HHS Public Access

Author manuscript

J Vis Exp. Author manuscript; available in PMC 2023 March 01.

Laser Microdissection for Species-agnostic Single-tissue Applications

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Abstract

Single-cell methodologies have revolutionized the analysis of the transcriptomes of specific cell types. However, they often require species-specific genetic "toolkits," such as promoters driving tissue-specific expression of fluorescent proteins. Further, protocols that disrupt tissues to isolate individual cells remove cells from their native environment (e.g., signaling from neighbors) and may result in stress responses or other differences from native gene expression states. In the present protocol, laser microdissection (LMD) is optimized to isolate individual nematode tail tips for the study of gene expression during male tail tip morphogenesis.

LMD allows the isolation of a portion of the animal without the need for cellular disruption or species-specific toolkits and is thus applicable to any species. Subsequently, single-cell RNA-seq library preparation protocols such as CEL-Seq2 can be applied to LMD-isolated single tissues and analyzed using standard pipelines, given that a well-annotated genome or transcriptome is available for the species. Such data can be used to establish how conserved or different the transcriptomes are that underlie the development of that tissue in different species.

Limitations include the ability to cut out the tissue of interest and the sample size. A power analysis shows that as few as 70 tail tips per condition are required for 80% power. Tight synchronization of development is needed to obtain this number of animals at the same developmental stage. Thus, a method to synchronize animals at 1 h intervals is also described.

Introduction

Nematodes—particularly the rhabditid nematodes related to the model system *Caenorhabditis elegans*—are a wonderful group of animals for evolutionary developmental biology (EDB) for many reasons[1, 2]. Advantages include their small number of cells, defined and consistent cell lineages, transparency, and ease of culture and husbandry. There are also many resources available, including high-quality genomes for multiple species, and for

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A complete version of this article that includes the video component is available at http://dx.doi.org/10.3791/63666.

Disclosures

All authors declare that they have no conflicts of interest.

C. elegans, extensive molecular genetic tools and knowledge about development, genetics, anatomy, and physiology[3, 4, 5, 6].

As with many other organisms, the ability to characterize transcriptome dynamics in single tissues or single cells has revolutionized the analysis of development in *C. elegans*[7, 8, 9, 10]. Being able to compare single-cell transcriptomes across nematodes would similarly transform EDB using these organisms. For example, such comparisons would provide insight into how gene regulatory networks have evolved for characters (traits) that have been conserved, for characters that have diverged, or for characters that evolved independently.

However, isolating particular tissues or cells from nematodes is one of the big challenges. For many organisms, single cells can be dissociated from tissues and harvested in an unbiased way or can be labeled with tissue-specific expression of a fluorescent protein and sorted by fluorescence-activated cell sorting (FACS)[11]. In *C. elegans*, high-throughput (HTP) isolation of cells has been limited mostly to embryos because the tough outer cuticle (and hydrostatic skeleton) has hampered cell isolation from larvae and adults. To get around this challenge, some methods have employed genetic tools in whole *C. elegans* worms, such as tissue-specific mRNA-tagging[12], and differential expression comparisons between wild-type and mutants affecting a cell type[13]. More recent methods have overcome the challenge by dissolving the cuticle to isolate nuclei[14] or entire cells[8, 9, 15]. Cell isolation and cell culture have the obvious disadvantages, however, that cells are removed from their natural developmental or anatomical context—e.g., away from cell-cell signaling and contact with the extracellular matrix—which are expected to impact the gene expression profile[15]. Moreover, the genetic tools and tissue-specific markers are species-specific (i.e., they can only be used in *C. elegans*).

LMD provides an alternative method for isolating tissues without disrupting the natural context of cells. Significantly for EDB, LMD also allows transcriptomes from homologous tissues of different species to be compared without the need for species-specific genetic toolkits if genome or whole transcriptome sequences of these species are available. LMD involves targeting tissues by direct microscopical observation and using a laser microbeam —integrated into the microscope's optics—to cut out and harvest (capture) the tissue of interest[16]. Limitations of LMD are that it is not conducive to very HTP approaches (although the transcription profiles for tail tips, as described in this protocol, were robust with ~70 samples), certain samples might be difficult to dissect out, and cuts are limited to the precision of the laser and what can be visualized in the microscope.

The purpose of the present protocol is to describe how LMD, followed by single-tissue RNA-Seq, can be used to obtain stage- and tissue-specific transcriptome data from nematodes. Specifically, it demonstrates LMD for isolating tail tips from fourth-stage larvae (L4) of *C. elegans*. However, this method can be adapted to other tissues and, of course, different species.

In *C. elegans*, there are 4 cells that make the tail tip in both males and hermaphrodites. During the L4 stage in males—but not in hermaphrodites—the tail tip cells change their shape and migrate anteriorly and inwardly. This process also occurs in some but not all

other rhabditid nematode species. Therefore, the tail tip is a good model for the evolution of sexual dimorphic morphogenesis. Because of its position, the tail tip is also easy to isolate by LMD.

To obtain transcriptome profiles from tail tips, the present protocol uses CEL-Seq2, an RNA-seq method developed for single cells[17, 18]. This method has several advantages for LMD-derived tissues. CEL-Seq2 is highly sensitive and efficient, using unique molecular identifiers (UMIs) to allow straightforward quantification of mRNA reads, *in vitro* transcription to ensure linear amplification, and barcoding that allows multiplexing of individual tissue samples. The only limitation of CEL-Seq2 is that recovered reads are biased to the 3' end of mRNAs, and most isoforms thus cannot be distinguished.

Protocol

1. Worm synchronization

NOTE: Two methods are described below to synchronize the development of *C. elegans* and other rhabditid species.

1. Synchronize by first larval stage (L1) arrest following alkaline hypochlorite (bleach) treatment.

   NOTE: This method was described previously in detail[19]. This method relies on two features of *C. elegans* that are also true for several other rhabditid species: (1) The eggshell is resistant to bleach, whereas the cuticle surrounding adult and larval worms is not. (2) First-stage larvae arrest development when kept without food[20].

   1. Treat gravid hermaphrodites (or females) with a diluted bleach solution to break up their cuticle and release embryos.
   2. Remove the embryos from the bleach and keep them without food until all L1 have hatched.
   3. Place the arrested L1 on food, where all resume development at about the same time.

      NOTE: Exit from L1 arrest can occur within one hour.

2. Synchronization with the "hatch-off" method (used here; Figure 1 top):

   NOTE: The hatch-off method allows for tight synchronization without disruption of development (L1 arrest affects the development even of later stages[21]).

