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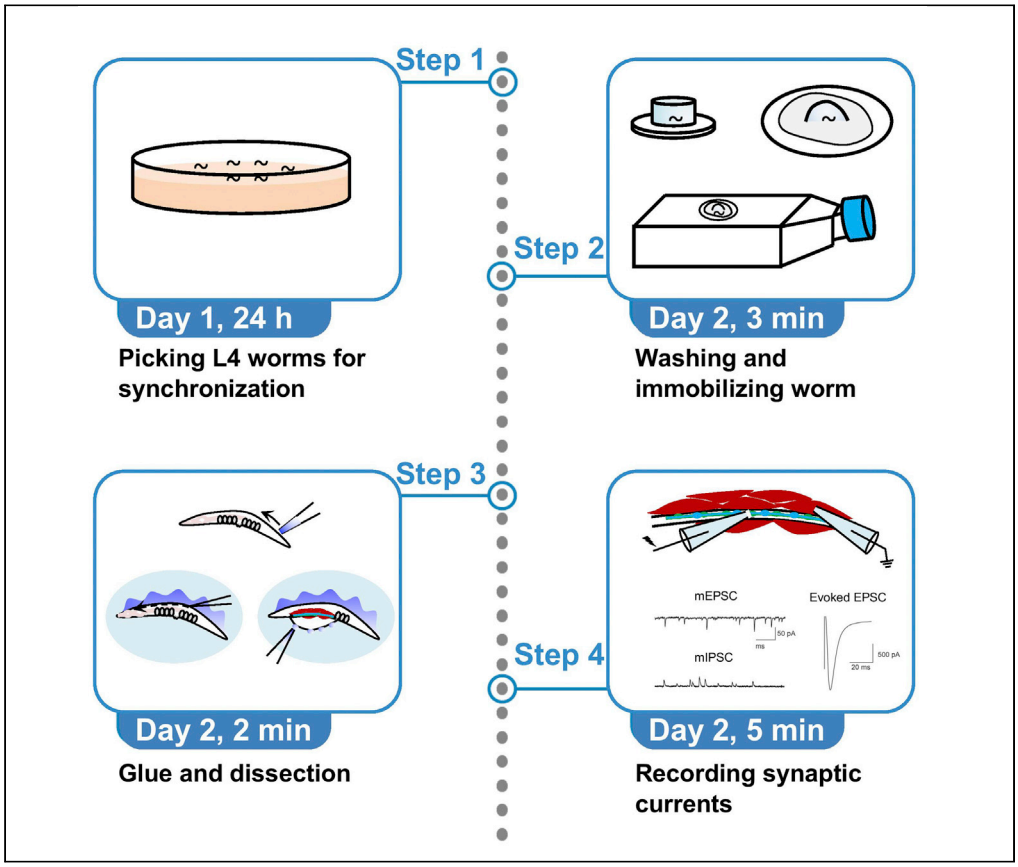
Protocols for electrophysiological recordings and electron microscopy at *C. elegans* neuromuscular junction



Release of neurotransmitters by synaptic vesicle exocytosis at presynaptic terminals is critical for neuronal communication within the nervous system. Electrophysiology and electron microscopy are powerful and complementary approaches used to evaluate the function of synaptic proteins in synaptic transmission. Here, we provide a protocol detailing the use of these two approaches at *C. elegans* neuromuscular junctions, including steps for worm picking and dissection, *in vivo* electrophysiological recording, and sample preparation for electron microscopy, followed by imaging and analysis.

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Highlights

In vivo electrophysiology at the *C. elegans* neuromuscular junction

Tips on immobilizing, gluing, dissecting, and recording processes

High-pressure freeze and freeze substitution electron microscopy in *C. elegans*

Tips on *C. elegans* sample sectioning and staining for electron microscopy

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Protocols for electrophysiological recordings and electron microscopy at *C. elegans* neuromuscular junction

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SUMMARY

Release of neurotransmitters by synaptic vesicle exocytosis at presynaptic terminals is critical for neuronal communication within the nervous system. Electrophysiology and electron microscopy are powerful and complementary approaches used to evaluate the function of synaptic proteins in synaptic transmission. Here, we provide a protocol detailing the use of these two approaches at *C. elegans* neuromuscular junctions, including steps for worm picking and dissection, *in vivo* electrophysiological recording, and sample preparation for electron microscopy, followed by imaging and analysis.

For complete details on the use and execution of this protocol, please refer to Liu et al. (2021) and Li et al. (2021).

BEFORE YOU BEGIN

Electrophysiology

Timing: around 3 h

1. Before the day of recording, the following needs to be prepared:
   a. Pick 20–30 L4 stage well-fed worms to a new NGM plate (nematode growth medium, please refer to http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html). These worms will be used for recordings in the next day.
   b. Internal pipette stock solution. It is recommended to make a 200 mL or 500 mL internal solution that can be aliquoted in 1.8 mL Eppendorf tubes, stored at −80°C and used for at least a year. The original pH value of the internal solution is around 3.5. Use CsOH to adjust the pH value to 7.2 prior to freezing.
   c. Collagenase digestion solution. First make 500 mL extracellular solution and bubble the solution with 5% CO_2 and 95% O_2 for at least 20 min. Dissolve 0.5 g collagenase powder into the extracellular solution to make a 1 mg/mL stock solution. Disperse into 1.8 mL eppendorf tubes, store at −80°C freezer. This can be used for at least two years.
   d. Prepare Sylgard 184 coated circular coverslips and cure over night at 60°C.
2. On the day of recording, the following needs to be prepared in advance:

a. Make 500 mL fresh extracellular solution and bubble the solution with 5% CO_2 and 95% O_2.
b. Prepare the ice bottle. Put three cell culture bottles filled with water into an ice box for at least 20 min. These bottles will be used to immobilize worms.
c. Pull all required pipettes, including recording pipettes which need to be polished, the stimulus pipettes (polish is not necessary), and the cutting pipettes which have sharp ends.
d. Thaw a tube of collagenase and internal pipette solution.

Electron microscopy

Timing: around 2 h

3. Before the day of high pressure freezing, the following should be done:
   a. Set out 3–7 NGM plates, previously seeded with *E.coli*, at RT (room temperature; 22°C) 2–3 days prior to day of freezing. This allows the bacteria to thicken, as these bacteria will be used as a cryoprotectant when freezing the worms.
   b. Day before the freeze pick 40–60 L4 stage, well-fed worms per strain being frozen to new plates.
   c. Prepare and sequentially number cryotubes for each strain being frozen (prepare extra) by putting 4 holes spaced around the center of the tube, as well as 2 holes in the lid. The holes allow for liquid nitrogen to enter the tube when submerged.

Note: The freeze and substitution processes require a large amount of liquid nitrogen. A tank should be ordered or secured prior to freezing and substitution.

4. On the day of freezing:
   a. Use a weighing spatula to scrape the bacteria to be used as the cryoprotectant into mounds on the surface of the plates. This will be used for picking worms and for filling the specimen carriers.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli OP50	Caenorhabditis Genetics Center (CGC)	OP50
Experimental models: organisms/strains		
C. elegans: Bristol N2 (adult stage, hermaphrodite)	Caenorhabditis Genetics Center (CGC)	N2
Chemicals, peptides, and recombinant proteins		
$CaCl_2$	Fluka Analytical	Cat#21114
$MgCl_2$	Fluka Analytical	Cat#63020
NaCl	Sigma-Aldrich	Cat#S9888
$NaHCO_3$	Sigma-Aldrich	Cat#S6014
Sucrose	Sigma	Cat#S9378
KCl	Sigma	Cat#P5405
$NaH2PO_4$	Sigma-Aldrich	Cat#S0751
Glucose	Sigma	Cat#G8270
CsCl	Sigma	Cat#C3309
CsF	Aldrich	Cat#289345
EGTA	Sigma	Cat#E3889
HEPES	Sigma	Cat#H4034
Na_2ATP	Sigma-Aldrich	Cat#2383
CsOH	Aldrich	Cat#C8518
Agarose	Sigma	Lot #SLBR6299V
All trans-Retinal	Sigma-Aldrich	CAS #116-31-4
Histoacryl Blue Topical Adhesive	McFarlane	CAS #1050044

