Experimental Methods

Last update on 9/26/2014

Small scale bleaching protocol

The bleaching was adapted from WormBook.[1]

- Objective

  To synchronize worms at L1 stage using a 1.5 mL centrifuge tube

- Procedure

  1. Before beginning, check plates for gravid adults and eggs
  2. Add 1 mL M9 buffer[1] to each well in a plate
  3. Wash plate with Pasteur pipette
  4. Transfer worm solution to 1.5 mL Ependorf centrifuge tube
  5. Centrifuge at 3,000 rpm for 30 seconds
  6. Aspirate to 0.3 mL
  7. Add 500 µL of 1M NaOH
  8. Add 200 µL of bleach
  9. Vortex immediately

     * Vortex every minute until adult worms begin to dissolve

  10. Centrifuge at 300 rpm for 30 seconds
  11. Aspirate above pellet
  12. Add M9 to 1 mL
  13. Vortex
  14. Centrifuge at 3,000 rpm for 30 seconds
  15. Repeat steps 10-13 three times
  16. Aspirate to 0.2 mL
  17. Add 2 mL of M9 buffer to one well of the sterile 6-well plate
  18. Add 2 µL of 5 mg/mL cholesterol to well
  19. Use a new Pasteur pipette to mix and transfer worm solution to well
  20. Incubate at 20°C overnight

References

[1] Stiernagle, T., Maintenance of *C. elegans*. WormBook, ed. The *C. elegans* Research Community. WormBook 2006. [link]

FUdR Dosing

- Objective

  FUdR dosing prevents eggs from hatching.[1]
- Procedure
  1. Calculate the amount of FUdR and deionized water needed for the experiment

     - We recommend a concentration of 25 µmol FUdR/L agar with a 100 µl dose per well of a 6-well plate
  2. Dose FUdR onto wells 49 hours after N2 eggs have been pipetted onto the wells.

References

[1] Gandhi, S.; Santelli, J.; Mitchell, D. H.; Wesley Stiles, J.; Rao Sanadi, D., A simple method for maintaining large, aging populations of *Caenorhabditis elegans*. Mech. Ageing Dev. 1980, 12 (2), 137-150.

Fitness assay

Experimental procedure

The fitness assay was adapted from Ramani et al.[1]

Day 0

- Conduct the small scale bleaching protocol to isolate eggs from gravid worms
- Incubate the eggs overnight at 20°C in M9 solution[2] containing cholesterol 5 mg/L
- Cultivate *E. coli* OP50 overnight at 37°C in Luria-Bertani (LB) medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L)[2]

Day 1

- Sonicate nanomaterial suspension solution for 2 min at 35% amplitude (total ~12,420 Joules applied) using a Vibra Cell sonicator
- To prepare samples with *E. coli* OP50, nanomaterial and worms
  - Add 250 µL of nanomaterial suspension solution at 4, 20, 100, or 200 ppm to each well in 24-well plates
  - Add 750 µL of *E. coli* OP50 solution at OD_{595} = 2.0 to each well

- Concentrate hatched L1 using a centrifuge
- Drop ~20 µL of worm solution containing ~50 worms into each well in the 6-well plates

- The following two controls were similarly prepared and tested in 96-well plates with 200 µL working volume per well
  - Nanomaterial with *E. coli* OP50 without worms
  - Nanomaterial without *E. coli* OP50, and without worms

  Note: these control plates could be copied from the test plates with *E. coli* OP50, nanomaterial and worms while preparing the test and control plates. This could be done by transferring certain amount of sample solution from the test plates to the control plates before adding *E. coli* OP50 or worms to the test plates
- Seal the plates with breathable films
- Place the plates at a 20°C shaker

Day 6

- Take OD measurements for nanomaterials where solvent is DI water, or SDS
  - Take 33 µL of sample solution per well to a 96-well plate
  - Dilute the sample by adding 67 µL of S-medium[1] per well
  - Measure OD_{595} using a microplate reader

