spell out journal names.

addressed

9) Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent.

Examples of commercial sounding language in your manuscript are Kramer , (Fisher Scientific #22037242, Metamorph (Molecular Devices Corporation), Axiovert Z, (CrestOptics, Photometrics prime, h X-Light V2, etc

1. Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

addressed:

5.9.3 A spinning disk confocal microscope that is suitable for exopher observation is the Zeiss confocal microscope equipped with Spinning Disk Confocal Unit, equipped with a 7-line LDI Laser Launch, a Scientific CMOS camera, and a computer controlled motorized stage.

10) Table of Materials: Please sort items alphabetically.

Addressed.

11) If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All graphs and tables are original and not published in another publication.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

"Quantitative Approaches for Scoring in vivo Neuronal Aggregate and OrganelleExtrusion in Large Exopher Vesicles" by Dr. Monica Driscoll et al. is a well-thought-out, detail-oriented, practical and comprehensive instruction protocol for visualization, scoring and analysis of neuronal exophers. With much attention to detail and numerous examples of what exophers look like (and no less important, what they don't look like), this article is a valuable and much-needed resource for the scientific community at large. I strongly recommend this manuscript for publication in JoVE.

Major Concerns:

None

Minor Concerns:

I have a few minor comments and/or suggestions aimed at improving clarity (or avoiding confusion) for a reader not familiar with C. elegans

Potentially inappropriate use of colloquialisms:

Frequent use of the words "garbage" and "trash" when describing cellular contents and/or phenotype

Addressed.

Inappropriate colloquialisms “garbage” and “trash” have been eliminated or replaced with “debris” or “cellular debris”

Addressed.

line 288: "chasing" (should be replaced with "following" or the like)

We have clarified that “chasing” means visually following animals under the microscope at line 288:

“Advantageously, immobilization treatments are not absolutely required, such that with a trained eye, neuronal identification and exopher presence can be scored by visually following crawling animals under the microscope (we term this procedure ‘chasing’) on the plate when determining whether or not an exopher has been produced.”

General comment: when discussing transgenic fluorescent reporters, authors call mCherry as "overexpressed", but GFP as "expressed". Both reporters are integrated and not endogenous to C. elegans. Hence the rationale for this differential description of transgene expression is unclear.

We have clarified that “overexpressed” has been replaced with “transgencially expressed” and mention that high copy numbers of a transgene construct led to the discovery of exophers.

very long sentence in lines 120-123 should be split into two.

We have rephrased the long sentence in lines 120-123:

“The early protrusion is formed as the PED begins to project outwards, forming a recognizable protruded bud. The late bud is defined when the widest diameter of the pre-exopher domain is approximately ⅓ larger than the diameter of the constriction of the soma-exopher neck **(Figure 1C)**.”

Figures:

Neuron mislabeling:

Figure 2B has two instances of "PML axon". Should be "PLM axon"

Addressed and corrected

Figures:

Figure 2B: all fluorescent images should indicate either A-P and D-V arrows or at least indicate what side of the animal is displayed. Addressed with label.

Figure 3A: Either in figure or legend indicate that the upper image is lateral view and lower image is ventral view. Addressed in figure.

Figure 3B: the remainder fluorescent signals should be annotated. What am I looking at: gut particle autofluorescence or "starry night" debris? What is the strong signal at the top, another exopher or AVM cell body? Addressed in figure.

Compliments: great idea about Figure 8E. Love it.

Suggestions for instructions:

2.2 Consider adding that once agar plates are solidified, these plates need to be left to dry for a couple of days (if left on the counter) or for a few hours (if drying in the hood) before being seeded with E. coli.

At step 2.2 we have added:

"Once agar plates solidify, plates need to be left to dry for at least 48 (if left at the bench) or for 2-4 hours (if dried in the hood) before being seeded with E. coli."

