Version 1

Sep 22, 2022

Synchronized C. elegans culture on NGM plates for FACS isolation of intestine cells V.1

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This protocol is published without a DOI.


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ABSTRACT

This protocol details the steps necessary to scale-up and synchronize *C. elegans* cultures for FACS isolation of intestine cells. The protocol utilizes agar-based NGM plates to reduce any confounding effects that may be introduced by large scale liquid culture. The protocol utilizes two rounds of mixed stage culture growth followed by two rounds of synchronized growth. After scale-up and synchronization, this protocol provides details for culture conditions necessary for intestine FACS of embryo, L1 or L3 stage experiments.

PROTOCOL CITATION

Robert TP Williams, Erin Osborne Nishimura 2022. Synchronized C. elegans culture on NGM plates for FACS isolation of intestine cells. **protocols.io** https://protocols.io/view/synchronized-c-elegans-culture-on-ngm-plates-for-f-b329qqh6

FUNDERS ACKNOWLEDGEMENT

National Institutes of Health
Grant ID: R35GM124877

Bridge to Doctorate at Colorado State University
Grant ID: 1612513

National Institutes of Health
Grant ID: R35GM124877

National Science Foundation

KEYWORDS

C. elegans, Intestine, FACS

CREATED

Jan 19, 2022

LAST MODIFIED

Sep 22, 2022

PROTOCOL INTEGER ID

57153

MATERIALS TEXT

Strains:

- OP50 E. coli
- 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:

- LB Broth Mix (Genesee 11-120)
- M9 buffer
  - 3 g KH_2PO_4 (Sigma-Aldrich P0662)
  - 6 g Na_2HPO_4 (Thermo Fisher S373)
  - 5 g NaCl (Sigma-Aldrich S9888)
  - 1 ml 1 M $MgSO_4$ (Sigma-Aldrich 208094)
  - H_2O to 1 liter
- NGM plates
  - (complete protocol: http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html#d0e214)
  - 3g NaCl (Sigma-Aldrich S9888)
  - 17g agar (Genesee 20-249)
  - 2.5g peptone
  - 975g H_2O (sterile and deionized)
  - 1ml 1M $CaCl_2$ (Sigma-Aldrich C3306)
  - 1ml 5mg/ml cholesterol (Fisher 501848291)
  - 1ml 1M $MgSO_4$ (Sigma-Aldrich 208094)
  - 25ml 1M KPO_4 Buffer pH 6.0 (108.3 g KH_2PO_4, 35.6 g K_2HPO_4, H_2O to 1 litre) (Sigma-Aldrich P0662, P3786
- Peptone enriched NGM: in recipe above use 20g peptone instead of 2.5g peptone
- Bleaching solution
  - Sodium Hypochlorite Solution, 6% available chlorine (Ricca Chemical, 7495.7-32)
  - 5N NaOH (Fisher S318-100)

Consumables:

- 150 mm petri dishes "large plates" (Corning 351058)
- 15 ml centrifuge tubes (Peak PS-695)

Equipment:

- Swinging bucket rotor refrigerated centrifuge (Eppendorf 5810R)
- Pipet-Aid (VWR 89166-464)
- 20°C incubator

Prepare OP50 seeded NGM plates

1 Using sterile technique, pick an OP50 colony and inoculate a 250 ml bottle of sterile LB.

2 Incubate OP50 liquid culture at 37°C overnight

3 Make 3 liters of Nematode Growth Media (NGM) with 150 mm petri dish **(hereafter referred to as "large plates")**. See the following WormBook page for NGM protocol: http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html#d0e214

3.1 ⚠

For FACS isolation of post-embryonic worm stages, prepare an additional 1 liter of peptone enriched NGM media. For peptone enriched NGM media, replace the normal 2.5 g peptone mass with 20 g.

4 Pour molten NGM into large plates. Each liter should make 20 plates, for a total of 60 large plates

5 Allow plates to dry overnight

6 Seed each NGM plate with 3 ml OP50 liquid culture.

7 Cover as much agar surface as possible by moving the plate in first a circular pattern, then a figure 8 pattern.

8 Dry the OP50 seeded plates at room temperature with the lids on for three to four days until there is no more excess liquid

Grow mixed stage cultures of cell sorting strain

9 Identify a 60 mm petri plate culture of the sorting strain that has recently exhausted the E. coli lawn

10 Chunk the plate into 5 equal pieces

11 Transfer each chunk to a fresh large NGM OP50 plate with the worm covered surface facing down

12 Place sorting strain cultures in 20°C incubator for 72-96 hours, until the E. coli lawn is exhausted 4d

Expand mixed stage cultures of cell sorting strain

13 Harvest the the mixed stage worm population from the 5 plates by washing each plate with ~10 ml of M9

14 Transfer the worm suspension to a 15 ml conical centrifuge tube

15 Pellet the worms by centrifuging for 1 min at 2,000 rcf

16 Wash with additional M9 by aspirating the supernatant and resuspending the worms in fresh M9 to a total volume of 15 ml

17 Repeat the M9 wash until the supernatant is clear

18 Measure the approximate concentration of worms in suspension such that the optimal density of worms are seeded onto the plate

18.1 Shake or vortex the tube to ensure the worms are evenly distributed in the suspension

18.2 Aspirate 2 ul of worm suspension with a p10 pipette. Pipette the worm suspension up and down at least four times before moving on.