Day 7

- Take OD measurements for nanomaterials where solvent is Tannic Acid
  - Take 33 µL of sample solution per well to a 96-well plate
  - Dilute the sample by adding 67 µL of S-medium per well
  - Measure OD_{595} using a microplate reader

Calculation of fitness

Fitness was defined similarly to Ramini et al.[1] Since nanomaterials in itself increased optical density in blank solution, measured OD_{595} in test solution was subtracted by OD_{595} contributed by nanomaterial, and the subtracted OD was designated as $(OD_{net})_{NP}$.

$$(OD_{net})_{NP} = OD_{+NP,+E.coli,+worms} - OD_{+NP,-E.coli,-worms}$$

Similarly,

$$(OD_{net})_{control} = OD_{-NP,+E.coli,+worms} - OD_{-NP,-E.coli,-worms}$$

$(OD_{net})_{NP}$: OD_{net} in test condition

$(OD_{net})_{control}$: OD_{net} in control

'+' sign in the 'NP', '*E. coli*', or 'worms': condition with nanomaterial, *E. coli*, or worms

'-' sign in the 'NP', '*E. coli*', or 'worms': condition without nanomaterial, *E. coli*, or worms

In the equation, a different control was used based on the type of background solution. If any nanomaterial was dissolved in 50 ppm tannic acid solution, 50 ppm tannic acid control solution that did not contain nanomaterial was used as its corresponding control. If any nanomaterial was dissolved in 0.0025% SDS, 0.0025% SDS control solution that did not contain nanomaterial was used as its corresponding control. Otherwise, DI water control solution that did not contain nanomaterial and also did not contain tannic acid or SDS was used as a corresponding control.

Fitness (*F*) was defined as the following equation.

$$F = \frac{\Delta OD_{NP}}{\Delta OD_{control}} = \frac{\left(OD_{net,\,Day\,0} - OD_{net,\,Day\,N}\right)_{NP}}{\left(OD_{net,\,Day\,0} - OD_{net,\,Day\,N}\right)_{control}}$$

ΔOD_{NP}: OD difference between day 0 and day N in test condition

$\Delta OD_{control}$: OD difference between day 0 and day N in control

$OD_{net,\,Day\,0}$: OD_{net} at day 0 (time zero)

$OD_{net,\,Day\,N}$: OD_{net} at day N

N was 6 for samples with water or SDS

N was 7 for samples with tannic acid.

When multiple wells were used, fitness (*F*) was defined as the average fitness in all wells.

$$F = \frac{1}{J}\sum_{j=1}^{J} F_j$$

$$F_j = \frac{\Delta OD_{NP,\,j}}{\frac{1}{B}\sum_{b=1}^{B}\left(\Delta OD_{control,b}\right)} = \frac{\left(OD_{net,\,Day\,0} - OD_{net,\,Day\,N}\right)_{NP,\,j}}{\frac{1}{B}\sum_{b=1}^{B}\left[\left(OD_{net,\,Day\,0} - OD_{net,\,Day\,N}\right)_{control,b}\right]}$$

F_j: Fitness in the *j*th well in test condition

j: *j*th well in test condition

J: Total number of wells in test condition

b: *b*th well in control
B: Total number of wells in control

Fitness in an individual control well was similarly calculated by the following equation.

$$F_q = \frac{\Delta OD_{control,q}}{\frac{1}{B}\sum_{b=1}^{B}\left(\Delta OD_{control,b}\right)} = \frac{\left(OD_{net,\,Day\,0} - OD_{net,\,Day\,N}\right)_{control,q}}{\frac{1}{B}\sum_{b=1}^{B}\left[\left(OD_{net,\,Day\,0} - OD_{net,\,Day\,N}\right)_{control,b}\right]}$$

F_q : Fitness in the *q*th well in control
q: *q*th well in control
b: *b*th well in control
B: Total number of wells in control

Creation of heat map of fitness

Multiple independent fitness assays were conducted. To combine results of multiple trials, fitness in an individual well was first calculated in each trial. Overall average fitness ($F_{overall}$) was then calculated by the following equation.