   The protocol is adapted from Pepper et al.[22]. The objective of this method is to collect L1 that have hatched over a specific period from a plate that only contains embryos.

   1. **Pick mothers:** On the evening before performing the hatch-off, pick ~30 gravid hermaphrodites onto a plate seeded with *E. coli* OP50.
   2. Incubate at 25 °C for egg-laying overnight.

NOTE: Choose a plate without cracks or bubbles where worms could get stuck. Avoid plates with a very thick bacterial lawn as it will be difficult to remove all worms later. If working with a temperature-sensitive strain, adjust the egg-laying time to account for longer embryogenesis. Pick mothers at the maximum egg-laying stage.

3. **Remove mothers and larvae:** the next morning, under the dissecting microscope (20x magnification), gently pipette 1-2 mL of M9 buffer against the wall of the plate without squirting; swirl the plate to dislodge the worms.

4. Remove and discard all liquid and worms by placing the pipette tip against the wall of the plate at the edge of the agarto avoid poking holes. Check that no worms (and only eggs/embryos) are left on the plate, especially not L1s; otherwise, repeat the wash.

5. Place the plate at 25 °C for 1 h and wait for some L1s to hatch.

6. **Collect newly hatched L1s:** carefully drop 1 mL M9 buffer onto the agar. Swirl the plate to dislodge L1 but not embryos. Gently pipette buffer and worms into a 1 mL centrifuge tube.

7. Centrifuge the tube for 1 min at ~18,000 × *g*. Remove the supernatant.

8. Pipette L1 directly onto the bacterial lawn of a seeded plate. Verify under the dissection microscope that no adult worms or embryos are present.

9. Keep worms at 25 °C until they have developed to the desired stage.

   NOTE: If conditions are optimal, two more batches of L1 can be collected from the same plate. Inspect the initial plate to make sure that no L1 are present. If necessary, wash again. Repeat steps 1.2.5-1.2.9.

10. **Check developmental timing.** Before proceeding to the downstream application, inspect some worms under a compound microscope at 400x magnification to confirm they reached the desired developmental stage, here L3.

    NOTE: Migration distance of distal tip cells or linker cells can be used as a guide, in addition to vulva development. For vulva development, Mock et al.[23] provide a useful guide, although timing in that study was determined at 20 °C. At 25 °C, wild-type *C. elegans* will undergo the L3-L4 molt 24 h after hatching.

2. Collecting L4 males and hermaphrodites and fixation

1. Prepare RNAse-free, cold (−20 °C), 70% methanol before fixation.

2. Under a dissection microscope at 30-50x magnification, begin picking males and hermaphrodites from the synchronization plates onto separate unseeded plates

as soon as the sexes can be distinguished (~21 h after hatching, Figure 2), and continue picking for 1-2 h or until 200 animals are collected.

3. Keep the worms at 25 °C until they reach the desired stage for the experiment.
4. Wash the worms off the plate with 1-2 mL of M9 buffer using a pipette tip prewashed with M9 buffer containing 0.01% detergent (to prevent worms from sticking to the tip).
5. Transfer the worms to a 1 mL centrifuge tube.
6. Spin for 1 min at 21,000 × *g* to pellet the worms. Remove the supernatant.
7. Add 1 mL of M9 buffer and mix to break up the pellet.
8. Spin for 1 min at 21,000 × *g* to pellet the worms. Remove the supernatant.
9. Repeat the wash.
10. Add 1 mL of ice-cold 70% methanol and mix well.
11. Spin for 1 min at 21,000 × *g* to pellet the worms. Remove the supernatant.
12. Repeat steps 2.10 and 2.11.
13. Add 500 µL of 70% methanol, mix, and store at 4 °C for 1 h to overnight.

3. Laser microdissection

NOTE: From here on, use RNase-free reagents and consumables; use filter tips.

1. If the CEL-Seq2 method is used to process the samples, prepare a master mix for each CEL-Seq2 primer (Supplemental Table S1): pipette 2 µL of CEL-Seq2 primer, 1 µL of 10 mM dNTP, and 9 µL of 1% β-Mercaptoethanol (in RNase-free water) into a labeled 200 µL tube.
2. Mounting on slide
    1. Under a dissection microscope, pipette 20 µL of the fixed worms (20-40 worms from step 2.13) onto the matte side of a polyethylene naphthalate (PEN)-membrane glass slide (where the membrane is).
    2. Wait for the methanol to evaporate. Use a slide warmer to speed up the evaporation.

        NOTE: Additional drops of methanol can be applied, and a pipette tip used to spread the worms out if they begin to clump as they dry. When worms are in clumps, they can be difficult to dissect.
3. Setting up the microscope

    NOTE: The following protocol is specific to the instrument listed in the Table of Materials. It needs to be adjusted if a different LMD microscope is used.

    1. Place a desktop humidifier behind the stage on the side of the LMD microscope. Ensure that the vapor is blowing directly onto the stage.

NOTE: The humidifier is helpful to reduce static electricity, which otherwise can prevent the small membrane section from falling into the tube cap.

2. Turn the key for laser power.
3. Turn stage control power on.
4. Turn the microscope control box on.
5. Open **Laser Microdissection** software.
6. Remove the plastic shield over the stage.
7. Click the **unload** button with the upward arrow for loading the membrane slides.
8. Make sure the slide is completely dry, flip so that the membrane is facing down.
9. Insert the slide and click **continue** in the **change specimen** window.
10. Replace the plastic shield.
11. On the bottom of the screen, choose which slide holder contains the slide.
12. To load the tubes, click the **unload** button with the downward arrow.
13. Pull the tray out and remove the tube block.

    NOTE: The tube block used for this experiment is for 500 µL PCR tubes.
14. Insert the tube caps of 500 µL PCR tubes into the holder and fold the tube under.
15. Return the block to the tray and slide the tray back into the microscope stage.
16. In the **change collector device** popup window, select **PCR tubes** and click **ok**.
17. Click on the empty tube location on the bottom left of the screen under **collector device tube caps**.
18. In the **Microscope control** panel, select **TL-BF** for transmitted light brightfield illumination.

4. Cutting

NOTE: This protocol is specific for the instrument listed in the Table of Materials.

1. Using the 2.5x lens, adjust the focus until the worms and the structure of the membrane are visible.

2. Switch to the 20x lens.

3. Move the stage to a region without worms. Adjust the focus such that the bubble-like structures in the membrane have a yellowish color (Figure 3A,B) to focus the laser on the correct focal plane.

4. Set the laser parameters; for tail tips, start with **Power 45, aperture 30,** and **speed 20**.

5. In the **Laser Control** panel, select **calibrate**. Follow the instructions.

   NOTE: The instrument will perform this step automatically. It will ensure that a shape drawn with the mouse on the screen is identical to the shape cut out by the laser.

