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TITLE:

Purification and Analysis of *Caenorhabditis elegans* Extracellular Vesicles

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flow cytometry, DI-8-ANEPPS, size exclusion chromatography, flow cytometry, large population *C. elegans* maintenance

SUMMARY:

This article presents methods for generating, purifying, and quantifying *Caenorhabditis elegans* extracellular vesicles.

ABSTRACT:

The secretion of small membrane-bound vesicles into the external environment is a fundamental physiological process of all cells. These extracellular vesicles (EVs) function outside the cell to regulate global physiological processes by transferring proteins, nucleic acids, metabolites, and lipids between tissues. EVs reflect the physiological state of their cells of origin. EVs are implicated to have fundamental roles in virtually every aspect of human health. Thus, EV protein and genetic cargos are being increasingly analyzed for biomarkers of health and disease. However, the EV field still lacks a tractable invertebrate model system that permits the study of EV cargo composition. *C. elegans* is well suited for EV research because it actively secretes EVs outside of its body into its external environment, permitting facile isolation. This article provides all the necessary information for generating, purifying, and quantifying these environmentally secreted *C. elegans* EVs including how to work quantitatively with very large populations of age-synchronized worms, purifying EVs, and a flow cytometry protocol that directly measures the number of intact EVs in the purified sample. Thus, the large library of genetic reagents available for *C. elegans* research can be tapped into for investigating the impacts of genetic pathways and physiological processes on EV cargo composition.

INTRODUCTION:

The secretion of membrane-bound extracellular vesicles (EVs) facilitates the global physiological processes by actively transporting specific protein, nucleic acid, metabolite, and lipid cargos between cells[1]. Cells secrete EVs that span a continuum of sizes ranging from 2 μm or greater to as small as 20 nm[2]. Small EVs (<200 nm) are increasingly studied because of their implication in pathological processes, including metabolic disorders, cancer, cardiovascular disease, and neurodegenerative diseases[3–5]. These pathologies have also been shown to influence the protein and genetic composition of EVs cargos of small EVs. Therefore, biomarker signatures of the pathology are increasingly being uncovered through EV cargo discovery methods such as LC-MS-MS and RNAseq[6–9].

C. elegans has been a useful invertebrate model for identifying evolutionarily conserved EV signaling pathways. For instance, a *C. elegans* flippase was first shown to induce the EV biogenesis in *C. elegans* embryos, and the human homolog was shown to influence EV release in human cells[10,11]. *C. elegans* EVs were reported to carry Hedgehog signals necessary for cuticle development. The delivery of Hedgehog and other morphogens was shown to play a major developmental role of EVs, and it is conserved in zebrafish, mice, and humans[12–15]. *C. elegans* is well suited for EV biomarker discovery because it secretes EVs outside of its body that function in animal-to-animal communication[16,17] (**Figure 1A**). However, the methodology established through a previous study cannot be used because the nematodes' *E. coli* food source also secretes EVs[18]. In this method, most of the sample is comprised of *E. coli* contamination, limiting the power of proteomic or RNAseq approaches for the discovery of *C. elegans* EV cargos. The methods described here were developed to yield highly pure *C. elegans* EVs at abundance levels characteristic of typical cell culture experiments and thus facilitate omics approaches for EV biomarker discovery.

Large populations of worms are needed to generate a sufficient number of EVs for cargo analysis. Therefore, methods for conducting quantitative cultivation of large populations of developmentally synchronized *C. elegans* are also included. Typically, when a large number of worms are needed for experiments, they are cultured in liquid media. While this is effective for generating large populations of worms, the physiology of the animals is considerably different from worms cultivated under standard conditions on nematode growth medium (NGM) agar plates. Animals cultured in liquid grow more slowly, are thinner, show developmental heterogeneity, and are subjected to a high degree of batch variability. Therefore, we present a simple but effective means for the quantitative cultivation of large populations of developmentally synchronized *C. elegans* using 10 cm high growth plates. The media composition of high growth plates includes more peptone than regular NGM plates and are seeded with the *E. coli* strain NA22 which grows more robustly than OP50.

Advances in flow cytometry technology (FACS) have enabled the direct analysis of individual EVs[20,21], permitting the quantification of EVs without the inherent limitations in other methods. Prior work has shown that protein is not a useful proxy for EV abundance because different purification methodologies result in significantly different EV-to-protein ratios[22]. Extremely pure EV fractions contain relatively little protein, making it difficult to quantify samples with BCA or Coomassie gels. Western analysis can identify relative differences of individual proteins but

cannot identify how many EVs are in the sample. Robust quantification of EV number by nanoparticle tracking analysis is hampered by its narrow signal-to-noise range, inability to differentiate between EVs and solid aggregates, and the lack of transferability of methods between instruments with different specifications[23]. Therefore, this article also contains a generalizable flow cytometric procedure for discriminating and quantifying EVs.

PROTOCOL:

1. Preparation

1.1. Add 2 g of gelatin to 100 mL of dH_2O and heat in the microwave until it starts to boil. Then stir and let it cool for 20 min.

1.2. Pass the solution through a 0.22 µm filter and dispense into sterile tubes. Treat pipette tips with gelatin immediately prior to the use by pipetting up and expelling the gelatin solution. Treated tips can either be used immediately or stored.

1.3. Cut out the membrane from a sterile cap filter.

1.4. Wet a 20 cm square of 5 µm nylon mesh fabric and place around the top of a sterile cap filter. Secure it with several thick rubber bands.

CAUTION: Carefully examine the fabric to ensure that there are no creases. These make air gaps that hinder suction.

2. Calculating large populations of worms (**Figure 1A**)

2.1. Vortex and pipette 10 µL of the worm suspension onto a worm cultivation plate lid or a glass slide. Repeat this process 3x. Record the number of animals in each drop.

2.2. Adjust the worm suspension to two animals per µL.

2.3. Vortex the suspension and pipette nine drops (10 µL) on a slide or a plate lid. Manually count the number of animals. Record the number of animals in each drop.

2.4. Calculate the mean and standard error of the mean (S.E.M.) as a percentage of each worm population.

2.5. Calculate the total number of animals in each population by dividing the mean value by 10 µL and then multiplying by the total volume of the worm suspension in µL.

[Place **Figure 1** here]

3. Cultivating *C. elegans* for EV purification

3.1. Generate a large population of developmentally synchronized *C. elegans*. To do so, follow the steps below.

3.1.1. Add a single animal on a 6 cm normal growth media plate. Incubate for two generations and then wash the animals onto two high growth medium plates.

3.1.2. Incubate until the worms have exhausted the food.

3.1.3. Filter L1 worms with 5 µm nylon mesh prepared in step 1.4. Place the screw cap on the sterile bottle, start the vacuum, and pour worms over the filter. Pass the same volume of the medium over the worm aggregates on the filter.

3.1.4. Quantify the worms and calculate the total number (see step 2). Recently starved (<24 h) worms grown on a high growth plate result in about 80,000 L1 larvae.

3.1.5. Centrifuge L1 larvae at 2,000 x *g* for 3 min. Resuspend to ~100,000 animals per mL.

3.1.6. Place ~50,000 animals per high growth plate. Cultivate the animals at 20 ˚C until they are gravid adults (about 72 h).

3.1.7. Bleach the gravid adults by standard means and allow embryos to hatch in 10 mL of S Basal solution with 2.5 µg/mL cholesterol.

3.1.8. Quantify L1 larvae (see step 2) and then add 50,000 worms to each high growth plate.

3.1.9. Cultivate plates at 20 ˚C until animals are young adults.

3.2. Prepare *C. elegans* for secretome generation.

3.2.1. Wash worms from plates by adding 15 mL of sterile M9 with 50 µg/mL carbenicillin to each high growth plate. Let the saturated plates sit for 5 min. Dislodge worms by circling the buffer around the plate. Avoid sloshing. Pour the buffer from each plate into a separate 50 mL conical tube. Repeat the wash step 2x more for a total of 45 mL of buffer per plate.

3.2.2. Let the worms settle for 15 min. Decant the supernatant and add 50 mL of fresh M9 buffer to the tube. Repeat for a total of 4x.

3.2.3. Float the worms on the top of a 30% sucrose step-gradient and recover animals in the S Basal solution[24].

3.2.3.1. Briefly resuspend the worms in 15 mL of ice-cold 100 mM NaCl, add 15 mL of ice-cold 60% sucrose, and invert to mix. With a glass pipette gently layer 5 mL of ice-cold 100 mM NaCl on the top of the sucrose mixture.

