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L1 stage C. elegans dissociation for FACS isolation and RNA-seq analysis of intestine-specific cells V.2

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We use this protocol and it's working

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Abstract

This protocol is for generating a single cell suspension suitable for isolation of intestine-specific cells through Fluorescence Activated Cell Sorting (FACS) from L1 stage *C. elegans*. This protocol utilizes treatment with SDS-DTT solution and Pronase E to disrupt the cuticle. Worms are mechanically homogenized with a dounce homogenizer.

Materials

Strains:

- FACS control *C. elegans* strain, i.e. N2
- FACS sorting *C. elegans* strain, i.e. JM149 *cals71*[elt-2p::GFP::HIS-2B::unc-54 3'UTR + rol-6(su1006)]

Reagents:

L15-10 solution

- Leibovitz's L-15 Medium (Thermo 21083027)
- Fetal Bovine Serum (heat inactivated) (Thermo 10438026)
- 100X Penicillin Streptomycin solution (Thermo 15140148)
- Sucrose powder

Stock solutions for egg buffer

- 2M NaCl
- 2M KCl
- 1M CaCl2
- 1M MgCl2
- 1M HEPES pH 7.2

Enzymes

- Pronase E, Protease from Streptomyces griseus (Sigma P8811-1G)

SDS-DTT

- 1M DTT
- 10% SDS
- 1M HEPES pH8
- 60% sucrose

Consumables:

- standard 1.5 ml tubes
- Stericup 0.2 micron filter (Fisher S2GPU05RE)
- 20-micron mesh filter (Fisher Scientific NC1004201)
- 35-micron nylon mesh filter caps (Stellar Scientific FSC-FLTCP)
- 5 ml sterile polypropylene round-bottom tube (STEMCELL Technologies 38057)
- Bio-Rad TC20 automated cell counting slide (Bio-rad 1450011)

Equipment:

- Fixed angle rotor centrifuge (Eppendorf 5424)
- Swinging bucket rotor refrigerated centrifuge (eppendorf 5810R)
- 15 ml tube and 1.5 ml tube adapter (eppendorf 022638704, eppendorf 022638742)
- Fluorescent microscope
- Nutating mixer
- Bio-Rad TC20 automated cell counter
- 2ml dounce homogenizer with pestle A (Sigma-Aldrich D8938)

Troubleshooting

Before beginning

1 Prepare reagents in advance

L15-10 Buffer: Mix 500 ml Leibovitz's L-15 Medium, 50 ml Fetal Bovine Serum (heat inactivated), 50 ul of 100x Penicillin-Streptomycin solution and 7.7 g sucrose. Filter with 0.2 micron pore filter. Store at 4ºC.

Egg Buffer: Mix 29.5 ml of 2M NaCl, 12 ml of 2M KCl, 1 ml of 1M CaCl2, 1 ml of MgCl2, 12.5 ml of 1M HEPES-NaOH pH 7.2 and 435 ml molecular grade water. Filter with 0.2 micron pore filter. Store at 4ºC.

Pronase E solution (15 mg/ml): Weigh 150 mg of Protease from Streptomyces griseus (Sigma P8811-1G) into a 15 ml tube. Dissolve the enzyme in 10 ml of Egg Buffer. Nutate the solution for approximately 10 minutes until dissolved. Prepare 1 ml aliquots in 1.5 ml tubes. Store aliquots at -20ºC.

SDS-DTT solution:

- To ensure treatment efficacy, the SDS-DTT solution is prepared fresh on the day of the experiment. Additionally, several aliquots of DTT powder in 1.5 ml tubes are prepared in advance and stored at -20ºC.
- To prepare the SDS-DTT solution, first resuspend 154 mg of DTT powder with 1 mL of Egg Buffer on the day of the experiment. Then, combine the following reagents in a 15 ml tube (final concentrations in parenthesis): 100 uL of 1M HEPES pH8 (20 mM), 125 uL of 10% SDS (0.25%), 1 mL of 1M DTT (200 mM), 250 uL of 60% sucrose (3%), and 3.525 mL of molecular grade water for a final volume of 5 mL.
- The SDS-DTT solution is one of the most important reagents to ensure successful dissociation of post-embryonic worms. To check that the SDS-DTT solution is working properly, incubate worms in freshly prepared SDS-DTT solution under a dissection microscope. When prepared properly, worms should dissolve within ~10 minutes of observation.

