Chapter 3

Lineage Tracing and Single-Cell RNA-seq in *C. elegans* to Analyze Transgenerational Epigenetic Phenotypes Inherited from Germ Cells

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Abstract

The last several years have seen an increasing number of examples of transgenerational epigenetic inheritance, in which phenotypes are inherited for three or more generations without changes to the underlying DNA sequence. One model system that has been particularly useful for studying transgenerational epigenetic inheritance is *C. elegans*. Their short generation time and hermaphroditic reproduction have allowed multiple transgenerational phenotypes to be identified, including aging, fertility, and behavior. However, it is still not clear how transgenerational epigenetic inheritance from the germline affects embryogenesis. Fortunately, the *C. elegans* embryo has a unique property that makes it ideal for addressing this question: they develop via an invariant lineage, with each cell undergoing stereotypical cell divisions to adopt the same cell fate in every individual embryo. Because of this invariant cell lineage, automated lineage tracing and single-cell RNA-seq can be employed to determine how transgenerational epigenetic inheritance from the germline affects developmental timing and cell fate. Unfortunately, difficulties with these techniques have severely limited their adoption in the community. Here, we provide a practical guide to automated lineage tracing coupled with single-cell RNA-seq to facilitate their use in studying transgenerational epigenetic inheritance in *C. elegans* embryos.

Key words *C. elegans*, Lineage tracing, Single-cell RNAseq, Epigenetic transgenerational inheritance, Germ cells

1 Introduction

The germline is a highly specialized tissue that produces gametes by the specialized cell division of meiosis. Gametes serve as the repository of all information that will be passed from one generation to the next. Predominantly, this information is encoded genetically in DNA. However, over the last few years, there have been increasing

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number of examples of transgenerational epigenetic phenotypes not encoded by changes in the DNA sequence itself. This much rarer mode of inheritance has been documented in organisms ranging from yeast to humans and has been proposed to occur via mechanisms like small non-coding RNAs, DNA methylation, and histone modifications [1, 2]. Studying epigenetic transgenerational inheritance is difficult in systems with a slow generational time, because of the time and effort it takes to monitor multiple generations. Studying epigenetic transgenerational inheritance can also be complicated by genetic variation, which can contribute to phenotypes not directly caused by transgenerational inheritance. One model organism that avoids these complications is the nematode *Caenorhabditis elegans* (*C. elegans*). *C. elegans* has a short generation time of 3 days and reproduces as self-fertilizing hermaphrodites, which limits genetic variation [3]. Partially as a result of these advantages, a large number of epigenetic transgenerational phenotypes have been observed in worms, including those that affect lifespan, fertility, and behavior [4–8].

Despite the examples of epigenetic transgenerational inheritance that have been identified, it remains unknown how heritable epigenetic transgenerational information causes phenotypes in resulting offspring. Fortunately, *C. elegans* has a unique property that makes it highly suitable for addressing this question. As originally identified by John Sulston, the *C. elegans* embryonic lineage is invariant [9], which means that the timing, cell movement, and cell fate of every cell remains the same between individual embryos, allowing the full lineage to be characterized. Because of this unique property, *C. elegans* can be used to investigate transcriptional and cell lineage defects at the single-cell level in the embryo [10]. In order to identify cell lineage defects, an automated cell tracking pipeline has been developed: StarryNite and AceTree [11]. These programs can be used to track each cell within an embryo and curate the cell lineage by utilizing live confocal imaging to follow mCherry- or GFP-labeled nuclei. By examining the cell lineage from mutant worms and comparing it with Wild Type (N2), it is possible to identify any defects in cell timing, cell migration, and inappropriate cell death, during all stages of embryogenesis. In addition, cell fate transformations can be identified by lineage conversion, as shown by the example reproduced from work by Boyle et al. [12] (Fig. 1). Along with automated lineage tracing, single-cell transcriptomics have been performed on N2 *C. elegans* embryos at all stages [13–15]. These experiments have defined the transcriptome of each cell in the *C. elegans* embryo, facilitating the identification of defects by comparison to N2.

By combining automated lineage tracing with single-cell RNA-seq (scRNA-seq) in the *C. elegans* embryo, it is now possible to determine how epigenetically inherited information affects the transcription and cell fate of embryonic cells at all embryonic stages.