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Collagenase	Sigma-Aldrich	CAS #C5138
Tannic acid		Cat#21700
Acetone, glass-distilled, electron microscopy grade	EMS	Cat#10015
Osmium tetroxide	EMS	Cat#19110
Non-stick Dry Film Lubricant Spray (w/Teflon)	DuPont	
Uranyl acetate	EMS	Cat#22400
Lead nitrate	EMS	Cat#17900
Sodium citrate	EMS	Cat#21140
KOH pellets	Fisher Scientific	CAS#1310-58-3
Eponate kit w/BDMA*	Ted Pella	Cat#18012
Software and algorithms		
ImageJ	NIH	RRID: SCR_002285
PatchMaster	HEKA Elektronik	V2x73.2
IGOR Pro	WaveMetrics	Version 7
SigmaPlot	Systat Software Inc.	Version 13.0
Prism	GraphPad	Version 9.0
Other		
Microscope cover glass	Fisher Scientific	Lot#060214-9
Glass capillaries	World Precision Instruments, Inc.	Lot#2009330
Sylgard	Dow Corning	3097366-1004
Collagenase	Sigma	C-5138
Histoacryl blue	McFarlane	15054BU
Topical tissue adhesive	GLUture	503763
Micropipette	World Precision Instruments	1B150F-4
Coverslip (22 mm in diameter)	eBay	N/A
High-vacuum silicone grease	Dow Corning	Z273554-1
Chamber	Made by workshop	N/A
MicroFil	World Precision Instruments	MF28G67-5
Silicone tubing	Local store	N/A
Specimen Carrier Type A (0.1 /02 mm)	Technotrade	241
Specimen Carrier Type B (0.3 mm)	Technotrade	242
2 mL Cryotubes	N/A	N/A
Flow-through rings	Mager Scientific	707157
Ring inserts D5×H15 mm	NCI, Inc	16702738
Universal chambers	Made by workshop	N/A
Plastic caps for chambers	NCI, Inc	16702747
50 mL Conical tubes	N/A	N/A
Disposable transfer pipettes	Fisherbrand	13-711-9AM
Polypropylene capsules, clear 26 mm	Ted Pella	21460
100 mm Disposable petri dishes	N/A	N/A
Glass slides	N/A	N/A
Parafilm	N/A	N/A
Fine-tip needles 30 g × $^1/_2$	BD	305106
Formvar/Carbon Slotted Grids	Electron Microscopy Sciences	FCF2010-CU
10 mL Syringe Luer-Lok Tip	BD	302995
25 mm Filter 0.2 µm, nylon, sterile	Fisherbrand	09–719C
Air table and Faraday cage	TMC Micro-g	TM63541
Microscope	Olympus	BX51WI
Moving stage	Mike's Machine Company	N/A
Amplifier	HEKA	EPC-10
Manipulator	Sutter Instrument	MPC-325
LED light source	Thorlabs	M470L3-C1
Stimulator	World Precision Instruments	A365
Pressure ejection device	Parker Instruments	Picospritzer III

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dissection scope	Olympus	SZ51
Micropipette puller	NARISHIGE	PC-10
Microforge	NARISHIGE	MF-830
HPM100	Leica	N/A
Dissecting light microscope	N/A	N/A
Top-light	N/A	N/A
Cryocanes	N/A	N/A
Unloading station	Made by workshop	N/A
AFS2	Leica	N/A
EM Ultramicrotome	Leica	N/A
Trimming tool 45	Diatome	TT-45
Diamond knife 45	Diatome	N/A
Perfect loop	Electron Microscopy Sciences	70944
Staining pads	Electron Microscopy Sciences	N/A
Molds for resin block	Electron Microscopy Sciences	N/A

Note: This protocol used Epon resin for ultrastructural, morphological analysis. Other resins can be used for sample preparation. For example, Lowicryl or LR White should be used for Immunological EM analysis.

MATERIALS AND EQUIPMENT

Materials for Electrophysiology
Sylgard
Collagenase
Histoacryl blue
Topical tissue adhesive
Micropipette
Coverslip (22 mm in diameter)
High-vacuum silicone grease
Chamber
MicroFil
Silicone tubing

Materials for Electron Microscopy
Specimen carrier type A (0.1/02 mm)
Specimen Carrier Type B (0.3 mm)
2 mL Cryotubes
Flow-through rings
Ring inserts D5×H15 mm
Universal chambers
Plastic caps for chambers
50 mL conical tubes
Disposable transfer pipettes
Polypropylene capsules, clear 26 mm
100 mm disposable petri-dishes
Glass slides
Parafilm
Fine-tip needles 30 g × $^{1}/_{2}$
Formvar/Carbon Slotted Grids
10 mL syringe Luer-Lok Tip
25 mm filter 0.2 μm, nylon, sterile

Equipment for Electrophysiology
Air table and Faraday cage
Microscope
Moving stage
Amplifier
Manipulator
LED light source
Stimulator
Pressure-ejection device
Dissection scope
Micropipette puller
Microforge

Equipment for Electron Microscopy
HPM100
Dissecting light microscope
Top-light
Cryocanes
Unloading station
AFS2
EM Ultramicrotome
Trimming tool 45
Diamond knife 45
Perfect loop
Staining pads
Molds for resin block

Extracellular recording solution (make fresh solution each time, use at room temperature)		
Reagent	Final concentration (mM)	Amount (g) for 1 L
NaCl	127	7.421
KCl	5	0.372
$NaHCO_3$	26	2.184
$NaH2PO_4$	1.25	0.172
Glucose	10	20 mL @ 0.5 M stock solution
$CaCl_2$	1	1 mL @ 1 M stock solution
$MgCl_2$	4	4 mL @ 1 M stock solution

Note: For low Ca^{2+} or Ca^{2+}-free bath solution, Mg^{2+} concentration is increased accordingly to maintain a total 5 mM of Ca^{2+} and Mg^{2+} (Liu et al., 2019).

Internal solution (store at −80°C, use for at least 2 years)		
Reagent	Final concentration (mM)	Amount (g) for 500 mL
CH_3O_3SCs	120	13.7
CsCl	4	0.335
CsF	15	1.135
$MgCl_2$	4	2 mL @ 1 M stock solution
EGTA	5	0.95
$CaCl_2$	0.25	125 µL @ 1 M stock solution
HEPES	10	1.19 g
Na_2ATP	4	1.1 g

Other stock solutions (store at −80°C, use for 6 months)		
Reagent	Stock concentration (mM)	Final concentration (mM)
Collagenase	1 mg/mL	0.5 mg/mL
Sucrose	N/A	1000
Acetylcholine	500	0.5
Levamisole	500	0.5
GABA	500	0.5

Note: It is recommended to remake the stock of the drugs (including acetylcholine, Levamisole, and GABA) every 6 months. The activated currents are likely to get smaller after 6 months.

STEP-BY-STEP METHOD DETAILS

Electrophysiological recordings

Washing and immobilizing adult worm

Timing: 3 min

1. Pick one adult worm from the NGM plate and place it into a small container (the cap of a 1.8 mL eppendorf tube is recommended) containing a small amount of bath solution. Use a tip-broken glass pipette (the open size is ~0.3 mm) to suck the worm into the pipette and then gently blow it out. Repeat this for 3–5 times to wash away the bacterial on the worm body (Figures 1A and 1B).
2. Suck the worm into the washing pipette and transfer the worm onto a Sylgard 184 coated circular coverslip. Make sure the worm is immersed in a small drop of bath solution (~20 μL). This prevents the worm from desiccating (Figure 1C).
3. Place the coverslip onto the cell culture bottle filled with cold water. The low temperature immobilizes the worm quickly (Figure 1D).

