Standard protocol

LIFESPAN and HEALTHSPAN ASSAY using WMICROTRACKER SMART

adapted from CITP STANDAR OPERATING PROCEDURE / Lifespan machine

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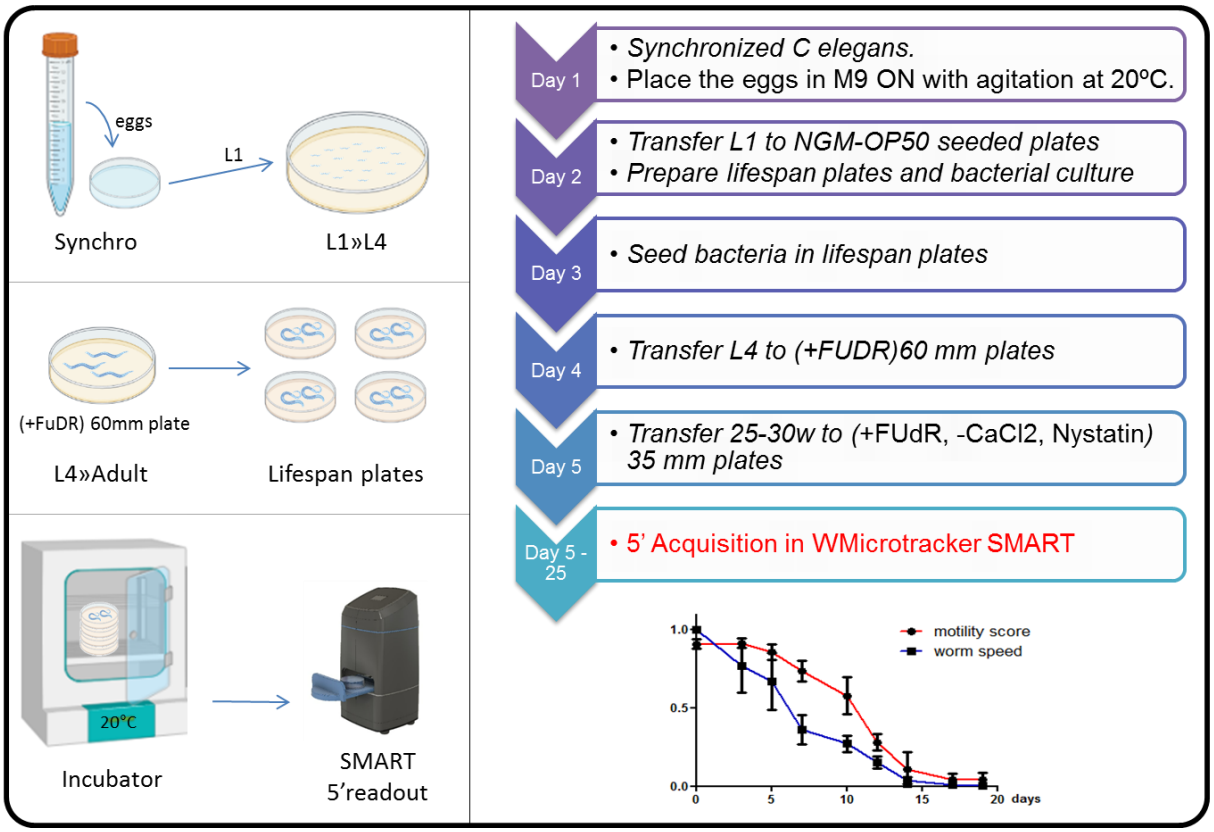
Graphical Abstract:



WHAT YOU NEED:

NGM-OP50 seeded plates	OP50 bacteria
Gravid adults worms	Parafilm
35 mm plates	Incubator
60 mm plates	Shaker
NGM medium	Mini Shaker
M9 Buffer	Plastic container
FUdR	**Wmicrotracker SMART**
Nystatin	Blue light chamber (Royal Blue LED x12)

Day 1

- Prepare age-synchronous cohorts by hypochlorite treatment (*See Obtaining synchronous cultures of C.elegans*). Eggs were washed in M9 buffer 4-6 times. Place the eggs in 3 ml M9 in a p35 with agitation (150rpm) at 20ºC.

Day 2

- 18:00 hs later, transfer L1 worms to NGM-OP50 seeded plates. We recommend transferring max 5000 worms per p100 plate to avoid overcrowding.

- Prepare the plates for lifespan assays: (+FUdR) and (+FUdR, -CaCl2, +Nystatin) (*see Lifespan Assay Plate Preparation*).

