Primary culture of *Caenorhabditis elegans* developing embryo cells for electrophysiological, cell biological and molecular studies

Kevin Strange, Michael Christensen & Rebecca Morrison

Departments of Anesthesiology, Molecular Physiology and Biophysics, and Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232, USA. Correspondence should be addressed to K.S. (kevin.strange@vanderbilt.edu).

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Cell culture is an invaluable tool for investigation of basic biological processes. However, technical hurdles including low cell yield, poor cell differentiation and poor attachment to the growth substrate have limited the use of this tool for studies of the genetic model organism *Caenorhabditis elegans*. This protocol describes a method for the large-scale culture of *C. elegans* embryo cells. We also describe methods for *in vitro* RNA interference, fluorescence-activated cell sorting of embryo cells and imaging of cultured cells for patch-clamp electrophysiology studies. Developing embryos are isolated from gravid adult worms. After eggshell removal by enzymatic digestion, embryo cells are dissociated and plated onto glass substrates. Isolated cells terminally differentiate within 24 h. Analysis of gene expression patterns and cell-type frequency suggests that *in vitro* embryo cell cultures recapitulate the developmental characteristics of L1 larvae. Cultured embryo cells are well suited for physiological analysis as well as molecular and cell biological studies. The embryo cell isolation protocol can be completed in 5–6 h.

INTRODUCTION

Genetic model organisms provide a number of powerful experimental advantages for defining the genes and genetic pathways involved in biological processes. The nematode *C. elegans* is a particularly attractive model system[1,2]. *C. elegans* is well suited for mutagenesis and forward genetic analysis and has a fully sequenced and well-annotated genome. Gene expression in nematodes is relatively easy and economical to manipulate using RNA interference (RNAi), knockout and transgenesis. Genomic sequence and many other biological data on this organism are assembled in readily accessible public databases and numerous reagents including mutant worm strains and cosmid and yeast artificial chromosome clones spanning the genome are freely available through public resources.

Despite these many experimental advantages, the small size of *C. elegans* and most of its somatic cells, and the presence of a tough, pressurized cuticle surrounding the animal have limited access for cell-specific physiological and molecular studies. A *C. elegans* cell culture system would provide direct access to individual cell types for functional and molecular analyses. Early attempts at large-scale culture of *C. elegans* embryonic cells were described by Bloom[3] and the feasibility of culturing differentiated worm neurons was demonstrated. However, Bloom noted significant problems with cell survival, attachment of cells to the growth substrate, cell differentiation and reproducibility of the methods. Initial attempts to patch-clamp cultured cells were unsuccessful. Buechner *et al.*[4] also reported that cultured *C. elegans* embryonic cells undergo morphological differentiation resembling neurons and muscle cells.

Bloom's studies led to the widely held belief in the field that *C. elegans* cells could not be cultured reliably *in vitro*. However, we have recently developed a robust approach for the large-scale culture of cells isolated from developing worm embryos[5,6]. Analysis of gene expression patterns and cell-type frequency suggests that *in vitro* embryo cell cultures recapitulate the developmental characteristics of L1 larvae. The protocol described here provides step-by-step details for culturing worms, isolating eggs and removing eggshells, dissociating embryos into single cells, placing embryo cells in culture and *in vitro* RNAi methods. We also describe fluorescence-activated cell sorting (FACS) methods that allow enrichment of specific cell types and video-enhanced microscopy methods required for electrophysiological analyses of cultured cells. Limitations of the method are the same as those of any other primary cell culture technique and include the loss of critical cell–cell interactions and factors that may be required for proper cell development and differentiation. Cultured *C. elegans* embryo cells are, however, suitable for a wide range of cell physiological and cell biological applications including studies of signal transduction, ion channel physiology and gene expression.

MATERIALS

REAGENTS

- Bacto Agar (Becton, Dickinson and Co., catalog no. 214010)
- Bacto Peptone (Becton, Dickinson and Co., catalog no. 211677)
- Bacto Tryptone (Becton, Dickinson and Co., catalog no. 211705)
- Bacto Yeast Extract (Becton, Dickinson and Co., catalog no. 212750)
- Cholesterol (Sigma Chemical Co., catalog no. C3045)
- NA22 *Escherichia coli* (*Caenorhabditis* Genetics Center)
- Chlorox bleach
- Chitinase (Sigma, catalog no. C6137 or C7809)
- L-15 cell culture medium (Invitrogen, catalog no. 21083-027)
- Heat-inactivated fetal bovine serum (Invitrogen, catalog no. 10082-139)
- Penicillin-streptomycin (Invitrogen, catalog no. 15140-122)
- Peanut lectin (Sigma, catalog no. L0881)
- Sucrose
- NaCl
- KCl
- $CaCl_2$
- $MgCl_2$
- HEPES
- NaOH
- $MgSO_4$
- KH_2PO_4

- Ethanol
- Milli-Q or equivalent water
- Poly-L-lysine (Sigma, catalog no. P1274)
- Propidium iodide (Molecular Probes, catalog no. P-1304)
- Commercially available fluorescent beads for FACS machine calibration (BD Biosciences and Duke Scientific Corporation)
- Concentrated nitric acid

EQUIPMENT

- Microcentrifuge (e.g., Eppendorf centrifuge 5415R)
- Osmometer (e.g., Wescore Vapro vapor pressure osmometer 5520)
- Tabletop centrifuge with swinging bucket rotor (e.g., Thermo IEC Centra-CL2)
- Laminar flow hood
- Inverted microscope with ×10 and ×20 objectives
- Hemocytometer
- Cell culture incubator (optional)
- 1–200 µl siliconized pipette tips (VWR International Inc., catalog no. 53503-794)
- Sterile 18 gauge needles (Becton Dickinson and Co., catalog no. 305196)
- Sterile 3 ml syringes (Becton Dickinson and Co., catalog no. 309585)
- 5.0 µm Durapore filters (Millipore Corporation, catalog no. SLSV025LS)
- Mat Tek dishes (35 mm diameter sterile plastic Petri dishes with a no. 0 15 mm diameter glass coverslip glued onto the bottom; Mat Tek Corp., model no. P35G-0-14-C)
- 12 mm diameter glass coverslips (Fisher Scientific, catalog no. 12-545-80)
- 3.5″ × 8″ sterilization pouches (VWR International, catalog no. 11213-237)
- Nunc four-well culture dishes (VWR International, catalog no. 62407-068)
- Lab-Tek chamber slides (Nalge Nunc International)
- Model R-26G bath chamber (Warner Instrument Corp.)