3.2.3.2. Centrifuge at 1,500 x *g* for 5 min. Worms will be concentrated at the interface between the NaCl and the sucrose.

3.2.3.3. Gently pipette out the animals from the interface into a new 50 mL conical tube. Add S Basal solution to 50 mL. Allow worms to settle 5 min.

3.2.3.4. Decant the supernatant and add fresh 50 mL of S Basal solution. Repeat this at least 3x.

3.2.4. Quantify the number of worms as described in step 2.

3.2.5. Resuspend the worms to a density of one animal per µL with sterile S Basal solution containing 2.5 µg/mL of cholesterol and 50 µM of carbenicillin.

3.2.6. To prepare the sample for secretome generation, add up to 400 mL of the suspension to a sterile 2 L bottom-baffled flask.

3.2.7. Place flasks on the circular rotator in a 20 ˚C incubator at 100 rpm for 24 h.

4. EV purification

4.1. Harvesting and fractionation

4.1.1. Remove the 2L flask of worms from the incubator and pellet the animals in 50 mL conical vials (500 x *g* for 2 min).

4.1.2. Pour the supernatant through a 0.22 µm vacuum filter to remove any particulate debris.

NOTE: At this point, the filtered supernatant containing the secretome and the vesicles can be stored at -80 ˚C.

4.1.3. Count the number of animals as described in step 2.

4.1.4. Determine the viability of the worms by placing a drop of suspended worms onto a bacterial lawn. Wait for 15 min and then score animals as moving, paralyzed, or dead.

4.1.5. Concentrate the 0.22 µm filtered supernatant to 700 µL using regenerated nitrocellulose 10 kDa filter units. Add 150 µL S Basal solution and then pipette over the filter and vortex. Repeat 2x.

4.1.6. Centrifuge the concentrated supernatant at 18,000 x *g* for 20 min at 4 ˚C and transfer the supernatant to a new tube. This step removes any potential debris or larger particles that might have accumulated during handling.

4.1.7. Add the protease inhibitor cocktail + EDTA per the manufacturer's instructions.

NOTE: At this point the concentrated supernatant containing the secretome and the vesicles can be stored at -80 ˚C.

4.2. Size exclusion chromatography

4.2.1. Pour 80–200 µm agarose resin (pore size fractionation range of 70,000 to 40,000,000 kDa for globular proteins) into an empty gravity flow column cartridge. Flow S Basal solution until the resin bed is packed at a final volume of 10 mL.

NOTE: If too much resin is poured into the column cartridge, then disperse the top of the column and remove the excess with a pipette.

4.2.2. Pass 40 mL of sterile filtered S Basal solution through the column and let it flow under gravity.

CAUTION**:** Check that the resin is not disturbed.

4.2.3. Let the buffer flow until the top of the resin is not submerged and then cap the bottom of the column cartridge. Place a collection tube underneath the column.

4.2.4. Remove the cap placed on the cartridge. Add the concentrated secretome obtained in step 2.1 dropwise to the top of the column bed. Pipette 1 mL of S Basal solution dropwise. Place a fresh collection tube under the column.

4.2.5. Slowly fill the upper column reservoir with 5 mL S Basal solution to not disturb the resin. Collect the first 2 mL of eluate then quickly change tubes and collect the next 4 mL. This is the fraction that is enriched for EVs.

4.2.6. Concentrate the eluate to 300 µL with a regenerated nitrocellulose 10 kDa molecular weight cut off (MWCO) filter and the transfer retentate to a low-binding microcentrifuge tube.

4.2.7. Wash filter membrane 2x with 100 µL of S Basal solution by vortexing for 20 s and pipetting the buffer across the filter. Add to the initial sample for a final volume of 500 µL.

4.2.8. Add protease inhibitor cocktail per the manufacturer's instructions.

4.2.9. Perform the downstream experiments immediately (RNAseq, LC-MS-MS, GC-MS-MS, Western, FACS, etc.) or store at -80 ˚C.

5. Flow cytometry quantification of EV abundance

5.1 Dye preparation

5.1.1. Prepare a 10 mM stock of DI-8-ANEPPS using fresh DMSO. Distribute into 10 µL aliquots and store at -20 ˚C.

5.1.2. Prepare a 1 mM working solution by adding 90 µL of sterile filtered PBS to a tube with 10 µL of dye stock.

5.2. Experimental sample preparation

5.2.1. Add 840 µL of S Basal solution into a microcentrifuge tube. Then add 60 µL of the purified EVs generated in section 4.2 and vortex.

5.2.2. Aliquot 300 µL of the sample into two new microcentrifuge tubes. There is now an experimental set of three tubes containing 300 µL of diluted EVs each. Label the tubes #1–3. To samples #2 and #3 add 7 µL of the DI-8-ANEPPS stock prepared in 5.1.2. Then add 7 µL of 1% Triton-X 100 to tube #3. Mix each tube by vortexing.

5.2.3. Sonicate sample #3 by placing the sonicator tip in the middle of the sample and pulsing 10 times at 20% power 30% duty cycle.

CAUTION: Ensure that the tip of the sonicator probe is in middle of the sample so that foam generation is kept to a minimum. Foam may compromise the sample by causing the EVs to aggregate.

5.2.4. Add 300 µL of S Basal solution to two microcentrifuge tubes. Add 7 µL of the DI-8-ANNEPS working reagent prepared in step 5.1 to the second tube and label “Dye Only”. Label the other tube “Buffer Only”.

NOTE: Prepare the Dye Only and Buffer Only control samples and the experimental samples with the same S Basal source.

5.2.5. Incubate the samples away from the direct light and at room temperature for 1 h.

5.3 Conduct FACS experiments.

5.3.1. Set the FACS excitation filter to 488 nm (blue) and the emission filter to 605 nm (orange). Set the flow rate to 1.5 µL per min.

5.3.2. Run nanobead FACS calibration mix (optional but recommended).

5.3.3. Run each sample on a FACS machine for 3 min (180 s). Note the exact running times in seconds.

5.4 Analyze the FACS data.

5.4.1. Open the files with FACS analysis software.

5.4.2. Switch the Y-axis to **488-Org (Area)** by clicking on the **Axis**, selecting from the drop-down menu.

5.4.3. Set a rectangular gate starting at the top of the plot, spanning from SALS level 10^2 to 10^4 (<300 nm sized particles). Extend the gate downwards until it contains 2.5% of the total events. Name the gate "DI-8-ANEPPS+" events.

5.5 Quantification of sample EV abundance

5.5.1. Copy this gate and paste it onto the other two samples in your experimental set as well as the Buffer Only and Dye Only controls. Export the analysis to a spreadsheet.

5.5.2. If the FACS samples were not run for the recommended 3 min (180 s), normalize all event counts in the sample to events per 180 s. For example, if a sample was run for 250 s, the DI-8-ANEPPS+ event number is scaled by 250 s/180 s.

5.5.3. Remove the dye background events by subtracting the number of DI-8-ANEPPS+ events in the Dye Only control from those in sample #2.

5.5.4. Remove isotype background events by subtracting the number of DI-8-ANEPPS+ events in sample #1.

5.5.5. Remove detergent-insensitive events by subtracting the number of DI-8-ANEPPS+ events in sample #3. This value is the number of bona fide EVs.

5.5.6. Calculate the number of EVs in 1 µL of sample by dividing the value calculated in step 5.5.5 by the volume analyzed in µL (4.5 µL analyzed as described in step 5.3) and multiplying by the dilution factor (10x as described in step 2.5). Multiply this value by the number of µL remaining in the EV preparation. This is the number of EVs available for downstream analysis.

[Place **Figure 2** here]

REPRESENTATIVE RESULTS:

A schematic for the processes necessary for generating and purifying EVs is shown in **Figure 1**. The typical times required to complete each step are shown underneath. **Figure 2** shows a schematic for preparing samples for FACS analysis using DI-8-ANEPPS (**Figure 2A**) as well as the calculations necessary to estimate total number of EVs in a sample (**Figure 2B**).

Representative results from 10 biological replicates are shown in **Figure 3A**. The variability between replicates is not significant and the typical S.E.M. of population size is a little over 10% (**Figure 3B**). Filtering the worms and cultivating on the fresh high growth plates will generate a large population of gravid adults suitable for bleach synchronization. A single starved plate

processed in this way can produce an experimental population of 1 x 10^6 or more synchronized progeny because each gravid adult will contain about 10 eggs. It is possible to maintain large populations of developmentally synchronized populations by filtering eggs and larvae through a 40 µm filter to obtain EVs from older animals. Retained adults are transferred to fresh high growth plates at a density of 20,000 animals per plate. This process was repeated every 2 days. **Figure 3C** shows three biological replicates of worm populations being moved across three plate transfers. The transferring of animals between plates results in about a 10% loss of animals (**Figure 3C**) while about 20% of the animals are lost in the sucrose flotation step (**Figure 3D**).