6. On the bottom of the screen at **collector device tube caps**, click on **position A**.

7. On the right side of the screen, select **single shape | Draw + Cut**. On the left side of the screen, select **PtoP**.

8. Draw a line.

9. Click **Start Cut** so that the laser cuts through the membrane.

   NOTE: It may also etch a line into the glass.

10. If this test-cut looks good (the membrane is cut, edges of cut look smooth), continue with the next step. Otherwise, adjust the focus and cut another line.

11. Find a worm. Switch to **Move + Cut** and use the mouse to cut through the tail.

    NOTE: If the laser does not cut through the tail, adjust the focus and increase the laser power. For thicker tissues, laser power may have to be set to 60.

12. Save the parameters: **File tab | Save Application Configuration;** for later retrieval, **Restore Application Configuration**.

13. To collect the sample, switch to the **Draw + Cut** setting with the **PtoP** function and draw a shape to complete the cut of a membrane section (Figure 3C).

    NOTE: Larger membrane sections and sections shaped like rectangles or triangles rather than circles or ovals are easier to locate in the collector tube cap.

14. Select the next tube at **Collector Device Tube Cap** on the bottom of the screen and cut the next tail tip.

15. Once four tails are cut, unload the tube rack (click **Unload** with downward arrow) and find the membrane sections under a dissecting microscope (Figure 3D).

    NOTE: The sections may be located in the middle of the tube cap or stuck to the side of the cap.

16. Continue with the downstream application. For CEL-Seq2, pipette 1.2 µL of a CEL-Seq2 primer master mix (from step 3.1) directly on top of the sample.

17. Close the tube, label with primer number, and immediately place the tube cap directly on a piece of dry ice to flash-freeze the sample and prevent RNA degradation.

18. Load more tubes, return the tube block to the stage, and cut more samples. Add a different CEL-Seq2 primer mix to each tail tip.

19. Store all tubes at −70 °C.

4. Single-tail RNA sequencing with CEL-Seq2

NOTE: For full details about the CEL-Seq2 protocol, see Yanai and Hashimshony[18].

1. Clean the lab bench area with RNase decontamination solution to prevent RNA degradation.

2. Prepare master mixes and keep them on ice.

    1. Prepare the reverse-transcription master mix: 0.4 µL of first strand buffer, 0.1 µL of 0.1M DTT, 0.1 µL of RNase inhibitor, and 0.1 µL of reverse transcriptase per sample.

    2. Prepare the second strand reaction master mix: 7 µL of water, 2.31 µL of second strand buffer, 0.23 µL of dNTP, 0.08 µL of *E. coil* ligase, 0.3 µL of *E. coli* DNA polymerase, 0.08 µL of RNaseH per sample.

3. Breaking open cells and annealing with primers (see Supplemental Table S1 for the full list of primers):

    1. Program the thermocycler and its lid to 65 °C.

    2. Retrieve the samples from −70 °C and incubate them in the thermocycler for 2.5 min.

    3. Spin at 21,000 × *g* for 30-40 s.

    4. Incubate at 65 °C for 2.5 min.

    5. Move them immediately to ice.

    6. Spin at 21,000 × *g* for 30-40 s and return them to ice.

4. Converting RNA to cDNA:

    1. Add 0.8 µL of the reverse transcription mix to each tail tip.

2. Incubate at 42 °C for 1 h.
3. Heat-inactivate at 70 °C For 10 min.
4. Move it immediately to ice.
5. Add 10 µL of the second strand mix to each tail tip.
6. Flick the samples.
7. Spin at 21,000 × *g* for 30-40 s.
8. Incubate at 16 °C for 2 h.

5. cDNA cleanup:
    1. Prewarm the DNA cleanup beads to room temperature.
    2. Pool up to 40 samples in a 1.5 mL centrifuge tube (up to 480 µL).
    3. Mix the beads until they are well dispersed and add 20 µL of beads and 100 µL of bead binding buffer for every 100 µL of the pooled sample (for 480 µL of sample add 480 µL of bead buffer and 96 µL of beads to a final volume up to 1,056 µL). Mix well by pipetting.
    4. Incubate at room temperature for 15 min.
    5. Place on a magnetic stand for at least 5 min until the liquid appears clear.
    6. Remove and discard all but 20 µL of the supernatant.
    7. Add 200 µL of freshly prepared 80% ethanol.
    8. Incubate for at least 30 s, remove the supernatant by pipetting it off without disturbing the beads. Discard the supernatant.
    9. Repeat steps 4.5.7 and 4.5.8 once.
    10. Air-dry the beads for 15 min or until they are completely dry.
    11. Resuspend the beads (~6.4 µL) with 6.4 µL of water. Mix thoroughly by pipetting the entire volume up and down ten times.
    12. Incubate at room temperature for 2 min.
    13. Go straight to *in vitro* transcription (IVT).

6. *In vitro* transcription and fragmentation:
    1. To the tube containing 6.4 µL of sample and the beads, add the following mix (9.6 µL total): 1.6 µL of 10x T7 Buffer, 1.6 µL of ATP, 1.6 µL of UTP, 1.6 µL of CTP, and 1.6 µL of GTP (each dNTP at 75 mM concentration) 1.6 µLof T7 enzyme.
    2. Incubate for 13 h at 37 °C with a 4 °C hold.
    3. Add 6 µL of exonuclease solution (final volume should be 22 µL).

4. Incubate for 15 min at 37 °C.
5. Place the tube back on ice and add 5.5 µL of fragmentation buffer (0.25 × reaction volume).
6. Incubate for 3 min at 94 °C.
7. Immediately move the tube to ice and add 2.75 µL of fragmentation stop buffer (0.5 × volume of fragmentation buffer added).
8. Remove the beads by placing the tube on the magnetic stand for at least 5 min until the liquid appears clear.
9. Transfer the supernatant to a new tube.

7. Amplified RNA (aRNA) cleanup:
    1. Prewarm the RNA cleanup beads to room temperature.
    2. Mix the beads until they are well dispersed.
    3. Pipette 55 µL of beads (1.8 × reaction volume).
    4. Incubate them at room temperature for 10 min.
    5. Place the tube on the magnetic stand for at least 5 min until the liquid appears clear.
    6. Remove and discard 80 µL of the supernatant.
    7. Add 200 µL of freshly prepared 70% ethanol.
    8. Incubate for at least 30 s, remove the supernatant by pipetting without disturbing the beads. Discard the supernatant.
    9. Repeat the ethanol wash two more times.
    10. Air-dry the beads for 15 min or until they are completely dry.
    11. Resuspend the beads with 7 µL of water. Pipette the entire volume up and down 10 times to mix thoroughly.
    12. Incubate at room temperature for 2 min.
    13. Place the tube with the beads on the magnetic stand for 5 min until the liquid appears clear.
    14. Transfer the supernatant to a new tube.