Applying glue and dissecting worm

Timing: 3 min

4. Once the worm is immobilized, remove extra solution using a micropipette connected by thin tubing to an Eppendorf pipette tip to allow mouth-controlled suction. Use the same pipette to adjust the worm body to an arc shape (the dorsal side is on the outer curve and the ventral side on the inner curve). A tip-broken pipette with a small amount of glue at the end is used to touch the dorsal side of the worm gently and quickly. The glue then automatically spreads along the worm body. Repeat this for several times at different spots on the dorsal side to solidify the connection between the worm and the glue (Figure 2A). A 15–20 μL bath solution is then quickly added back onto the worm.
5. Use another pipette with a very sharp tip (called a cutting pipette) to cut the cuticle from a point a little behind vulva to the bulb along the dorsal side (Figure 2B). After dissection, an extraction pipette (about 5–10 μm of the open size) controlled by mouth suction via thin tubing is used to remove all the internal organs, including the intestine, gonads, and eggs. The cut-edge of the cuticle on the ventral side is glued down with a new pipette to expose the ventral nerve cord and the body wall muscles. During gluing positive pressure is applied to the pipette to prevent the solution entering the glue tip and polymerizing the glue before it has been dispensed (Figure 2C).
6. Take the coverslip off the ice bottle and secure it into the inner well of the recording chamber with pre-applied silicone grease. Using the cap of a 15 mL centrifuge tube to apply gentle pressure to the coverslip and ensure that the seal between the coverslip and the chamber is good.

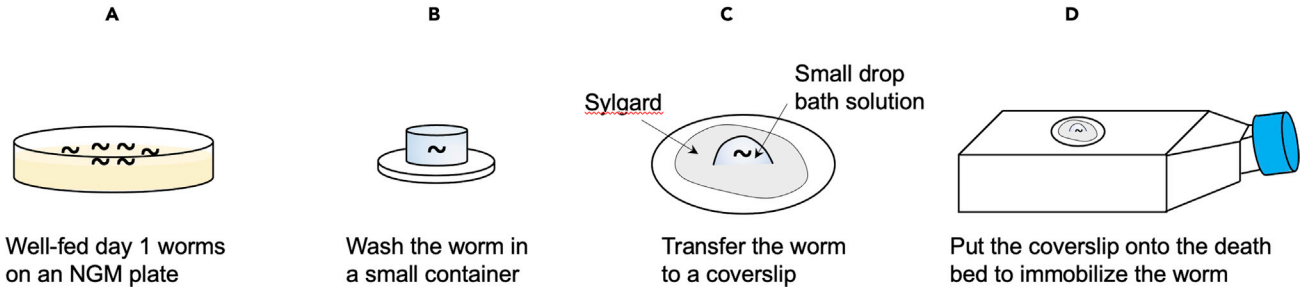


Figure 1. Washing and immobilizing worm for dissection

(A and B) Pick 1 adult worm from the plate to a bath container for cleaning. (C) The cleaned worm is immersed in a drop of bath solution on a sylgard pad. (D) Immobile the worm on a cold cell culture bottle.

7. Add a 20 μL collagenase (1 mg/mL dissolved in extracellular solution) to the small drop of solution on the worm to digest the cells for 30 s (the final concentration of the collagenase applied to the cells is therefore about 0.5 mg/mL). The collagenase tube should be kept on ice at all times.
8. Transfer the chamber to the inner holder of the moving stage and adjust this to the correct position from which both the stimulus and patch pipettes can reach the neuromuscular junctions easily. Wash several times with the bath solution via the perfusion system to remove the collagenase completely. Finally add 1.5–2 mL bath solution into the chamber. The preparation is now ready for recording.

⚠ CRITICAL: It is important to leave a very small volume of bath solution under the worm before the first glue application. This prevents the glue crossing to the ventral side.

Note: This dissection step is the most important because the quality of the dissection directly determines the ability to obtain good data. Damage to the cell usually comes from shaking of the cutting pipette. We hold the pipette (about 1 cm from the sharp tip) between the thumb and index finger and place the hand with the pipette onto the iced bottle tightly before the cuticle incision. This allows us to hold the pipette in position without shaking, thereby avoiding unnecessary damage in the dissection process. Usually, this step requires a few weeks or months of practice, although it takes even longer to master the dissection of smaller worms.

Recording synaptic currents

Timing: 5–10 min

9. Position the recording chamber on the moving stage. This allows both the recording pipette and the stimulus pipette (or the pressure-ejection pipette) to be placed on proper position. Patch the muscle using the standard patch-clamping technique. The patch pipette (4–5 megohm resistance) is pressed gently against the muscle cell (at the mid-point) and suction is applied to form a high resistance seal (gigaohm seal). Changing the holding potential to −60 mV and applying brief mouth suction ruptures the patch, thereby forming the whole-cell patch.
10. Once the whole-cell patch clamp is formed, miniature excitatory postsynaptic currents (mEPSCs) are observed at the −60 mV membrane potential (the reversal potential of GABA receptor channels) (Figures 3A and 3B). Switch the membrane potential to 0 mV (the reversal potential of acetylcholine receptor channels) to record miniature inhibitory postsynaptic currents (mIPSCs) (Figures 3A and 3B).
11. To record electrical stimulus-evoked EPSCs, a second pipette (4–5 megohm resistance, filled with bath solution) is placed above the ventral nerve cord (one muscle anterior distance from the recording pipette). Press the stimulus pipette gently onto the cord and apply a 0.4 ms, 85 uA square pulse to depolarize the neurons (Stimulator: WPI A365), which will produce a large

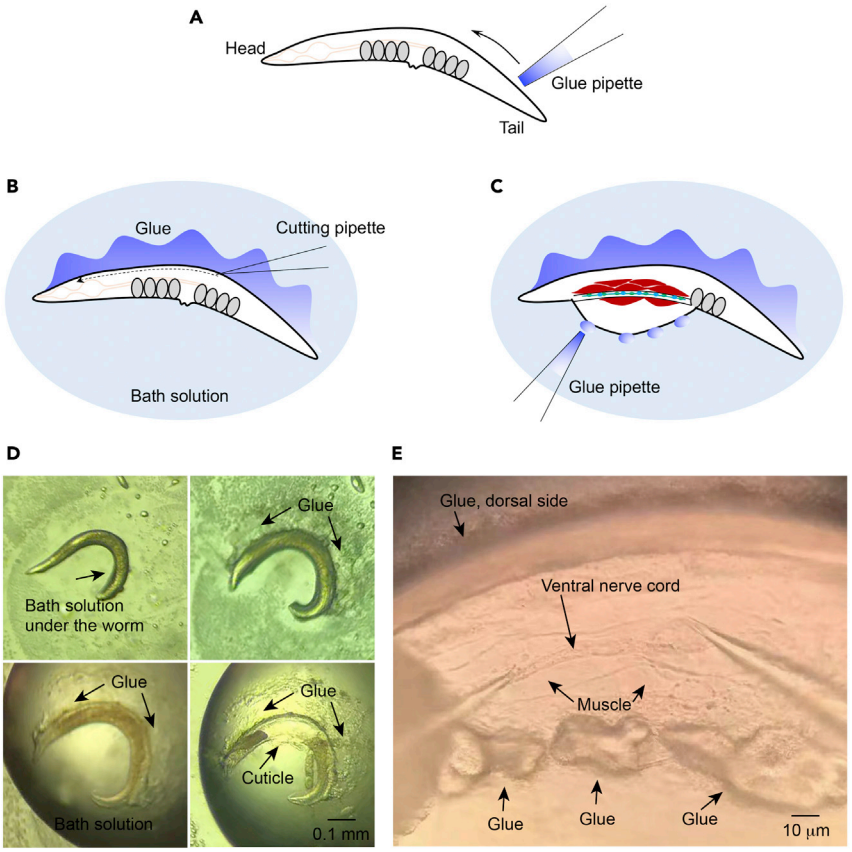


Figure 2. Dissecting worm to expose the ventral nerve cord and body wall muscles

(A–C) Cartoons describing each step during the dissection.

(D) Snapshots of the key steps in a real dissection.

(E) Zoomed view showing ventral nerve cord and muscle.

and synchronized evoked excitatory postsynaptic current (evoked EPSC) (Figures 3A and 3B). A typical wild-type evoked EPSC is around 1.5–2.5 nA in amplitude.