Type	***Volume of plate***	***Used for***
Medium (60mm)	10 mL	+FUdR: used for lifespans assays
Small (35mm)	4 mL	+FUdR, -CaCl2, +Nystatin: used for lifespans assays

Plates should be allowed to solidify before replacing lids. Allow plates to dry for 24 hours with the lids on.

- Prepare OP50 bacterial culture (*see Bacterial culture preparation*).

Day 3

- The next day, “seed” the center of each plate with liquid OP50 suspension and spread the bacteria lawn in the center, away from the edges of the plate.

Scanner plates should be seeded in a sterile hood and left lid-off until the lawns is dry.

Type of Plate	***Amount of OP50-1 (1X) liquid culture***
Medium (60mm)	100 µL
Small (35mm)	20 µL

Once dry, transfer plates to a standard box, and store lid up in a 20°C incubator for another 24 hours.

Store plates lid down in a tightly-covered plastic bin at 4°C for up to three weeks.

Day 4

- Transfer late L4-larve to standard plate containing (+FUDR) 60mm plate.

Day 5

- Transfer (25-30) d0 adults to small (+FUdR, -CaCl2, +Nystatin) 35 mm plates in triplicate (i.e., 100 animals per strain, per condition, to start an experiment). Seal each plate with parafilm.

Take care not to gouge the agar or leave the plate exposed to open air any longer than necessary. Check that the plates are labeled on the sides, not the lid or bottom, which would interfere with the software's ability to properly track the worms.

- Keep plates lid-down in a plastic container at 20ºC. Recreate a wet chamber inside the plastic container placing a moist paper sheet inside; keep the plates non in contact with the paper).

- Incubate the plates at least 1 hour at the desired temperature and then proceed with their acquisition en WMicrotracker SMART.

SMART ACQUISITION

- Open SMART Software and adjust the focus of the plate.

- Immediately before acquisition, expose each plate to blue light (10'').

- Acquire 5 minutes in the WMicrotracker SMART

Acquire each plate once every 2/3 days.

Notes on acquisition

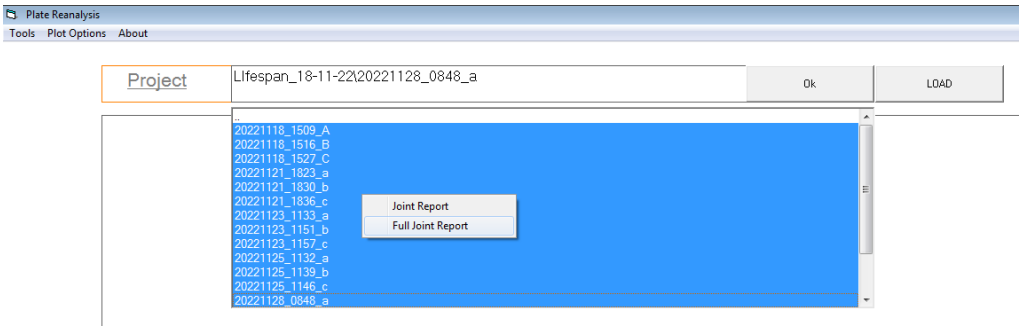
• Observe the plates in the magnifying glass every 4 days (always after the acquisition in the WMicrotracker SMART).

• You might have to replace parafilm once a week or when you observed is riven.

• Additional transfers may be necessary if fungal contamination is observed. If bacterial contamination is observed, score all worms as lost. If a control plate is censored, then the remaining plates in that technical replicate should also be censored.

Plot and analysis of the data:

- Obtain the Full Joint report of the whole lifespan experiment. In check results menu, select the lifespan project and with shift click on all the acquisition to select them simultaneously, right click and select the option Full Joint Report.



- The joint report with the parameters of all the acquisitions will be generated:

	A	B	C	D	E	F	G	H
1	Report File	##########						
2	Folder	G:\SMART2\Lifespan_18-11-22						
3	Acquisition L	5						
4								
5	**Acq_Name**	**Acq_Date**	**Acq_Time**	**#Particles**	**Average_spe**	**Travelled_Di**	**Motility_Sco**	**Rotation Index**
6	A	18/11/2022	15:09	23	0.058	20.35	0.96	5.85
7	B	18/11/2022	15:16	23	0.078	29.2	0.92	5.92
8	C	18/11/2022	15:27	21	0.122	39.08	0.95	5.93
9	a	21/11/2022	18:23	19	0.059	27.84	0.85	5.31
10	b	21/11/2022	18:30	21	0.067	25.69	0.95	5.91
11	c	21/11/2022	18:36	17	0.053	26.94	0.85	5.47
12	a	23/11/2022	11:33	15	0.059	26.18	0.94	5.38
13	b	23/11/2022	11:51	21	0.046	20.82	0.89	4.73
14	c	23/11/2022	11:57	19	0.049	18.26	0.92	5.46

- Graph the motility score in function of the days.