        NOTE: Stopping point: samples can be kept at −70 °C.

8. Optional: Check the aRNA amount and quality with an automated electrophoresis system following the manufacturer’s protocol.

9. Library preparation:
    1. To 5 µL of RNA, add 1 µL of 100 µM random hexamer RT primer (see the Table of Materials) and 0. 5 µL of 10 mM dNTP.

2. Incubate at 65 °C for 5 min.
3. Add 4 µL of the following mix at room temperature: 2 µL of First Strand buffer, 1 µL of 0.1 M DDT, 0.5 µL of RNase inhibitor, 0.5 µL of reverse transcriptase.
4. Incubate at 25 °C for 10 min.
5. Incubate at 42 °C for 1 h (in a hybridization oven or a preheated thermal cycler with the lid set to 50 °C).
6. Incubate at 70 °C for 10 min.
7. Transfer 5 µL to a new tube (keep the rest of the reaction at −20 °C). Add 5.5 µL of ultrapure water, 1 µL of RNA PCR Primer (RP1), 1 µL of indexed RNA PCR Primer (RPIX), and 12.5 µL of PCR mix.
8. Use the following program on the thermocycler: 30 s at 98 °C, 11 cycles of: (10 s at 98 °C, 30 s at 60 °C, 30 s at 72 °C), 10 min at 72 °C, hold at 4 °C.

   NOTE: Stopping point: samples can be kept at −20 °C.

10. Library cleanup:
    1. Prewarm the DNA cleanup beads to room temperature.
    2. Mix the beads until they are well dispersed.
    3. Add 25 µL of the beads to the PCR reaction. Mix well by pipetting.
    4. Incubate at room temperature for 15 min.
    5. Place the tube on the magnetic stand for at least 5 min until the liquid appears clear.
    6. Remove and discard 45 µL of the supernatant.
    7. Add 200 µL of freshly prepared 80% ethanol.
    8. Incubate for at least 30 s, remove, and discard the supernatant without disturbing the beads.
    9. Repeat the ethanol wash once.
    10. Air-dry beads for 15 min or until they are completely dry.
    11. Resuspend them with 25 µL of water. Mix well by pipetting.
    12. Incubate at room temperature for 2 min.
    13. Place the tube on the magnetic stand for 5 min until the liquid appears clear.
    14. Transfer 25 µL of supernatant to a new tube.
    15. Repeat steps 4.10.2-4.10.10 once.
    16. Resuspend with 10.5 µL of water. Mix well by pipetting.

17. Incubate at room temperature for 2 min.
18. Place the tube on the magnetic stand for 5 min until the liquid appears clear.
19. Transfer 10 µL of the supernatant to a new tube and store at −20 °C.

11. Assess the library quality and quantity according to the requirement of the sequencing facility.

Representative Results

Following laser capture microdissection, individual tail tips of males and hermaphrodites at 4 time points (L3 22 h after hatch; L4 24, 26, and 28 h after hatch) were prepared for RNA sequencing using the CEL-Seq2 protocol. CEL-Seq2 primers contain unique barcodes that enable sequencing reads from a particular sample (in this case an individual tail tip) to be identified bioinformatically. Sequencing data were generated with this method for a total of 557 tail tips (266 hermaphrodites and 291 males across 4 developmental time points, 59-78 tails per sex and time point). CEL-Seq2 barcodes were recovered for 97% (i.e., 543) of these tail tips (Supplemental Table S2). For most libraries, the recovery rate was 99-100%; however, it was 88% for one male time point. It is worth noting that about half of the male tail tips from the 22, 24, and 28 h time points were stored at −80 °C for ~4 months due to COVID-19-related delays. This demonstrates that, while it is ideal to prepare sequencing libraries shortly after sampling, it is possible to store dissected samples for a longer time before library preparation.

The CEL-Seq2 primers also add a UMI to each mRNA transcript. This enables PCR duplicate removal and precise quantification of gene expression in the sample. The number of UMIs varied dramatically across tail tips (Figure 4; male mean = 92,560; male min. = 155; male max. = 1,183,998; hermaphrodites mean = 67,597; hermaphrodites min. = 132; hermaphrodites max. = 630,427). For UMI counts per tail tip, see Supplemental Table S3. Due to the low amount of input RNA for single-cell library preparation, single-cell sequencing data are known to have a large amount of technical noise. Hence, it is recommended to filter samples that have very low or very high UMI counts before analysis[24].

The R package powsimR[25] was used to assess the statistical power and sample size requirements for reliably detecting differentially expressed (DE) genes in single-cell or bulk RNA-seq experiments. Parameters for the simulations were based on a sequencing dataset of 70 individual male tail tips (at the 24 h time point) obtained with the method described here. Expected log-fold changes were based on results from a separate RNA-seq experiment that pooled 80-100 tail tips. The simulations determined that the single-tail-tip data have sufficient power (True Positive Rate = TPR) to detect DE genes, except for genes that have a very low mean expression value (top of Figure 5; dashed line represents 80% TPR). Adding more simulated tail tips per time point increased the power somewhat for lowly expressed genes. A similar pattern is seen for the False Discovery Rate (FDR). FDR is high (>0.10) for the lowly expressed genes; however, for more highly expressed genes, it falls at or below the nominal 0.10 cutoff (dashed line for FDR in the bottom of Figure 5). In summary, increasing

the number of tails sampled per time point above 70 would do little to lower the FDR or increase power. However, 70 tail tips provide a much lower FDR and stronger power than 30 tail tips.

Discussion

Critical steps of the method

If performed correctly, the method described here will obtain robust RNA profiles with a relatively small number of laser-dissected samples (70 tail tips in this example). However, for samples from developing animals, tight synchronization is critical to reducing the variability between samples. For this reason, the protocol recommends the hatch-off method for worm-synchronization. Here, the researcher can determine and precisely control the age difference between individuals (1 h in the present protocol). In addition, the hatch-off method is applicable to any species, even if the embryos are sensitive to bleach, L1 do not arrest, or recovery from L1 arrest is variable. For a successful synchronization by hatch-off, the washing steps are crucial: all adults and larvae must be removed at the beginning of the hatching period, and no embryos should be washed off along with the newly hatched L1 at the end of the hatching period. This only succeeds if the agar surface of the plate is undamaged by cracks, holes, or bubbles, the bacterial lawn on the plate is fresh and not too thick, and the liquid is added and agitated only very gently.