12. For estimating the readily releasable pool size, place a pipette containing 1 M sucrose solution at the end of the patched muscle cell (around half muscle distance from the recording pipette), and a 20 psi, 2 s pressure pulse was applied by Picospritzer (Parker) to create a rapid jump in osmolarity at the neuromuscular junctions (< 200 msec latency on average) (Figures 3C and 3D).
13. To measure the function of receptors on the muscle membrane, apply drugs (e.g., acetylcholine, GABA, and levamisole, dissolved in bath solution) by pressure-ejection them directly onto the body of the muscle. The pipette filled with drug is placed at the end of the patched muscle cell. Pressure-ejection parameters are kept the same for these drugs (12 Psi, 100 ms) (Figures 3C and 3D).

⚠ CRITICAL: It is important to place the stimulus pipette in the correct position (usually one muscle distance from the recording pipette) as the evoked current will be small if it is too far away or the gigaohm seal will be damaged if it is too close.

Note: It is recommended to record all types of synaptic currents from the same muscle, and only one muscle is recorded in each dissected worm. The recording of drug-activated currents can be repeated several times, but the solution has to be replaced and wait for at least 1 min before the next application of drugs. *C. elegans* muscles fire classical all-or-none action

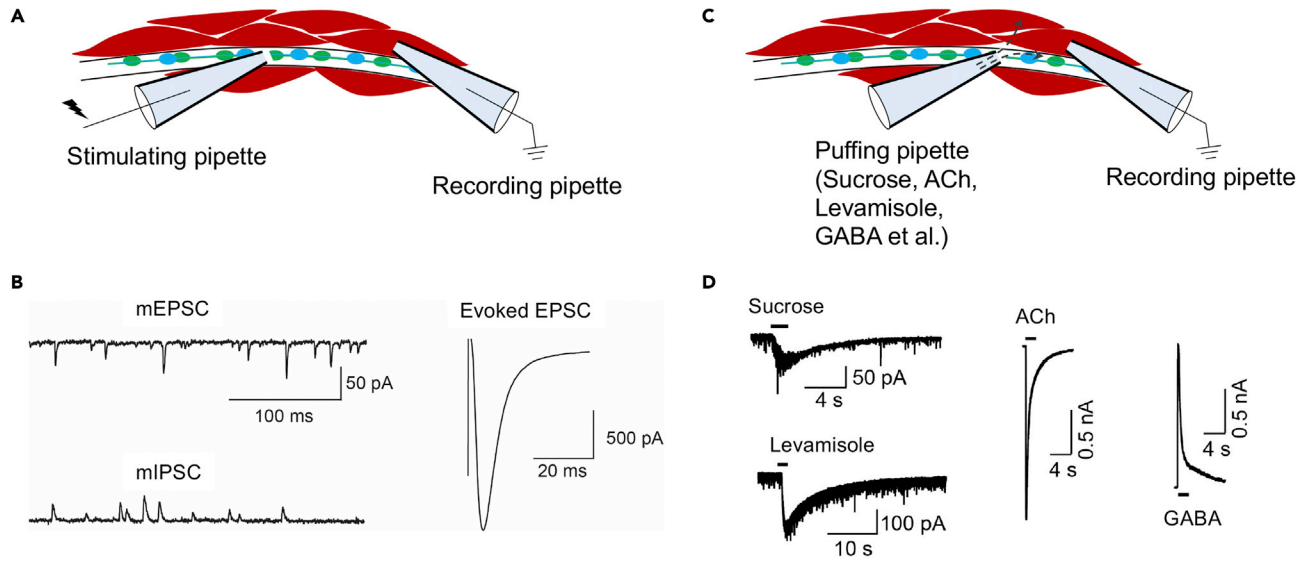


Figure 3. Recording various synaptic currents at the *C. elegans* NMJ

(A) Schematic showing the position of the recording pipette and the stimulating pipette. (B) Example traces of mEPSC, mIPSC, and evoked EPSC recorded from a wild-type worm. (C) Schematic showing the position of the recording pipette and the puffing pipette. (D) Representative traces of synaptic currents triggered by sucrose, levamisole, acetylcholine (ACh), or GABA.

potentials. Please refer to the recent papers from the Wang and Zhen lab for details (Gao and Zhen, 2011; Liu et al., 2011).

Analyzing electrophysiological data

The Igor (WaveMetrics) software is used to analyze all electrophysiology data.

14. Analyze the minis using an Igor macro called "Amperometric Spike Analysis" originally written by Dr. Eugene Mosharow from Columbia University. The software "Mini Analysis" is also recommended. A proper threshold needs to be set before running the macro. In most cases, a 4 pA minimum threshold is used, above which the majority of the mini events are accurately selected and analyzed (Figure 4) (Li et al., 2018). An averaged mEPSC current is integrated in Igor to calculate the charge transfer (the pink area, Figure 5).
15. For sucrose-evoked currents, the integrated charge transfer is computed as a function of time for the entire sucrose response (the pink area in Figure 6), and the charge accumulation is corrected for the baseline holding current and spontaneous fusion events prior to sucrose application.
16. For all other recordings (e.g., stimulus-evoked EPSC, and drug-activated current), we use the Igor built-in macros to quantify all the parameters, including amplitude, charge transfer, rise-time, and inactivation decay. The charge transfer of the evoked EPSCs is calculated in the same way as the averaged mEPSC.

High-pressure freeze, substitution, and electron microscopy

Timing: Freeze: 3 h–5 h depending on number of strains to be frozen; Substitution: 10d total process (program in Figure 7), around 12 h–15 h active

17. Freezing Process: Filling carriers for freezing
    a. Place a small drop of ddH_2O in the middle of the shallow side (100 μm) of the A-type specimen carrier.

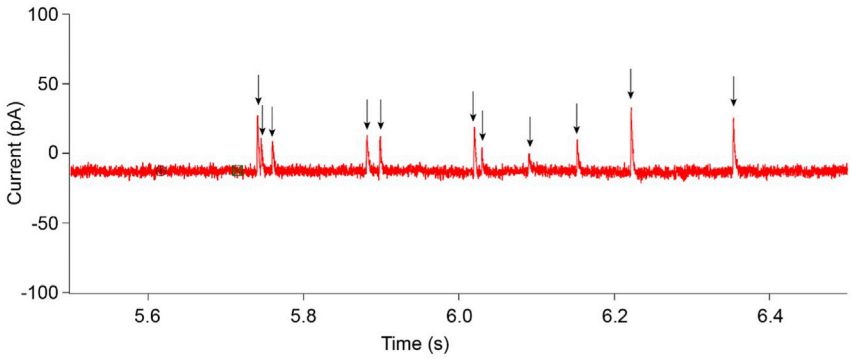


Figure 4. Analysis of the mini events in Igor

Above the threshold of 4pA, almost all events are selected, and the frequency and amplitude of each mini trace are calculated.

b. Scoop up a dollop of the OP50 so that it hangs from the worm pick and can be easily dropped into the water. Spread the OP50 and water around in the carrier that so the bottom is completely covered but do not overfill as the mixture will leak out of the carrier. Work quickly so that the bacteria mixture does not dry out.
c. Pick 20–30 young adult worms and place them in the A-type carrier. The carrier should be completely full so as to eliminate any air bubbles that may occur.
d. Place the B-type specimen carrier flat side down on top of the A-type carrier to form a "sandwich."
e. After high-pressure freezing the first "sandwich" repeat the process in order to have a total of 40–60 worms frozen per strain.

18. Freezing, removing and storing carriers
    a. When using the Leica HPM100, insert the sandwiched specimen carrier into the holder and activate the freezing function.
    b. Repeat this process with the second carrier from the same strain.
    c. Remove the HPM100 liquid nitrogen storage dewar containing the carriers from the two rounds of freezing a single strain and pour them into an open container full of liquid nitrogen.
    d. Remove each carrier from the holder being extremely careful to ensure the carriers remain submerged in liquid nitrogen throughout the removal process.
    e. Place the two frozen carriers into a single numbered cryotube that is submerged and filled with liquid nitrogen then cap the cryotube.
    f. Once the carriers are in the appropriate cryotube, quickly place the tube in a cryocane submerged in liquid nitrogen. When this process has been completed for a second strain, the two cryotubes should be placed in the two bottom slots of a cryocane and then immediately placed in a transfer dewar full of liquid nitrogen.
    g. Samples are typically stored in a transfer dewar that is kept in a 4°C cold room overnight before the substitution process begins the following day. Be sure the dewar is full of liquid nitrogen before leaving.
    h. Fill the AFS with liquid nitrogen, start the preprogrammed substitution protocol (Figure 7) and then hit pause so that the AFS can cool down to −90°C before samples are placed within the machine for substitution. Place the AFS ventilation tube in the fume hood for the remaining substitution protocol.