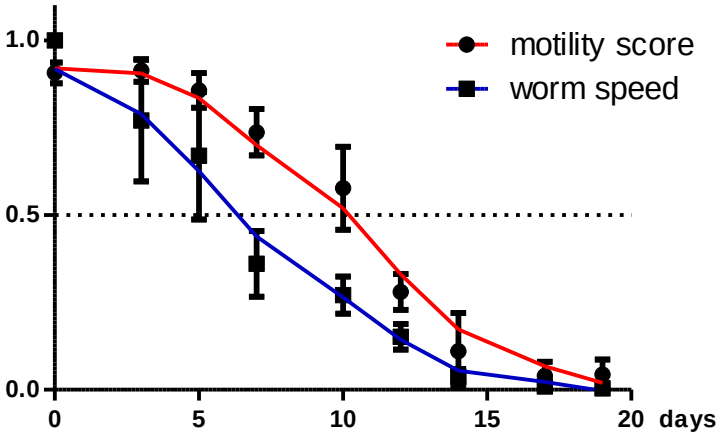
Note: *Lifespans (ages) are calculated with the egg lay as day zero of adult.*

To graph the motility score in function of the days, create a new colum: <u>day number</u> = acq_date (of the day) – $acq date (day 0).

5	Day number	Acq_Name	Acq_Date	Acq_Time	#Particles	Average_spe	Travelled_Di	Motility_Sco	Rotation Index
6	-	A	18/11/2022	15:09	23	0.058	20.35	0.96	5.85
7	-	B	18/11/2022	15:16	23	0.078	29.2	0.92	5.92
8	-	C	18/11/2022	15:27	21	0.122	39.08	0.95	5.93
9	3.00	a	21/11/2022	18:23	19	0.059	27.84	0.85	5.31
10	3.00	b	21/11/2022	18:30	21	0.067	25.69	0.95	5.91
11	3.00	c	21/11/2022	18:36	17	0.053	26.94	0.85	5.47
12	5.00	a	23/11/2022	11:33	15	0.059	26.18	0.94	5.38
13	5.00	b	23/11/2022	11:51	21	0.046	20.82	0.89	4.73
14	5.00	c	23/11/2022	11:57	19	0.049	18.26	0.92	5.46
15	7.00	a	25/11/2022	11:32	20	0.031	13.15	0.76	4.31
16	7.00	b	25/11/2022	11:39	15	0.026	12.57	0.85	4.55
17	=+C17-C$6	c	25/11/2022	11:46	20	0.026	9.83	0.62	4.26
18	10.00	a	28/11/2022	8:48	15	0.019	8.61	0.74	3.08
19	10.00	b	28/11/2022	8:55	14	0.025	7.14	0.68	4.86

- Graph the motility score in function of the days.

<u>Results:</u>



N2 worms lifespan was evaluated during 24 days. Worms were allowed to grow at 20ºC. Each plate was acquire using SMART Wmicrotracker during 5 min. Data represents the average and SEM of the motility score ( ) and worm speed ( ) of each individual plate (3 replicates).

APPENDIX

1. Obtaining synchronous cultures of C.elegans

(Adapted from the book: C.elegans – A Practical Approach)

Bleaching solution.

Final Volume	*5 ml*
Household bleach 5%	1,9 ml
NaOH 1N	2,5 ml
H2O	0,6 ml

Make this solution fresh just before use!