REAGENT SETUP

2× YT medium Dissolve 16 g Bacto Tryptone, 10 g Bacto Yeast Extract and 5 g NaCl in 1 liter of Milli-Q water. Adjust pH to 7.0 with NaOH and autoclave. Medium can be stored for several weeks at room temperature (21–22 °C).

Nematode growth medium agar plates Dissolve 3 g NaCl, 2.5 g Bacto Peptone and 17 g Bacto Agar in 1 liter of Milli-Q water and autoclave. After cooling to 55 °C, under sterile conditions add the following solutions in the given order, while swirling: 1 ml of cholesterol solution (5 mg cholesterol per ml ethanol), 1 ml of 1 M $CaCl_2$ solution, 1 ml of 1 M $MgSO_4$ solution and 25 ml of 1 M KH_2PO_4 (pH 6) solution. Pour liquid agar medium into 10 cm Petri dishes. ▲ CRITICAL $CaCl_2$, $MgSO_4$ and KH_2PO_4 stock solutions should be sterile-filtered before use.

Enriched peptone agar plates Dissolve 1.2 g NaCl, 20 g Bacto Peptone and 25 g Bacto Agar in 1 liter of Milli-Q water and autoclave. After cooling to 55 °C, under sterile conditions add the following solutions in the given order, while swirling: 1 ml of cholesterol solution (5 mg cholesterol per ml ethanol), 1 ml of 1 M $MgSO_4$ solution and 25 ml of 1 M KH_2PO_4 (pH 6) solution. Pour liquid agar medium into 10 cm Petri dishes.

Egg isolation solution Mix together 1 ml fresh Chlorox bleach, 0.25 ml 10 N NaOH and 3.75 ml sterile H_2O. ▲ CRITICAL This solution must be made fresh before each use. If you store bleach in smaller volumes on the bench, the container should be light tight. Replace bleach stored in smaller containers at least once a week.

Egg buffer Contains 118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES, pH 7.3 and should have an osmolality of 340 mOsm. ▲ CRITICAL The osmolality of egg buffer should be measured with an osmometer and must be 340 ± 5 mOsm for cell viability.

Chitinase solution Contains 1 U chitinase per ml sterile egg buffer. ▲ CRITICAL Sigma chitinase no. C7809 is more expensive, but works faster than chitinase no. C6137. ▲ CRITICAL Prepare chitinase solution under aseptic conditions in ice-cold egg buffer. Store aliquots at −80 °C and thaw immediately before use.

Cell culture medium L-15 cell culture medium without phenol red, containing 10% (v/v) heat-inactivated fetal bovine serum, 50 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin. ▲ CRITICAL The osmolality of L-15 culture medium varies from lot to lot. It is therefore important to measure the osmolality of each bottle of medium and adjust to 340 ± 5 mOsm by addition of an appropriate amount of sucrose. Sterile-filter the medium after osmolality is adjusted.

Peanut lectin solution Dissolve peanut lectin in its original vial. Add sterile water to fill the vial and store it overnight at 4 °C. Transfer this material aseptically to a sterile 50 ml tube and rinse the vial with additional measured volumes of sterile water to ensure full recovery of the lectin and a final peanut lectin concentration of 0.5 mg ml^{-1}. Mix well and transfer 1 ml aliquots into sterile Eppendorf tubes. Store aliquots at 4 °C. The aliquots remain usable for many months. ▲ CRITICAL Stored aliquots will occasionally contain small amounts of insoluble lectin. Leave this material in the bottom of the tube when coating culture vessels. Attempting to redissolve this material promotes cell clumping. ▲ CRITICAL Peanut lectin solutions cannot be filter-sterilized or autoclaved. Sterilize small bottles of peanut lectin using gamma irradiation if possible. The dosage and time of exposure vary with the gamma source. Discuss the proper sterilization procedure with the person who oversees your institution's gamma irradiation facility. Alternatively, lectin-coated culture vessels can be UV-irradiated in a laminar flow hood for several hours to overnight. The actual length of irradiation time will depend on the age of the UV lamp.

Poly-L-lysine solution Prepare poly-L-lysine in sterile water to a final concentration of 40 µg ml^{-1} and sterilize by sterile filtration.

EQUIPMENT SETUP

Acid-washing glass coverslips Glass coverslips must be acid-washed prior to coating with lectin or poly-L-lysine. Acid-washing removes manufacturing residues, dirt and/or fingerprints and is necessary to maximize cell attachment, differentiation and survival. In a fume hood, drop 100–200 coverslips individually into 50 ml of concentrated nitric acid in a 100 ml beaker and allow them to soak overnight. Decant the nitric acid into an appropriate waste container. Wash the coverslips in the beaker several times to remove the majority of the nitric acid. To ensure complete removal of the acid, remove single coverslips with forceps and dip them into a series of three beakers containing 400–500 ml of Milli-Q water. Lay coverslips on clean Kim Wipes to dry. When dry, place coverslips into sterilization pouches and autoclave. Store opened pouches under sterile conditions. ! CAUTION Use all standard laboratory safety procedures when handling and disposing of concentrated nitric acid. ▲ CRITICAL It is essential to remove completely all nitric acid from the coverslips. Residual acid will harm or kill cells.

Coating culture vessels with peanut lectin In a laminar flow hood, aseptically pipette a small volume of the peanut lectin solution onto the glass substrate so that it covers about 80% of the surface. Incubate the substrate with the lectin solution for 10–20 min and then remove completely by aspiration. Discard the lectin solution or, alternatively, you can pipette the solution onto another growth substrate to conserve the lectin. If the peanut lectin was not sterilized by gamma irradiation, the lectin-coated growth substrates should be sterilized by UV irradiation. Peanut lectin-coated culture vessels and coverslips can be stored for weeks under sterile conditions. ▲ CRITICAL It is essential to completely remove the lectin solution. Excess lectin on the growth substrate causes cell clumping. Make sure to remove any lectin solution that may have crept underneath glass coverslips.

Coating culture vessels with poly-L-lysine for FACS experiments Cover the glass growth substrate with poly-L-lysine solution for 1 h at room temperature. Aspirate the solution, wash three times with sterile water and air-dry. ▲ CRITICAL Excess poly-L-lysine can be toxic to cells. It is therefore important to aspirate completely the poly-L-lysine solution from the culture vessel and to thoroughly wash the vessel after coating.