If data are to be obtained separately for males and hermaphrodites/females, reliable identification of the sexes is also important. Distinguishing L3 larvae by sex (see Figure 2) requires experience. It is recommended to practice picking L3 males and hermaphrodites/females and check the success rate after the animals have developed into adults and the sexes are easily distinguished. After single-tissue RNA-Seq, the outliers can also be identified by principal component analysis and removed, if necessary.

For successful recovery of laser-cut samples, it is important to reduce static electricity as much as possible. Charged PEN-membrane pieces often do not drop into the tube cap but stick to the slide or any other part of the microscope. One remedy is raising the humidity in the room and specifically around the microscope by placing a small humidifier next to the stage. Additionally, the membrane slides can be treated with UV light. To do this, incubate slides in a UV-C (254 nm) crosslink chamber and deliver at least 1 joule of energy, or expose the slides to the UV light in a laminar air flow bench for 30 min.

Since the goal of the protocol is RNA-Seq, keeping an RNase-free working environment is critical. Beginning with the fixation solution, reagents, containers, and consumables should be RNase-free, the work surface should be decontaminated, and the researchers should wear clean gloves. The dissected samples should be frozen as soon as possible and kept at −70 °C until further processing. It is also recommended to use low-retention tubes and tips for the CEL-Seq2 part of the protocol.

The present article provides only a basic outline of the CEL-Seq2 protocol, which was previously published by its developers with helpful notes and tips[17, 18]. It is recommended that these publications are consulted before using the CEL-Seq2 method.

The LMD-RNA-Seq data can be validated by single-molecule-RNA fluorescent *in-situ* hybridization (smRNA FISH)[26, 27, 28]. smRNA FISH has been extensively used in *C. elegans* and is amenable to other nematode species, different from immunostaining with existing antibodies (which may not crossreact) or the introduction of transcriptional reporters through transgenesis. The latter works well in *C. elegans* and some related *Caenorhabditis* species[29], but transgenesis can be more challenging in other nematode species[30, 31].

Limitations of the method

The method described here works very well for collecting tail tips, a thin tissue at the end of a worm. Dissecting tissues in the thicker middle of older larvae or adults is more challenging. The software of the instrument used here includes a setting for multiple cuts placed at subsequently deeper levels in the tissue. This setting can be used for cutting thicker areas of the animal. Because the worms need to be fixed before dissection, structural details are difficult to see, which prevents the precise dissection of specific small structures. As mentioned above, LMD-RNA-Seq is not an HTP method. However, 50-70 samples can be dissected in one afternoon.

Significance of the method with respect to existing/alternative methods

LMD-RNA-Seq can be used in any species even if no transgenic tools are available. Other methods rely on FACS sorting of fluorescently labeled cells[8, 9, 32] or isolation of labeled nuclei[33, 34] and thus require transgenic animals. Methods that dissociate and isolate cells in postembryonic *C. elegans* tend to miss the tissues at the two ends of the worm (Dylan Rahe, personal communication). These caveats are overcome by combining single-cell RNA-Seq with cryosectioning of entire worms (RNA tomography)[35]. This method was used to compare spatial gene expression between *C. elegans* and another rhabditid nematode, *Pristionchus pacificus*[36]. Alternatively, one can experiment with formalin-fixed paraffin-embedded (FFPE) worms. Such material has been successfully used for RNA-Seq following LMD of mammalian tissue samples[37]. However, RNA tomography and LMD of FFPE worms are limited to the analysis of only a handful of animals. They are, therefore, not as well suited for the study of dynamic gene expression in developing tissues as LMD-RNA-Seq.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by NIH (R01GM141395) and NSF (1656736) grants to DF and NIH fellowship (F32GM136170) to AW. Figure 1 was created with the help of BioRender.com.

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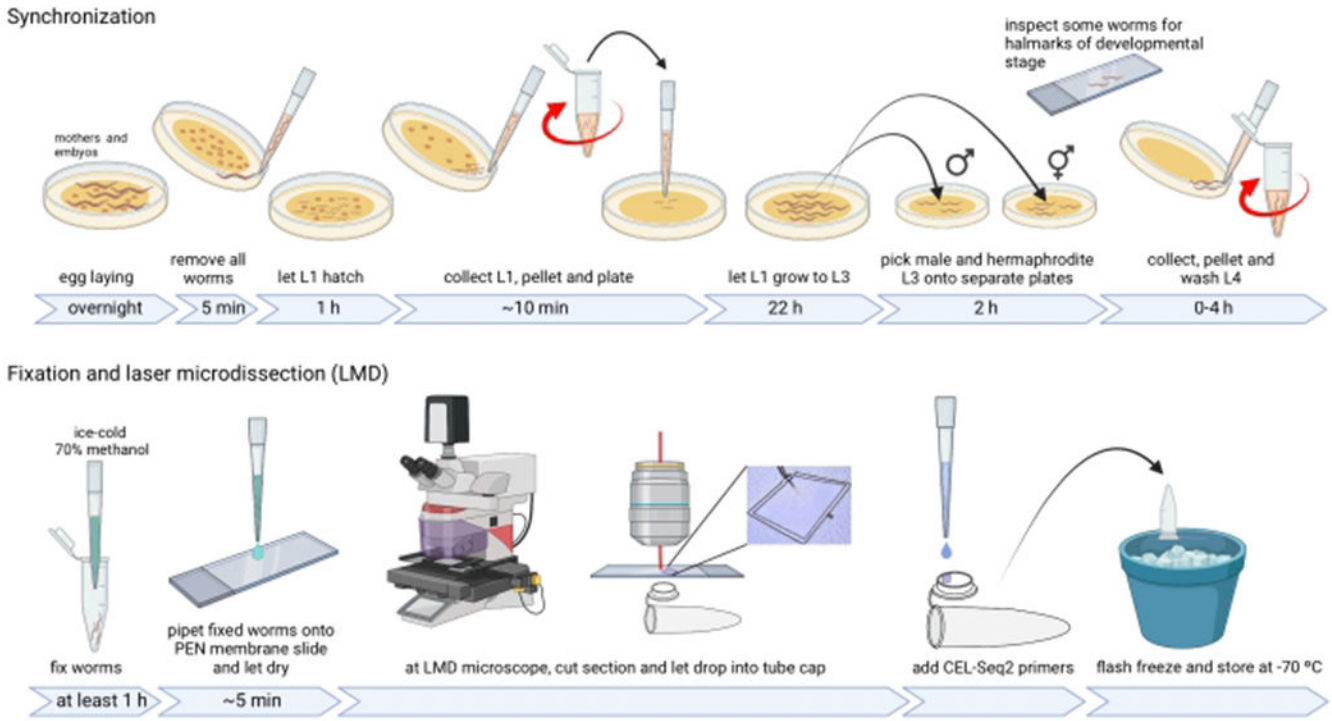

Figure 1: Procedure overview for synchronization of *Caenorhabditis elegans* with the hatch-off method and laser microdissection of tail tips.

