**Make RNAi Media**

1. Prepare 1L of medium 100 mm NGM plates (see end of protocol)
2. Streak RNAi strains on LB-amp plates. Place streaked plates in plastic bin and incubate at 37C overnight
   1. Re-streak RNAi feeding strains for each experiment
   2. Streak the following strains:
      1. L4440
      2. POP-1
      3. ELT-2 RNAi
      4. ELT-7 RNAi
3. Pick a single colony into LB-carb media (50 ug/ml). Incubate shaking for 16 hours at 37C. Culture 10x the volume of E. coli that will be seeded. Example below is for a single experiment
   1. L4440: 10 ml
   2. POP-1: 10 ml
   3. ELT-2: 15 ml
   4. ELT-7: 15 ml
4. Concentrate E. coli liquid culture by spinning at top speed for 10 minutes in swinging bucket centrifuge
5. Decant LB media and resuspend the E. coli pellet with M9. Below is for a single experiment
   1. L4440: 1 ml
   2. POP-1: 1 ml
   3. ELT-2: 1.5 ml
   4. ELT-7: 1.5 ml
6. Mix the ELT-2 and ELT-7 feeding strains
   1. 0.5 ml ELT-2 RNAi + 0.5 ml ELT-7 RNAi
7. Seed the NGM RNAi plates with the following volumes of resuspended E. coli
   1. L4440: 1 ml
   2. POP-1: 1 ml
   3. ELT-2: 1 ml
   4. ELT-7: 1 ml
   5. ELT-2; ELT-7: 1 ml
8. Let dry at room temperature

**Culture worms**

**Initial growth**

1. Chunk recently starved small plate to large NGM/OP50 plates
2. Incubate for 48-72 hours, until gravid adults are visible

**Synchronization**

1. Visually confirm a large population of gravid adults
2. Wash plates with M9 a 10 ml pipette. Transfer worms to a 15 ml tube
3. Centrifuge worms for 1 min at 2000 rcf in swinging bucket centrifuge
4. Resuspend worm pellet in fresh M9
5. Continue washing the worm pellet 2-3x with fresh M9 to remove excess E. coli
6. Once the worm suspension is free of E. coli, centrifuge again and remove all but 8 ml of M9 supernatant from the worm pellet
7. Add 0.9 ml of Sodium Hypochlorite Solution (Ricca Chemical, 7495.7-32) and 1.44 ml of 5N NaOH
8. Vortex the worm pellet to resuspend and incubate at room temperature for 6 to 8 minutes.
   1. While incubating shake the tube or place on a nutator
9. Monitor the progression of the hypochlorite treatment
   1. Larval worms should dissolve, adult worms will begin to break at the vulva and release embryos
   2. 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
10. Once the worms are sufficiently dissolved, centrifuge the tube for 30 seconds at 2,000 rcf in swinging bucket centrifuge
    1. Ensure there is as little worm debris as possible, as hatching L1s can eat this and will not be synchronized
11. Decant the supernatant and resuspend the embryo pellet in 15 ml of M9 buffer to quench the hypochlorite treatment
12. Decant the supernatant and resuspend the embryo pellet in 15 ml of M9 buffer. Repeat for a total of three M9 washes
13. After final wash, resuspend embryos in 1 ml M9
14. Seed 1 ml of embryos on fresh NGM/OP50 plates
15. Incubate for ~48-72 hours, until worms are L4 stage

**RNAi Treatment**

1. Visually confirm worms are L4 stage. Worms should have a developed gonad arms but are not gravid.
2. Wash worms from plate with M9 using 10 ml pipette. Transfer worms to 15 ml tube.
3. Centrifuge worms for 1 min at 2000 rcf in swinging bucket centrifuge
4. Resuspend worm pellet in fresh M9
5. Continue washing the worm pellet 2-3x with fresh M9 to remove excess E. coli
6. Measure the approximate concentration of worms in suspension
   1. Shake or vortex the tube to ensure the worms are evenly distributed in the suspension
   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.
   3. Dispense the worm suspension on a clean microscope slide
   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.
   5. Determine the concentration and total number of worms
7. Transfer 200-500 synchronized L4 worms to RNAi treatment plates
   1. POP-1
   2. L4440
   3. ELT-2
   4. ELT-7
   5. ELT-2;ELT-7
8. Incubate for 24 hours at 20C, until worms become gravid adults