Preparation of enriched peptone agar plates seeded with NA22 *E. coli* When seeded onto enriched peptone agar plates, these bacteria form a thick lawn that will support the growth of large numbers of gravid adult worms required for the preparation of isolated embryo cells; spread each enriched peptone agar plate with 0.4 ml of NA22 *E. coli* cultured overnight (12–16 h) in 2× YT medium at 37 °C with shaking at 250 r.p.m. After the liquid has absorbed into the agar, invert the plate and then incubate overnight at 37 °C.

PROCEDURE

Synchronization of worm cultures ● TIMING approximately 45–60 min

1| It is essential to synchronize worm cultures in order to obtain large numbers of gravid adult animals for subsequent embryo cell isolation and culture. To synchronize cultures, isolate eggs as described below (Steps 8–17) and resuspend in 100–200 µl of sterile water and seed onto a nematode growth medium agar plate without bacteria.

TABLE 1 | Time required for growing N2 wild-type L1 larvae at various temperatures to the gravid adult stage.

Temperature (°C)	Incubation time (h)
24	48
20	64
16	96

2| Allow the eggs to hatch by incubating the plate at 16–24 °C for 12–16 h. Without food, worms will arrest at the L1 larval stage.
▲ CRITICAL STEP Do not incubate wild-type worms for longer than 16 h. With longer incubations, worms burrow into the agar, which reduces the final yield.

3| After hatching, rinse the L1 larvae off the plate into a 15 ml conical tube with sterile water.

4| Pellet the worms by centrifugation at ~350*g* for 3 min at room temperature.

5| Remove the liquid and repeat the wash and pellet step a second time to eliminate dauer pheromone that may have accumulated during the starvation period. Using first a plastic transfer pipette and finishing with a 100 µl pipettor, remove as much of the final wash liquid as possible to create a dense pellet of larvae.

6| Using a siliconized pipette tip, pipette 8 µl of larvae onto each 10 cm enriched peptone agar plate seeded with NA22 *E. coli*.

7| Grow larvae into young gravid adults. The time to reach this end point varies with temperature, worm strain, etc. For N2 worms, the incubation temperatures and times listed in **Table 1** work well.

Egg isolation ● TIMING **approximately 45 min**

8| Wash adult worms off agar growth plates with water into a 15 ml conical tube and pellet by centrifugation at ~350*g* for 3 min in a swinging bucket rotor in a tabletop centrifuge at room temperature.

9| Wash pelleted worms with water 1–3× until the supernatant is clear of bacteria. Recentrifuge the worms as described in Step 8 after each wash. If you are using multiple plates of worms, the washing and lysis steps will be more efficient if you also use multiple 15 ml tubes.

10| After the last wash, lyse the worm pellet(s) by adding to each 15 ml conical tube 5 ml of freshly prepared egg isolation solution. Rock worms gently by hand during the lysis. The progress of the lysis reaction is monitored with a dissecting microscope by viewing 5–10 µl of the worm suspension pipetted onto a glass slide. The lysis reaction should be stopped when ~50% of the worms are lysed.
▲ CRITICAL STEP Lysis time is critical. Under no conditions should the lysis reaction exceed 5 min.

11| Stop the lysis reaction by filling the tube with egg buffer and immediately pellet the eggs and lysed worms by centrifugation at ~350*g* for 3 min at room temperature.

12| Remove the supernatant using a sterile plastic transfer pipette and then wash 3× with 10–12 ml of egg buffer. Repellet the lysed material after each wash by centrifuging at ~350*g* for 3 min at room temperature.
▲ CRITICAL STEP Make sure the pellet is completely resuspended in the egg buffer during each wash.

13| After the last centrifugation, carefully remove the buffer by sterile plastic transfer pipette.

14| Resuspend the pelleted eggs and lysed worms in 5 ml of sterile water and then add 5 ml of a sterile 60% (w/v) sucrose stock. Mix this solution well by shaking vigorously.

15| Separate the eggs from lysed worms and other debris by centrifugation at ~350*g* for 4 min at room temperature using a swinging bucket rotor in a tabletop centrifuge; eggs will float in the sucrose solution and collect at the solution meniscus and just below the meniscus.

16| Using a sterile plastic transfer pipette, transfer the eggs at the meniscus into sterile 15 ml conical tubes. Eggs that stick to the tube can be recovered by gently washing down the sides with a small volume of the sucrose solution and then removing them with a plastic transfer pipette.
▲ CRITICAL STEP You must use a plastic pipette to remove eggs as they will stick to glass.
▲ CRITICAL STEP Collect no more than 3–4 ml of the egg/sucrose suspension in each tube.

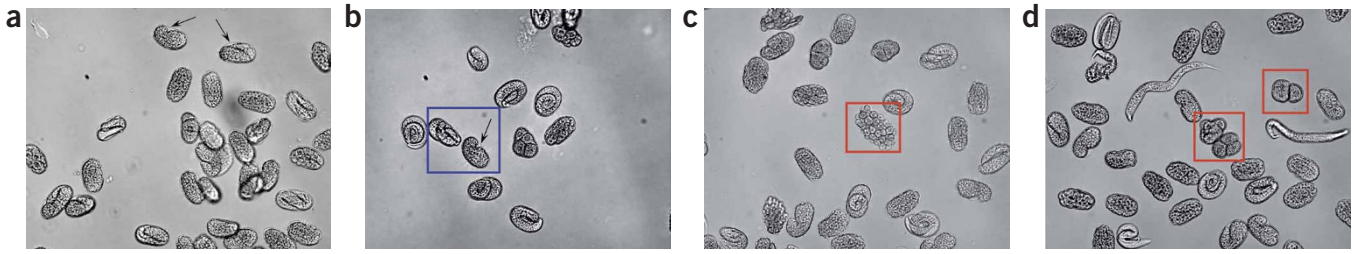

Figure 1 | Appearance of isolated *C. elegans* embryos during chitinase digestion of the eggshell. (**a**) Isolated embryos before exposure to chitinase. The eggshell is readily apparent (arrows). Also note the relatively uniform elongated oval shape of the eggshell-encased embryos. (**b**) Isolated embryos during early stages of chitinase digestion. Embryos in the blue box have a largely intact eggshell. The eggshell is clearly visible (arrow) and the embryos retain their elongated oval shape. The other embryos in the field still have an intact eggshell; however, their eggshell is weakening, as evidenced by the loss of the elongated oval shape and rounding of the embryos. (**c**,**d**) Isolated embryos during late stages of chitinase digestion. Eggshell has been completely digested away from embryos in red boxes. In **c**, the outlined embryo shows a "grape cluster"-like appearance, which is indicative of complete loss of the eggshell. Presence of free larvae (**d**) in the preparation is also indicative of eggshell digestion. Embryos were imaged at ×20 using an inverted microscope and conventional bright-field optics.

