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Measure 40 worm feeding with bioluminescent bacteria after drug treatment

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Protocol status: Working

We use this protocol and it's working

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Abstract

Protocol uses bioluminescently labelled bacteria (Gregor et al, 2008, Addgene Plasmid #107879, <https://www.pnas.org/content/115/5/962>) to measure population feeding of 40 adult *C. elegans* worms following serotonin or naloxone drug treatment.

Materials

bioluminescence bacteria: Addgene Plasmid #107879

IVIS Spectrum imaging system (Caliper LifeSciences)

Living Image software (v 4.3.1)

Serotonin creatinine sulfate monohydrate (Sigma H7752)

Naloxone (Sigma PHR1802)

ampicillin sodium salt (Sigma A9518)

LB broth and agar

NGM and low peptone NGM agar

M9 buffer

laminar flow hood (Heraguard)

spectrophotometer (Jenway7315)

centrifuge (Hettich Universal 320)

vortex mixer (Vortex-Genie 2, Scientific Industries)

styrofoam box

glass pipette

manual click counter



Bleach synchronise worms (Day -7 to -4)

- 1 Bleach synchronise animals from three 60mm plates, making sure lots of gravid hermaphrodites are present. Leave on rotator at 20 °C until use.

Protocol

NAME

Bleach synchronisation of *C. elegans*

CREATED BY

Ida Barlow

Preview

- 1.1 Wash hermaphrodites off plate with several ml of M9 solution and transfer to 15ml falcon tube (Fisher Scientific-Falcon 352096)
- 1.2 Fill falcon tube up to 15ml with M9 solution
- 1.3 Centrifuge for 2 minutes at 1500 rpm (RCF:210, ascending 9; descending 7) – program 1

Program 1 retains the worms as pellets and the bacteria is suspended as the supernatant

The descending is slow as the worm pellet is loose at this stage which we don't want to break
- 1.4 Remove supernatant using a plastic Pasteur pipette taking care not to disturb pellet
Leave atleast 0.5ml M9 to avoid disturbing the pellet
- 1.5 Fill the tube with M9 upto 15ml
- 1.6 Spin program 1
- 1.7 *Repeat steps 4-6*



- 1.8 On final wash remove as much supernatant as possible and add M9 upto 4ml
- 1.9 Add 4ml 2X Bleach solution (From here onwards try to work as quickly as possible to avoid over-exposure of the worms to the bleach)

USE FRESHLY PREPARED BLEACH EVERYTIME**Note****2X Bleach solution:**

5% Sodium hypochlorite solution - 4ml
Sterile water - 3.5 ml
1M NaOH solution - 2.5 ml
TOTAL - 10 ml

- 1.10 Vortex on maximum setting for 4 min (no more as this will damage the eggs)

Make sure the vortex forms

After vortexing, top up the tube with M9 till 15ml
- 1.11 Centrifuge for 2 mins at 2500rpm (RCF:590, ascending 9; descending 7) – program 2

(Always check the program on the centrifuge before using it)
- 1.12 Remove supernatant by pouring into waste bottle – pellet should be compact and yellow in colour at bottom of falcon, but be careful not to lose
- 1.13 Add 15ml M9
- 1.14 Centrifuge at program 2
- 1.15 Repeat steps 12-14 four more times

The number of washes is crucial here as we need to get rid of all the bleach
- 1.16 After final wash add 15ml M9 and store eggs/larvae in the falcon on the rotator that is constantly spinning at 20°C, until feeding

**Note**

L1 arrested larvae can be starved for up to 5 days before refeeding

1.17 Centrifuge larvae on program 2 to pellet

1.18 Remove supernatant with plastic Pasteur pipette

The pellet is loose here so make sure not to disturb it

1.19 Add 15ml M9, spin to wash

1.20 On final wash leave 0.5ml M9 in falcon

1.21 Resuspend the pellet by gently tapping the tube/flicking it

1.22 Place droplet containing larvae onto seeded plate and allow to grow to desired developmental state (ie. 2 days for L4s, 2.5 days for young adults)

Use glass pipette to place the droplet onto seeded plate, avoid using plastic pipette as larvae will stick to it



Note

Development times at 20°C:

- 2 days for L4s
- 2.5 days for young adults

Note:

- If you feed larvae within 12hrs of bleaching then they develop faster than the longer arrested ones
- It is a good practice to bleach in two tubes in parallel
- If you drop the tube at any point of the process, make sure to transfer the contents into a new tube as the dropped tube may get cracked resulting in loss of worms during centrifugation/vortexing
- Any unused larvae can be topped up with M9 and stored spinning in the rotator to be re-used
- Use clean autoclaved rubber bulbs for the refeeding everytime to avoid contamination
- Put the used bulb in the box labelled 'Used Teets'

Stages	Grown at 20 C from L1	Grown at 25 C from L1
L1 division	11.7hrs	9hrs
Mid L1	16.9hrs	13hrs
First L2 division	22.1hrs	17hrs
Between L2 divisions	23.4hrs	18hrs
Second L2 divisions	24.3hrs	19hrs
Mid L2	29.9hrs	23hrs
L3 division	32.5hrs	25hrs
Mid L3	37.7hrs	29hrs
L4 division	42.9hrs	33hrs
Mid L4	49.4hrs	38hrs
Early adult	55.9hrs	43hrs
Adult	62.4hrs	48hrs

Table of Development times for different temperatures

Re-feed worms (Day -3)

- 2 Re-feed worms using a glass pipette. Incubate at 20 °C.

**Note**

Monitor these plates until use to ensure worms do not run out of food.

Prepare drug plates (Day -1)

3

Make up drug plates and store in the dark at  4 °C overnight.

Protocol

NAME

Make serotonin and naloxone drug plates

CREATED BY

Serena Ding

Preview

- 3.1 First identify the drugs (serotonin and naloxone) to be used in the study and ensure that they are correctly labelled and handled.
- 3.2 Get six 35 mm petri dishes and label three as serotonin (20 mM) plates and three as naloxone (10 mM) plates.
- 3.3 Since these are powered compounds, calculate the desired weight required for 10 mL of serotonin agar (at 20 mM final concentration), and for 1 mL of 10X naloxone stock solution (10X is 100 mM, for 10 mM final concentration). These volumes will give 3 drug plates each.



3.4 Make up 500 mL low peptone NGM agar and put it on autoclave.

Note

Do not attempt to use a smaller volume even though not much agar is required for drug plate preparation, as chemical weights will be too small to be measured accurately.

3.5 Inside a fume hood, with an analytical balance weigh out the desired amount of serotonin and leave aside. Weigh out the desired amount of naloxone and transfer the powder into a 1.5 mL Eppendorf tube labelled with the compound and the final concentration. To the naloxone tube, add 1 mL sterile water to make up the 10X stock solution, and close the lid of the Eppendorf tube.

3.6 When the autoclave cycle is done, add salts as normally required.

3.7 Remove 10 mL of molten low peptone NGM agar, take it to the fume hood and add the pre-weighed serotonin to achieve 20 mM final concentration. Mix well. Dispense 3 mL into each of three pre-labelled plates using a 10 mL serological pipette. Let serotonin plates set.

3.8 Take the naloxone stock solution tube out of the fume hood. To ensure the compound is dissolved fully, vortex the Eppendorf tubes (ensuring the lid is firmly in place) at setting 10 for 30 seconds.

3.9 Using a different 10 mL serological pipette, draw up 10 mL of molten agar (without serotonin) and dispense 3 mL each into a different set of three pre-labelled plates. Let the plates set (these will become the naloxone plates).

3.10 Add 300 μ L of 10X naloxone stock solution on top of each plate from the last step. Let these plates dry in a laminar flow hood for 3 hours.

3.11 Wrap drug plates in foil and leave them overnight at 4°C to let the drugs diffuse. Use for experiments immediately the next day.

Grow bacteria overnight

4 Day -2

Streak out bioluminescent bacteria frozen stock onto an LB plate containing 50 μ g/mL ampicillin and incubate overnight at  37 °C .

Note

This step does not need to be performed every time. A streaked plate can be re-used within 1 month, so long as single colonies are still available. Otherwise re-streak onto a new LB/ampicillin plate for single colonies.

5 Day -1 ~5pm

To a 300 mL volumetric flask, add 100 mL LB broth, 100 μ L ampicillin stock solution (50mg/mL), and a single colony of bioluminescent bacteria from the streaked plate. Place on  37 °C incubator shaking at 220 rpm overnight.