**Harvest embryos and L1 synchronization**

1. Wash gravid adults from RNAi treatment plates with 3 ml M9
2. Transfer to 1.5 ml tube
3. Wash 3x with M9 until supernatant is free of excess E. coli
4. Embryo prep RNAi treated worms
   1. Make 10 ml Bleach Solution: 8 ml H2O, 0.9 ml of Sodium Hypochlorite Solution (Ricca Chemical, 7495.7-32) and 1.44 ml of 5N NaOH
   2. Pellet worms, 2000 x g for 30 sec
   3. Aspirate supernatant and resuspend in 1 ml Bleach solution
   4. Rock at room temperature for 6-8 mins
   5. Pellet worms, decant supernatant and resuspend in M9. Repeat for a total of 3 washes
5. Incubate in 1 ml tube at 25C for 24 hours

**RNAi treated L1 harvest and fixation**

PBS containing 0.1% Tween: add 50 ul of Tween-20 to 50 ml of PBS.

1. In a tabletop centrifuge, spin down worms at 2000 x g , 30 seconds.
2. Decant supernatant. Be careful to try to retain as many of the worms as possible.
3. Immediately submerge the tube in liquid nitrogen for exactly 1 minute.
4. Remove tube from nitrogen and immediately add 1 ml -20C MeOH. Gently vortex to resuspend worms and store in -20C for 10 mins
5. Remove MeOH and wash once with PBST by rocking for 5 min
6. Pellet worms by spinning at 2000 xg for 30 seconds, remove supernatant
7. Resuspend worms in 750 ul PBST
8. Crosslink worms by adding 50 ul fresh 16% microscopy grade Formaldehyde
9. Rock samples at room temperature for 30 mins
10. Add 40 ul 2.5 M glycine to quench reaction
11. Wash 2x with PBST, rocking for 5 min each

**Mount worms to slide**

1. Microwave valap for 2-3 minutes, until melted
2. Place on heat block on high setting
3. Transfer worms to slide
   1. For high worm concentration sample: Transfer 10 ul of worm to center of slide of 1 ml worm suspension
   2. For low worm concentration sample: Pellet worms and resuspend in 100 ul PBS. Transfer 10 ul of worm suspension to slide
   3. For extremely low concentration sample: Pellet worms and resuspend in 10 ul PBS. Transfer 10 ul of worm suspension to slide
4. Place coverslip on sample, avoid making bubbles
5. Seal with a thin layer of melted valap

**Media and Solutions**

**NGM Media for RNAi**

* Add the following to a 2L Flask:
  + 3 g NaCl
  + 17 g Agar
  + 2.5 g peptone
  + 975 ml dH2O
* Autoclave on 40 min liquid cycle
* Allow to cool in 55C hot bath for ~45 minutes
* Once cooled and before pouring add the following:
  + 1 ml 1M CaCl2
  + 1 ml 5 mg/ml cholesterol
  + 1 ml 1 M MgSO4
  + 25 ml KPO4 buffer pH 6.0
  + 1 ml 10 mg/ml Nystatin
  + 1 ml 1M IPTG
  + 1 ml 50 mg/ml Carbenicillin
* Pour into 100 mm plates, this should make approximately 50 plates
* Allow to cool overnight
* Package and store plates in 4C cold room

**Carbenicillin Stock Solution (50 mg /ml)**

**0,5 g Carbenicillin**

**10 ml H2O**

* Mix thoroughly in a 15 ml conical vial.
* Filter sterile the solution through a 0.22 um filter.
* Freeze in 1 ml aliquots.

**1 M IPTG**

**2.38 g Isopropyl-β-D-thiogalactopyranoside (IPTG)**

**10 ml H2O**

* Mix thoroughly in a 15 ml conical vial.
* Filter sterilize through a 0.22 um pore filter.
* Freeze 1 ml aliqiuots.

**PBST**

PBS containing 0.1% Tween: add 50 ul of Tween-20 to 50 ml of PBS.

**10X PBS**

25.6 Na2HPO4\*7H2O

80 g NaCl

2 g KCL

2 g KH2PO4

Bring to 1 L with H2O, Autoclave liquid cycle