Method

- Use *C.elegan*s plates that have many gravid hermaphrodites.
- Wash the plates with sterile M9 buffer. Pipette the buffer across the plate several times.
- Collect the liquid with worms in a sterile 15 ml conical centrifuge tube with a cap.
- Let the worms settle by decantation. Throw out the supernatant (use a sterile Pasteur pipette) taking care not to disturb the pellet.
- Perform a wash with 5 ml of M9 buffer. Briefly shake or invert the tube.
- Repeat the decantation step. Aspirate most of the supernatant and retain worms in the tube.
- Add 5ml of bleaching solution to the centrifuge tube containing the worms.
- Vortex the tube for no more than 4 minutes. It is important to observe the disintegration of worms.
- Centrifuge in a table-top centrifuge for 60 seconds at 1500 rpm (≈300g) to pellet the released eggs.
- Throw out the supernatant taking care not to disturb the pellet.
- Add sterile M9 Buffer to 14 ml. Vortex or manually shake the tube until the eggs are resuspended in the buffer.
- Repeat the wash step (step 9-11) two times more.
- After last resuspension, centrifuge in a table-top centrifuge for 60 seconds at 1500 rpm (≈300g) to pellet the eggs. Aspirate the buffer with a sterile Pasteur pipette to 0.1 ml.
- Use a sterile Pasteur pipette to transfer the eggs in the remaining 0.1 ml of liquid to the edge of a clean NGM plate seeding with bacteria (OP50).
- Incubate the plate overnight at 20ºC.The next day the eggs will have hatched and the larvae will have crawled into the *E.coli* OP50 lawn.

2. Preparation of plates:

For 200ml of media, add the following to a 500ml Erlenmeyer flask. Put a clean stir bar in each flask to help with mixing reagents after autoclaving.

Ingredient	Amount per 200ml	Amount per liter
Agar	4.6 g	23.0 g
NaCl	0.6 g	3.0 g
Meat Peptone	0.5 g	2.5 g
DI Water	200 ml	1000 ml

- Autoclave this nematode growth media on a liquid cycle (121°C, 15 psi minimum) for 20 minutes.
- When autoclave cycle is complete, move media immediately to 55°C water bath to cool.

- After media cools to 55°C, add the following ingredients in the order shown below. Use previously added stir bar to mix between each addition.

Ingredient	Stock Concentration	Vol. added to 100ml media	Vol. added to 200ml media	Vol. added to 1L media	Final concentration
Potassium phosphate buffer KP04 (pH 6.0)	1 M	2.5 ml	5 ml	25 mL	24 mM
Magnesium sulfate (MgSO4)	1 M	0.1 ml	0.2 ml	1 mL	1 mM
Calcium chloride (CaCl2)	1 M	0.1ml	0.2 ml	1 mL	1 mM
Cholesterol	5 mg/mL	0.1 ml	0.2 ml	1 mL	5 mg/L
FUdR (if required)	25 mM	0.3 ml	0.6 ml	3 ml	75 uM
Nystatin (if required)	5mg/ml	0.2 ml	0.4 ml	2 mL	10 µg/ml

- Pour plates with the desired volume of agar using a plate-pouring machine. Plates should be poured in a sterile hood and the agar should be allowed to solidify before replacing lids.

Type	Volume of plate	Used for
Medium (60mm)	10 mL	+FUdR: used for lifespans assays
Small (35mm)	4 mL	+FUdR, -CaCl2, +Nystatin: used for lifespans assays

- Allow plates to dry for 24 hours at room temperature or in hood with the lids on.

3. Bacterial culture preparation:

1. Use a flamed wire loop to select a single colony from an OP50 streak plate to inoculate 50 mL of sterile LB broth in a 250 mL Erlenmeyer flask.

2. Grow culture overnight at 37°C and 250 rpm for 16 hours. Store the OP50 suspension in a 50 mL conical tube at 4°C for up to 1 week.

3. Scanner plates should be seeded in a sterile hood and left lid-off until the lawns is dry.

Type of Plate	*Amount of OP50-1 (1X) liquid culture*
Medium (60mm)	100 µL
Small (35mm)	20 µL

At the moment to seed the assay plates, take care not to spread the bacteria lawn all the way to the edges of the plate; keep the lawn in the center. The worms tend to spend most of the time in the bacteria. If the lawn extends to the edges of the plate the worms may crawl up the sides of the plate, dry out and die

4. Reagent Stock Preparation

Reagent	Solute amount	Solvent	Solvent volume	Conc.	Filter sterilize	Autoclave	Change monthly
Magnesium Sulphate	2.4 g	H20d	20 ml	1 M	No	Yes	Yes
Calcium Chloride (CaCl2.2H20)	2.94 g	H20d	20 ml	1 M	No	Yes	Yes
Cholesterol	100 mg	EtOH	20 ml	5 mg/ml	No	No	Yes
Potassium Phosphate Buffer	KH2PO4: 10,83g K2HPO4: 3,56 g	H20d	100 ml - Titrate to a pH of 6.0	1 M	No	Yes	Yes
FUdR	6,22 mg	H20d	1ml	25 mM	No	No	No (Store aliquots at -20ºC)
Nystatin	5 mg	DMSO	1 ml	5 mg/ml	No	No	No (Store aliquots at -20ºC)