[Place **Figure 3** here]

Protein-to-animal ratio is a useful metric for characterizing an EV preparation. This metric can provide a quick means for determining the consistency between EV preparations. On average, 1,000 wild type young adult animals will secrete 1 µg of proteins larger than 10 kDa into their environment (**Figure 4A**). When this fraction is further separated by the size exclusion chromatography, the total protein elution profile shows a small protein peak between 2–6 mL and a large peak after 8 mL. The EVs are contained in the first 5 mL of the column eluate (**Figure 4B**). Nanoparticle tracking analysis the first 5 mL of the column eluate contains a monodisperse population of small, ~150 nm EVs (**Figure 4C**). Transmission electron microscopy of size exclusion fractions reveals abundant EVs in the 2 mL–6 mL fraction of eluate but not in the later eluate volumes. EVs are known for their cup-like shape and are therefore easy to discriminate from solid particles that appear as punctate bright dots when prepared under the negative staining conditions (**Figure 4D**).

[Place **Figure 4** here]

To directly compare the flow cytometry characteristics between *C. elegans* and human EVs, we purified EVs from the conditioned media of cell cultures of human neurons with this method. Flow cytometry separates particles based on light scattering. The small-angle light scattering (SALS) roughly correlates with the size, and the long-angle light scattering (LALS) correlates with internal membrane structure. The majority of the total sample events from both cell culture and *C. elegans* EV preparations are tightly focused in a cluster centered around 10^3 SALS and 10^4 LALS (**Figure 5A**). A histogram of *C. elegans* EV sample events sorted by SALS shows that all preparations peak at ~10^3 (**Figure 5B**).

[Place **Figure 5** here]

The DI-8-ANEPPS robustly labels *C. elegans* EVs, consistently highlighting a majority of the total events in both the *C. elegans* and cell culture derived samples. The calibration beads and controls are presented in **Figure 6A**. The individual FACS scatter plots are shown from a *C. elegans* sample prepared from 200,000 animals (#1) and two prepared from 500,000 animals (**Figure 6B**). EVs from either 100 mL (#1) or 200 mL (#2,3) of cell culture were also analyzed (**Figure 6C**). Both *C. elegans* and cell culture derived samples showed abundant fluorescent events that disappeared when treated with 0.05% Triton-X 100 and light sonication. This demonstrates that the labeled

events are detergent labile phospholipid structures (vesicles) rather than detergent-insensitive solid lipoprotein aggregates. The EV abundance is calculated as the difference between the number of events in the DI-8-ANEPPS+ gate between the fractions without detergent (-) and with detergent (+) minus the events in the Dye Only control. For clarity, the data presented individually in **Figure 6B** and **Figure 6C** is summarized as bar graphs in **Figure 6D** and **Figure 6E**. The abundance of the purified EVs was not significantly different between the *C. elegans* and the cell culture derived preparations (**Figure 6F**).

[Place **Figure 6** here]

Table 1 contains the raw experimental values for the full 4.5 µL of each sample type analyzed. The total number of events collected from the EV fractions purified from 500,000 animals was 10–20x over the background number of particles collected by buffer alone while the fraction purified from 200,000 animals contained about 2x as many events as buffer alone. The number of events within the DI-8-ANEPPS+ gate is also shown for each sample. These metrics can be imported into a spreadsheet to calculate the number of dye-positive, detergent-sensitive EVs as described above. For example, the number of EVs in 1 mL of the first *C. elegans* biological replicate shown would be calculated like this:
(18,974 – 3,853 - 1,487) x (10/4.5) x 1,000 = 30,297,778.

[Place **Table 1** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Procedure overviews for generating, quantifying, and purifying *C. elegans* EVs. (**A**) Schematic for counting large populations of animals. (**B**) Schematic for generating worms for harvesting EVs. (**C**) Schematic for purifying EVs from *C. elegans* supernatent.

Figure 2: Schematic of flow cytometry sample preparation to quantify EV abundance. (**A**) The EV preparation is divided into three identical samples. Sample # 1 is the No Dye negative control. DI-8-ANEPPS is then added to samples #2 and #3. Triton-X 100 is then added to sample #3. The green arrow indicates that only sample #3 is subsequently sonicated. The data from the flow help determine the absolute number of detergent sensitive EVs in the FACS samples and then calculate the abundance of EVs in the total preparation. The gate for determining dye-positive events is set with sample #1, which contains no dye. The red writing on the spreadsheet data is to illustrate that dye-positive events from the detergent-treated sample are subtracted from the sample with dye. (**B**) Equations for quantifying EVs in a FACS analyzed sample and calculating the total number of EVs in the sample.

Figure 3: Quantification of large populations of *C. elegans* for EV analysis. (**A**) A comparison of the number of L1 larval stage worms isolated from a single recently starved (<24 h) high growth plate. The values of each of 10 biological replicates is plotted. The summation of all the data points in the 10 replicates is also presented as a violin plot and bar with S.E.M. This shows that a single plate of recently starved worms will yield ~80,000 L1 larval stage worms. (**B**) The S.E.M. of the 15 different plate population measurements in panel A plotted as individual points.

Generally, the S.E.M. is slightly greater than 10%. (**C**) Three biological replicates of worm populations were estimated after each of two transfers between plates every 4 h. This transfer of animals between plates did not result in a significant decrease in population size. This indicates that the methodology presented here for moving worms does not result in a significant loss of animals. (**D**) Population measurements taken along the course of three EV experimental replicates: The numbers of animals that were estimated for the original L1 larvae that started the experiment, the number of young adult animals recovered from the sucrose float and ready for the 24 h incubation period, the number of animals counted from EV preparation upon recovery of the secretome. There is a small but not significant decrease of population size between the initial population estimation at the L1 larval stage and later when the animals are young adults. The mean L1 population measurement was designated as 100% and all other measurements were scaled by their relation to this value. Error bars S.E.M. * = p value < 0.05, N.S. = not significant in a parametric paired t-test.

Figure 4: Purification of *C. elegans* EVs from total secreted biomass. (**A**) The ratio of the total proteins larger than 10 kDa to the number of worms incubated in the preparation for 10 biological replicates was plotted. Most preparations contained 1 µg of protein per 1,000 animals. (**B**) Protein elution profile from a 10 mL resin column loaded with 1 mL of the concentrated secretome. The fractions that are consolidated in further analyses are shaded into groups. (**C**) Nanoparticle tracking analysis of the consolidated resin eluted fractions 2–6 mL. The 95% confidence interval is shaded gray. (**D**) TEM analysis of the consolidated resin eluted fractions. The starting material had both vesicle-like particles and non-vesicle particles. The consolidated 2–6 mL elution fraction was enriched for vesicles with few non-vesicle particles. The 7–11 mL consolidated fractions contained few vesicle-like particles but many non-vesicle particles. The 12–15 mL elution fractions contained only non-vesicle particles. All micrographs are shown at the same magnification. Scale bar = 200 nm.

Figure 5: *C. elegans* EVs are clearly defined by their light scattering properties. (**A**) Histogram of SALS events in three biological replicates of *C. elegans* EVs. ~80% of the total sample events are contained within a tight, clearly defined population. Similar to nanoparticle tracking analysis, it shows that the size distribution of *C. elegans* EVs is monodisperse. The histogram of the S Basal buffer used to dilute the samples is shown for comparison. (**B**) Comparison of refractive properties of *C. elegans* and human cell culture EVs purified with the methods described in this text. Both EV types consistently present comparable short and long-angle light scattering distributions (SALS and LALS).

Figure 6: Examples of flow cytometry data used to calculate EV abundance. (**A**) To ensure that the number of events can be directly compared between samples, the Buffer Only and the Buffer and Dye Only controls need to be run at the same flow rate and collection time as the EV sample fractions. There are very few events in the DI-8-ANEPPS gate. (**B**) Representative results of the DI-8-ANEPPS and detergent treatment protocol from *C. elegans*. Three biological replicates are shown. Biological replicate #1 was prepared from 200,000 animals while #2 and #3 were prepared from 500,000 animals. The disappearance of EVs with the detergent treatment is clear in all cases. (**C**) Representative results of the DI-8-ANEPPS and detergent treatment protocol

using human cell culture conditioned media. Biological replicates #1 and #2 were prepared from 200 mL of conditioned media while biological replicate #3 was prepared from 100 mL. (**D**) Bar graph of the data collected from the three biological replicates of *C. elegans* EVs. Error bars = S.E.M. Paired two-tailed t-tests between treatments. *** = p value < 0.001. (**E**) Bar graph of the data collected from the three biological replicates of cell culture derived EVs. Paired two-tailed t-tests between treatments. *** = p value < 0.001 (**F**) The number of dye-labeled detergent-sensitive events per µL was calculated for all the biological replicates of *C. elegans* and cell culture. The values between the two EV sample sources is not significantly different. Paired two-tailed t-tests between treatments p value = 0.685.