Abbreviations: L1-L4 = larval stages 1 to 4; PEN = polyethylene naphthalate; LMD = laser microdissection. Please click here to view a larger version of this figure.

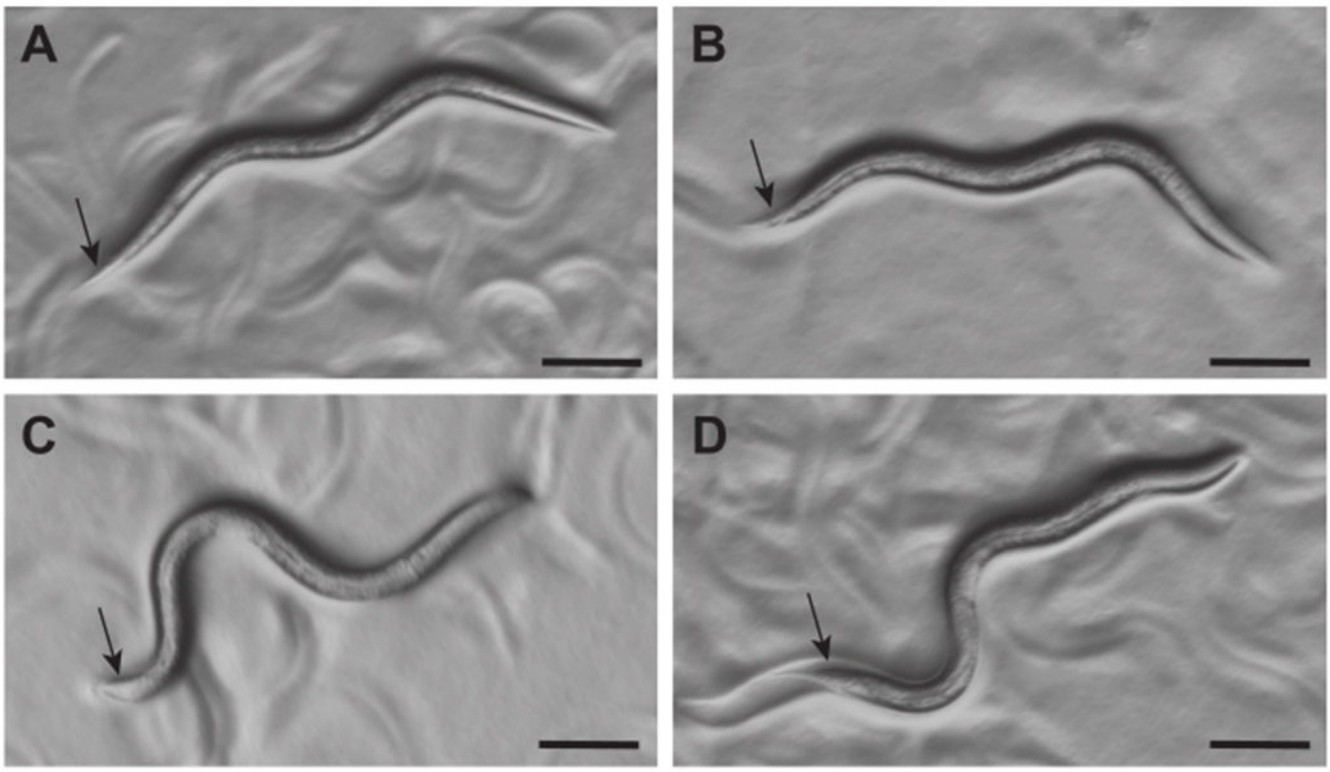

Figure 2: Appearance of *C. elegans* L3 hermaphrodites and males under a dissection microscope. Hermaphrodites (**A, B**) and males (**C, D**) at 21-23 h after hatch can be distinguished under a dissection microscope (~50x magnification) by the morphology of their tails (arrows). The tail of hermaphrodites is narrow, while that of males is swollen and appears clear. Scale bars = 0.1 mm. Please click here to view a larger version of this figure.