Dispense the worm suspension on a clean microscope slide

18.3

18.4 With a cell counter, count the number of worms on the slide under a dissection microscope. Dilute the worm suspension if there are too many to count.

18.5 Determine the concentration and total number of worms

$$\frac{\text{worms in 2ul drop}}{\text{2ul}} \approx \frac{\text{worms}}{\text{ul}}$$

$$\frac{\text{worms}}{\text{ul}} \times \text{worm suspension ul} \approx \text{total \# of worms}$$

19 Seed 20 fresh large NGM OP50 plates with 5,000 worms per plate

20 Incubate for 72 hours in 20°C incubator, until there is a large number of gravid adults

First embryo synchronization with hypochlorite solution

21 At the beginning for the day, chunk one recently starved 60 mm N2 plate to a fresh large NGM OP50 plate. This is important to do, and will serve as the negative GFP control for cell sorting.

22 Harvest mixed stage sorting strain worms from all 20 plates by washing each plate individually with ~10 ml of M9 1h

22.1 Transfer mixed stage worm suspension to a 15 ml conical centrifuge tube

22.2 Pellet the worms by centrifuging for 1 minute at 2,000 rcf

22.3 Aspirate the supernatant

22.4 Harvest worms from another plate as outline above

22.5 Resuspend the worm pellet with worm suspension from the newly washed plate

22.6 Repeat this process until worms have been harvested from all 20 plates into a single 15ml tube

23 Once all plates have been harvested, continue washing the worm pellet with fresh M9 to remove excess E. coli by pelleting and resuspending in fresh M9. The final worm pellet yield should be 1 to 2 ml.

24 Once the worm suspension is free of E. coli, centrifuge again and remove all M9 supernatant from the worm pellet

25 Resuspend the worm pellet in 8 ml of H2O

26 Add 0.9 ml of Sodium Hypochlorite Solution (Ricca Chemical, 7495.7-32) and 1.44 ml of 5N NaOH to the worm suspension

27 Resuspend the worm pellet with brief vigorous vortexing

28 

Incubate at room temperature for 6 to 8 minutes. While incubating shake the tube or place on a nutator. The time to bleach the worms depends on the worm pellet volume, with larger worm pellets taking longer. Do not incubate for longer than 8 minutes.

29 Monitor the progression of the hypochlorite treatment. Larval worms should dissolve, adult worms will begin to break at the vulva and release embryos. I typically monitor the treatment

by looking through the tube under a dissection microscope. Aliquots of the worm suspension can also be taken throughout the process and viewed on a microscope slide.

30 Once the worms are sufficiently dissolved, centrifuge the tube for 30 seconds at 2,000 rcf

31 Decant the supernatant and wash the embryo pellet by adding 15 ml of M9 to quench the hypochlorite treatment

32 Wash the bleached embryos a second time. Centrifuge the tube for 30 seconds at 2,000 rcf to pellet the embryo suspension. Decant the supernatant and resuspend the embryo pellet in 15 ml of M9.

33 Wash the bleached embryos a third time. Centrifuge the tube for 30 seconds at 2,000 rcf to pellet the embryo suspension. Decant the supernatant and resuspend the embryo pellet in 15 ml of M9.

34 Wash the bleached embryos a fourth time. Centrifuge the tube for 30 seconds at 2,000 rcf to pellet the embryo suspension. Decant the supernatant and resuspend the embryo pellet in 15 ml of M9.

35 Measure the approximate concentration of embryos in suspension

35.1 Shake or vortex the tube to ensure the embryos are evenly distributed in the suspension

35.2 Aspirate 2 ul of embryo suspension with a p10 pipette. Pipette the embryo suspension up and down at least four times before moving on.

35.3 Dispense the embryo suspension on a clean microscope slide

35.4 With a cell counter, count the number of embryos on the slide under a dissection microscope. Dilute the embryo suspension if there are too many to count.

36 Seed 20 large NGM/OP50 plates with 5,000 embryos. Incubate at 20°C for approximately 72 hours until worms are gravid.

Second embryo synchronization with hypochlorite solution

37 Harvest both synchronized sorting strain worms and mixed stage N2 worms by washing each plate with ~10 ml of M9

38 Transfer the worm suspension to a 15 ml conical centrifuge tube

39 Pellet the worms by centrifuging for 1 minute at 2,000 rcf

40 Discard the supernatant

41 Resuspend the worm pellet with worm suspension from another large plate

42 Repeat this process until worms have been harvested from all 20 plates

43 Once all plates have been harvested, continue washing the worm pellet with fresh M9 to remove excess E. coli. The final worm pellet yield should be 1 to 2 ml.