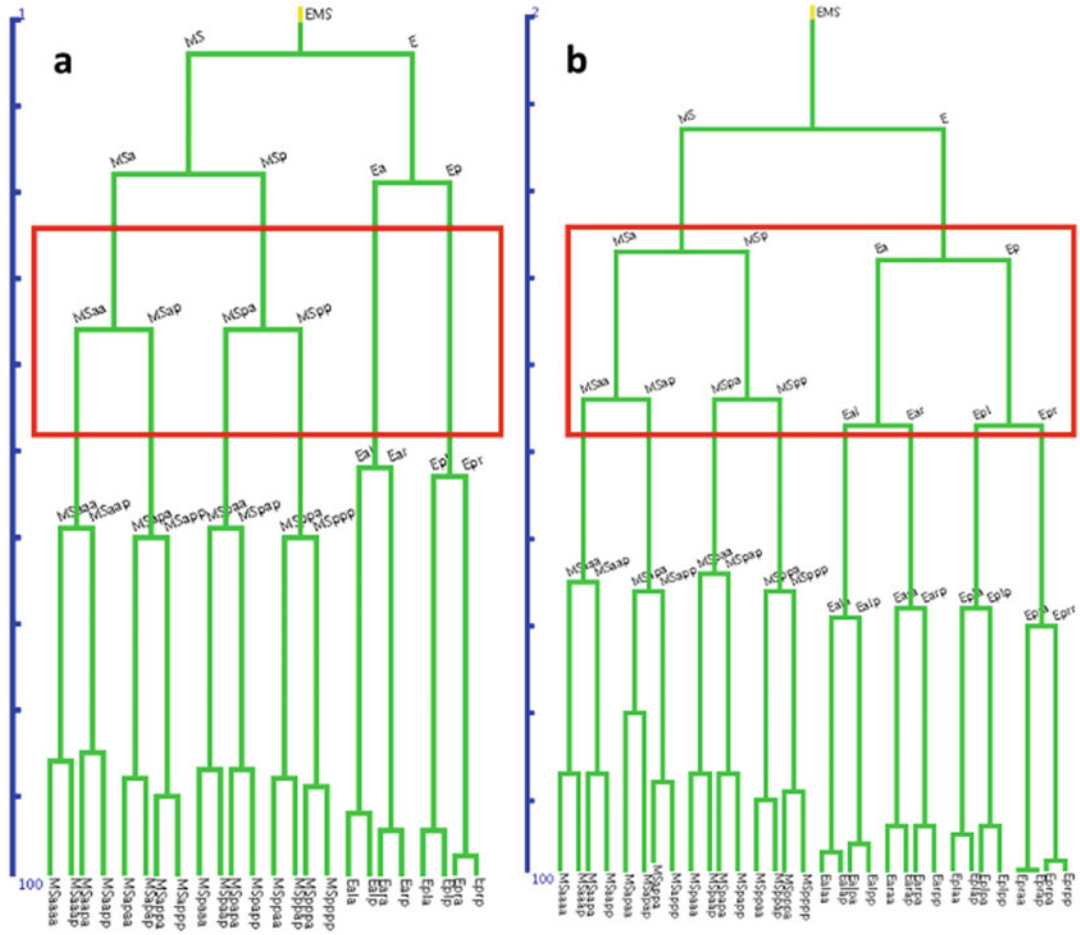

Fig. 1 Example of a lineage conversion. (**a**) the Wild-Type (N2) and (**b**) the *lit-1(RNAi)* EMS sub-lineage. Red squares highlight the difference in cell division timing between the MS (muscle) and E (intestine) lineages which are eliminated in *lit-1(RNAi)* animals. This result suggests that the E lineage adopted the cell fate of the MS. This result was previously shown by Boyle et al. [12] and colleagues, and is useful as a positive technical control

Considering the number of transgenerational phenotypes that have been identified in *C. elegans*, we believe that this will be a powerful new approach. By comparing N2 versus mutant embryos, it may be possible to determine how defects in the previous germline can give rise to phenotypes in the subsequent generation. In order to facilitate this analysis, this Methods in Molecular Biology chapter provides a practical guide for carrying out scRNA-seq and automated cell lineage tracing. The protocols that these methods are based on were originally developed and described in the following publications: scRNA-seq in Packer et al. [14], and lineage tracing in Murray et al. [11].

2 Materials

2.1 Growing Worms

1. Agar plates: 1 L Nematode Growth Media (NGM): 3 g $NaCl_2$, 20 g Agar, 2.5 g Bacto peptone dissolved in 975 mL with diH_2O. The remaining ~25 mL will be added after autoclaving. Be sure to add a stir bar to the solution before autoclaving. Autoclave for 1 h. Cool the flask by stirring on a heat/stir plate until the flask is ~55 °C (cool enough to briefly touch) and add the rest of the reagents: 25 mL of 1 M KPO_4 buffer pH 6.0 (108.3 g KH_2PO_4, 35.6 g K_2HPO_4 in a total of 1 L H_2O, autoclaved) (25 mM final), 1 mL 1 M $MgSO_4$ (1 mM final), 0.5 mL of 1 M $CaCl_2$ (0.5 mM final), 1 mL of 5 mg/mL Cholesterol (0.005 mg/mL final). Continue to stir and heat using a heat/stir plate while pouring into the 60 mm Petri dishes. The heating will prevent the agar from solidifying. It is recommended to use a plate pouring machine so that plates are all the same height, which eliminates the need to refocus when looking through multiple plates. Leave the plates lid-side up to dry for 2–4 days, then store lid-side down at 4 °C. Plates can be stored for several months.
2. Seeding the agar plates with bacteria for worm growth. Make Luria Broth (LB), which is used to grow *E. coli* (OP50 strain) that *C. elegans* feed upon. 1 L LB: 10 grams of Tryptone, 10 g of NaCl, and 5 g of yeast extract. Dissolve in distilled water, up to 1 L, and split into 10 glass flasks; each one should have approximately 100 mL. Autoclave. Inoculate 100 mL of LB media with a single colony of *E. coli* OP50 obtained from the Caenorhabditis Genetics Center (CGC); incubate overnight at 37 °C. From this culture, spot three OP50 drops onto each 60 mm NGM plate using a 5 mL serological pipet. The OP50 culture can be stored at 4 °C and used for several weeks.

2.2 scRNA-seq

1. M9 buffer: 22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, and 1 mM $MgSO_4$ (store at room temperature). Prepare 1 L. Sometimes the $MgSO_4$ will precipitate out after autoclaving. In this case, an alternative approach is to add the $MgSO_4$ after autoclaving.
2. Embryo collection: Bleach solution: M9 buffer containing 20% Bleach and 10 M NaOH (store at 4 °C). Prepare 500 mL. The bleach solution can be stored for up to 1 month. After this, the bleach solution should be remade from bleach stock purchased at least every 6 months to prevent a loss of efficacy. Note: the anti-splash additive now included in many commercial bleaches is not good for bleaching worms, so do not use bleach containing anti-splash additive.