Table 1: Tabulated flow cytometry data. The data shown in **Figure 6** is presented as a spreadsheet. For each sample the total events and DI-8-ANEPPS+ gated events are shown. Each biological replicate is arranged in the order of samples #1–3 in the protocol. These metrics reveal that the total number of events collected from the *C. elegans* samples prepared from 500,000 animals or 200 mL of cell culture conditioned media was 10–20x over the background number of particles collected by buffer alone while the biological replicate purified from 200,000 animals contained about 2x as many total events as buffer alone. The number of events within the DI-8-ANEPPS+ gate is also shown for each sample. These metrics can be exported to a spreadsheet to calculate the total number of EVs.

DISCUSSION:

A fundamental challenge of the EV field is separating the wide variety of EV subtypes[2]. The methods described here use filtration, differential centrifugation, and size-exclusion chromatography to generate a pure population of small EVs because ~100 nm EVs were previously shown to be secreted into the external environment and function in physiologically relevant communication pathways[16]. EVs purified through size-exclusion chromatography may still be a mixture of exosomes and small microvesicles because these EV subclasses are similarly sized. Vertebrate EV subclasses are commonly separated by immunoprecipitation because this method isolates EVs through binding to selective membrane protein markers. The methods described facilitate the identification of *C. elegans* EV membrane marker proteins. Therefore, in the future it may be possible to further separate small EV subclasses through analogous immunoprecipitation methods. In theory, immunoprecipitation could isolate EVs from well-fed worms because *E. coli* EVs should not interact with the antibody. Researchers interested in identifying the protein and genetic cargos of larger EV subclasses (>200 nm) can do so by skipping the 0.22 µm filtration step and then analyzing the pellet rather than the supernatant. Uncovering the protein and genetic cargo abundances of large EVs will establish a more comprehensive understanding of the physiological processes that function through the *C. elegans* secretome. In addition to being secreted into the environment, *C. elegans* EVs are transferred internally between tissues. Therefore, these methods only isolate a subset of total *C. elegans* EVs. However, cargo discovery of internal EVs is not possible because there is no method for separating EVs from lysed worms. Cargo analysis of this externally secreted EV subset provides a means to identify potential general EV protein markers capable of labeling internal EVs as well.

The scale of the EV preparation will depend on the demands of the experiment. A total of 500,000 young adults provides enough EVs for conducting flow cytometry, LC-MS-MS, and RNAseq analysis in parallel. This number can be scaled up or down depending on the experimental needs. The largest practical factor for scaling up is the number of 10 cm high growth plates required. High growth plates prepared with 500 µL of 20x concentrated overnight NA22 culture will support a population of ~50,000 adults from the L1 larval stage until the first day of adulthood. Therefore, to conduct an experiment with 500,000 adults, 13 high growth plates are needed: one plate to generate the population of L1s, two plates to generate the adults for bleaching, and 10 plates for the growth of the experimental animals. These metrics are contingent on the seeding and bacterial growth conditions of the high growth plates. Therefore, it is recommended to make all plates in a standardized way and then calibrate with known quantities of animals.

Worms are counted at two stages during the cultivation process: 1) before seeding worms on plates and 2) before generating the conditioned media. Animal cultivation density has been shown to impact behavior, development, and stress responses[15–18] and may, therefore, also influence EV cargos. Therefore, for consistent cultivation density it is necessary to accurately estimate worm populations. Quantifying the number of worms in nine drops gives statistical confidence of population estimations. It is essential to treat each drop individually, vortexing between each drop and inserting the pipette the same distance into the worm suspension. Taking these precautions will ensure S.E.M. values of ~5–10% of the total population. If the S.E.M. for a worm population is higher than 20%, then something went awry.

After the 24 h bacteria-free incubation period, young, wild type *C. elegans* crawl immediately upon addition to a bacterial lawn. This indicates that the incubation does not severely impact their health. However, this step does influence the physiology of the animals and therefore may influence the composition of the EV cargos. Therefore, when working with very sick genotypes or older animals, it is essential to check viability after the incubation step. If all animals do not survive the incubation, then increase the experimental population size and decrease the time of incubation. When working with large volumes of conditioned media, it is more practical to use a stirred-cell concentrator with a filter made of regenerated nitrocellulose. EVs do not bind to this filter chemistry as strongly as other filter types[14].

The ratio of the total protein recovered from the conditioned media to the number of animals incubated to make the preparation is a useful metric. If this ratio is much higher than expected, then it is possible that the population estimations were off or that animals died and deteriorated in the conditioned media. If this ratio is much lower than expected, then some protein may have been lost on the filters during the concentration process. While the FACS methods will directly quantify the EVs in the preparation, this metric is useful because it can highlight sample anomalies early in the process. Another useful method for verifying the quality of the EV preparation is transmission electron microscopy, as shown in **Figure 4D**. Although the protocol for transmission electron microscopy is not formally included in this methods article due to length, it can be conducted by standard methods. It is recommended to conduct this type of analysis, at least initially, as a complimentary method to FACS for assessing the quality of EV preparations. For best results use freshly discharged formvar-carbon grids and stain the samples

with 2% phosphotungstic acid instead of uranyl acetate.

DI-8-ANEPPS was chosen because it has been shown to quantitatively label EV membranes, outperforming other general EV dyes with human biopsy samples and liposomes[25]. The measurements are quick, taking only 3 min of collection time for each sample. The quantification of detergent-sensitive EVs has direct functional significance because it capitalizes on a fundamental physical distinction between EVs and solid lipoprotein aggregates. Importantly, this method is not influenced by the amount of total protein or number of non-EV aggregates and therefore can quantify EVs obtained through different purification methods in an unbiased manner. The FACS-verified EV metric we describe here would be especially helpful for studies that demonstrate physiological impacts of purified EVs. It could also benefit the EV field in general to establish a FACS methodology as a universal metric so that absolute EV abundances can be directly compared across studies. Vital dyes can also label *C. elegans* EVs, but they are not as bright as EVs labeled with Di-8-ANEPPS.

This purification approach capitalizes on the active secretion of EVs outside the bodies of intact, living worms. This permits *C. elegans* EVs to be isolated at comparable purity and abundance as from cell culture, specifications that are sufficient for identifying hundreds of protein and RNA cargos via LC-MS-MS and RNAseq analysis[26]. Thus, the large library of reagents available for *C. elegans* research can be leveraged for investigating the influence of the genetic and physiological perturbation on EV cargo composition.

ACKNOWLEDGMENTS:

We gratefully acknowledge Nick Terzopoulos for worm plates and reagents; the *Caenorhabditis* Genetics Center (CGC) at the NIH Office of Research Infrastructure Programs (P40 OD010440) for the N2 nematode line; Lucia Vojtech, PhD, for assistance with the nanoparticle tracking analysis; Jessica Young, PhD, and Marie Claire, MD, PhD, for hiPSC-derived neuronal conditioned cell media; Wai Pang for assistance with TEM imaging. This work was supported by NIH grant P30AG013280 to MK and NIH grant AG054098 to JCR.

DISCLOSURES:

The authors declare no competing interests.