17| To remove the sucrose and debris, fill each tube containing the isolated eggs with 10–12 ml of sterile water and repellet by centrifuging at ~350*g* for 3 min at room temperature. Using first a plastic transfer pipette and finishing with a 100 µl pipettor, remove as much of the final wash liquid as possible to recover a dense egg pellet.
▲ CRITICAL STEP When isolating eggs for establishing synchronized worm cultures, it is essential to remove debris produced during the worm lysis. If not removed, hatched larvae may feed on this material and fail to arrest.
▲ CRITICAL STEP After egg isolation, all subsequent steps are carried out in a laminar flow hood under sterile conditions.
? TROUBLESHOOTING

Preparation of dissociated embryo cells ● TIMING approximately 50–110 min

18| Using a pipettor, add 100 µl of chitinase solution to the egg pellet in the 15 ml tube. Resuspend the eggs and then transfer them into a new sterile Eppendorf tube. To ensure maximal egg recovery, rinse the 15 ml tube and pipette tip 4× using 100 µl of additional chitinase solution. Transfer each 100 µl aliquot of chitinase solution into the same sterile Eppendorf tube. The final volume of chitinase solution in the tube should be 500 µl. Mix the egg suspension in the Eppendorf tube by rocking gently at room temperature for 20–80 min.

19| When ≥80% of the eggshells have been digested, add 800 µl of L-15 cell culture medium to the Eppendorf tube. The progress of eggshell digestion should be monitored by viewing samples of the egg suspension under a microscope at ×10–20 magnification. **Figure 1** shows examples of the appearance of an isolated egg preparation before and during chitinase digestion.
▲ CRITICAL STEP Note that each lot of chitinase tends to vary in potency. Consequently, incubation times will have to be assessed for each lot.

20| Pellet the eggs by centrifugation at ~900*g* for 3 min at 4 °C in a microcentrifuge.

21| Carefully remove the supernatant and add 800 µl of fresh L-15 cell culture medium.

22| Gently dissociate the cells by repeatedly pipetting the cell suspension against the side of the Eppendorf tube using a 100–1,000 µl pipettor. Monitor the degree of dissociation by periodically placing an ~10 µl drop of the suspension on a microscope slide and viewing at 20×. Continue the dissociation until at least 50% of the cellular material in the visual field is single cells. The preparation will also contain some intact eggs, undissociated embryos, clumps of cells and hatched larvae (see **Fig. 2a**).
▲ CRITICAL STEP Use of excessive force during the dissociation step will damage the cells. It should be stressed that one should not attempt to prepare a homogeneous population of dissociated cells. In our experience, doing so typically causes excessive cell damage.

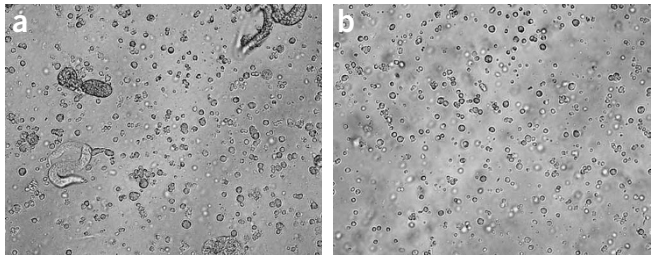

Figure 2 | Appearance of embryo cells during various isolation steps. (**a**) Embryo cells immediately following dissociation step. The majority of material in the preparation is single cells. Intact eggs, hatched larvae, cell clumps and debris are also present. (**b**) Filtered embryo cells. Preparations were imaged at ×20 using an inverted microscope and conventional bright-field optics.

23| Pellet the dissociated cell suspension by centrifugation at ~900*g* and 4 °C for 3 min.

24| Remove the supernatant, which may be somewhat cloudy, and resuspend the pellet in 500 µl of L-15 cell culture medium.
▲ CRITICAL STEP It is important to remove as much of the supernatant as possible before resuspending the cells in L-15 medium. The fine debris in the supernatant tends to clog the filter used in the filtration step described below.

Filtration of dissociated embryo cells ● TIMING **approximately 15–20 min**

25| Hatched larvae in the cultures will eat isolated embryo cells. Consequently, cell suspensions must be filtered to remove these larvae as well as cell clumps. Attach a sterile 18 gauge needle to a sterile 3 ml syringe and draw 1 ml of L-15 medium into the syringe.

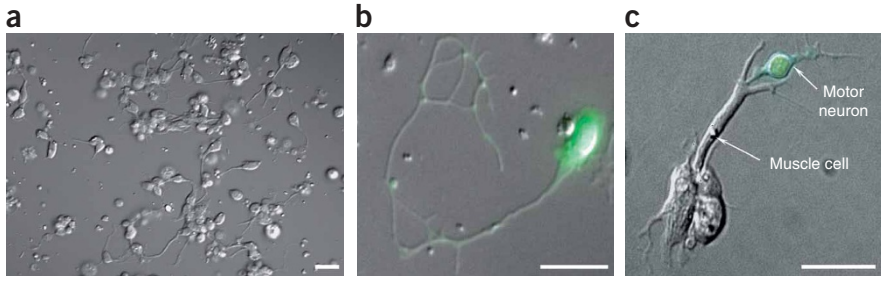

Figure 3 | Morphological and GFP reporter expression characteristics in cultured terminally differentiated *C. elegans* embryo cells. (**a**) DIC image of a typical culture of differentiated *C. elegans* embryonic cells 4 days after isolation and plating. Neurons and muscle cells are the most predominant cell type in the visual field. Scale bar, 5 μm. (**b**) Combined DIC and fluorescence micrograph of a *unc-119*::GFP-expressing neuron. *unc-119* encodes a conserved metazoan protein that may function in signaling pathways that control axonal patterning. (**c**) Combined DIC and fluorescence micrograph of a *unc-4*::GFP-expressing cholinergic motor neuron physically interacting with a body wall muscle cell. *unc-4* encodes a homeodomain transcription factor. Scale bars, 10 μm (**b**,**c**). Reproduced from Christensen *et al.*[6] with permission of Elsevier, copyright 2002.

26| Draw the cell suspension from Step 24 slowly into the syringe. ▲ CRITICAL STEP Draw the cell suspension up slowly enough so that it does not mix with the L-15 medium in the syringe.