3. Egg Buffer: 118 mM NaCl, 48 mM KCl, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 5 mM HEPES pH 7.2 (store at room temperature). Prepare 2 L, then add BSA to 100 mL, stored in 50 mL conical tubes (Egg Buffer +1% BSA, store at −20 °C).
4. 60% sucrose in H_2O (store at 4 °C). Prepare 100 mL and store in a sterilized container.
5. PBS (Corning 21-040-CV) (store at room temp).
6. Egg Buffer +1% BSA (−20 °C).
7. Chitinase (Sigma Aldrich C6137-5UN, ≥200 units/g solid) stored at −20 °C.
8. Pronase (Sigma Aldrich-10165921001, 1G) stored at 4 °C.
9. RNAse Zap (Thermofisher AM9782).
10. Autoclaved sterile glass Pasteur pipettes.
11. Individual sterile plastic transfer pipets.
12. 3 cc syringe and 21½ G needle.
13. 10 μM filter.
14. Trypan Blue.
15. Glass hemocytometer.

2.3 Preparation of Solutions and Equipment for Microscopy

1. Boyd's buffer with methylcellulose: 60 mM NaCl, 32 mM KCl, 3 mM Na_2HPO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$; 5 mM Hepes, 0.2% Glucose, 1% methylcellulose. This buffer can be used when dissecting worms and to make the 20 μm beads dilution. Prepare 1 L (store at 4 °C).
2. M9 buffer (see Subheading 3.1).
3. Frosted microscope slides 25 × 75 × 1.0 mm (Fisherbrand: 12-550-15).
4. Coverslip 18 mm × 18 mm (sigma Aldrich: 12-548-A).
5. Two 25G × 5/8 Needles (BD:305122).
6. 10 well-cutting glass plate (Fig. 4).
7. 20 μm beads (5 mL from Polyscience, 18329).
8. Confocal microscope (Zeiss LSM 510, or other) equipped with temperature-controlled stage (Brook Industries, Lake Villa IL). Setting the temperature-controlled stage at 20 °C mimics standard laboratory conditions for *C. elegans*.
9. Petroleum jelly (1.75 ounce jar of Vaseline brand purchased from a drug store).
10. Mouth pipet with a capillary glass tube (VWR 51608) Length 75 mm ±0.05, Column 75 μL.

2.4 Website to Download Lineage Program Software

1. StarryNite launcher: Integration of StarryNite and AceTree: https://waterston.gs.washington.edu/ [11] (see **Note 1**).
2. StarryNite: https://wormguides.org/starry-nite/
3. AceTree: https://github.com/zhirongbaolab/AceTree [11].

3 Methods

3.1 Cell Isolation for scRNA-seq

For scRNA-seq cell isolation, you will perform 3 separate synchronization steps. First, you will roughly synchronize the first generation. Second, you will collect their progeny for more precise synchronization. Finally, you will collect young embryos at the desired stage for performing scRNA-seq. For complete resuspension of the chitinase enzyme, please *see* **Note 2**.

1. Grow worms on 20 plastic 60 mm petri dishes, seeded with OP50 bacteria (*see* Subheading 2): place 3 L4-stage worms per plate (1 on each drop). All worms are grown in a 20 °C incubator. 20 plates of N2 will ultimately yield approximately 29,000 embryos at the ~100 cell stage (2.9×10^6 total cells). Certain mutants may be less fertile than N2 and will require starting with a larger number of worms.
2. Wait until each plate is confluent with gravid adults (worms containing two rows of embryos), but not starved (~3–4 days for N2, but the timing may be different in mutants). Using a plastic transfer pipet, rinse worms off plates with M9 buffer (by squirting onto a tilted plate ~10–20 mL for 20 plates). Collect the worms into a 50 mL conical tube, and let worms settle to the bottom by gravity (~10 min). Wash with M9 three times by removing the supernatant with a 50 mL plastic transfer pipet and adding 50 mL of M9 each time.
3. After the last wash, carefully pipet out most of the M9 with a plastic transfer pipet and add 25–30 mL of bleach solution. Place the tube onto a platform rocker and monitor degradation by looking in the tube under a dissecting light microscope (under 3–5× magnification) until most of the carcasses first disappear and embryos remain (~10 min, but this varies widely depending on the bleach, concentration of worms, etc.). Be careful not to let the embryos sit excessively in bleach as this will damage them.
4. After only embryos remain, bring the volume to 50 mL with M9 and centrifuge for 1 min at 450 rcf (Eppendorf Centrifugation 5810 R table top centrifuge). Carefully remove the supernatant using a serological pipet but leave a little bit of volume so the pellet won't be discarded by accident. Resuspend pelleted worms by bringing the volume to 50 mL with

Egg Buffer and gently mix by inversion. Centrifuge for 2 min at 450 rcf. During this time prepare a 15 mL conical tube with 5 mL of 60% sucrose (stored at 4 °C). After centrifugation is completed, discard the supernatant using a 50 mL plastic serological pipet.

5. Resuspend the embryos with 5 mL of Egg Buffer by pipetting up and down with a 5 mL plastic transfer pipet and transfer to a 15 mL conical tube with the 60% sucrose; vortex for 5 s and centrifuge for 5 min at 3220 rcf.
6. After centrifugation, two layers will form. The embryos will be in the top layer. Transfer the embryos to a 50 mL conical tube by using a glass Pasteur pipet (do not use plastic because the embryos and cells will stick to the plastic). Bring the volume to 40 mL with Egg Buffer and centrifuge for 2 min at 1260 rcf. Remove the supernatant with a 50 mL plastic serological pipet, leaving a little bit behind so the embryo pellet won't be discarded by accident. Resuspend in 1–1.5 mL of Egg Buffer (final volume, ~2 mL total). Then transfer the resuspended embryos onto 8–10 60 mm unseeded plates with a glass Pasteur pipet.
7. Allow the embryos to hatch overnight at 20 °C on unseeded plates. Without food, worms arrest at the L1 larval stage, so hatching onto unseeded plates synchronizes L1 larvae. After hatching overnight on unseeded plates move the synchronized L1 larvae to seeded plates by rinsing off the plates by squirting ~1–2 mL M9 per plate onto a tilted plate, using a glass Pasteur pipet.
8. Following the first L1 larval synchronization, proceed with a second synchronization. This second synchronization limits the number of worms you will obtain, but is necessary to make the synchronization tighter. Allow the L1 larvae to grow at 20 °C for approximately 46 h. This produces young adult worms with the first embryos in the gonad. After 46 h (this time may differ for mutant strains), repeat the bleach synchronization starting at **step 2** of Subheading 3.2.