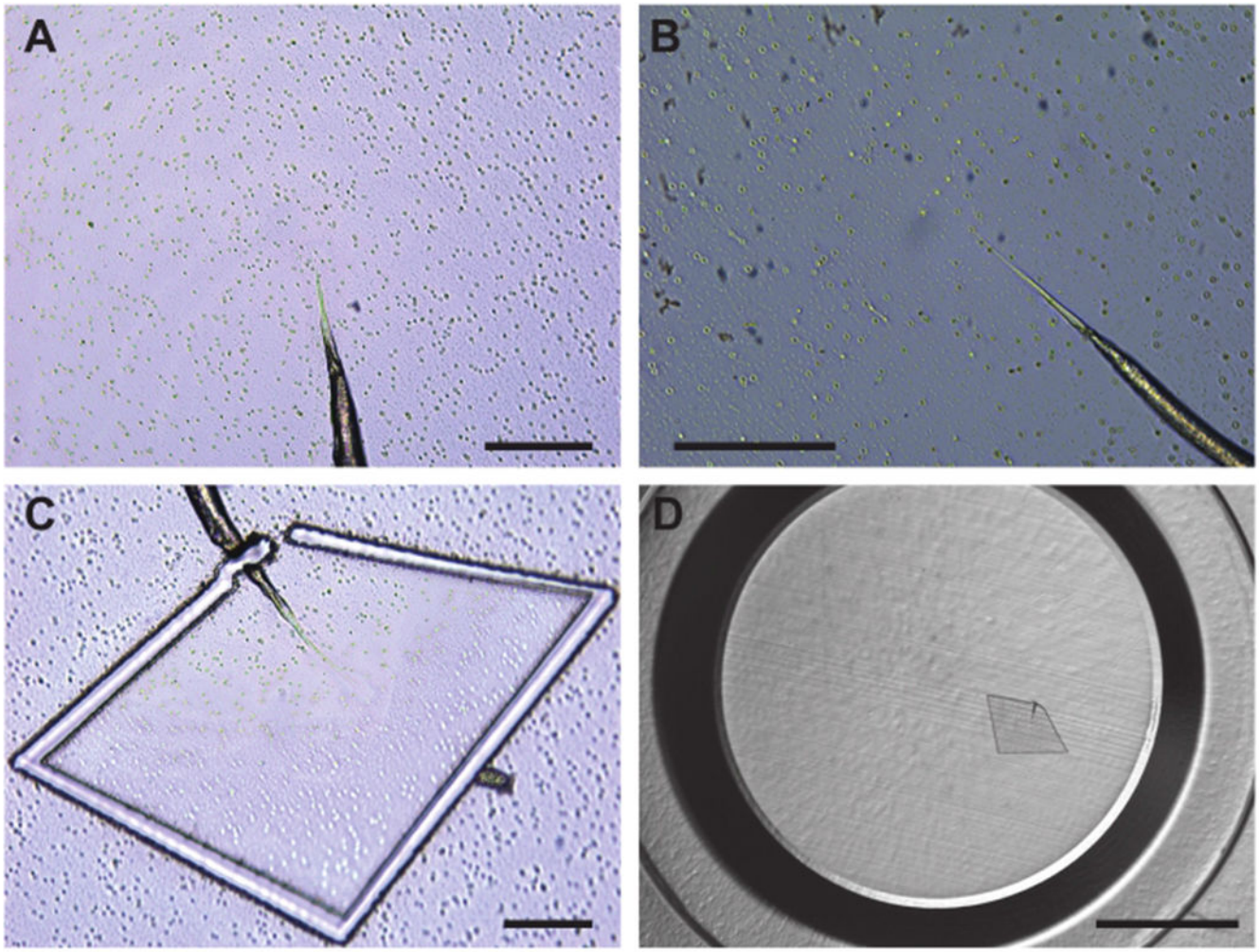

Figure 3: Appearance of the PEN membrane slide structure and worm tail.
Focus is correct for dissection of the tissue viewed with the 20x (**A**) and 40x (**B**) lens at the microscope. (**C**) Dissected tail and partially cut out PEN membrane. After closing the gap in the cut, the membrane piece will drop into the tube cap below the slide. (**D**) Tube cap with a PEN membrane section containing a dissected tail tip. Scale bars = 0.1 mm (**A-C**), 1 mm (**D**). Please click here to view a larger version of this figure.

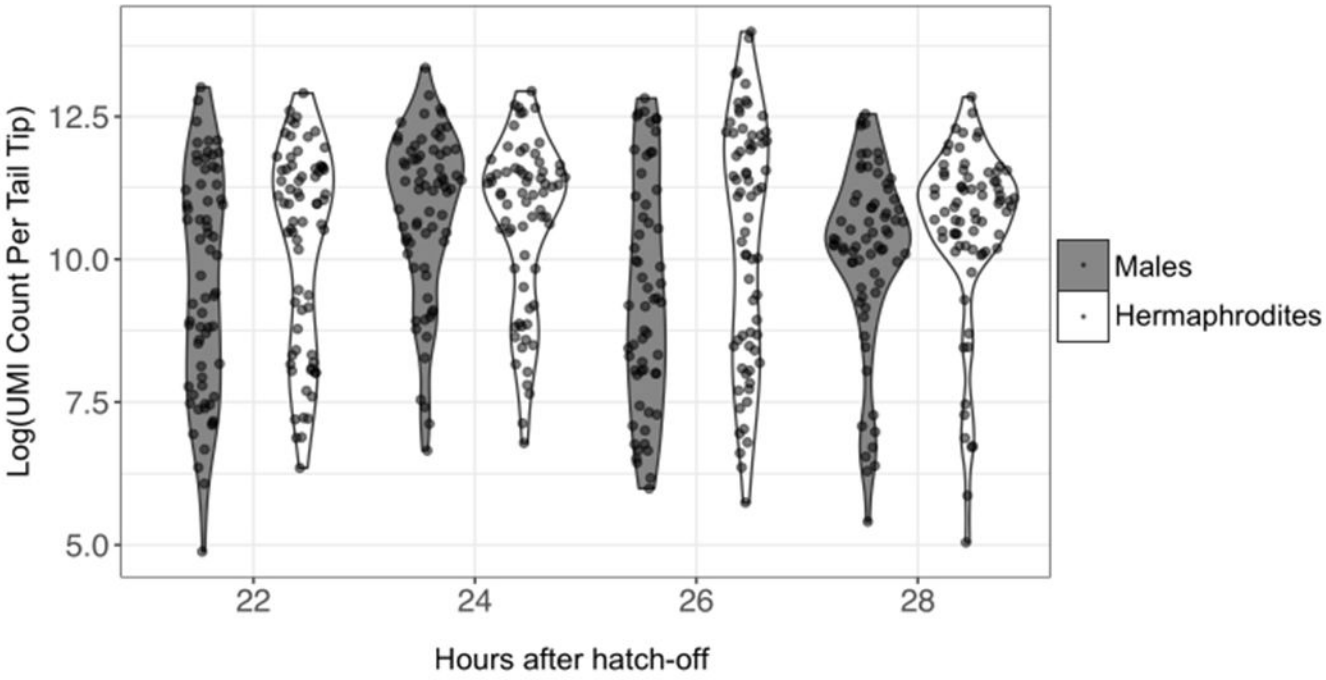

Figure 4: Natural log-transformed UMI counts per individual tail tip for different time points and sexes.

RNA from individual tails was prepared for sequencing using the CEL-Seq2 method; 557 tails were sequenced in total, with 59-78 tails per sex and time point. Extremely low and high UMI outliers would be removed from the data before analysis. Abbreviation: UMI = unique molecular identifier. Please click here to view a larger version of this figure.