⚠ CRITICAL: It is important that once the carrier has been frozen all steps are carried out with the samples submerged in liquid nitrogen. The samples should not be exposed to ambient room temperatures or be removed from liquid nitrogen at any point during this process.

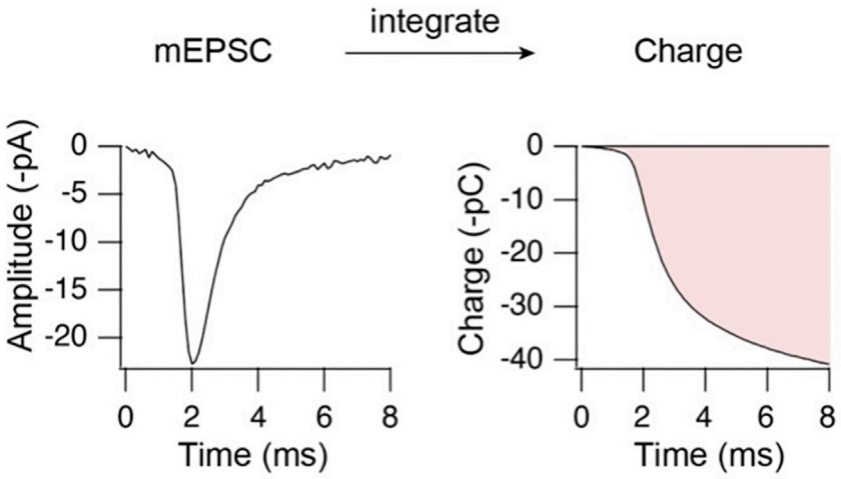


Figure 5. Analysis of the charge of mEPSCs

All the mini events from one 15 s trace are averaged to produce an average mEPSC, the charge of which is calculated by the integration function in Igor. The charge of the average mEPSC reflects the neurotransmitter content of a single vesicle.

19. Substitution Process: Preparing chambers for samples
    a. Place a disposable ring-insert tube for each frozen strain into the flow-through ring. Each ring holds 10 individual inserts.
    b. Cut a notch into one ring-insert tube to indicate where the first strain will be placed. Samples should be placed sequentially into respective insert tubes in a clockwise manner.
    c. Place the ring containing the solution ring-inserts into a universal chamber. A diagram made for each filled chamber is important to track strain placement.
    d. An apparatus specified for use as an unloading station (Figure 7) should be cooled and filled with liquid nitrogen. Continue to top off liquid nitrogen throughout the transfer of samples.

20. Preparing a 0.1% tannic acid solution for substitution
    a. Measure .05 g tannic acid (TA).

    Note: Static can cause TA to move through the air, use caution when weighing.
    b. Add the TA to a 50 mL conical tube.
    c. Add 50 mL of acetone (EMS – 100% glass distilled, for EM) to the tube. This does not have to be exact but do not overfill.
    d. Invert the tube until the TA is dissolved.

21. Transferring samples from cryotubes to tannic acid chamber

    Note: Have a dewar or container of liquid nitrogen on hand in order to ensure samples are constantly submerged during transfer process.
    a. Fill the universal chamber containing the ring with the tannic acid solution using a plastic pipette to add the solution to the center of the ring for even distribution.
    b. Fill to ~50% of the insert tube height. The solution will expand when frozen.
    c. Place the universal chamber into the liquid nitrogen filled unloading station.
    d. The TA solution will freeze, and will likely make a crackling sound.
    e. Remove the cryocane containing the first sample cryotube from the transfer dewar and place it in the unloading station so that cryotubes are submerged in the liquid nitrogen.
    f. Precool all forceps tips in the liquid nitrogen

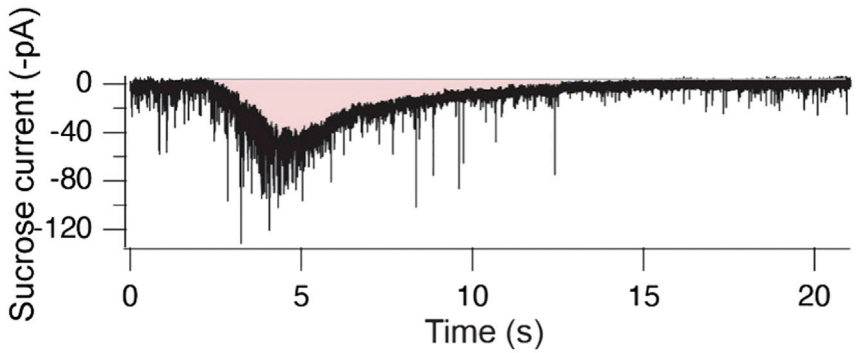


Figure 6. Calculating the total charge of the sucrose-evoked current

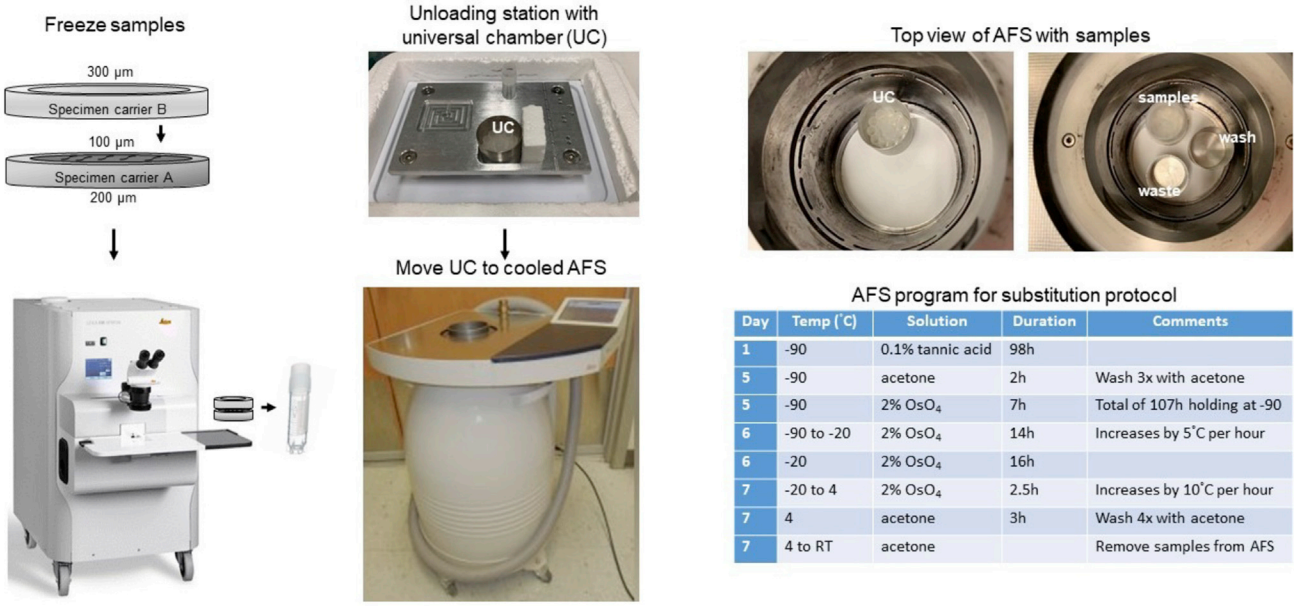
g. Remove the cryotube with the first strain from the cryocane and place the specimen carriers from the cryotube into the first notched insert tube of the universal chamber. Be extremely careful to keep the carriers submerged in liquid nitrogen throughout the process. The movement of the carrier from the unloading station to the solution insert should be very quick.
h. Repeat steps f-g for each strain placing the carriers in each sequential l insert tube in a clockwise manner.
i. Place the universal chamber into the AFS, cover with a disposable lid and restart the substitution protocol. Note the time the protocol is started.
j. After 1 h, check to ensure the samples are submerged in the TA. Residual TA and liquid nitrogen can be evaporated in a fume hood.
k. Before leaving check liquid nitrogen levels in the AFS and fill if necessary.