2 On day of protocol:

Cool swinging bucket centrifuge to 4ºC
Thaw pronase aliquots at room temperature
Place L15-10 and egg buffer on ice
Wash and sterilize dounce homogenizer and pestle A (1ml H20, 1ml 70% EtOH)

3 Starting material:
Worm suspension in 15 ml tube (material generated from **this protocol**)

Strains: N2, fluorescent sorting strain

Perform this protocol on both strains in parallel

Note: The volumes for chemical and enzymatic treatments in this protocol require an L1 pellet less than 200 uL. If L1 pellet exceeds 200 ul, utilize 2x the L1 pellet volume.

Harvest L1 Worms

4 Harvest the synchronized L1 worms by washing the plates with fresh M9.

5 Pass the harvested L1 suspension through a 20 micron filter. This will filter any contaminating debris (agar chunks, partially bleached worm chunks) and any unhatched or dead embryos.

6 Pellet the worms in a 15 ml tube for 1 min at 2,000 rcf. Discard the supernatant and repeat M9 washes until the supernatant is free of visible E. coli.

SDS-DTT Treatment

7 Once the worm suspension is free of E. coli, centrifuge worm suspension at 2,000 rcf for 1 minute

8 Resuspend the worm pellet in 1 ml of M9 and transfer to a 1.5 ml tube.

9 Pellet the worms again at 2,000 rcf for 1 minute.

10 Transfer 10 ul of L1 worm pellet to 1ml of Qiazol and store at -80°C for downstream RNA analysis

11 Resuspend the worm pellet in 200 ul of fresh SDS-DTT solution

12 Incubate for 2 min room temperature to digest the cuticle
NOTE:
- Worms should look ruffled, and pharynx should change from thin and elongated to short and round
- Incubation exceeding 2 min will damage the sample

13 Quench reaction by adding 800 ul of ice cold egg buffer

14 Pellet worms at 13,000 rcf for 15 seconds at 4ºC

15 Decant the supernatant and replace with 1 ml of ice-cold egg buffer

16 Repeat the egg buffer wash for a total of 5 washes

Pronase E Treatment

17 Resuspend the SDS-DTT treated worms in 200 ul of 15 mg/ml Pronase E

18 Incubate for 30 minutes at room temperature nutating to digest the cuticle

19 Quench the Pronase E treatment by adding 1 ml of L15-10

20 Pellet the worms at 13,000 rcf for 15 seconds at 4°C

21 Decant the supernatant and resuspend the worms in 1 ml of L15-10

22 Pellet the worms at 13,000 rcf for 15 seconds at 4°C

23 Resuspend the worms in 1ml of L15-10

Cell dissociation

24 Transfer the worm suspension to a 2 ml glass dounce homogenizer

25 Perform 100 strokes with dounce pestle A to generate a worm slurry

26 Visually confirm worm dissociation by viewing a 2 ul sample of worm slurry on a fluorescent microscope

27 Harvest the cells

27.1 Transfer worm slurry to 1 ml centrifuge tube

27.2 Pellet undissociated worms at 100 rcf for 1 minute at 4ºC in swinging bucket centrifuge

NOTES:
- This step will separate the dissociated cells from intact worms
- Cells will remain in the supernatant
- Intact and partially dissociated worms will remain in the pellet
- Ensure your cell type of interest is not lost during this step.
- Visually confirm fluorescent cells **are present** in the supernatant.
- Visually confirm fluorescent cells **are not present** in the pellet.
- You may need to reduce the centrifuge speed and/or time if fluorescent cells are in the pellet of this step.

27.3 Aspirate 1 ml of the cell-containing supernatant. Keep the pipette away from the pelleted worm debris.

27.4 Dispense the cell suspension though a 35-micron nylon mesh filter into a 5 ml flow cytometry tube

28 Resuspend remaining worm slurry in 1ml of L15-10

29 For the sorting strain, perform an additional round of dissociation and cell harvest (Steps 24-28). You will perform a total of two to three homogenization cycles, until few intact worms remain.

Total cell suspension volumes:

- Control strain = 1ml
- Sorting strain = 2-3ml

30 - Transfer 70 ul of cells to 1 ml of Qiazol and store at -80°C for downstream RNA analysis
- Continue to step 31
- Retain the remaining ~2ml of cells for **FACS**

Measure approximate cell concentration

31 Load 10 ul of cell suspension to a Bio-Rad TC20 automated cell counting slide. Also can use a hemocytometer.

32 This protocol should yield between 2×10^6 to 4×10^6 total cells

33 Dilute the sample to $1 \times 10^6 \frac{\text{cells}}{\text{ml}}$ if above this concentration with L15-10

34 Microscopically confirm fluorescent cells are present in the cell counting slide

35 If the total cell yield is less than 1×10^6 cells and many intact worms remain, repeat the SDS-DTT, Pronase E and dounce homogenization steps

36 Move on to **FACS protocol**