27| Keeping the syringe held vertically, remove the needle and secure a 5.0 μm Durapore filter to the syringe. Force the cell suspension and L-15 medium through the filter with "medium" pressure into a sterile Eppendorf tube. ▲ CRITICAL STEP Excessive force during the filtration step can damage the cells. Use of too little force will reduce cell yield.

28| To maximize the yield of single cells, rinse the filter with additional L-15 medium as follows: remove the filter unit from the syringe and attach a new, sterile needle. Draw an additional 1.0–1.5 ml of L-15 medium into the syringe, remove the needle and replace with the filter. Force the L-15 medium through the filter into a second sterile Eppendorf tube.

29| Pellet the cells by centrifugation of both Eppendorf tubes (from Steps 27 and 28) at ~900*g* and 4 °C for 3 min.

30| Remove the supernatants and resuspend the cells in 25–200 μl of L-15 cell culture medium. The actual volume depends on the size of the cell pellet. Small pellets should be resuspended in small volumes of medium.

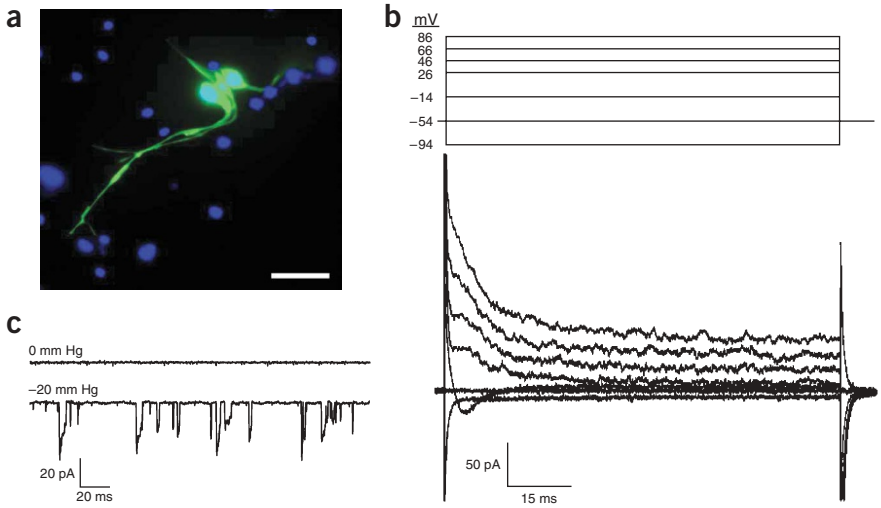

Figure 4 | Whole-cell and cell-attached currents recorded from *C. elegans* mechanosensory neurons. (**a**) Fluorescent micrograph of cultured mechanosensory neurons expressing *mec-4*::GFP. *mec-4* encodes an amiloride-sensitive Na^+ channel or "degenerin" protein[20]. Cell nuclei are labeled with DAPI and shown in blue. Scale bar, 10 μm. (**b**) Whole-cell currents in a cultured mechanosensory neuron. Currents were elicited by clamping membrane voltage from −94 to 86 mV. (**c**) Cell-attached patch currents recorded from a mechanosensory neuron. Holding potential is −60 mV. Channel activity is not detected in the absence of mechanical force (0 mm Hg). Application of suction (−20 mm Hg) to the patch pipette activates inward currents. The currents rapidly and repeatedly activate and inactivate throughout the recording period. *x* and *y* axes in scale bars are milliseconds (ms) and picoamperes (pA), respectively.

31| After resuspension, pool the two samples. **Figure 2b** shows a field of cells after filtration.

? TROUBLESHOOTING

Setting up cultures ● TIMING **approximately 30–150 min**

32| Prepare appropriate dilutions of the cell suspension in L-15 medium and determine cell density using a hemocytometer. Useful counting dilutions range from 1:10 to 1:200. For our experimental purposes, we count only "large" and "medium" cells and ignore any small cells. ▲ CRITICAL STEP Plating density depends on the nature of the experiments that are going to be performed. For patch-clamp and optical experiments, we find that a seeding density of ~230,000 cells per cm^2 is optimal. In addition, we have used seeding densities of 8,000,000 cells per well of a one-well chamber slide for western analyses and 800,000 cells per well of a four-well chamber slide for immunocytochemistry.

BOX 1 | TREATING *C. ELEGANS* CELL CULTURES WITH DOUBLE-STRANDED RNA TO INDUCE RNAi ● TIMING APPROXIMATELY 140 MIN

Gene expression can be silenced in cultured embryo cells using RNAi[7]. RNAi is induced by adding dsRNA directly to the culture medium. If gene knockdown does not affect fertility, worms can also be first fed dsRNA-producing bacteria to maximize the RNAi effect. However, it should be noted that the conditions under which RNAi feeding is performed[8] do not support the same degree of worm growth as those outlined earlier. Therefore, to isolate sufficient quantities of cells, it is necessary to culture more and/or larger plates of worms.

Most of our RNAi experiments are performed using cells grown on 12 mm diameter coverslips for electrophysiological studies. The protocol described below was designed to minimize the amount of dsRNA and volume of culture medium used.

1. To silence gene expression in cultured embryo cells by adding dsRNA directly to the culture medium, seed embryo cells onto 12 mm diameter glass coverslips in individual wells of four-well culture plates.
2. Incubate cells with 100 μl of L-15 cell culture medium containing 15 μg ml^{-1} of gene-specific dsRNA.
3. After 2 h, increase the culture medium volume to 300 μl. We typically use our dsRNA-treated cells within 2–3 days after plating. Therefore, we add 100 μl of L-15 medium containing 5 μg ml^{-1} dsRNA to the cultures on each subsequent day of culturing. However, it should be noted that excessive amounts of culture medium may limit O_2 diffusion to the cells.

33| Plate cells onto lectin-coated glass growth substrates using the following methods, according to the type of culture vessel used: use option A for 12 mm diameter glass coverslips and Mat Tek dishes, and option B for chamber slides:

(A) Plating cells onto 12 mm diameter glass coverslips or Mat Tek dishes

(i) For 12 mm diameter glass coverslips, place single coverslips into 35 mm diameter Petri dishes.

(ii) Pipette 75–100 μl of cell suspension onto the center of each 12 mm coverslip or the center of the Mat Tek dish coverslip and allow the cells to settle and attach for a minimum of 2 h.

(iii) Add an additional 2 ml of L-15 medium to each Petri or Mat Tek dish.