$$F_{overall} = \frac{1}{W}\begin{pmatrix} F_{Trial\,1,\,Well\,1} + F_{Trial\,1,\,Well\,2} + \cdots \\ + F_{Trial\,2,\,Well\,1} + F_{Trial\,2,\,Well\,2} + \cdots \\ + F_{Trial\,3,\,Well\,1} + F_{Trial\,3,\,Well\,2} + \cdots \end{pmatrix}$$

$F_{overall}$: Overall average fitness in all trials
$F_{Trial\,k,Well\,h}$: Fitness in the *h*th well in the *k*th trial
W: Total number of wells in all trials

The overall average fitness ($F_{overall}$) was represented in color gradient in the heat map.

Statistical analysis

- Student t-test was performed to calculate p-value using Java Apache Commons Math 2.2 API, or in PHP using the Al-Kashi library version 5.0.

References

[1] Ramani, Arun K.; Chuluunbaatar, T.; Verster, Adrian J.; Na, H.; Vu, V.; Pelte, N.; Wannissorn, N.; Jiao, A.; Fraser, Andrew G., The majority of animal genes are required for wild-type fitness. Cell 2012, 148 (4), 792-802.
[2] Stiernagle, T., Maintenance of *C. elegans*. WormBook, ed. The *C. elegans* Research Community. WormBook 2006. [link]

Lifespan assay on solid media

The lifespan assay was conducted similarly as described.[1]

Experimental procedure

Day 0

- Conduct the small scale bleaching protocol to isolate eggs from gravid worms
- Incubate the eggs overnight at 20°C in M9 solution[2] containing cholesterol 5 mg/L

Day 1

- Sonicate nanomaterial suspension solution for 2 min at 35% amplitude (total ~12,420 Joules applied) using a Vibra Cell sonicator
- Drop 100 µL nanomaterial suspension solution at 20, 50, 100, or 200 ppm onto each well in a 6-well NGM agar plates seeded with *E. coli* OP50 (6 mL agar/well)
- Concentrate hatched L1 using a centrifuge at 3,000 rpm for 1 minute
- Transfer ~70 worms into each well in the 6-well plates
- Incubate the worms at 20°C

Day 3 (worms at L4)

- Dose the plate with FUdR at 25 µmol/L agar to prevent eggs from hatching

Day 4 and onwards

- Scan plates every day until all worms are dead

Image analysis

- WormLifespan software[1] was used to count living worms.

Calculation of lifespan

Mean lifespan ($\overline{N}$) was defined as the average of normalized lifespan of worms as shown in below.

$$\overline{N} = \frac{1}{J}\sum_{j=1}^{J}\overline{N}_j$$

$\overline{N}$: Mean lifespan of worms (normalized) in test condition

$\overline{N}_j$: Normalized lifespan of the *j*th worm in test condition

J: Total number of worms in test condition

where an individual worm's lifespan (N_j) was normalized by dividing the mean lifespan of the N2 control as shown in below.

$$\overline{N}_j = \frac{N_j}{\frac{1}{B}\sum_{b=1}^{B}C_b}$$

N_j: Lifespan of the *j*th worm in test condition

$\overline{N}_j$: Normalized lifespan of the *j*th worm in test condition

C_b: Lifespan of the *b*th worm in control

B: Total number of worms in control

In the equation, a different control was used based on the type of background solution. If any nanomaterial was dissolved in 50 ppm tannic acid solution, 50 ppm tannic acid control solution that did not contain nanomaterial was used as its corresponding control. If any nanomaterial was dissolved in 0.0025% SDS, 0.0025% SDS control solution that did not contain nanomaterial was used as its corresponding control. Otherwise, DI water control solution that did not contain nanomaterial and also did not contain tannic acid or SDS was used as a corresponding control.