3.2 Staging Worms and Collecting ~100 Cell Embryos

1. To obtain worms at approximately the 100-cell stage, allow the twice-synchronized L1 larvae to grow at 20 °C for approximately 46 h. This produces young adult worms with the first embryos in the gonad.
2. Pick individual young adults with the first embryos in the gonad onto a 60 mm plate containing a single OP50 drop from a 5 mL serological pipet. Even though the worms have been synchronized twice, picking worms is necessary to get the correct stage. It is important to pick all of the young adults within 1 h. scRNA-seq requires 10,000 cells, which generally requires starting the process with 14,000 cells. Therefore, if

embryos are at the ~100 cell stage, you will need 140 embryos. Obtaining ~140 embryos generally requires starting from 20 or more 60-mm plates of confluent worms.

3. Rinse plates with ~1–2 mL M9 (for 20 plates) by squirting onto a tilted plate with a plastic transfer pipet and collect worms into a 15 mL conical tube. Let the worms sink in the conical tube by gravity (~10 min) and wash the worm pellet with M9 three times by removing the supernatant with a 10 mL plastic serological pipet and adding 10 mL of M9 each time. The liquid should become clearer with each wash as the bacteria is removed.
4. After removing the last wash, add 7–10 mL of bleach solution, vortex for 20-s and place the 15 mL conical tube on a platform rocker for ~10 min. Monitor the worm degradation with a dissecting light microscope (3–5× magnification), vortexing occasionally, until at least 80–90% of embryos have been released and the carcasses disappear.
5. After confirming that embryos are released, bring the volume to 50 mL with M9 and centrifuge for 1 min at 450 rcf. After centrifugation is completed, carefully remove supernatant, but leave some volume behind so that the pellet won't be discarded by accident. Resuspend the pellet by bringing the volume to 50 mL with Egg Buffer and centrifuge for 2 min at 450 rcf. During this time, prepare a 15 mL tube on ice and add 5 mL of cold 60% sucrose solution. After the centrifugation is complete, discard the supernatant using a 50 mL plastic serological pipet.
6. Resuspend embryos with 5 mL of Egg Buffer and transfer to the 15 mL conical tube containing cold 60% sucrose. Vortex for 5 s to mix, then centrifuge for 5 min at 3220 rcf. The embryos should be in the top layer.
7. Transfer the embryos from the top layer to a 50 mL conical by with a glass Pasteur pipet. Bring the volume to 40 mL with Egg Buffer. At this point, you can let the embryos develop in Egg Buffer until the target cell stage is reached (*see* **Note 3**).
8. Once the target stage is reached, centrifuge for 2 min at 450 rcf., remove the supernatant using a 50 mL plastic serological pipet and resuspend the embryos in 1–1.5 mL Egg Buffer. Transfer the resuspended embryos to a 12-well plastic cell culture plate with a glass Pasteur pipet, then proceed with the cell membrane and cell isolation step.

3.3 Eggshell Removal and Single-cell Suspension

1. Add a ratio of 1 mL chitinase (1 U/mL) to 0.5 mL embryo suspension and incubate at room temperature for 20–30 min (if the desired embryos are greater than the 300-cell stage, *see* **Note 4**). Monitor eggshell removal under a dissecting light microscope (3.5–55×). It is very important to keep monitoring the cell suspension during the incubation time to observe the

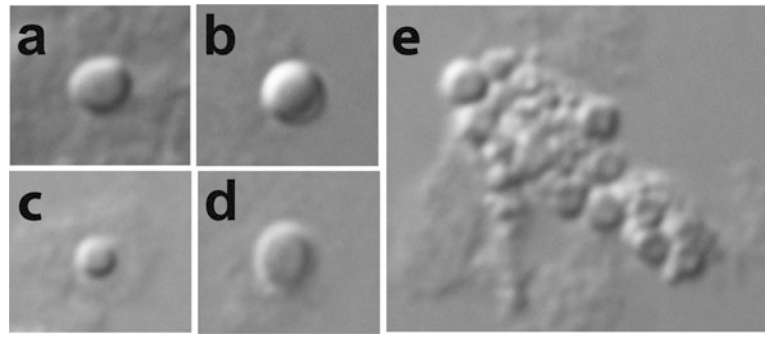

Fig. 2 Verification of single cells. DIC images of embryonic cells. (**a**–**d**), isolated single cells and (**e**) clump of cells. Images were taken at 100×

removal of the eggshell, so that the reaction does not proceed past the removal of the eggshell. Proceeding past the initial removal of the eggshell can result in damage to the individual cells. In the meantime, thaw the Egg Buffer with 1% BSA (stored @ −20 °C) and place a new 15 mL conical tube on ice for **step 4**.