BOX 2 | ENRICHING SPECIFIC CELL TYPES BY FACS ● TIMING APPROXIMATELY 4–6 H

FACS can be used to isolate specific cell types from embryo cell cultures. These cells can in turn be used for cell-specific biochemical, molecular, DNA microarray and proteomic studies. Alternatively, sorted cells can be recultured. This approach may be useful for enriching cultures with cell types that interact with each other *in vivo* (e.g., muscles cells and motor neurons; see **Fig. 3c**).

For FACS studies, cells are cultured as described above, but using poly-L-lysine-coated growth substrates. As removal of cells from peanut lectin-coated substrates is extremely difficult and results in excessive cell damage and death, we have experimented with a variety of different coating materials including fibronectin, collagen IV, laminin and poly-L-lysine. Adherence of cells to poly-L-lysine-coated glass surfaces is sufficient for morphological differentiation to occur, but is weak enough to allow cell removal. Zhang *et al.*[9] have also reported that cultured embryo cells can be removed for FACS analysis from glass substrates coated with 3-aminopropyltriethoxysilane.

To enrich embryo cells expressing fluorescent reporter genes, cells loaded with fluorescent dyes or cells labeled with antibodies tagged with fluorophores, unlabeled cells must be used to determine background autofluorescence in each experiment. In addition, fluorophore-positive controls are required to accurately set the fluorescence gates on the FACS instrument. These gates are used to differentiate and sort labeled cells. Therefore, each experiment must include both unlabeled and labeled cells. Alternatively, commercially available fluorescent beads with a diameter of 2 μm, which is similar to that of cultured embryo cells, can be used to set fluorescence gates.

1. To isolate specific cell types from embryo cell cultures by FACS for further analysis or culturing, culture cells as described above (Steps 32–34) using poly-L-lysine-coated culture vessels (see EQUIPMENT SETUP).
2. Remove cultured cells from poly-L-lysine-coated growth substrates by repeated gentle washings with L-15 culture medium.
3. Transfer the dissociated cells to an Eppendorf tube and pellet by centrifugation at ~900*g* and 4 °C for 3 min.
4. Remove the L-15 culture medium and resuspend the cells in ice-cold sterile egg buffer to a final concentration of $\sim 10 \times 10^6$ cells per ml. Keep the cells on ice until they are sorted.
5. Add propidium iodide (PI) to each sample at a final concentration of 5 μg ml^{-1}.

▲ CRITICAL STEP PI staining is used to identify and remove dead cells from the preparation during sorting. However, with long exposures, PI will also stain live cells. Cells should be exposed to PI for no more than 3 h before sorting is begun.

6. Power up the cell sorter at least 15 min before loading the first sample. Sterilize the instrument's fluidics using a 10% (v/v) sodium hypochlorite solution.

▲ CRITICAL STEP To maintain viable cells throughout the sorting procedure, osmotically compatible buffers must be used in all steps. Therefore, the fluidics of the cell sorter must be thoroughly flushed with sterile egg buffer before use to replace any incompatible solutions that could harm the cells.

7. Load the unlabeled cells onto the cell sorter and adjust the forward and side scatter to compensate for the relatively small size of *C. elegans* embryo cells (~2–6 μm in diameter). Adjust the gain of each fluorophore channel to minimize background autofluorescence.
8. Using either fluorescently labeled embryo cells or fluorescent beads, adjust the gain settings on the respective fluorescence channels so that the positive cells/beads are clearly demarcated from the unlabeled cells by at least one log in fluorescence intensity. This must be carried out for each fluorophore used. Set the gate for sorting cells that are PI negative (viable) and fluorophore positive. When the instrument has been fully adjusted, flush the fluidics.
9. Resuspend the primary sample of cells and load them into the cell sorter. If needed, make any required adjustments to the gain and/or sorting gate to maximize cell yield. Sort the positively labeled, PI-negative cells into sterile collection tubes containing 1.5 ml of sterile egg buffer and pellet by centrifugation. The enriched cells are ready for further analysis or cell culture.

BOX 3 | PATCH-CLAMP ELECTROPHYSIOLOGY

Patch-clamp electrophysiological analysis of cultured *C. elegans* embryo cells is relatively straightforward and uses conventional electrophysiological methods[10]. However, because the cells are small, we have found that high-resolution and high contrast microscope imaging methods greatly improve the rate of successful seal formation.

For patch-clamp studies, we image cells using video-enhanced differential interference contrast (DIC) microscopy. Our electrophysiology rigs are built around Nikon TE300 or TE2000-U inverted microscopes. The microscope components we use for DIC imaging include a long working distance 0.52 NA condenser lens, an extra long working distance PlanFluor 60 × 0.70 NA dry objective lens, matched DIC prisms for objective and condensers lenses, and polarizer and analyzer filters. A dry objective lens avoids the mess and trouble associated with using immersion oil. For video imaging, we use a Hamamatsu C2400 CCD camera with detail enhancement and 9″ monochrome video monitors. The primary microscope image is magnified onto the camera using a 5× transfer lens. Additional magnification is achieved by extending the camera out from the transfer lens using a 2″ C-mount tube.

The specific components used in an imaging system can vary. However, it is essential to use high-quality DIC optics and high NA objective and condenser lenses with sufficient working distance to allow access for patch-clamp electrodes and to image cells through two coverslips (see below). A good-quality CCD camera with contrast controls and a detail or edge enhancement feature improves image quality. Final video image magnification is important. For example, cultured *C. elegans* intestinal cells, which have diameters of ~4–6 μm, appear with diameters of 1.5–2.5 cm on a 9″ video monitor in our system.