Creation of heat map of lifespan

Multiple independent lifespan assays were conducted. To combine results of multiple trials, an individual worm's lifespan was first normalized in each trial. Overall mean lifespan ($\overline{N}_{overall}$) was then calculated by the following equation.

$$\overline{N}_{overall} = \frac{1}{W}\begin{pmatrix} \overline{N}_{Trial\,1,\,Worm\,1} + \overline{N}_{Trial\,1,\,Worm\,2} + \overline{N}_{Trial\,1,\,Worm\,3} + \cdots \\ + \overline{N}_{Trial\,2,\,Worm\,1} + \overline{N}_{Trial\,2,\,Worm\,2} + \overline{N}_{Trial\,2,\,Worm\,3} + \cdots \\ + \overline{N}_{Trial\,3,\,Worm\,1} + \overline{N}_{Trial\,3,\,Worm\,2} + \overline{N}_{Trial\,3,\,Worm\,3} + \cdots \end{pmatrix}$$

$\overline{N}_{overall}$: Overall mean lifespan (normalized) in all trials

$\overline{N}_{Trial\ k,Worm\ h}$: Normalized lifespan of the *h*th worm in the *k*th trial

W: Total number of worms in all trials

The overall mean lifespan ($\overline{N}_{overall}$) was represented in color gradient in the heat map.

Statistical analysis

- LogRank p-value between two survival curves was calculated using homemade Java software (Download LogRankP.java).

References

[1] Jung, S.-K.; Aleman-Meza, B.; Riepe, C.; Zhong, W., QuantWorm: A comprehensive software package for *Caenorhabditis elegans* phenotypic assays. *PLoS ONE* **2014,** *9* (1), e84830.
[2] Stiernagle, T., Maintenance of *C. elegans*. WormBook, ed. The *C. elegans* Research Community. WormBook 2006. [link]

Locomotion assay on solid media

This assay was conducted in conjunction with the lifespan assay as described.[1]

Experimental procedure

Day 0

- Conduct the small scale bleaching protocol to isolate eggs from gravid worms
- Incubate the eggs overnight at 20°C in M9 solution[2] containing cholesterol 5 mg/L

Day 1

- Sonicate nanomaterial suspension solution for 2 min at 35% amplitude (total ~12,420 Joules applied) using a Vibra Cell sonicator
- Drop 100 µL nanomaterial suspension solution at 20, 50, 100, or 200 ppm onto each well in a 6-well NGM agar plates seeded with *E. coli* OP50 (6 mL agar/well)

- Concentrate hatched L1 using a centrifuge
- Transfer ~70 worms into each well in the 6-well plates
- Incubate the worms at 20°C

Day 3 (worms at L4)

- Dose the plate with FUdR at 25 µmol/L agar to prevent eggs from hatching

Day 4 (worms at 1 day of adulthood)

- Take videos for 30 seconds for each well

Image analysis

- WormLocomotion software[1] was used to measure worm speed.

Calculation of speed

Average worm speed ($\bar{v}$) was defined as the average of normalized average speed in detected tracks as shown in below.

$$\bar{v} = \frac{1}{J}\sum_{j=1}^{J}\bar{v}_j$$

$\bar{v}$: Average worm speed (normalized) in all videos in test condition

$\bar{v}_j$: Normalized average speed of the *j*th track in all videos in test condition

J: Total number of tracks in all videos in test condition

where average speed in each track (v_j) was normalized by dividing the average speed of the N2 control as shown in below.

$$\bar{v}_j = \frac{v_j}{\frac{1}{B}\sum_{b=1}^{B}V_b}$$

v_j: Average speed of the *j*th track in all videos in test condition

$\bar{v}_j$: Normalized average speed of the *j*th track in all videos in test condition

V_b: Average speed of the *b*th track in all videos in control

B: Total number of tracks in all videos in control

In the equation, a different control was used based on the type of background solution. If any nanomaterial was dissolved in 50 ppm tannic acid solution, 50 ppm tannic acid control solution that did not contain nanomaterial was used as its corresponding control. If any nanomaterial was dissolved in 0.0025% SDS, 0.0025% SDS control solution that did not contain nanomaterial was used as

its corresponding control. Otherwise, DI water control solution that did not contain nanomaterial and also did not contain tannic acid or SDS was used as a corresponding control.