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Figure 1

Click here to access/download;Figure;Fig 1

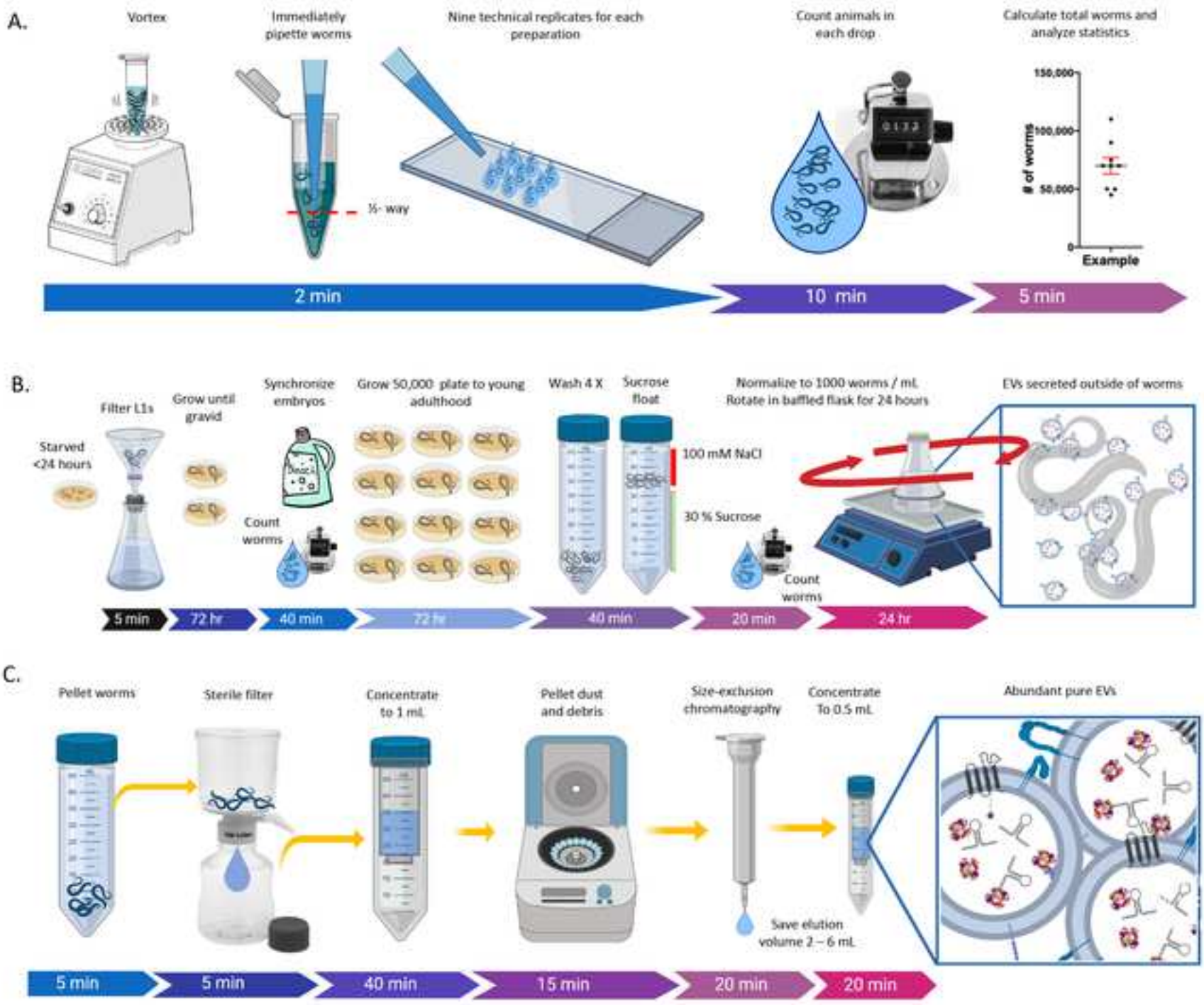
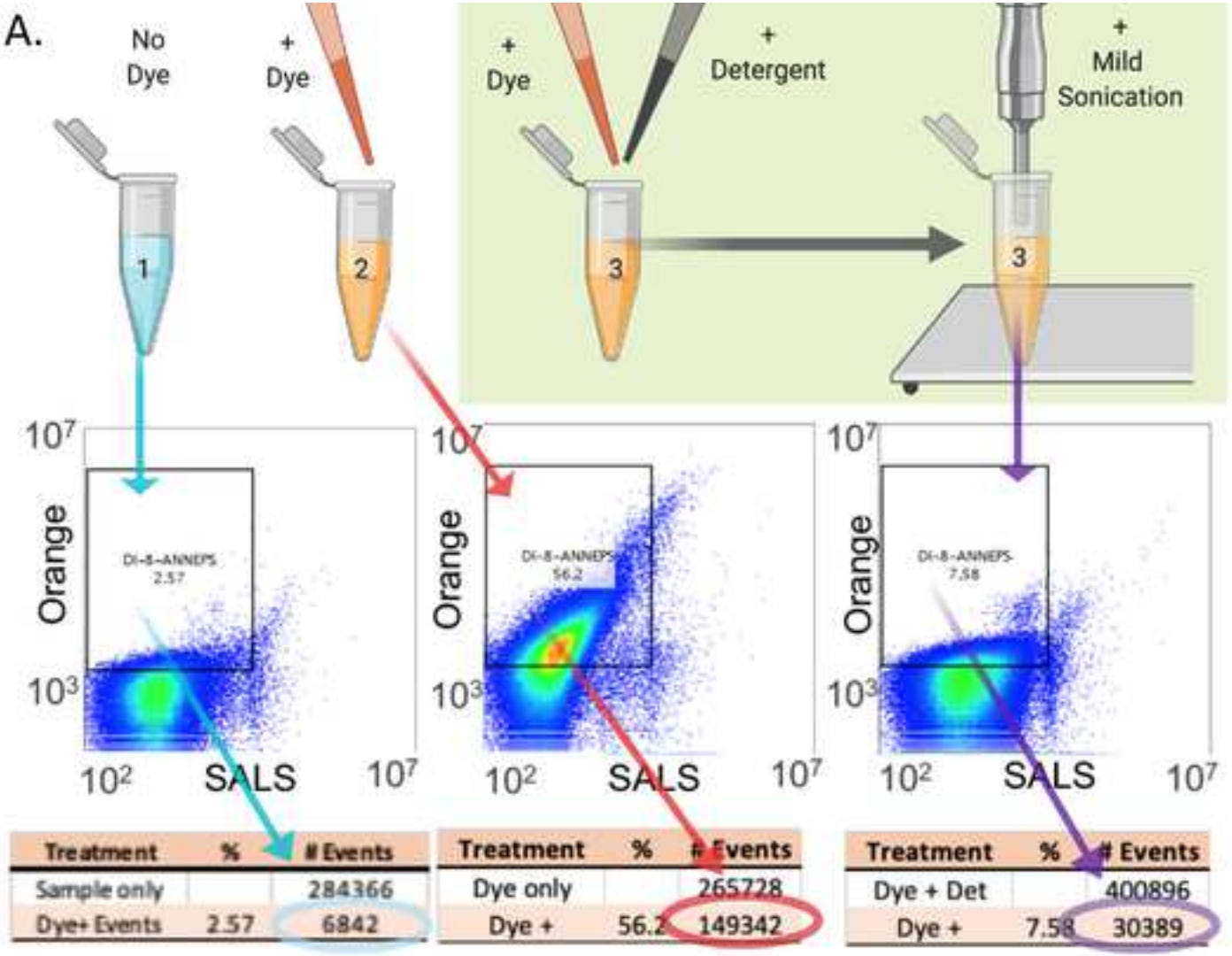


Figure 2



B.

EV abundance in FACS sample = Sample + dye - Sample + Det - Sample + no dye

Total EVs = (EV abundance FACS) * Dilution factor * (1 / volume analyzed) * (Total sample volume)