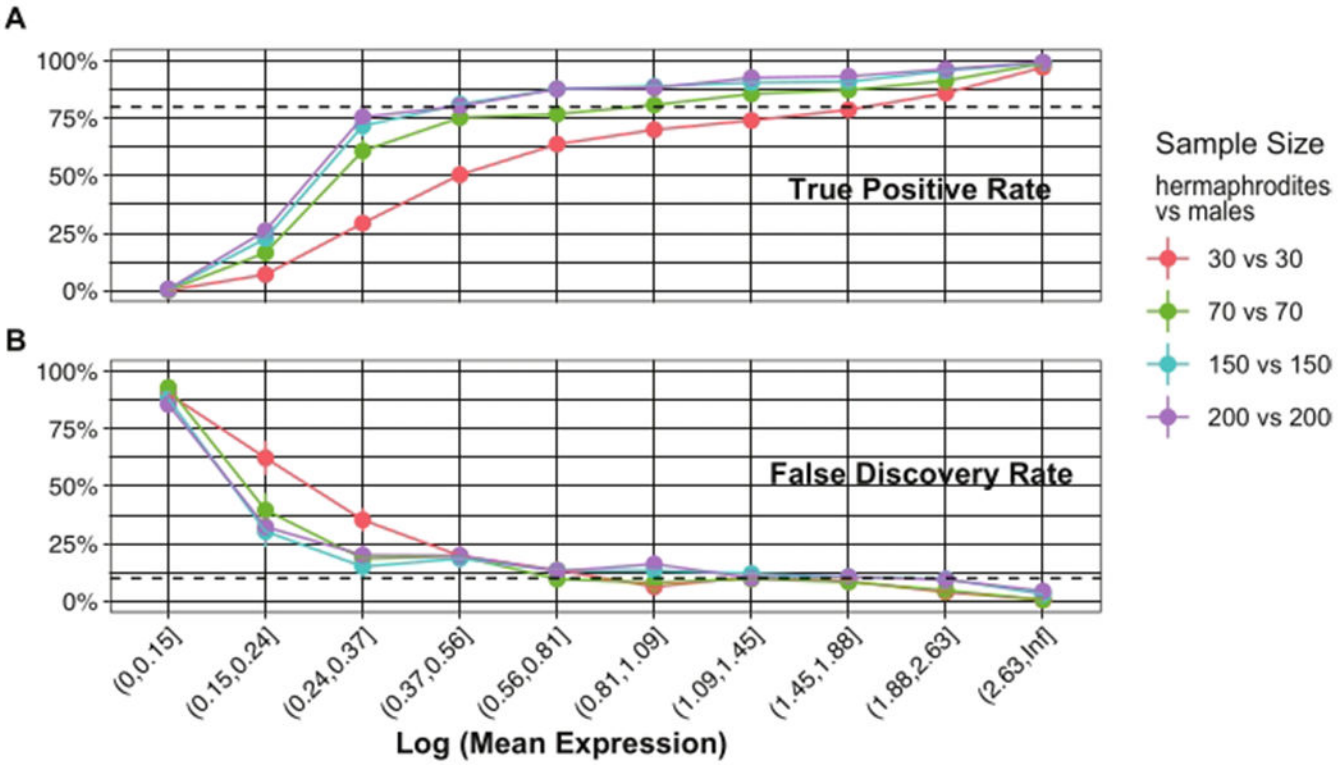

Figure 5: Results of an *a posteriori* power analysis using simulations with powsimR.
The powsimR software determines the number of independent samples required to detect DE genes at various expression levels. Genes are binned by mean expression transformed as the natural log of UMI counts. (**A**) Power (TPR) to detect DE genes between two conditions (here, male vs hermaphrodite) for four different simulations (different colored graphs) incorporating different sample sizes (numbers of individual tail-tips) per condition. Dashed line indicates 80% TPR. (**B**) FDR in the same four simulations as in (**A**), dashed line indicating 10% FDR. The graphs show that a sample size of 70 tail tips (green) per condition is sufficient for detecting DE genes, except for genes with very low expression levels. That is, the power and false discovery rate for such genes cannot be greatly improved by increasing the sample size beyond 70. Abbreviations: DE = differentially expressed; UMI = unique molecular identifier; TPR = true positive rate; FDR = false discovery rate. Please click here to view a larger version of this figure.

Materials

Name	Company	Catalog Number	Comments
0.5 µM PEN membrane glass slides RNase free	Leica	11600288	for LMD
500 µL PCR tubes (nuclease-free)	Axygen	732-0675	to cut the tail tips into
Compound microscope with 40x objective and DIC	any		to check age of worms
Desktop humidifier	any		
Dissection microscope with transmitted light base	any		for all worm work
glass pasteur pipets	any		handle of worm pick
glass slides and coverslips	any		to check age of worms
LMD6 microdissection system	Leica	multiple	to cut tail tips
LoBind tubes 0.5 mL	Eppendorf	22431005	
M9 Buffer			Recipe in WormBook
Methanol 99.8%	Sigma	322415	to fix worms
NGM growth medium	US Biological	N1000	Buffers and salts need to be added: Recipe in WormBook
P10 pipette variablle volume	e.g. Gilson		
P1000 pipette variable volume	e.g. Gilson		
P2 pipette variable volume	e.g. Gilson		
Pipette tips 1,000 µL	any		
Pipette tips 1-10 µL filtered	any		
platinum iridium wire	Tritech	PT-9010	to make worm pick
sterile and nuclease-free 1 mL centrfuge tubes	any		
Tween 20	Sigma	P9416	Add a very small amount to M9 buffer to prevent worms from sticking to the pipet tips
vented 6 mm plastic Petri dishes	any		
For CEL-Seq2			
4200 TapeStation System with reagents for high-sensitivity RNA and DNA detection	Aligent		automated electrophoresis system
AMPure XP beads	Beckman Coulter	A63880	DNA cleanup beads
Bead binding buffer 20% PEG8000, 2.5 M NaCl			
CEL-Seq2 primers (see Table S1)	Sigma Genosys	6335000020	Thermal cycler with programmable lid and block for 200 µl tubes.
	Mastercycler Nexus GX2 Eppendorf		
DNA Polymerase I (*E. coli*)	Invitrogen	18052-025	
dNTP mix 10 mM	any		
E. coli DNA ligase	Invitrogen	18052-019	
Ethanol			
ExoSAP-IT For PCR Product Clean-Up	Affymetrix	78200	exonuclease solution
MEGAscript T7 Transcription Kit	Ambion	AM1334	For step 4.6.1

Name	Company	Catalog Number	Comments
Nuclease-free water	any		
Phusion High-Fidelity PCR Master Mix with HF Buffer	NEB	M0531	PCR mix step 4.9.7
random hexamer RT primer GCCTTGGCACCCGAGAATTCCANNNNNN	IDT		a primer with 6 nucleotides that are random
RNA Fragmentation buffer	NEB	E6150S	
RNA Fragmentation stop buffer	NEB	E6150S	
RNA PCR Index Primers (RPI1–RPI48)	Illumina, NEB, or IDT		RPIX in protocol step 4.9.7, sequences available from Illumina
RNAClean XP beads	Beckman Coulter	A63987	
RNase AWAY Surface Decontaminant	Thermo Scientific	7000TS1	or any other similar product
RNaseH (*E. coli*)	Invitrogen	18021-071	
RNaseOUT Recombinant Ribonuclease Inhibitor	Invitrogen	10777-019	
Second strand buffer	Invitrogen	10812-014	
Superscripit II	Invitrogen	18064-014	reverse transcriptase