1. Culture cells on 12 mm diameter glass coverslips as described in Steps 18–34. Place a coverslip with adherent cultured cells into a model R-26G bath chamber. The chamber floor consists of a 22 × 40 mm glass coverslip. Movement of the coverslip during solution changes can be prevented by placing a small spot of vacuum grease on the chamber floor. The grease is placed so that it sticks to the 12 o'clock edge of the cell coverslip and does not obscure cells to be imaged.
2. Identify a cell of interest and position it in the center of the field of view. Cell identification is typically carried out using cell-specific GFP reporters or morphological features (see **Figs. 3–5**) (ref. 6).
3. Place the patch electrode in the bath and move it toward the bottom of the bath chamber and the center of the field of view. Initial electrode positioning is carried out by observation through the microscope eyepieces using ×4 and ×10 objectives.
4. Once the electrode is in place, switch to the ×60 objective and lower the condenser to its proper working distance for Kohler illumination. Position the cell of interest and the electrode tip in the center of the video monitor.
5. At this point, the electrode should still be well above the cell. Watching the video monitor, focus slightly below the electrode tip and move the electrode down into focus. Repeat this process until the electrode is close but not touching the cell. Adjust the position of the electrode so that it is above the cell center. Monitor electrode resistance while moving the electrode onto the cell surface and during gigaohm seal formation.
▲ CRITICAL STEP It is essential that final movement of the electrode onto the cell surface be carried out while monitoring electrode resistance and not the video screen. Placing the electrode tip onto the cell under visual control usually damages the cell and/or reduces the rate of successful seal formation.
6. The bath chamber is typically not perfused continuously while recording. To change bath solution composition, we use Warner Instruments pinch valves. Most experiments are carried out at room temperature. However, if bath temperature control is necessary, we use a Warner Instruments model SC-20 dual in-line heater/cooler, a model CL-100 bipolar temperature controller and a PHC series heater/cooler jacket for the bath chamber.
▲ CRITICAL STEP Bath solution composition will be dictated by the nature of the experiments being performed. However, it is important that bath osmolality is the same as that of the cell culture medium (i.e., 340 ± 5 mOsm). The bath chamber solution should be changed frequently to avoid increases in osmolality owing to evaporation.

(B) Plating cells onto chamber slides

(i) Add 2.5 ml of cell suspension to a one-well chamber slide or 600 μl of cell suspension to each well of a four-well chamber slide.

(ii) A cell settling period is not needed for chamber slides and they can be placed in the incubator immediately after seeding.
▲ CRITICAL STEP The type of culture vessel used will depend on the type of experiments being performed. For electrophysiology experiments, we culture cells on 12 mm diameter acid-washed glass coverslips. For high-resolution differential interference contrast (DIC) and fluorescence microscopy studies, we usually culture cells in Mat Tek dishes. If larger numbers of cells are needed, we have used one- and four-well Lab-Tek chamber slides.
▲ CRITICAL STEP Isolated embryo cells must adhere tightly to the growth substrate for differentiation to occur and cells do not attach well to coated or uncoated plastic growth substrates.
Peanut lectin coating of glass growth substrates is therefore used to promote cell adhesion (see EQUIPMENT SETUP).
? TROUBLESHOOTING

34| Keep cultures in small sealed Tupperware containers lined with wet paper towels during seeding and throughout the culture period to prevent evaporation of culture medium. Keep Tupperware containers in a humidified incubator at room temperature and ambient air.

▲ CRITICAL STEP Prevention of culture medium evaporation is critical. As a general rule, we do not use cultures that are older than 5 days. If cultures are kept longer than this, it may be necessary to replace the culture medium periodically.

35| To carry out optional applications of cell cultures such as RNAi, FACS or patch-clamp electrophysiology, see **Box 1, 2** or **3**, respectively, for procedures.

● TIMING

Steps 1–7, synchronization of worm cultures: approximately 45–60 min
Steps 8–17, egg isolation: approximately 45 min
Steps 18–24, preparation of dissociated embryo cells: approximately 50–110 min
Steps 25–31, filtration of dissociated embryo cells: approximately 15–20 min
Steps 32–34, setting up cultures: approximately 30–150 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
8-17	Low egg yield	Too little starting material	Increase number of enriched peptone plates used. Make sure worms are gravid before lysis
		Incomplete worm lysis	Allow lysis reaction to proceed for full 5 min
		Failure to completely remove eggs from sucrose spin	Make sure eggs are not sticking excessively to the transfer pipette. Wash sides of tube more thoroughly. Remove clumped eggs that may be located just below the solution meniscus
18-31	Low yield of dissociated cells	Incomplete chitinase digestion of eggshell	Make sure that $\geq$80% of the eggshells have been digested before beginning dissociation step. Increase digestion time. Replace chitinase stocks
		Loss of cells during filtration step	Be sure to remove debris from the dissociated cell preparation by centrifugation prior to filtering. Increase number of filter washes
32-33	Cells fail to attach to growth substrate	Substrate has not been properly prepared	Make sure coverslips are thoroughly acid–washed and rinsed. Make sure growth substrate has been coated properly with lectin
		Cells have been damaged during dissociation	Use gentler pipetting to dissociate cells. Make sure to monitor the degree of dissociation by periodically observing ~10 µl aliquots of the preparation at 20X
	Cells exhibit excessive clumping	Failure to completely remove excess lectin from growth substrate	Aspirate excess lectin solution from growth substrate more thoroughly. For 12 mm diameter coverslips, make sure that no lectin solution has crept underneath the coverslip
		Cell preparation has sat to long after filtration and counting	Gently pipette cells up and down 2-3 times prior to plating to ensure a homogeneous suspension

ANTICIPATED RESULTS

Embryo cells terminally differentiate within 24 h after isolation (see **Fig. 3**) and exhibit developmental characteristics and gene expression patterns that resemble those of the L1 larva. Our limited studies suggest that postembryonic cell differentiation does not occur in embryo cell cultures[6]. Identification of specific cell types *in vitro* is greatly facilitated by culturing cells from transgenic worm strains expressing cell-specific GFP reporters (see **Figs. 3b**,**c**, **4** and **5**). It is also possible to identify certain cell types by specific morphological features. Cell survival is excellent for 2–3 weeks[6]. However, it should be stressed that these are primary cultures and they may dedifferentiate with time. We typically prepare cells weekly and do not use cultures for physiology experiments that are older than 5 days.

Except for their small size, cultured embryo cells present no serious challenges for patch-clamp studies. *C. elegans* cell culture has been exploited recently to characterize ion channel and transporter function and regulation[5,6,11–19]. **Figure 4** shows examples of whole-cell and cell-attached patch currents recorded from cultured *C. elegans* mechanosensory neurons expressing *mec-4*::GFP (see **Fig. 4a**). *mec-4* encodes an amiloride-sensitive Na^+ channel or "degenerin" protein[20]. The whole-cell currents shown (see **Fig. 4b**) are largely due to the activity of K^+ and Ca^{2+} channels (M .Christensen & K. Strange, unpublished observations) and resemble those recorded from *C. elegans* mechanosensory neurons *in vivo*[21]. Inward currents activated by application