2. Optional: Another way to confirm eggshell disruption is to place 2 μL of the sample on a slide with a 2% agarose pad and examine under differential interference contrast (DIC) microscopy at 40× to verify the single-cell isolation (*see* Fig. 2). If there are still clumps of cells, pass the suspension through the 21½G needle again multiple times. Clumps can clog the 10× Chromium capture mixer (10× Genomics) and/or result in cell doublets within a single GEM droplet.
3. After confirmation of the disruption of the eggshell, pass the embryos repeatedly through a $21^{1/2}$ G needle ~20 times to generate a single-cell suspension in one well of a 12-well cell culture plate. Incubate at room temperature for 5 min. The 12-well plate makes it easier to pass through the syringe multiple times and to monitor the single-cell isolation under a dissection light microscope (10–20×). Then move to **step 5** if the embryos are less than the 300-cell stage. If the desired embryonic stage is greater than 300 cells, proceed with the Subheading 3.3, **step 4**. Place 10 μL of the single-cell suspension on a 2% agarose pad and look under the DIC microscope at 40–100× to verify the single-cell isolation (*see* Fig. 2). If there are still clumps of cells, pass the suspension through the 21½G needle again multiple times. Clumps can clog the 10× Chromium capture mixer (10× Genomics) and/or result in cell doublets within a single **G**el bead in **EM**ulsion (GEM) droplet.
4. Stop the enzymatic reaction by adding 3–4 mL of Egg Buffer with 1% BSA (thawed in **step 1**) to the well of the 12-well plate.
5. Transfer to a chilled 15 mL tube by passing the cells through a 10 μm filter on a 3 cc syringe. Filtering through the 10 μm filter

on a 3 cc syringe removes almost all of the debris and intact embryos, while all of the single cells pass through. As a result, this filtering step should be included.

6. Centrifuge at 2500 rcf for 5 min at 4 °C. The cells will be in the pellet. In the original protocol, this centrifugation was performed at a slower speed to pellet the debris. However, after a slow speed spin, many single cells were found in the pellet with the debris and were lost during this step. The inclusion of the filtering in **step 6** makes pelleting the debris unnecessary. As a result, a slightly stronger spin (2500 rcf for 5 min at 4 °C) can be used to pellet all of the single cells in this step.
7. Using a glass Pasteur pipet, carefully remove the supernatant and wash the pellet 3× by centrifuging at 2500 rcf for 3 min. Resuspend pellet in 1 mL Egg Buffer with 1% BSA by pipetting up and down with a glass Pasteur pipet. After the first resuspension, transfer to a nonstick polypropylene Eppendorf tube for the second and third washes (*see* **Note 5**).
8. Following the final wash, remove most of the supernatant and add 1 mL of Egg Buffer with 1% BSA. Then centrifuge at 4 °C at 2500 rcf for 5 min.
9. Using a glass Pasteur pipet, carefully remove the supernatant and resuspend the pellet by adding 100 μL of ice-cold Egg Buffer and pipetting up and down with a glass Pasteur pipet. Then proceed with cell counting (*see* **Note 6**).
10. Following resuspension, count the number of cells using a hemocytometer (Fig. 3). During the counting, keep the resuspended cell on ice all the time. Clean the glass hemocytometer

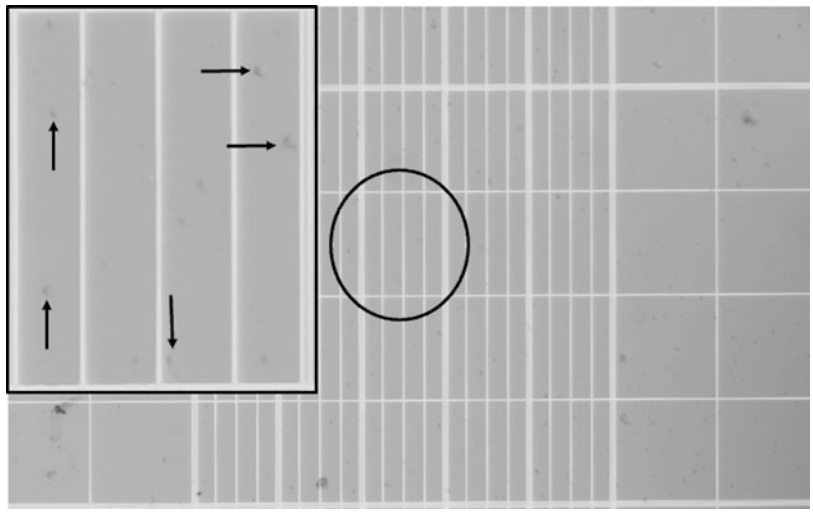

Fig. 3 Cell counting. A volume of 10 μL was loaded into the hemacytometer. The boxed inset shows a zoom in of the region of the hemacytometer that is circled. The arrows point to isolated cells. Image was taken at 20×

and coverslip with clean ethanol. Place the coverslip onto the hemocytometer, the coverslip should cover both chambers. Gently flick the Eppendorf tube that has the cells. Then take 10 μL and apply it into the loading area of the hemocytometer, underneath the coverslip. Wait around 30–60 s for the cells to settle. Finally, count the cells at 10× or 20× magnification using a phase contrast microscope. The cells can be counted by quadrant. For example, you could count the four outside edge quadrants and the one in the center. Then the formula that would apply to the cell counting is: number of cells counted in each quadrant multiplied by the dilution factor (if you diluted the cells, i.e., with Trypan blue – *see* **Note 7**) equals the number of cells $10\times^4$ cells/mL, divided in the # of quadrants, in this example the # of quadrants is 5. This information will be important for the 10× Genomics protocol. During the cell counting process, keep cells on ice and proceed directly, as quickly as possible, to the 10× Genomics protocol for RNA isolation, cDNA conversion, library preparation, and sequencing guidelines (s*ee* **Note 8**).