6 Day 0 ~10am

Take the bacteria overnight culture off of the shaking incubator and leave at room temperature on the bench until use that afternoon.

Note

Do not put bacteria culture at 4 °C to save for later, as this will abolish the signal. Instead, a fresh overnight culture should be grown for each day of experiment.

Initialise the IVIS imaging system (Day 0 ~4.30pm)

7 Initialise the IVIS system via the LivingImage software.

8 Set up imaging parameters within the LivingImage software interface. Set stage temperature to 20 °C, designate auto-saving directory for acquired images, and set up acquisition sequence parameters.

Note

Bioluminescence acquisition parameters: subject height: 0.5 cm; field of view: C; exposure: 1 second; frame interval: 6 minutes; excitation: block; emission: open; luminescence binning: 4; photograph binning: 4; luminescence F/stop: 1; photograph F/stop: 1; batch sequence: true; sequence 1 length: 99; sequence 2 length: 35.

These imaging parameters have been determined in advance for the experiment and are saved as a .xsq configuration file, which can be loaded into the imaging software.



Seed bacteria for imaging (Day 0 ~4.30pm)

- 9 Take drug plates prepared from the day before out of the  4 °C cold room, let them incubate at  Room temperature for 30 minutes to warm up.
- 10 Give the flask containing the bacteria overnight culture a swirl to resuspend the bacteria cells that may have settled during the day.
- 11 Seed 20 µL of the bacteria culture onto the centre of each 35 mm imaging plate. Dry the plate in a laminar flow hood (setting II) for 30 minutes.
- 12 Measure bacteria culture optical density with a spectrophotometer for the record.

Inoculate worms and drug exposure for experiment (Day 0 ~5.30pm)

- 13 Retrieve imaging plates from the flow hood and label them with sample name.
- 14 Harvest synchronised adult worms using 2 mL of M9 buffer and transfer them into a 15 mL tube. Wash them twice with M9 buffer by topping up the tube with M9, spinning at 1500 rpm/210 rcf (ascending 9, descending 7), and then aspirating the supernatant leaving the loose pellet intact. After the last wash, leave the loose worm pellet.

Note

This spinning programme separates worms (pellet) from bacteria (supernatant), so bacteria can be removed. Soft brake prevents the loose worm pellet from breaking up.

- 15 Using a glass pipette with a medicine dropper bulb attached, transfer all worms onto an unseeded NGM plate in order to starve the worms for  01:00:00 .
- 16 After 1 hour, harvest the starved worms again using M9 into a 15 mL tube. Pellet worms by spinning at 1500 rpm/210 rcf (ascending 9, descending 7) and aspirating the supernatant leaving the loose pellet intact.
- 17 Using a glass pipette with a medicine dropper bulb attached, transfer 40 starved worms to each imaging plate. Use a manual click counter to count 40 worms.

**Note**

Try to keep the droplet small so it gets absorbed into the media in a reasonable amount of time (a couple of minutes), although this makes accurate counting more difficult so it's worth practicing worm transfer beforehand to find the optimal worm transfer techniques.

Note

It's handy to have a P20 pipette in hand with a few microliters of M9 already inside. To remove excess worms, lower the tip near the worms and carefully suck worms back out. It's not usually precise as to how many worms are removed, so repeat counting with a manual counter again after removing worms.

- 18 Place the lid back onto the imaging plate. When all worms are transferred, put all the samples inside a cardboard box and then inside a styrofoam box, and take them to the IVIS imaging system.

Note

Carry carefully, as some liquid in which worms are transferred may not have dried completely and may disturb the bacterial lawn if it spills over.

- 19 Let the worms be exposed to drugs for  01:00:00 at  20 °C .

Image (Day 0)

- 20 Vortex imaging samples for 10s on the lowest setting of a vortex mixer to disperse any existing aggregates in order to randomise the initial position of worms.
- 21 Place imaging samples inside the IVIS chamber inside the laser grid guidelines. Close the chamber door, and start acquisition via the software exactly 1 min after the vortex start.

Signal extraction and export (Day 1)

- 22 After image sequence acquisition, read signal across the acquisition sequence using the LivingImage software by measuring radiance (photons/s) for a defined region of interest.



23 Export signal as a tab-delimited text file for downstream analysis.