44 Once the worm suspension is free of E. coli, centrifuge again and remove all M9 supernatant from the worm pellet

45 Resuspend the worm pellet in 8 ml of H2O

46 Add 0.9 ml of Sodium Hypochlorite Solution (Ricca Chemical, 7495.7-32) and 1.44 ml of 5N

NaOH to the worm suspension

47 Resuspend the worm pellet with brief vigorous vortexing

48 Incubate at room temperature for 6 to 8 minutes. While incubating shake the tube or place on a nutator. The time to bleach the worms depends on the worm pellet volume, with larger worm pellets taking longer. Do not incubate for longer than 8 minutes.

49 Monitor the progression of the hypochlorite treatment. Larval worms should dissolve, adult worms will begin to break at the vulva and release embryos. I typically monitor the treatment by looking through the tube under a dissection microscope. Aliquots of the worm suspension can also be taken throughout the process and viewed on a microscope slide.

50 Once the worms are sufficiently dissolved, centrifuge the tube for 30 seconds at 2,000 rcf

51 Decant the supernatant and resuspend the embryo pellet in 15 ml of M9 to quench the hypochlorite treatment

52 Wash the bleached embryos a second time. Centrifuge the tube for 30 seconds at 2,000 rcf to pellet the embryo suspension. Decant the supernatant and resuspend the embryo pellet in 15 ml of M9.

53 Wash the bleached embryos a third time. Centrifuge the tube for 30 seconds at 2,000 rcf to pellet the embryo suspension. Decant the supernatant and resuspend the embryo pellet in 15 ml of M9.

54 Wash the bleached embryos a fourth time. Centrifuge the tube for 30 seconds at 2,000 rcf to pellet the embryo suspension. Decant the supernatant and resuspend the embryo pellet in 15 ml of M9.

55 The final embryo yield should be approximately 0.25 ml for the wildtype N2 stain and 1 ml for the fluorescent sorting strain.

56 For embryo stage FACS experiments, move on to the embryo dissociation protocol: https://www.protocols.io/view/embryo-stage-c-elegans-dissociation-for-facs-isola-b35vqq66

57 For post-embryonic stage FACS experiments, move on to step 63 for L1 stage experiments or step 70 for L3 stage experiments

Synchronized L1 culture

58 Incubate the synchronized embryos in 15 ml M9 overnight rotating in 20°C incubator for 24 hours

59 Feed the synchronized L1 sorting strain worms six hours before beginning the L1 dissociation protocol. This is to reduce any observable starvation-induced responses in the measured transcriptional data. Negative control N2 worms can remain incubating in M9 suspension.

60 Pellet the L1 sorting strain worms and resuspend in 500 ul of M9. Transfer equal volumes of the synchronized L1 sorting strain worms onto two large peptone enriched NGM OP50 plates

61 Feed the synchronized L1 worms for 6 hours in a 20°C incubator.

62 Harvest the synchronized L1 worms by washing the plates with fresh M9. Pellet the worms for 1 min at 2,000 rcf. Discard the supernatant and repeat M9 washes until the supernatant is free of visible E. coli.

63 For both fluorescent sorting strain and wildtype strain, 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.

64 Move on to the L1 stage dissociation protocol: https://www.protocols.io/view/l1-stage-c-elegans-dissociation-for-facs-isolation-b36aqrae

Synchronized L3 culture

65 Synchronize the embryos to the L1 stage by incubating the embryos for 24 hours suspended in M9, rotating in a 20°C incubator

66 Measure the approximate concentration of L1 worms in suspension

66.1 Shake or vortex the tube to ensure the worms are evenly distributed in the suspension

66.2 Aspirate 2 ul of worm suspension with a p10 pipette. Pipette the worm suspension up and down at least four times before moving on.

66.3 Dispense the worm suspension on a clean microscope slide

66.4 With a cell counter, count the number of worms on the slide under a dissection microscope. Dilute the embryo suspension if there are too many to count.

67 Seed the plates with a worm suspension volume between 100 and 500 ul. Concentrate or dilute the worm suspension accordingly. To avoid generating stressed clumps of developmentally delayed worms, spot the worm suspension evenly across the OP50 lawn.

67.1 For the sorting strain worms, seed at least 10 large peptone enriched NGM OP50 plates with 30,000 L1 worms.

67.2 For the wildtype worms, seed at least 5 large peptone enriched NGM OP50 plates with 30,000 L1 worms

68 Incubate the worms for 48 hours in a 20C incubator until worms visibly reach the L3 stage.

69 Move on to the L3 stage dissociation protocol: https://www.protocols.io/view/l3-stage-c-elegans-dissociation-for-facs-isolation-b363qrgn