Figure 3

Click here to access/download;Figure;Fig 3.tif

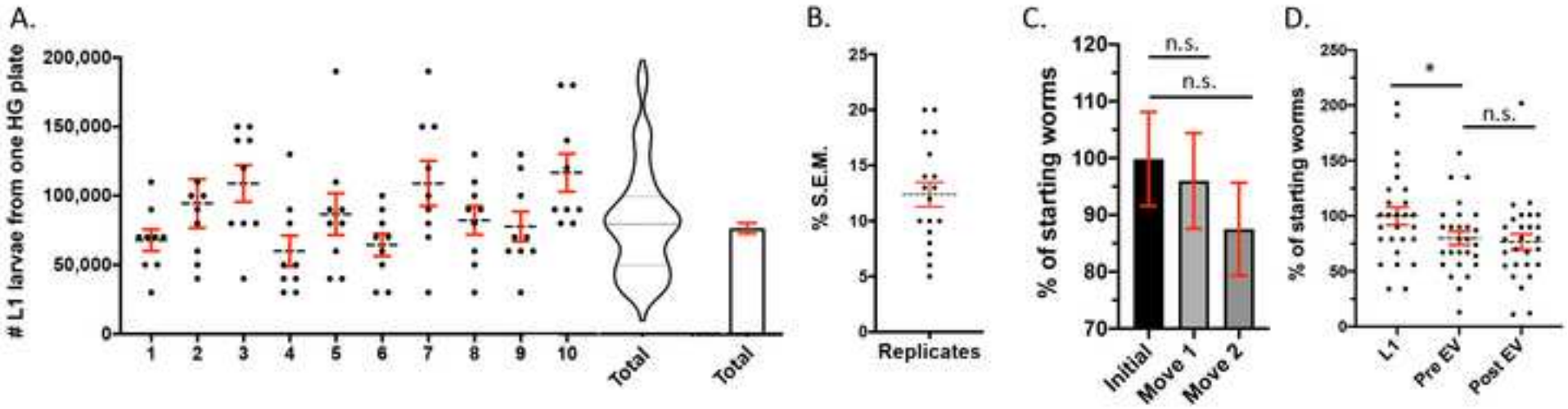


Figure 4

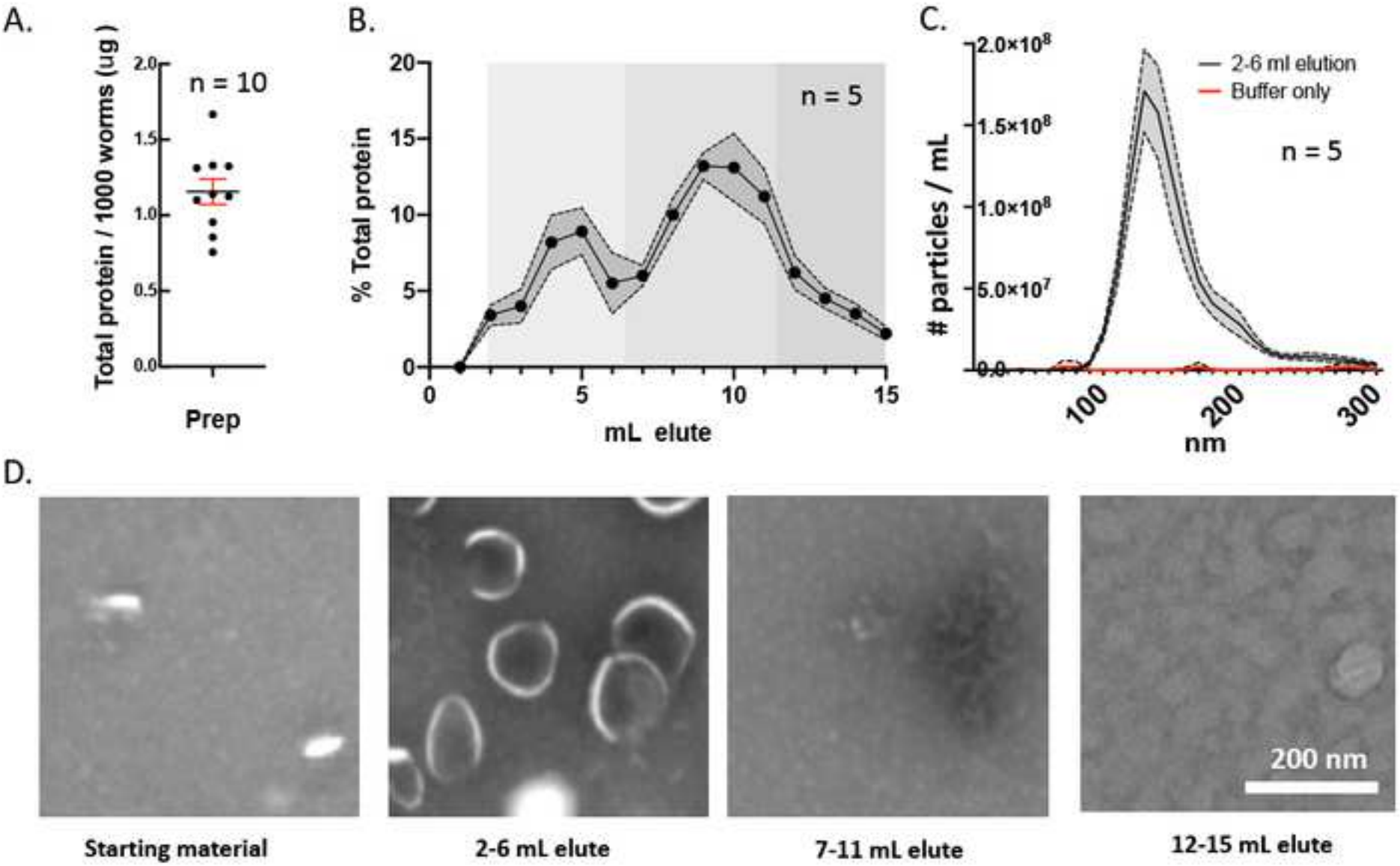


Figure 5

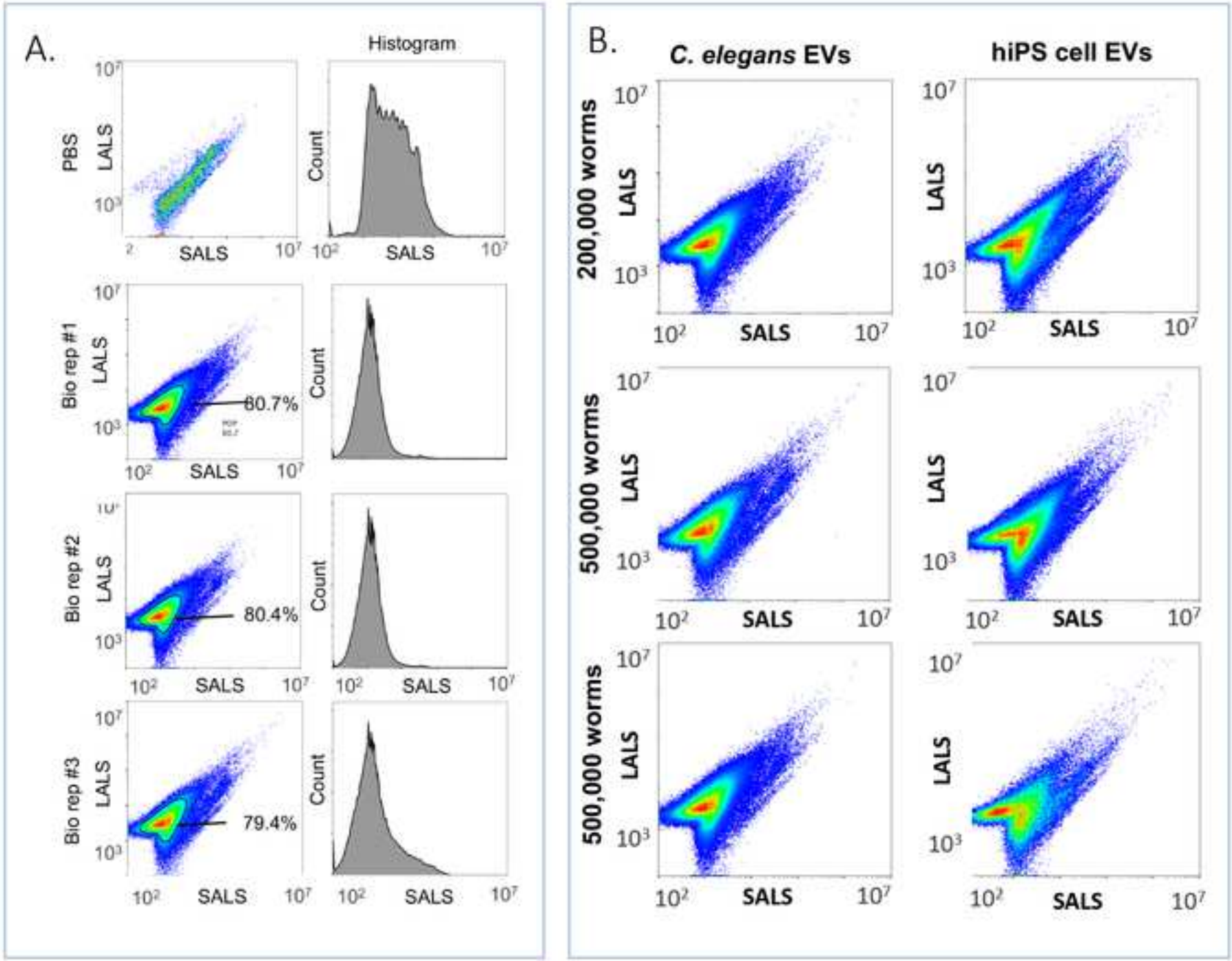


Figure 6

Click here to access/download;Figure;Fig 6.tif

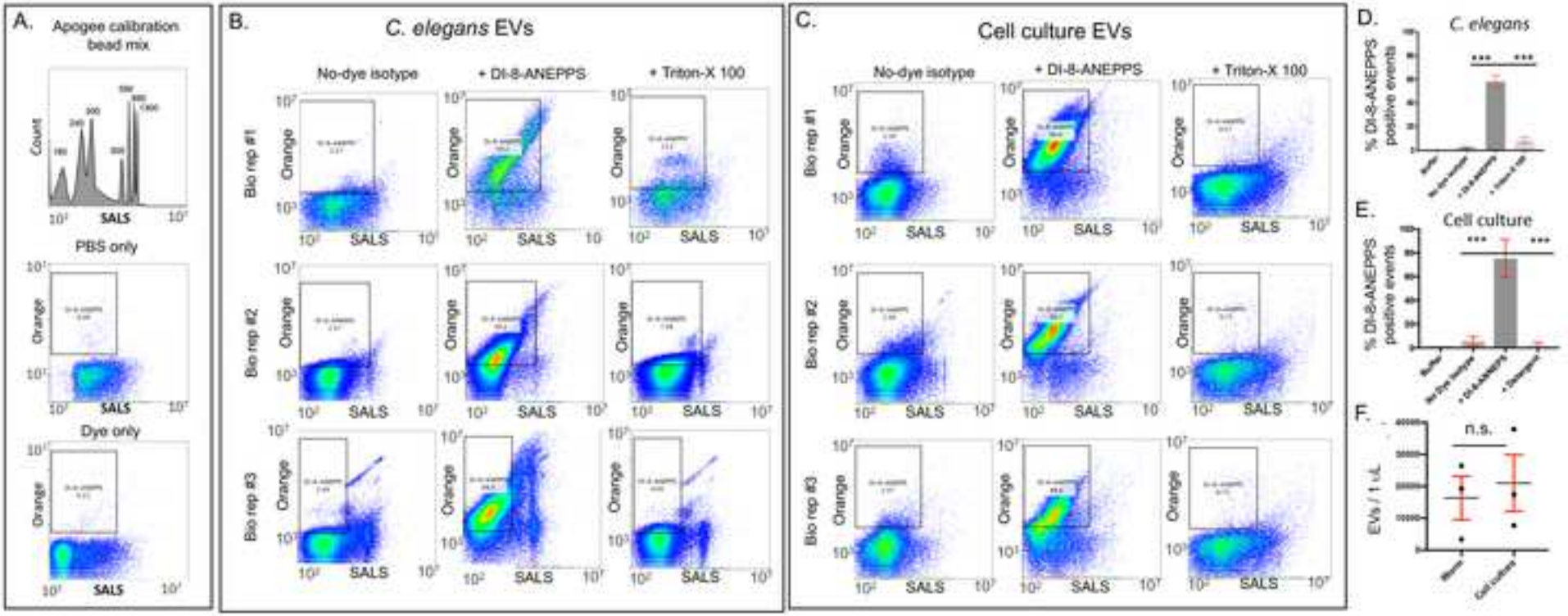


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.22 filters units	Genesee	25-233	For clairifying the conditioned media from debris
1% solution of Triton X-100	ThermoFisher	HFH10	Add this to 0.05 % to lyse EVs
10 cm high growth plates	N/A	N/A	For cultivating large populations of worms
2-liter bottom baffled flasks	ThermoFisher	4110-2000PK	For conducting size exclusion separation of EVs
40 µm mesh	Amazon	CMY-0040-C/5PK-05	Use this to separate adult worms from larval stages
5 µm mesh	Amazon	CMN-0005-C	Use this to separate L1s from other worm stages
6 cm normal growth medium worm cultivation plate	N/A	N/A	For cultivating small populations of worms
Amicon Ultra-15 Centrifugal Filter 10 kD mwco	MilliporeSigma	C7715	For concentrating the size exclusion elute
Amicon Ultra-4 Centrifugal Filter 10 kD mwco	MilliporeSigma	C78144	For concentrating the conditioned media
Apogee A50 flow cytometer	Apogee		This flow cytometer is specialized for resolving nanometer size particles
ApogeeMix	Apogee	1493	Assess light scatter and fluorescence performance of FACS
DI-8-ANEPPS	ThermoFisher	D3167	Add this at 20 uM to label EVs
Disposable chromatography Column	BioRad	7321010	For conducting size exclusion separation of EVs
Geletin	MilliporeSigma	G9391-100G	To treat pipette tips so that worms do not stick
HALT protease inhibitor	ThermoFisher	87785	Add to EV sample to prevent protein degradation
Low-protein binding collection tubes	ThermoFisher	90410	Use these for EV samples
NaCl	Sigma	S7653-1KG	For sucrose floatation of worms
S Basal buffer	N/A	N/A	Recipe in WormBook
Sepharose CL-2B resin	MilliporeSigma	CL2B300-100ML	For conducting size exclusion separation of EVs
Small orbital shaker	ABC Scientific	83211301	Use this for worm S Basal incubation
Sucrose	Sigma	S8501-5KG	For sucrose floatation of worms

September 4th 2019

Dear Editor and Reviewers,

Thank you very much for your feedback. We have considered all of the suggestions in the revised manuscript. In this introduction we more clearly place these methods within the context of previous *C. elegans* research (52-65) explaining why we developed this method rather then use previously established ones. We also clairify why this protocol was developed to isolate small EVs. In the discussion we discuss other larger EV classes and suggest opportunities for future development (401-423). Text was changed throughout for appropiate syntax and consistency. We hope that these revisions have highlighted the key distinctions and innovation of the protocols and made them easier for others to adopt. Please let us know if we could further clarify our new method.

Sincerely,
Russell *et al.*

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Noted

2. Please include at least 6 keywords or phrases.

Noted

3. Please ensure that the long abstract is within 150-300-word limit.

205 words

4. 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.”
Fixed

5. Please do not use commercial terms in the manuscript. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example Sepharose, Amicon Ultra, FloJo software. , etc.
Fixed