11. After counting, immediately follow the 10× Genomics protocol for RNA isolation, cDNA conversion, library preparation and sequencing. Details related to the 10× Genomics protocol are not included here because the 10× Genomics protocols are constantly changing. However, it should be noted that for troubleshooting and smaller samples, currently it is recommended to use the Chromium Next GEM Single Cell 3′ LT v3.1 low throughput kit (PN-1000325). For full samples, use the Chromium Next GEM Single Cell 3′ LT v3.1 kit (PN-1000128).This kit is more expensive but the cell recovery is much higher. In addition, more cells can be analyzed in a single assay using this kit.

3.4 Synchronization and Worm Dissection to Obtain 2-Cell Embryos for Lineage Tracing

1. The automated lineage tracing was designed for use with the Zeiss LSM 510 microscope. It may be possible to use other confocal microscopes, but we encountered difficulty when we tried to use a Leica SP8 (*see* **Note 9**).
2. Embryos should start to be imaged at the 2–4-cell stage, to allow the tracking software to function correctly. The program to track the cells is StarryNite (*see* **Note 10**).
3. Pick around 20–30 L4 worms and place them on a seeded plate (*see* **Note 11**). Use the JIM113 strain: *ujIs113 [pie-1p::mCherry::H2B::pie-1 3'UTR + nhr-2p::his-24::mCherry::let-858 3'UTR + unc-119(+)]*. The goal is to compare Wild Type to certain mutants, so mutations will need to be crossed into the JIM113 genetic background.

Fig. 4 A 10-well glass plate. This plate can be used to clean the worms and perform the dissection

4. After 20–24 h, worms should have the first embryos in the uterus.
5. Prepare the imaging setup prior to placing the worms into the cutting glass plate (*see* **Note 12**). Place 3–4 worms into one well of a 10-well-cutting glass plate (Fig. 4) filled approximately half way with M9. Move the worms into a new well 3× to rinse and remove the bacteria.
6. Cut the worms by using two needles and slicing the worm at the boundary between the uterus and the gonad (approximately in the middle of the worm).
7. Using a mouth pipet, move ~4–6 (as many as you can find) 2–4 cell embryos into a new well of a 10-well-cutting glass plate containing M9. This helps to reduce any remaining bacteria contamination. It is best if the embryos are at the 2-cell stage or even the occasional fertilized egg at the 1-cell stage.
8. Create the bead mount: Place 3 μL of 1:20 dilution of 20 μm beads in Boyd's buffer (final concentration of beads, 1 μM) onto a microscope slide and mouth pipet 2–4 embryos from the 10-well-cutting glass plate at the 2–4 cell stage (pick the earliest staged embryos available). Using a worm pick, gently lower the coverslip onto the embryos to avoid damaging them.
9. Seal the edges of the coverslip with enough petroleum jelly to cover the edges using a brush (e.g., a cleaned nail polish brush). Sealing the coverslip prevents evaporation. Do not use nail polish, as the acetone in the nail polish can kill the embryos (*see* **Note 13**).

10. Place the slide into the previously prepared temperature-controlled stage and start live-imaging with a Zeiss LSM 510 (*see* **Note 14**).
11. Live imaging must start with 2 or 4 cell-stage embryos and 200 min is typically sufficient to reach the 100 cell-stage. It requires ~13 h to image until the embryo hatches. At the earlier stages (2–16 cell-stage), the laser power can be high (>80). However, as cells start to divide, the signal intensity increases as nucleus size decreases, which makes it difficult for StarryNite to track the cells. As a result, the StarryNite program makes more mistakes, which requires extensive manual correction. Murray and colleagues [11] suggest setting the software to automatically adjust the laser power and other parameters in different time blocks during the imaging, but some versions of the ZEN software for running the Zeiss LSM 510 lack that option. If automatically changing the laser power and other parameters is not an option, the laser can be adjusted manually by decreasing the laser power by 20–40% after 1–2 h of live imaging. Images should be collected using a 63×, 1.4NA oil Plan-APOCHROMAT objective (*see* **Notes 15** and **16**).
12. When using the Zeiss LSM 510, follow the microscope settings and parameters listed in Murray et al. 2006 [11]. Images should be exported in the 8-bit TIFF format. The confocal generates individual images for every focal plane at every time point. In order to run the images on StarryNite, they must be grouped by timepoints. Murray and colleagues [11] suggest using Matlab to group the images. However, an alternative way to create folders with images grouped by timepoints is to use a Mac command. To do this use a command written in AWK to compile each image by timepoint, and group each timepoint in a separate folder (*see* **Note 17**). When images are grouped by timepoints, use an ImageJ macro command to compress all of individual Z-stacks into a single 8-bit TIFF file, which can then be imported to StarryNite (*see* **Note 17**).
13. StarryNite produces multiple outputs. Open the XML file (contained in the ZIP file) in AceTree.
14. AceTree is used to visualize the lineage and can be used to manually correct any cell division mistakes or add any cells that failed to be tracked. The most common error made by StarryNite is missing a daughter cell from the previous division. To correct this error in AceTree, click on the "edit" option and select "edit tools." This step opens two new windows, "Edit Tracks" and "Adjust or Delete Cells." Select the mother cell by right-clicking on the cell in the embryo image window that contains the error, then click on use active cell from the "Edit Track" window. In the "Edit Track" window, check the box