22. Washing TA and exchanging for OsO_4
The first wash will be done 98 h after starting the AFS protocol.
Prior to the wash:
a. Precool a universal chamber filled with acetone and another universal chamber for waste liquids in the AFS.
b. Set up waste and wash plastic pipettes
c. Cool acetone for osmium tetroxide (either −20°C or 4°C is acceptable)

23. Beginning at 98 h into the substitution protocol is a series of washes:
a. Precool pipettes by first holding them in the chamber and then pipetting cold acetone in and out of them. Do NOT bring acetone into the pipette above the level of the AFS chamber.
b. Pipette waste out from external region of the specimen containing flow-through ring while pipetting fresh acetone into center hole of the flow-through ring.
c. Use the waste pipette to rotate the ring to help distribute the solution.
d. Repeat this process 3× until acetone covers ~60% of inserts. This completes one wash.
e. Perform two sequential washes over a 1-h time period following steps a-d. Prepare the OsO_4 solution after the 1-h wash period.

Note: OsO_4 is toxic, take proper safety precautions when preparing the solution (at minimum, wear a lab coat and gloves and work in a fume hood).

24. Prepare 2% osmium tetroxide
a. Set up conical tubes in a fume hood.
i. A 50 mL conical tube should contain vegetable oil or Vitamin C used to deactivate OsO_4 waste.
ii. A 50 mL conical tube should contain 5 mL cold acetone.



AFS program for substitution protocol

Day	Temp (°C)	Solution	Duration	Comments
1	-90	0.1% tannic acid	98h	
5	-90	acetone	2h	Wash 3x with acetone
5	-90	2% OsO_4	7h	Total of 107h holding at -90
6	-90 to -20	2% OsO_4	14h	Increases by 5°C per hour
6	-20	2% OsO_4	16h	
7	-20 to 4	2% OsO_4	2.5h	Increases by 10°C per hour
7	4	acetone	3h	Wash 4x with acetone
7	4 to RT	acetone		Remove samples from AFS

Figure 7. Freezing and substitution

b. Set up a plastic bag for solid waste.
c. Clean OsO_4 ampoule with acetone.
d. Cover with tissue or plastic breaker to break ampoule top, if top is empty discard into waste tube (if not empty follow steps for crystal containing ampoule).
e. Using forceps place the ampoule with OsO_4 crystals into the acetone. Pipette acetone in and out of ampoule until crystals dissolve.
f. Transfer OsO_4 solution into a universal chamber.
g. Place OsO_4 containing chamber into AFS to reach −90°C and cover.
h. Deactivate residual OsO_4 solution with either vegetable oil or ascorbic acid and place into the waste conical tube for later disposal. Also clean forceps with vegetable oil or ascorbic acid followed by acetone.

25. Exchange acetone for OsO_4
    a. Two hours after the washing period began, use the washing method described above to exchange the acetone for OsO_4. Cover the samples with a disposable lid.
    b. Discard of liquid waste in vegetable oil or ascorbic acid and use one of the oil to deactivate any solid waste and discard in the waste bag.
    c. Wash the universal chamber used for OsO_4 first with vegetable oil or ascorbic acid and then acetone.
    d. The samples are left in OsO_4 for 39.5 h as the temperature is gradually raised.

26. Acetone wash
    Once samples have been in OsO_4 for 39.5 h and the temperature has come up to 4°C the OsO_4 should be washed out.
    a. 30 min prior to the first wash put 4°C stored acetone into a universal chamber and place in the AFS.
    b. Wash 2× with cooled acetone following the wash protocol listed above.
    c. After 30 min do a 3rd wash.
    d. Prepare an embedding resin according to directions, shake or rotate the bottle for ~30 min to mix.
    e. Make 50% resin by filling a 50 mL conical tube with 10 mL cold acetone and adding 10 mL resin.

f. Fill a universal chamber with the mixture and store at 4°C or in the AFS.
g. After 1 h has passed from the 3rd wash, do a 4th and final wash.

27. Acetone to 50% resin
    a. One hour after the final acetone wash remove the samples from the AFS to room temperature.
    b. Exchange the acetone for the 1:1 acetone:resin mixture.
    c. Let this sit for 4 h at RT.
28. Transfer samples to polypropylene capsules with 100% resin
    a. Number a polypropylene capsule disc for each strain.
    b. Place a small drop of resin into the capsule.
    c. Using a pair of forceps to stabilize the flow through ring and another pair to grasp the insert tube, remove the tube with your palm facing up and invert to transfer the carrier into the resin.
    d. Be sure the samples are fully submerged in the resin, add more resin if needed.
    e. Leave samples in resin for 18 h or overnight in a 4°C refrigerator.
29. Sample Isolation and Thin-embedding Process
    a. The morning that embedding begins, slides and embedding dishes need to be prepared and assembled. Additionally, put resin out to warm to RT.
30. Top Slide Preparation
    a. Prepare top slides for thin-embedding by laying out 1 slide per strain, plus extras, on paper towels.
    b. Heavily coat one side of each slide with Teflon spray.
    c. Let dry for 5–10 min.
    d. Repeat for 3–4 coats so that one side of the slide is completely covered in Teflon.
31. Embedding Dish Preparation
    a. Cut thin strips of parafilm and make small stacks 3 strips deep, two stacks per strain.
    b. Set out one 100 mm petri dish per strain. Number the dishes.
    c. Place two toothpicks parallel in the bottom of the dish.
    d. Label a slide for each strain. Place the slide on top of the toothpicks.
    e. Place two stacks of parafilm on either end of the slide. These determine the thickness of the embedding resin,

32. Isolation and Embedding
    a. Use a microscope with a light source underneath and set up a top light.
    b. Place the polypropylene capsule containing the resin-infused samples into a working petri dish.
    c. Use fine syringe needles (30 gauge) to gently pick apart the bacteria cryoprotectant to remove the worms from the bacteria. Two needles are helpful as one can be used to stabilize the whole sample while the other is used to gently separate the worms from the bacteria.

Note: Separation of the worms from the bacteria is difficult as the osmium tetroxide causes both the bacteria and the worms to turn dark brown or black (Figure 8). Be extremely careful to not damage the worms in this process.

    i. Start from the middle of the sample as worms are frequently located near the edges of the carrier.
    ii. Wipe off needles or use new ones between strains.
    d. Place a small drop of resin onto the labeled slide (~0.5–1 cm diameter).
    e. Transfer separated worms to the resin, taking care to space them out so they can be individually cut out later with a razor blade.
    f. Angle a Teflon-coated slide (be sure Teflon is facing down!) slightly offset from the bottom slide and cover.
    g. Place petri dishes in a 60°C incubator for 2 days.

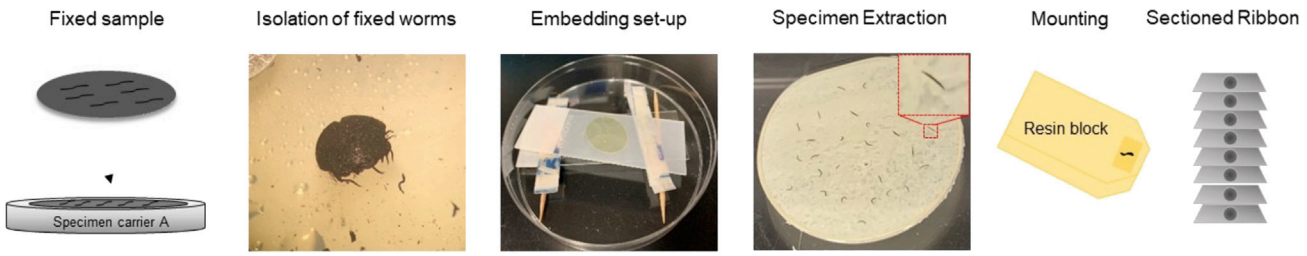


Figure 8. Isolation, embedding and sectioning

h. Use remaining resin to fill block molds to be used for mounting worms for sectioning. Cure these blocks in the 60°C incubator for 2 days.