Creation of heat map of locomotion

Multiple independent locomotion assays were conducted. To combine results of multiple trials, overall average speed ($\bar{v}_{overall}$) was calculated by the following equation.

$$\bar{v}_{overall} = \frac{\frac{1}{I}\sum_{i=1}^{I} v_i}{\frac{1}{M}\sum_{m=1}^{M} V_m} = \frac{M}{I}\frac{\left(\begin{array}{l} v_{Trial\,1,\,Track\,1} + v_{Trial\,1,\,Track\,2} + v_{Trial\,1,\,Track\,3} + \cdots \\ + v_{Trial\,2,\,Track\,1} + v_{Trial\,2,\,Track\,2} + v_{Trial\,2,\,Track\,3} + \cdots \\ + v_{Trial\,3,\,Track\,1} + v_{Trial\,3,\,Track\,2} + v_{Trial\,3,\,Track\,3} + \cdots \end{array}\right)}{\left(\begin{array}{l} V_{Trial\,1,\,Track\,1} + V_{Trial\,1,\,Track\,2} + V_{Trial\,1,\,Track\,3} + \cdots \\ + V_{Trial\,2,\,Track\,1} + V_{Trial\,2,\,Track\,2} + V_{Trial\,2,\,Track\,3} + \cdots \\ + V_{Trial\,3,\,Track\,1} + V_{Trial\,3,\,Track\,2} + V_{Trial\,3,\,Track\,3} + \cdots \end{array}\right)}$$

$\bar{v}_{overall}$: Overall average speed (normalized) in all trials

v_i: Average speed of the *i*th track in all videos in all trials in test condition

V_m: Average speed of the *m*th track in all videos in all trials in control

I: Total number of tracks in all videos in all trials in test condition

M: Total number of tracks in all videos in all trials in control

The overall average speed ($\bar{v}_{overall}$) was represented in color gradient in the heat map.

Statistical analysis

- Student t-test was performed to calculate p-value using Java Apache Commons Math 2.2 API, or in PHP using the Al-Kashi library version 5.0.

References

[1] Jung, S.-K.; Aleman-Meza, B.; Riepe, C.; Zhong, W., QuantWorm: A comprehensive software package for *Caenorhabditis elegans* phenotypic assays. *PLoS ONE* **2014,** *9* (1), e84830.

[2] Stiernagle, T., Maintenance of *C. elegans*. WormBook, ed. The *C. elegans* Research Community. WormBook 2006. [link]

Body size assay on solid media

The body size assay was conducted as described.[1]

Experimental procedure

Day 0

- Conduct the small scale bleaching protocol to isolate eggs from gravid worms
- Incubate the eggs overnight at 20°C in M9 solution[2] containing cholesterol 5 mg/L

Day 1

- Sonicate nanomaterial suspension solution for 2 min at 35% amplitude (total ~12,420 Joules applied) using a Vibra Cell sonicator
- Drop 100 µL nanomaterial suspension solution at 20, 50, 100, or 200 ppm onto each well in a 6-well NGM agar plates seeded with *E. coli* OP50 (6 mL agar/well)
- Concentrate hatched L1 using a centrifuge at 3,000 rpm for 1 minute
- Transfer ~70 worms into each well in the 6-well plates
- Incubate the worms at 20°C

Day 4 (worms at 1 day of adulthood)

- Collect worms from each well with M9 solution and transfer to unseeded 6-well plates
- Kill the worms by adding 15 µL of 1 M sodium azide into each well
- Leave the plates for ~1 hr
- Scan the plates

Body size assay in aqueous media

The body size assay was conducted as described.[1]

Experimental procedure

Day 0

- Conduct the small scale bleaching protocol to isolate eggs from gravid worms
- Incubate the eggs overnight at 20°C in M9 solution[2] containing cholesterol 5 mg/mL
- Cultivate *E. coli* overnight at 37°C in Luria-Bertani (LB) medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L)[2]