6. We cannot have paragraphs of text in the protocol section. Please number all the steps in JoVE style. 1 should be followed by 1.1. and 1.1.1. and so on. Please do not use bullets, hyphens or alphabets.
Fixed

7. Please do not use personal pronouns like you, we, your, our etc. in the protocol section of the manuscript.
Fixed

8. The Protocol should contain only action items that direct the reader to do something.
Fixed

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.
Noted

10. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
Fixed

11. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less 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. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next.
Noted

12. Please use complete sentences throughout.

Fixed

13. Please ensure you answer the “how” question, i.e., how is the step performed?

Noted

14. Lines 97-136: Please make a subheading “preparation” and then make all the steps as numbered steps.

Fixed (93-101)

15. Lines 138-Line 151: We cannot have paragraphs of text in the protocol section. Either move this part to the introduction/discussion or convert it to numbered action steps describing how each step is being performed.

Paragraphs of text were removed from the protocol section

16. 1.1.4: Do you count before resuspending? If yes how?

No the are counted in 1.1.3 and they all should be retained as the pellet from the subsequent centrifugation step.

17. 1.2.2: Super = supernatant?

Fixed

18. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less 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. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Noted and 2.75 pages of text selected.

19. Please discuss all figures in the Representative Results. However, for figures showing the

experimental set-up, please reference them in the Protocol. Please ensure that the figures are discussed in the order of their numbering.
Done

20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
No figures were reused from previous publications

21. As we are a methods journal, please ensure that the Discussion 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
Noted

22. Please do not abbreviate the journal titles in the reference section.
Fixed

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This methodological manuscript is a well-prepared article for the EV field that has recently gained extensive attention. The significance of this article is the application of nematode C. elegans for EV purification and the subsequent analysis, a previously unappreciated EV investigation model system. The report provides a very detailed description of the collection, purification, analysis, and interpretation of EV secreted by nematodes. However, some issues still need to be addressed by the authors.

Major Concerns:

1. The author should add some comments on the purification and identification of different types of EVs in the text, and present some thoughts concerning the future development of associated methods.
Text added to the introduction and discussion. (410-426)

2. In addition to being secreted into the environment, EV is often transmitted between tissues, and its physiological significance could more critical. The author should also discuss this in the text.
Text added to the discussion. (426-431)

Minor Concerns:
3. Line 147-148: for NA22, a brief description should be given.
NA22 is the standard bacteria for use with HG plates therefore we decided to not explicitly mention the bacteria in the HG plate.