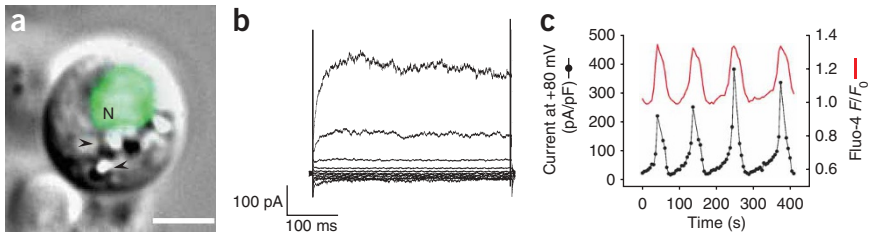

Figure 5 | Patch-clamp electrophysiology and intracellular Ca^{2+} imaging in cultured *C. elegans* intestinal cells. (**a**) Combined fluorescence and DIC micrographs of a cultured intestinal cell expressing the intestine-specific reporter *elt-2*::GFP (ref. 33) in the cell nucleus (labeled N). *elt-2* encodes a GATA transcription factor. Scale bar, 2.5 μm. Arrowheads denote refractile granules that are a characteristic feature of the worm intestine. (**b**) Whole-cell ORCa channel currents recorded from a cultured intestinal cell. Pipette was buffered with 10 mM 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). Currents were elicited by stepping membrane voltage from −100 to +100 mV in 20 mV steps from a holding potential of 0 mV. *x* and *y* axes in scale bars are milliseconds (ms) and picoamperes (pA), respectively. **a** and **b** reproduced from Estevez *et al.*[11] with permission of The Rockefeller University Press, copyright 2003. (**c**) Simultaneous measurement of ORCa channel current and intracellular Ca^{2+} in a cultured intestinal cell. Pipette solution contained 1 mM BAPTA and 30 μM fluo-4, a Ca^{2+}-sensitive fluorescent probe. Fluo-4 fluorescence intensity changes are plotted as the ratio of F/F_0, where F_0 is the fluorescence intensity measured at time 0. Current density on the *y* axis is reported as picoamperes/picofarad (pA/pF) and was recorded at a membrane potential of +80 millivolts (mV). Reproduced from Estevez and Strange[15] with permission of Blackwell Publishing, copyright 2005.

of suction to the patch pipette are observed in cell-attached patches (see **Fig. 4c**). The channels carrying these currents activate and inactivate rapidly and repeatedly in the presence of constant pipette suction. Channel inactivation observed in the presence of continuous mechanical force is referred to as "adaptation" and allows mechanoreceptors to respond to transient and dynamic stimuli[22]. Rapid adaptation has also been observed in whole-cell mechanoreceptor currents recorded *in vivo* from worm mechanosensory neurons[21].

Cultured embryo cells are also well suited for studies using optical probes of signal transduction and ion activity. Cells can be cultured from worms expressing cell-specific genetically encoded fluorescent indicators[23,24] or cells can be loaded *in vitro* with fluorescent dyes. **Figure 5** shows an example of simultaneous measurement of whole-cell current and intracellular Ca^{2+} in a cultured *C. elegans* intestinal cell (see **Fig. 5a**). These cells express an outwardly rectifying Ca^{2+} (ORCa) channel (see **Fig. 5b**) (see ref. 11). When cells are patch-clamped with solutions containing low concentrations of Ca^{2+} buffers, channel activity oscillates. Oscillating channel activity in turn gives rise to intracellular Ca^{2+} oscillations (see **Fig. 5c**) (see ref. 15). Such oscillating channel activity may play an important role in driving intestinal Ca^{2+} oscillations that control the *C. elegans* defecation rhythm[25–27].

Gene expression can be silenced by RNAi in *C. elegans* cell culture simply by adding double-stranded RNA (dsRNA) to the culture medium[6,28]. **Figure 6a** shows an example of a whole-cell current in a cultured worm intestinal cell that is activated by depletion of Ca^{2+} from endoplasmic reticulum (ER) Ca^{2+} stores[11]. This store-operated Ca^{2+} channel or SOCC requires the function of the ER transmembrane protein STIM-1 (ref. 28) and the plasma membrane protein ORAI-1 (ref. 29). Addition of *stim-1* or *orai-1* dsRNA to the culture medium for 2–3 days inhibits SOCC activation >90% (see **Fig. 6**) (see refs. 28,29).

Fluorescence-activated sorting of *C. elegans* cultured cells has to date been used to enrich mechanosensory[9], motor[30,31], and thermosensory and olfactory neurons[32] expressing cell-specific GFP reporters. Isolation of cell populations with purities for these neurons of 40–90% has been reported. These enriched cell populations have in turn been used for cell-specific gene expression studies[9,30–32].

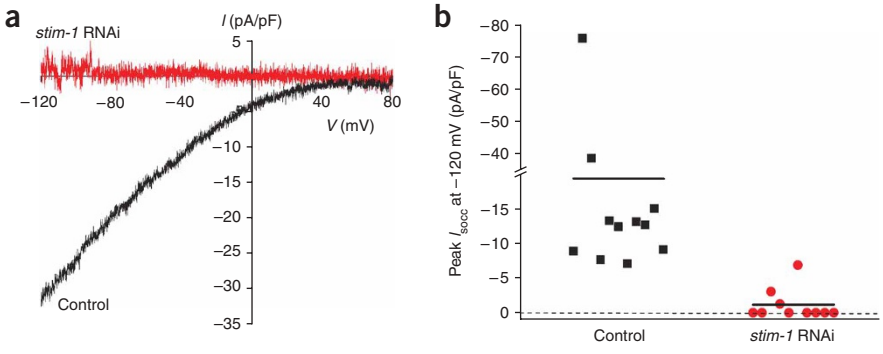

Figure 6 | Effect of *stim-1* RNAi on whole-cell SOCC currents recorded from cultured *C. elegans* intestinal cells. (**a**) Current-to-voltage relationships of whole-cell Ca^{2+} currents detected in cultured *C. elegans* intestinal cells after depletion of ER Ca^{2+} stores. Currents are peak currents recorded 5–6 min after store depletion was induced. Calcium stores were depleted by dialyzing cells with a pipette solution containing 10 μM IP_3, 10 mM BAPTA and a free Ca^{2+} concentration of ~18 nM. SOCC current fails to activate in cells treated with *stim-1* dsRNA for 2–3 days. The *y* axis is current (*I*) reported as picoamperes/picofarad (pA/pF). The *x* axis is membrane voltage (*V*) expressed as millivolts (mV). (**b**) Effect of *stim-1* dsRNA on peak SOCC current measured 5 min after obtaining whole cell access. Solid lines are the mean currents for the cells shown. Reproduced from Yan *et al.*[28] with permission of The Rockefeller University Press, copyright 2006.

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