33. Preparing the Sample for Sectioning

Remove the Teflon top slide and select a worm to be mounted from your embedded samples.

a. Using a fresh razor blade, cut out a piece of resin containing the worm of interest being careful to leave resin all around the worm to prevent accidental cutting, breaking or damaging the worm.
b. Use sandpaper to rough the mounting end of the flat side of a resin block.
c. Apply a small drop of super glue and place the resin encased worm, with the worm facing down onto the block and quickly orient the worm head toward the front of the block for ease of access when sectioning.
d. Hold the worm in place in the super glue until it has begun to stick.
e. Cover the entire piece of resin containing the worm with super glue.
f. Allow the glue to dry overnight before preparing for sectioning.

34. Trimming

a. Replace the trimming/sectioning knife holder stage with the chuck holder stage on the ultramicrotome.
b. Place the worm block into the chuck and firmly tighten, being careful to ensure the block is secure but do not tighten to the point of cracking the block. Place the chuck into the chuck holder and tighten to secure the chuck.
c. Using a fresh razor blade, angled ~45°, trim away excess resin from the block underneath the embedded worm using a downward motion. Take care to avoid cutting too close to the worm to prevent damage. This initial rough trim will eliminate wear of the trim tool as well as reduce the time spent on achieving the trapezoid face shape needed to create a ribbon when sectioning.
d. Remove the chuck from the stage and place it into the specimen holder on the ultramicrotome.
e. Adjust the angle of the block so that the area of interest of the worm will be perpendicular to the sectioning knife.
f. Rotate the chuck to 90° and trim the bottom and top of the block with the edge of the trim tool.
g. Trim the face using the top of the trim tool.
h. Rotate the chuck back to 0° and return the angle of the chuck to 0°.
i. Move the stage to the angle that corresponds with the angle used to trim the bottom, top and face of the block.
j. Rotate the chuck 5° to the left and trim the left side of the block using the right edge of the trim tool.
k. Rotate the chuck 5° to the right and trim the right side of the block using the left edge of the trim tool.

l. Repeat these trim steps until the block is a trapezoid shape with the top and bottom flush with the worm. The block should be wide enough to allow for the width of ~$1^1/_2$ - 2 worm diameters on either side of the sample. The block face should be angled perpendicular to the area of interest. Example: To examine the ventral nerve cord anterior to the vulva trim away 250 μm from the tip of the nose in 1 μm steps to reach the area of interest for collecting sections, ensuring the angle of the face of the block is exactly flush with the angle of the worm at the area of interest.
m. After reaching the area of interest and achieving the trapezoid face shape be sure the face of the block is free of any imperfections or debris. The face should be shiny and reflective. If the face is not perfect trim off a couple more sections until it is shiny.

35. Alignment and Sectioning
    a. Replace the trim tool with the diamond sectioning knife.

    Note: Diamond knives are easily damaged and expensive to sharpen so this step requires prior training and practice.
    b. Turn off the top light and use the reflection of the knife on the block face to align the knife to the exact angle of the block. Use 0.3–0.5 μm steps to check the distance of the knife from the left and right sides of the block face, and the top then bottom and make small adjustments to the block angle to match top and bottom distances and change the angle of the knife to make left/right adjustments until it takes the same number of steps on both sides to reach the block face. You can determine this by monitoring the reflection of the knife on the face of the block, when the reflection goes black, the knife is near the block face.
    c. When the block and knife are perfectly aligned, step the block back slightly and set the automated cutting parameters on the ultramicrotome using the reflection on the bottom of the block as the starting point and on the top as the end point.
    d. Orient the specimen arm so that the block is below the knife to start. Turn the top light on again.
    e. Slightly overfill the boat with ddH_2O so that a dome of water is just above the boat and water is covering the blade.
    f. Allow the blade to soak up water for between 10–15 min.
    g. When the blade is wet and with the block still positioned below the knife, adjust the water level until the water surface is reflective, appears silver and is level with the edge of the knife blade.
    h. Raise the block to be slightly above the knife blade.
    i. Start the automated movement of the ultramicrotome specimen arm. If the alignment is accurate a ribbon of sections should form. If a ribbon does not begin to form, remove water from the boat and repeat alignment steps.
    j. Stop the automated movement after ~25–30 sections and using an eye lash brush gently move the ribbon of sections away from the blade.
    k. Carefully collect the sections on a grid.
    l. Repeat until the desired number of sections is collected.

    Note: Clean the diamond knife according to instructions. Never leave residual resin or sample on the blade!

36. Post-staining with Uranyl Acetate and Lead Citrate
    a. Set up a staining pad with 1 drop of 2% uranyl acetate per grid and a series of drops of CO_2-free ddH_2O for washing. CO_2-free ddH_2O can be purchased or ddH_2O can be boiled and capped immediately. A fresh bottle should be used for staining.
    b. Place the grids on the Uranyl Acetate for 4 min.
    c. Use a perfect loop to move the grids through the series of washes.

d. Remove the grids and use lens paper cut into triangles to wick away any excess water or allow the grids to dry before moving to the next staining.
e. Set up a petri dish containing KOH pellets to create a CO_2-free chamber and place the staining pad on top.
f. Set up the staining pad with 1 drop of Lead Citrate and a series of drops of CO_2-free ddH_2O for washing. (Lead Citrate will need to be made beforehand using CO_2-free ddH_2O. Alternatively, EMS now offers a CO_2-free lead citrate.)
g. Place each grid on Lead Citrate for 2 min.
h. Use a perfect loop to move the grids through the series of washes.
i. Remove the grids and use lens paper cut into triangles to wick any excess water or allow the grids to dry before being placed in the grid storage box.

37. Imaging.

Note: High-pressure freeze, freeze substitution and imaging techniques described in this protocol were performed to acquire serial 40 nm sections for transmission electron microscopy used to study synaptic ultrastructure in the referenced paper. This is one of many possible applications resulting from the use of this protocol (Rostaing et al., 2004; Weimer et al., 2006).

a. Acquire images using a JEOL JEM-1220 transmission electron microscope operated at 80 kV and a side-mounted Gatan digital camera at a magnification of 100 k (1.8587 pixels/nm).
b. Collect images from the ventral nerve cord in the region anterior to the vulva.

38. Analysis

Note: Analysis described in the referenced paper was performed to study synaptic ultrastructure. This is merely an example of an analysis application resulting from the described high-pressure freeze and freeze substitution protocol (Rostaing et al., 2004; Weimer et al., 2006).

a. Perform analysis using the NIH ImageJ software package.
b. Annotate regions of interest and measure areas or diameters based on pixels/nm of acquired images. For the referenced study, cholinergic synapses were identified on the basis of their typical morphology (White et al., 1986).
c. Define a synapse as a series of sections (profiles) containing a dense projection as well as two flanking sections on either side without dense projections.
d. Identify synaptic vesicles as spherical, light gray structures with an average diameter of ~30 nm.
e. Identify dense core vesicles as spherical, dark black structures with thick membranes and an average diameter of ~60–70 nm.
f. Measure distances of docked synaptic vesicles to dense projections (DP) from closest membrane edge of vesicle to closest edge of DP (vesicles are considered docked when distance to plasma membrane is 0 nm). Raw data is statistically analyzed and graphed in Prism 9 software. One-way ANOVA followed by Tukey's multiple comparisons test are used to determine statistical significance.

EXPECTED OUTCOMES

The quality of the electrophysiology data is directly determined by the quality of the preparation. A good dissection allows to acquire high quality of mEPSC, mIPSC, and evoked EPSC (Figure 3) (Li et al., 2019), or sucrose- or drug-activated currents (Figure 3) (Li et al., 2021). For wild-type worms, the mEPSC and mIPSC frequencies vary from 20 Hz–80 Hz, with an average of around 45 Hz. The amplitudes of the evoked EPSCs vary from 1.5 nA–3 nA, with an average of around 2 nA. Please note that all recordings are performed at room temperature (22°C). For temperature sensitive experiments, a temperature control chamber should be used.