5.8.1 Is not clear enough. Specify that one may need to alternate mixing and microwaving (low power for few seconds) to achieve even mixing. Inclusion of air bubbles on agar pads is common mistake and should be avoided. To that end, once the boiling hot, mounting agar needs to be left on a heating block to allow all the air bubbles reach the surface. When using Pasteur pippette to draw agar for making slides, draw from well below the surface of molten agar where there are no air bubbles.

To clarify instruction we have added two additional steps to section 5.8

5.8.2 To achieve an agar pad of sufficient quality alternate mixing and microwaving at low power for less that 20 seconds. Avoid the inclusion of air bubbles within the pad by placing boiling agar on a heating block and allowing the bubbles to rise to the surface.

5.8.3 Use a Pasteur pipette to draw agar from deep within the molten solution below the risen bubbles.

Either in 5.8.9 or in discussion consider including a comment about temperature of the room where animals are mounted on slides, examined and/or imaged under the microscope. Main suggestion is that change in temperature is also form of stress and therefore temperature of the microscope room shouldn't differ too much from the temperature animals are raised in. For example, if animals are kept at 20C, they shouldn't be imaged in a room at 25C.

We have added warnings regarding different conditions between culture, lab bench, and experimental environments:

3.4 **Temperature.** For basal conditions, keep animals at a constant temperature of standard 20 °C because rearing animals at variable temperatures (even acute changes in temperature) can cause modest variations in the timing of maximal exopher production.

Temperature variability limited to culture conditions. Temperatures during experiments or at the lab bench can be impactful. For example, temperatures within a microscope room should not differ dramatically from the culture incubator or lab bench.

Potentially confusing instructions:

5.8.6 Instructs "to remove the top slide..." and then 5.8.7 "to use pads within 30 minutes..." This sequence creates an impression that once opened gel pads can be used within 30 minutes. Please specify that agar plates can be used within minutes as long as they are sandwiched between two glass slides. Once the top slide is removed, the gel pad should be used immediately for worm mounting.

We have added instructions to clarify:

5.8.8 Remove the top slide by sliding **(Figure 6E)** andavoid agar pads that contain bubbles. Agar pad dry quickly and are best used within minutes. Once the top slide is removed, the gel pad should be used immediately for mounting animals.

5.8.9 Agar pads can be saved up to 30 minutes if kept encased between the two glass slides. Dried agar causes animals to clump together and dessicate. Mount animals within 2-15 µl of paralytic or microbeads and cover with coverslip **(Figure 6)**.

Reviewer #2:

Manuscript Summary:

This is a well written protocol describing methods for quantifying the properties of a new cell biological entity called the exopher. Exophers are an exciting discovery and provide a new paradigm for the study of neurobiology and neurodegenerative diseases. Given that exophers were discovered in and are best characterized in C. elegans, these protocols provide an essential toolkit for identifying these structures and maintaining consistency across experiments and between labs. The figures and text provide an excellent description of these structures, caveats that may interfere with their identification, and factors that could lead to inconsistency in exopher production. I really enjoyed reading this outstanding manuscript!

Major Concerns:

None

Minor Concerns:

Some formatting issues with the references were noted, ie reference #s occuring after punctuation (for example, line 42 reference 1).

We have addressed the issue of references occuring after punctuation.

I was also wondering if the authors might be able to comment on whether there is any effect of the HT115 bacteria itself on exopher production? While RNAi was discussed, it was not clear to me if there were differences in the rate of exopher production or #s on this different food source as compared to the standard OP50 methodology.

Addressed:

HT115 bacteria can be used when scoring for exophers in feeding RNAi experiments does not significantly change baseline exopher production from the standard OP50 *E. coli* strain.

Reviewer #3:

Manuscript Summary:

The manuscript extends technical approaches for the study of exophers, a mechanism used by neurons to discard protein aggregates and other malfunctioning or toxic cellular components, by extruding them in large vesicles. Such extrusion of toxic aggregates is critical for maintenance of proteostasis and is involved in a range of neurodegenerative disease. This protocol expands on the technology presented by the authors' recent groundbreaking publication. As the release of exophers emerges as a critical phenomenon is cell biology, this protocol can be extremely useful for other researchers seeking to study this process further. The manuscript provides clear and detailed description of the methodology, and is therefore of high value to the research community.