4. Line 151: the order of Fig 1A and 1B should be adjusted in accordance with the text.
Changed

5. Line 158: it is recommended to use the full number instead of the abbreviation K.
Changed

6. Line 182-189: the description is not quite clear. It is recommended to briefly explain the purpose of the method steps, such as why a 0.22um filter is used. The description of this paragraph is very confusing.
Changed for clairity

7. The abbreviations in many places throughout the article lack the necessary explanations, especially in the steps. Since nematode EV purification and research is an emerging field, authors should give appropriate background to the readers.
Added

Reviewer #2:
Manuscript Summary:
The manuscript entitled "Purification and analysis of Caenorhabditis elegans extracellular

vesicles" described thoroughly detailed materials and methods that are required for generating, purifying, and quantifying extracellular vesicles (EVs) from tractable invertebrate model system, C. elegans. The authors also determined the abundance of C. elegans EVs by comparing flow cytometry characteristics of C. elegans EVs with those of human ones. This is an excellently written method paper and will greatly benefit the EV biology in C. elegans research field. I have several suggestions to improve this paper.

Major Concerns:

1) This paper will be improved if the authors add 'caution' points and 'troubleshooting'. It will be also better to mention 'stop point' or availability of storage.

We have added these throughout the text.

2) The isolation of EVs is a very important step. Explain 'Size exclusion chromatography' section more in detail for this purpose.

We had added some more specific details to this section. We feel that these are sufficient for conducting the protocol but would be glad to include other details if it protocol still seems too vague.

Minor Concerns:

1) Briefly explain the purpose of using certain reagents or chemicals in the main text in addition to the Table of Materials.

JoVE does not want to have specific products mentioned in the main text. However we did discuss why regenerated nitrocellulose filters are used for the concentration steps and why DI-8-ANEPPS was choses as a dye.

2) They need to remove ambiguity in the text. For example, in "Dilute worms so that you get about 20 in a 10 µL drop.", the authors may intend '20' as 20 animals or worms. "EV prep" should be "EV preparation". In Figure 1A, "Grow 50,000 plate to young adulthood 20,000 after that" seems wrong. "plate 50,000 worms per HG plate" is unusual. What is the amount of protease inhibitor cocktail needed?

We fixed these ambiguities in the figure and text.

3) Consider adding an actual example that calculated the number of total EVs to help readers to understand the procedure better.

Added an example at line 302

4) I think the resolution of all figures will be improved for the final publication but currently the resolution is too low to review.
We changed the TEM micrographs so that the EVs can be identified even at low resulution

5) Make bold fonts, italics, superscripts, and subscripts consistent. For example, "Add" should not be bold in "Gel 1.1 Add 2 g of gelatin to 100 mL dH20 and heat in microwave until it starts to boil." '10e2 to 10e4' in 3.4.3 should be corrected too.
Fixed

6) The authors may add Gel 1.3 to explain how to use aliquots of gelatin solution for pipette tip treatment in detail before pipetting.
Added (92-95)

7) Avoid tautology like "sterile 15 mL sterile tubes". "Lower numbers of animals will reduce the accuracy of the calculations while having much more than animals per drop makes it difficult to count worms reliably." in 'Calculating large populations of worms' and "If there are more than 20 animals, then it's difficult to count accurately and lower numbers of animals will not provide statistical power to confidently estimate the worm population to within 10 %." in 'Count 1.2' are also duplicated.
Fixed

8) How many plates are needed to support 500,000 young adults or 3,000,000 L1 larval stage animals in 'Experimental planning'?
This section was moved to discussion with the number of plates needed explicity stated (436-437)

9) Specify the number of HG plates that used for sampling in 1.1.4.
Do you mean 1.2.4? The sampling in 1.2.4 is to be conducted on the worms collected from all the plates.

10) Change 1.1.9 to 1.1.8.
Corrected (129)

11) It will be better to describe the reason of using starved animals for the description of Figure 1.

We are not clear on what this comment is asking. Should we add a reason for using starved worms in the figure title or legend? We have added some text in the introduction to explaining that EVs from well-fed worms can not be purified away from *E. Coli* EVs. (61-64)

12) Please mention 'tube #1' in 3.2.

Done

13) Is there any alternative for DI-8-ANEPPS?

Yes, vital dye (Calcein-AM, Vybrant- CFDA) can also be used. We added this to the discussion. (490)

Reviewer #3:

Manuscript Summary:

Russell et al. detail methods to purify extracellular vesicles from worms cultured on plates and then starved in liquid media. The protocol appears to nicely purify a subpopulation of EVs, but appropriate controls and/or discussion of these limitations is missing from the manuscript.

Major Concerns:

The protocol uses two rounds of starvation to synchronize animals and a third round to secrete EVs, which is likely to alter the content and source of the EVs. The authors acknowledge that stress responses can alter EV cargo, but do not discuss this in terms of their own protocol.

Added (459-460)

The use of an 0.22 µm filter will remove the larger subpopulation of EVs. In some cell types, the average diameter of EVs is 0.2 µm (i.e. Brisson et al., Platelets 2017), which would lead to a considerable loss of potentially relevant EV populations. Have the worm EVs been analyzed for their typical size in an unbiased fashion? This major limitation of the method was not discussed.

Text added to discussion (410-431) To the best of our knowledge there has been no study that has examined externally secreted EV size in an unbiased fashion. The secretate has been shown to contain metabolites that are different between genetic lines that have mitochondrial mutations that result in lengthened or shortened lifespans[1]. It is currently unclear if these

metabolites are freely secreted or contained in specific classes of EVs. A comprehensive unbiased characterization of the secretate could shed light on this interesting result.

Also, state the pore size of the chromatography matrix.
Added (160, 322)

The electron micrographs in Fig. 4D are too small to see the shape of the vesicles. The MISEV2018 checklist indicates that a zoomed image showing only a few vesicles is also necessary to demonstrate that the protocol purified vesicles.
Fixed

Minor Concerns:
Abstract: "However, the EV field still lacks a tractable invertebrate model system in which fundamental questions related to EV biology can be explored." "The facile genetics and huge library of reagents and disease models developed for this widely-used genetic model can now be employed to investigate the organismal pathways and processes that impact EV signaling." These sentences should be rephrased. Researchers have been publishing work on EVs in invertebrate models for over a decade. In particular, purifying and analyzing EVs from C. elegans culture has already been reported (Wang J et al., Curr Biol 2014), so the claim at novelty here is overstated.
This has be rephrased so that the claims are focused on EV cargo composition.The Wang et al method washed worms off seeded plates and then pelleted at 200,000 X G for 2 hours. This process results in mostly *E. coli* EVs. While their method is suitable for immuno- analysis (immune-TEM or Western) as well as their functional studies the preparation is not pure C. elegans EVs. Therefore it is not suitable for EV cargo discovery using -omic methodologies. We added this distinction in the text. (61-63)

Line 65-66: "not suitable for conducting genetic screens. An invertebrate model for studying EV biology would therefore complement current EV research approaches."
The method being described in this manuscript is also not suitable for conducting genetic screens, so this is irrelevant.
Sorry for the confusion! We did not mean to imply this method is suitable for conducting forward screens. However the large library of mutants and disease models avalible for *C. elegans* research can be used for screening genetic pathways and disease models.

Line 85: What kind of media was used for HG plates? What size petri dishes were used?
Added

Lines 117-119: Duplicated text from Count 1.2
Deleted

Lines 302-304: Define SALS and LALS
Added (273-274)

Lines 329-330: Duplicated text.
Deleted

Line 360: Duplicated text.
Deleted

Line 493: CGC acknowledgements should include their funding information:
NIH Office of Research Infrastructure Programs (P40 OD010440).
Added

Reviewer #4:
Manuscript Summary:
The manuscript provides a detailed and very thorough methodology for purifying EVs from C. elegans. The methods are very clear and detailed, and the protocols are well supported by background material.

Major Concerns:
None

Minor Concerns:
1. I find the description on lines 153-165 is not perfectly matched to the left-hand part of Figure 1A, and this lead to some confusion on my part (e.g., why does the figure say "20,000 after that"). Please make sure the method for scaling up worm numbers is very clear in the flow chart in 1A (ie. include all the steps that are described).

Filtration step added to Figure 1 so that the text and figure match.

2. Explain 'HG plates'

We decided to get rid of the HG abbreviation and use “high growth” throughout. They are explained in 435-437

3. Some parts of text have syntax/grammar/spelling errors; eg. lines 147-148, 161, 277-278, 336, 346, 358, 360 (duplicated statement).

Fixed

References

Butler, Jeffrey A., Robert J. Mishur, Shylesh Bhaskaran, and Shane L. Rea. "A metabolic signature for long life in the Caenorhabditis elegans Mit mutants." *Aging cell* 12, no. 1 (2013): 130-138.

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Title of Article:	Purification and Analysis of Caenorhabditis elegans Extracellular Vesicles
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