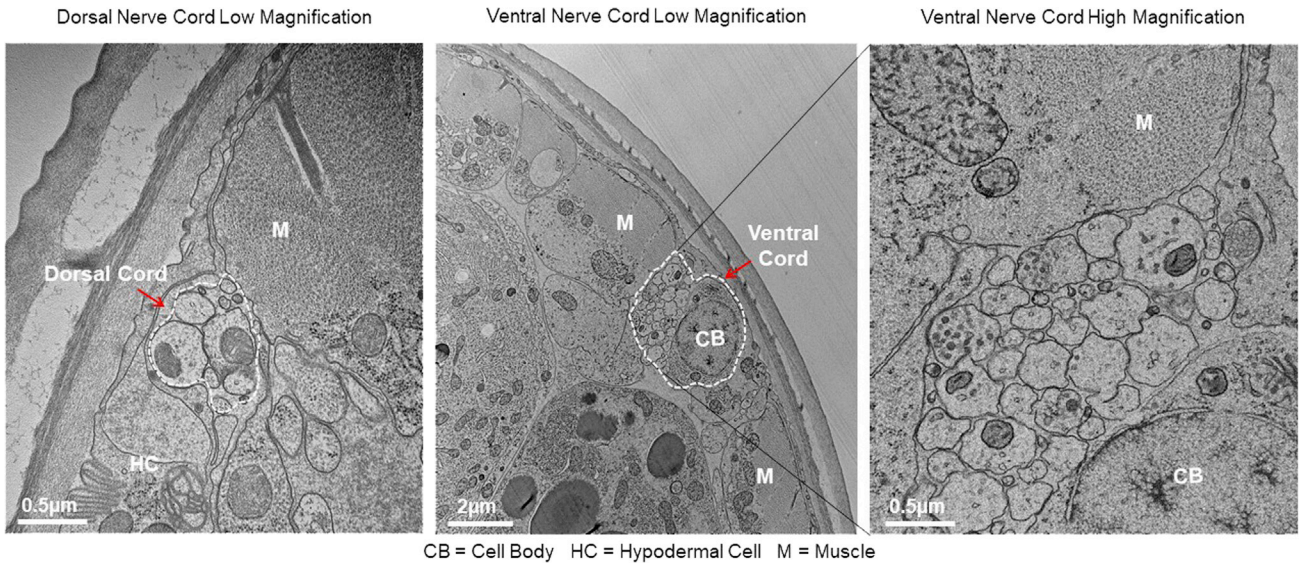


Figure 9. Expected imaging outcomes

The quality of electron micrographs and subsequent data analyses is dependent on the quality of the freeze and substitution process. A successful preparation should yield dark worms that have retained their shape and ultrastructural morphology (Figures 9 and 10). Though the protocol (described in steps 37–38 and shown in Figures 9 and 10) was utilized for the ultrastructural analysis of neuromuscular synapses, the preparation allows for the analysis of any regions of interest, step 38 (shown in Figure 10) merely provides an example of the type of analyses conducted in the original referenced study.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were statistically analyzed in Prism 9 software. Normality distribution of the data was determined by the D'Agostino-Pearson normality test. When the data followed a normal distribution, an unpaired student's t-test (two-tailed) or one-way ANOVA was used to evaluate the statistical significance. In other cases, a Mann-Whitney test or one-way ANOVA following Kruskal-Wallis test was used. All electrophysiology and EM data are presented as mean ± SEM (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).

LIMITATIONS

The electric stimulus-evoked IPSC cannot be obtained using the current protocol. One possibility is that the processes for the GABAergic motor neurons are relatively shorter than the cholinergic motor neurons. The gigaohm seal is damaged by the stimulus pipette if it is too close to the patch pipette.

TROUBLESHOOTING

Problem 1

It is difficult to cut the cuticle, and the worm separates from the glue during the dissection (step 5).

Potential solution

It is recommended to make 10–15 holes along the dorsal cord by penetrate the sharp end of the cutting pipette into the cuticle. This takes longer time but makes the cutting easier.

Problem 2

It is hard to form the gigaohm seal (step 9).

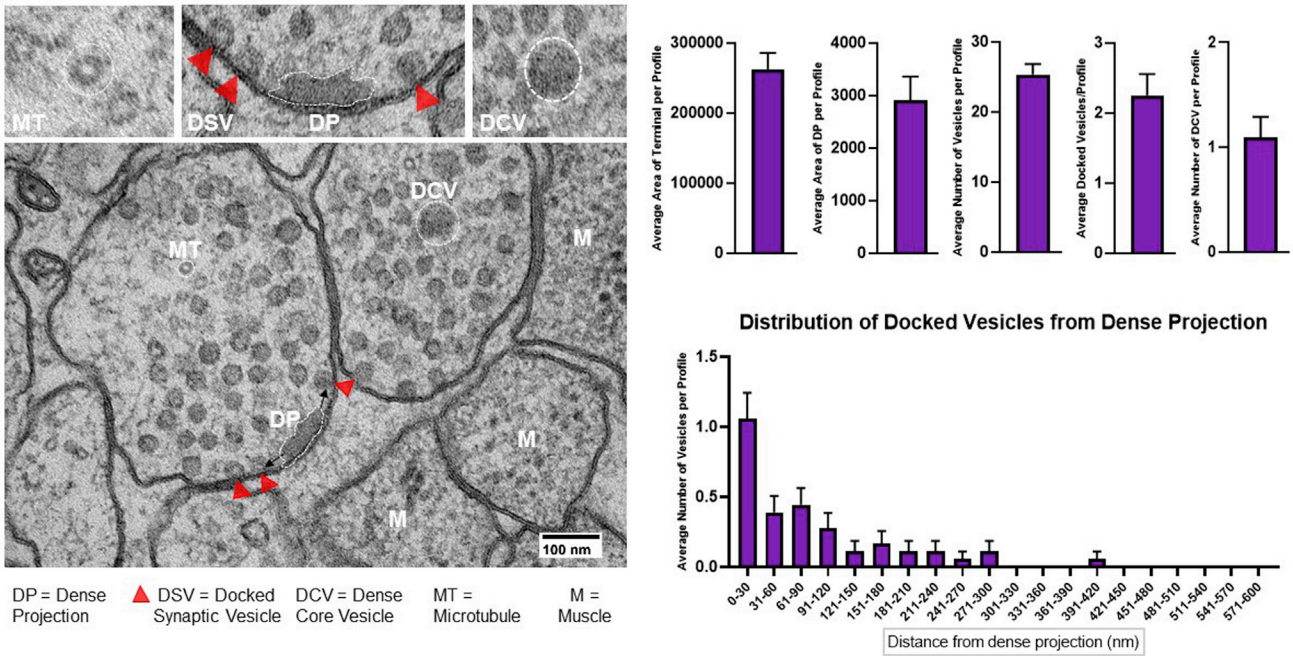


Figure 10. Morphological analysis

Potential solution

First check the osmolarity of both the internal (~315 osm) and external solution (~330 osm). The difference between these two solutions is key for gigaohm seal. Next consider using a new stock of collagenase.

Problem 3

Precipitate formation on grids when staining (step 36).

Potential solution

Precipitate can form on grids when post-staining with Lead Citrate. Make sure the Lead Citrate solution and ddH_2O are CO_2-free. It is important to keep from breathing on the samples while staining. Be sure to keep the grids covered when possible throughout staining and washes.

Problem 4

Poor sample and/or image quality (step 37).

Potential solution

Image quality may be poor if the worm sample or sections are damaged. Damage may be due to a poor freeze or substitution process. Quality of the freeze may vary among and between strains. Check another worm from the same strain, if quality of a second worm is poor check a worm from another strain. In some rare cases a freeze and substation process will need to be repeated.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhitao Hu (z.hu1@uq.edu.au).

Materials availability

This study did not generate unique materials or reagents.

Data and code availability

This study did not generate unique datasets or codes.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.L., L.L., M.K., S.S., and Q.Z.; investigation, H.L., L.L., M.K., S.S., and Q.Z.; writing – original draft, Z.H., J.E.R., and M.K.; writing – review & editing, Z.H., J.E.R., H.L., M.K., and J.C.; funding acquisition and supervision, Z.H. and J.E.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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