Major Concerns:

None

Minor Concerns:

1) Since there are several forms of extrusion of vesicles of different sizes, it might be helpful to give some description of that in the introduction, so that the reader can differentiate if this protocol is the right one for the phenomenon that she/he is studying.

Edited for more emphasis lines 22-25:

C. elegans adult neurons that transgenically express aggregating proteins can extrude large (~4µm) membrane-surrounded vesicles that can include the aggregated protein, mitochondria, and lysosomes. These large vesicles are called “exophers” and are distinct from exosomes (which are about 100X smaller and have different biogenesis...

2) The protocol focuses on mCherry; it will be useful to comment in the introduction (in addition to Table 1 and other text in the Protocol section) on how similar will it be to follow other aggregating proteins in which other researchers might be interested.

Added in lines 29-31:

While exophers have been mostly studied in animals that express high copy transgenic mCherry within touch neurons. These protocols are equally useful in the study of exophergenesis using fluorescently tagged organelles or other proteins of interest in various classes of neurons.

3) Other Minor issues:

L44: location of citation #3 should be moved.

We have moved the citation:

"The neurotoxic challenges of aggregates and dysfunctional mitochondria have long been considered to be cell-intrinsic, but more recently it has become clear that misfolded human disease proteins originating in one neuron can also spread to neighboring cells, causing pathology[1]."

L65: font issue: Aβ

Addressed:

"Protein stress, such stress induced by *hsf-1* genetic disruption, autophagy knockdown, MG132-mediated proteasome inhibition, or transgenic expression of human disease proteins such as Huntington's disease-associated expanded polyglutamine Q128 or Alzheimer's disease-implicated fragment $A\beta_{1-42}$, can increase the numbers of neurons that produce exophers."

L135-136: "ASE... and amphid neurons" - ASE is also an amphid neuron.

Addressed:

"Exophers have been reported to be generated by *C. elegans* dopaminergic neurons PDE and CEP, ASE and ASER sensory neurons, and dye-filling amphid neurons [5]."

L154: add : ….; dye-filling protocol is outlined in Ref 13

Addressed:

"1.3. Alternatively exophers can be visualized with a dye-filling assay of the amphid head neurons, which are open to the environment and amenable to backfilling[5]. A dye-filling protocol is outlined in reference [13]."

L162: If you go into the trouble of explaining how to pure plates, it might also be useful to mention the use of automatic dispenser for plate homogeneity, such as Wheaton UniSpense.

Addressed:

"2.2. To prepare plates, under sterile conditions, pour approximately 15 mL of molten NGM into sterile 60 mm diameter petri dishes and allow to cool and solidify. Pouring can be done manually or with an automatic dispenser. "

L254: 3000RPM? In what rotor? Or give g.

addressed

L406 & forward: the text refers to the touch neuron's "axon". I am not sure if this is the ideal term here ; is the neuronal process studied here purely axonal, or is it sensory/dendritic? If not purely axonal, you might want to consider alternative terms like "neuronal process" or "neurite".

We have changed axon to neuronal process in each case

L414-415: the structure of the sentence is not clear, especially the context of "the animal is sitting on the side".

Addressed:

"6.6.2 The **AVM,** a nearby ventral neuron, can help assign animal orientation. If the AVM neuron is in the same plane as the ALM then the animal is resting upon its side and the neuron outside that plane is the ALMR . If the AVM neuron is not in the same plane as the ALM in question, the closest touch neuron to the focal plane is ALML."

L646 Ref#10 is a pre-print ; is this allowed by JoVE?

Is this allowed by JoVE?

4) The order of authors on the editorial front page is different than that on the manuscript's front page.

Will address this when we submit again.