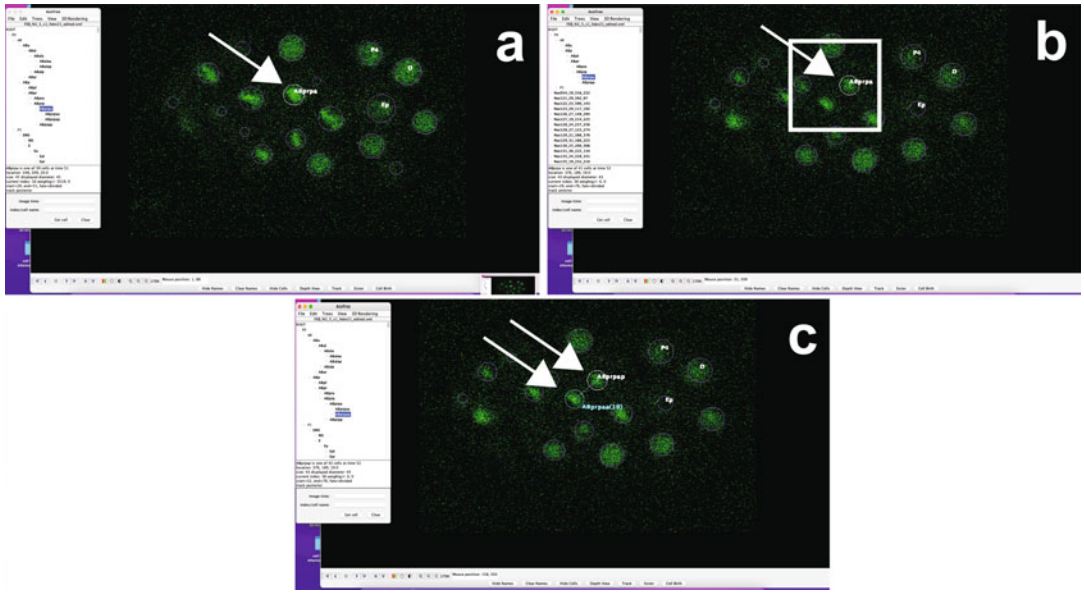

Fig. 5 Using AceTree editing tools to correct an error with StarryNite. Wild-Type (N2) embryo is shown using the AceTree program. (**a**) Mother cell (arrow). (**b**, **c**) The cell division (daughter cells) from the mother cell in a. In **b** (boxed in white), StarryNite didn't track the division. In **c**, the editing tool from AceTree was used to correct the cell division

that says "is early set correctly?" Then move to the next time-point by clicking on the forward arrow in the embryo image window and click on the unlabeled daughter cell that the software has failed to track. A new circle will form around the cell. Finally, click "use active cell" and "apply" from the "Edit Track" window. Now the two daughter cells will be linked to their mother cell (Fig. 5 for an example of this common mistake). Until you fix the tracking errors, AceTree will show many untracked cells as Nuc# instead of as the correct cell lineage name. Once you correct the error, AceTree will automatically assign the correct Sulston lineage name to the fixed cell.

4 Notes

1. Initially, SNlauncher software downloaded from the original website (Subheading 2.4, **item 1**) did not function. After communication with Bao lab members, an alternative version of AceTree was obtained (Subheading 2.4, **item 2**) and this version was successfully installed on a Mac OS version 12.2.1. Therefore, if SNlauncher downloaded from Subheading 2.4, **item 1** does not work, try downloading from Subheading 2.4, **item 2** as an alternative.

2. The chitinase purchased from Sigma Aldrich often does not dissolve completely, leaving very small particles that can only be observed under phase-contrast microscopy. The remaining small particles interfere with the single-cell experiment by replacing actual cells in the single GEM droplet during 10× Genomics RNA isolation. To avoid this problem, perform an ultracentrifuge step to separate the chitinase enzyme from the solid undissolved particles. Centrifuge for 30 min at 48,000 rpm at 4 °C. After centrifugation, remove the supernatant containing the particle-free chitinase enzyme and transfer to a new tube for use.
3. It typically takes ~40 min to isolate embryos via bleaching and pelleting over the sucrose cushion. To target the ~100-cell stage, let the embryos develop in Egg buffer for an additional 30–45 min. The amount of time has been empirically determined by monitoring under a dissecting light microscope (10–20× magnification) for N2 at room temperature. As a result, the amount of time will need to be separately determined for every mutant strain. This is the step before the chitinase process (Subheading 3.3).
4. If the desired embryos are greater than the 300-cell stage: The original protocol for isolating single cells from the *C. elegans* embryo incorporated both a chitinase and Pronase step. However, if targeting an embryonic cell stage of less than 300 cells, only the chitinase step is necessary, because using both chitinase and Pronase on embryos at earlier stages affects the viability of the cells. If the desired embryos are greater than the 300-cell stage, use Pronase to remove the vitelline layer of the eggshell: Add 100 μL per mL of 15 mg/mL Pronase (final concentration 1.5 mg/mL) to the sample. Using a 3 cc syringe, pass embryos repeatedly through a 21½ G needle ~20 times to generate a single-cell suspension in one well of a 12-well cell culture plate. Incubate at room temperature for 5 min. The 12-well plate makes it easier to pass through the syringe multiple times and to monitor the single-cell isolation under a dissection light microscope (10–20×).
5. Pay attention to the pellet because cells can stick to the tube. If you find that the cells are sticking to the tube, you can try centrifuging for a shorter amount of time (e.g., 30–60 s).
6. Do not use an automated cell counter instrument because the cell counting is likely to be very inaccurate. These kinds of instruments are typically designed for mammalian cells. The *C. elegans* embryonic cells are very small, often resulting in the instrument counting incorrectly. Instead, use a traditional hemocytometer to count the cells manually at 20× magnification or greater (Fig. 3).