Day 1

- Sonicate nanomaterial suspension solution for 2 min at 35% amplitude (total ~12,420 Joules applied) using a Vibra Cell sonicator
- To prepare samples with *E. coli* OP50, nanomaterial and worms
  - Add 250 µL of nanomaterial suspension solution at 4, 20, 100, or 200 ppm to each well in 24-well plates (use the same amount of background solution for control without nanomaterial)
  - Add 750 µL of *E. coli* OP50 solution at OD_{595} = 1.0 to each well
  - Concentrate hatched L1 using a centrifuge at 3,000 rpm for 1 minute
  - Drop ~20 µL of worm solution containing ~200 worms into each well in the 6-well plates
- Seal the plates with breathable films
- Place the plates at a 20°C shaker

Day 4 (worms at 1 day of adulthood)

- Cool down the plate in ice water for 15 min
- Aspirate solution up to ~100 µL
- Transfer worm solution to unseeded 6-well plates
- Kill the worms by adding 15 µL of 1 M sodium azide into each well
- Leave the plates for ~1 hr
- Scan the plates

Image analysis

- WormLength software[1] was used to measure body length

Calculation of body length

Body length ($\overline{L}$) was defined as the average of normalized body length of worms as shown in below.

$$\overline{L} = \frac{1}{J}\sum_{j=1}^{J}\overline{L}_j$$

$\overline{L}$: Body length of worms (normalized) in test condition

$\overline{L}_j$: Normalized body length of the *j*th worm in test condition

J: Total number of worms in test condition

where an individual worm's body length (L_j) was normalized by dividing the average body length of the N2 control as shown in below.

$$\overline{L}_j = \frac{L_j}{\frac{1}{B}\sum_{b=1}^{B}C_b}$$

L_j: Body length of the *j*th worm in test condition

$\overline{L}_j$: Normalized body length of the *j*th worm in test condition

C_b: Body length of the *b*th worm in control

B: Total number of worms in control

In the equation, a different control was used based on the type of background solution. If any nanomaterial was dissolved in 50 ppm tannic acid solution, 50 ppm tannic acid control solution that did not contain nanomaterial was used as its corresponding control. If any nanomaterial was dissolved in 0.0025% SDS, 0.0025% SDS control solution that did not contain nanomaterial was used as its corresponding control. Otherwise, DI water control solution that did not contain nanomaterial and also did not contain tannic acid or SDS was used as a corresponding control.

Creation of heat map of body length

Multiple independent body size assays were conducted. To combine results of multiple trials, an individual worm's body length was first normalized in each trial. Overall average body length ($\overline{L}_{overall}$) was then calculated by the following equation.

$$\overline{L}_{overall} = \frac{1}{W}\begin{pmatrix} \overline{L}_{Trial\,1,\,Worm\,1} + \overline{L}_{Trial\,1,\,Worm\,2} + \overline{L}_{Trial\,1,\,Worm\,3} + \cdots \\ + \overline{L}_{Trial\,2,\,Worm\,1} + \overline{L}_{Trial\,2,\,Worm\,2} + \overline{L}_{Trial\,2,\,Worm\,3} + \cdots \\ + \overline{L}_{Trial\,3,\,Worm\,1} + \overline{L}_{Trial\,3,\,Worm\,2} + \overline{L}_{Trial\,3,\,Worm\,3} + \cdots \end{pmatrix}$$

$\overline{L}_{overall}$: Overall average body length (normalized) in all trials

$\overline{L}_{Trial\,k,Worm\ h}$: Normalized body length of the *h*th worm in the *k*th trial

W: Total number of worms in all trials

The overall average body length ($\overline{L}_{overall}$) was represented in color gradient in the heat map.

Statistical analysis

- Student t-test was performed to calculate p-value using Java Apache Commons Math 2.2 API.

References

[1] Jung, S.-K.; Aleman-Meza, B.; Riepe, C.; Zhong, W., QuantWorm: A comprehensive software package for *Caenorhabditis elegans* phenotypic assays. *PLoS ONE* **2014,** *9* (1), e84830.
[2] Stiernagle, T., Maintenance of *C. elegans*. WormBook, ed. The *C. elegans* Research Community. WormBook 2006. [link]