7. Trypan blue dye is used to determine the viability of cells because dead cells take up the dye. For 10× Genomics, it is highly recommended to start with a cell population with viability of >90%, because transcripts tend to be degraded in dead cells.
8. Additional reagents not provided by the 10× Genomics kit will be needed. It's recommended that all the additional reagents be made fresh on the day of the protocol.
9. It may be possible to use a confocal other than a Zeiss LSM 510. For example, automated lineage tracing has been published using a Leica SP5 [16]. However, the StarryNite program failed to track early cell divisions from Z-stack time series generated with a Leica SP8. This may be due to differences with the metadata or with StarryNite parameters.
10. The first cell divisions in the *C. elegans* embryo occur approximately every 15 min. To slow down this process, store the buffer M9 and cutting glass at 4 °C. This will provide more time for the embryo cell stage selection, slide preparation and the microscope setup.
11. Picking L4 larvae 20–24 h prior to dissection of the adults will increase the number of worms that have 2–4 cell stage embryos 20–24 h later.
12. The temperature stage should be prepared prior to the cutting of the worms by filling the tank with distillated water and setting the temp to 20 °C.
13. Seal the slide with warm petroleum jelly. Warm petroleum jelly can be maintained permanently by storing in a glass test tube in a heat block at >65 °C.
14. Sometimes embryos will settle during the live-imaging process, which changes the focal plane and disrupts StarryNite's ability to track the lineage. If this happens, consider the following solutions:
    (a) Setup additional Z-stacks beyond the 3 recommended by Murray and colleagues [11].
    (b) Use a modified agar plate to prevent embryos from sinking: Using an empty 60-mm plastic petri dish, make a small opening with a heated surgical blade (sigma Aldrich:2976, No. 11) on the bottom of the plate; embryos will be placed on the exposed agar in the opening (Fig. 6). Then add 10–15 mL of hot NGM agar. Place the hole over a coverslip when pouring the NGM to prevent leakage (10–15 mL is more than needed to fill the plate, in

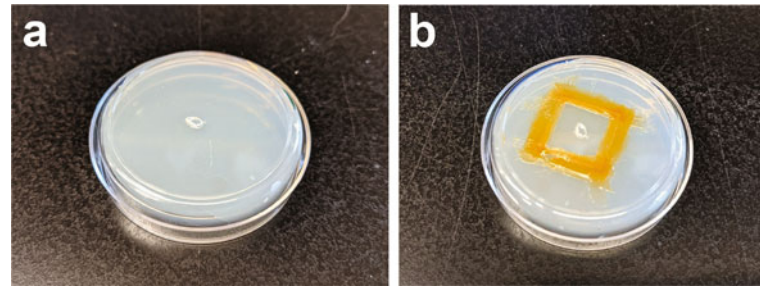

Fig. 6 Alternative method to prevent the embryo from settling during live imaging. Plate designed to prevent settling (sinking) of embryos. (**a**) NGM plate with hole where embryos will be placed. (**b**) Plate after the coverslip has been added and sealed with petroleum jelly (for details, see video link: https:/www.youtube.com/watch?v=q9GlxGfvEiQ)

case of leakage). Let the NGM agar solidify overnight on the bench and use the next day. If you are using this technique, mouth pipet the embryos into the hole (instead of onto a slide, Subheading 3.4, **step 7**) and gently cover embryos with a glass coverslip; use a worm pick to gradually lower the coverslip at a diagonal. Note, when using this technique, there are no beads and no liquid on the agar. Seal the coverslip with enough petroleum jelly to prevent airflow (identical to Subheading 3.4, **step 8**). Place the plate on the temperature stage to start live imaging (a video link showing this process is provided in Video 1).

15. Use an upright confocal microscope rather than an inverted microscope to prevent embryos from settling out of the focal plane when inverted.
16. Any 63× objective (including oil, glycerol, or water) can be used for imaging. However, the standard 63× oil lens yielded a weaker signal. Signal intensity was improved slightly by a glycerol lens. However, a strong signal was only obtained by using the Zeiss C-Apochromat 63×/1.20 W Corr UV-VIS-IR water objective. Poor signal prevents StarryNite from correctly assigning cell fate, which greatly increases the amount of time that will be needed for manual correction.
17. The script used for image grouping is included in Fig. 7 (AWK code in Linux). The ImageJ macro for formatting the images prior to running StarryNite is included in Fig. 8.

```
ls –lt > qc1

ls –1 | sed 's/.tif//g' | sed 's/_/ _/g' | awk '{print $6}' | sed 's/_/
sort –k 1,1 | uniq | grep –v "(" | grep t | awk '{print "mkdir "$1}' >
directory.sh

sh directory.sh

ls –lt > qc2

ls –1 | grep tif | sed 's/_/ /g' | sed 's/.tif/ .tif/g'| awk '{print
$6"\t"$7}' | sort –k 1,1 | uniq | awk '{print "mv *"$1$2" "$1"/"}' > mv

sh mv.sh
```

Fig. 7 This Linux AWK command can be used to group images by each timepoint

```
/*
 * Macro template to process multiple images in a folder
 */

#@ File (label = "Input directory", style = "directory") input
#@ File (label = "Output directory", style = "directory") output
#@ String (label = "File suffix", value = ".tif") suffix

// See also Process_Folder.py for a version of this code
// in the Python scripting language.
setBatchMode(true);
processFolder(input);

// function to scan folders/subfolders/files to find files with correct suffix
function processFolder(input) {
        list = getFileList(input);
        list = Array.sort(list);
        for (i = 0; i < list.length; i++) {
                if(File.isDirectory(input + File.separator + list[i]))
                        processFolder(input + File.separator + list[i]);
                if(endsWith(list[i], suffix))
                        processFile(input, output, list[i]);
        }
}

function processFile(input, output, file) {
        // Do the processing here by adding your own code.
        // Leave the print statements until things work, then remove them.

        print("Processing: " + input + File.separator + file);
   open(input + File.separator + file);
        outfile = output + File.separator + file;
        ofile="[" + outfile + "]";

        run("Image...  ", "outputfile=&ofile display=&ofile");
        //run("Image...  ", "outputfile=[&outfile] display=&file"); only does 1
        print("file:" + file);
        close();
}
setBatchMode(false)
```

Fig. 8 ImageJ macro for formatting the images prior to running StarryNite

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