STAR Protocols

Protocol

A maze platform for the assessment of *Caenorhabditis elegans* behavior and learning

Step 1 — **5-15h**
Design and 3D-print Molds
Design maze
Design mold
3D-print mold

Step 2 — **1.5h-overnight**
Maze Fabrication and Maze Baiting
Immerse mold in liquid NGM
NGM solidification & drying
Baiting

Step 3 — **10-40min**
Introduce and Record *C. elegans*
Behavioral assay
Video recording

Step 4 — **1h**
Analyze Data
Statistical analysis
Index calculation

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Highlights

Design and 3D-print the maze mold and fabricate the maze plates

Bait the mazes and insert *C. elegans* nematodes

Record nematode behavior and analyze data

Tips on steps that require extra caution

Mazes are broadly used to investigate animal decision-making and spatial learning. However, they have been sparsely employed to explore *C. elegans* behavior and training-improved performance. This protocol describes a highly reproducible, low-cost maze platform, made of the standard, agar-based, nematode culturing material. It can be used to reliably assess *C. elegans*' maze behavior, and we have recently applied this protocol to establish multisensory learning. Limitations include challenges in locomotion tracking and in distinguishing learning formation versus retrieval.

Protocol

A maze platform for the assessment of *Caenorhabditis elegans* behavior and learning

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SUMMARY

Mazes are broadly used to investigate animal decision-making and spatial learning. However, they have been sparsely employed to explore *C. elegans* behavior and training-improved performance. This protocol describes a highly reproducible, low-cost maze platform, made of the standard, agar-based, nematode culturing material. It can be used to reliably assess *C. elegans'* maze behavior, and we have recently applied this protocol to establish multisensory learning. Limitations include challenges in locomotion tracking and in distinguishing learning formation versus retrieval.

For complete details on the use and execution of this protocol, please refer to Gourgou et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the steps required to generate T-shaped mazes in a Nematode Growth Medium (NGM) plate (Figure 1). We have used the same process to generate mazes of various designs, as shown in Figure 2. The molds were printed in a Form2 FormLabs 3D printer, but any Fusion Deposition Modeling (FDM) or stereolithography 3D printer with sufficient resolution could be used.

The produced mazes are made of the exact same hydrogel used to culture worms in the lab (NGM) thus minimizing stress due to environment change and unfriendly material.

Maze mold design

⏱ Timing: depends on the design complexity; est. 2–7 h

1. Use a computer-aided design (CAD) software to design the 3D molds. We used SolidWorks, Dassault Systèmes, France. Any CAD software that can produce 3-dimensional .STL files that can be read by the 3D -printer available can be used.
   a. Consider adding auxiliary areas (see note) to provide the space needed to successfully place the worms in and to pipette the liquid attractant of choice (bacterial food or other), as shown in Gourgou et al. (2021), Figure 1.
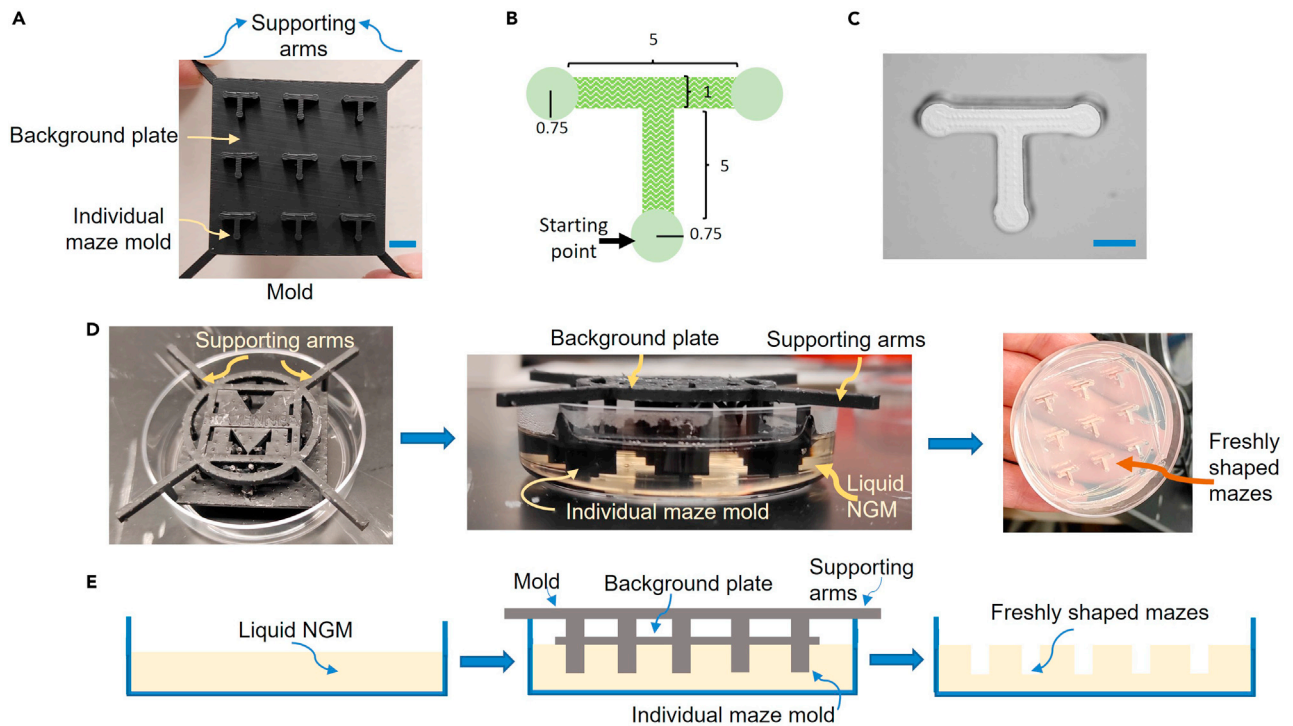
Figure 1. Maze fabrication process

(A) The 3D-printed mold. Nine copies of individual maze molds are included, 5–7 mm apart, with the supporting arms of the entire structure branching out in a X-like configuration. The square-shaped background plate prevents liquid agar from forming a meniscus around each maze mold (Figure 2). Scale bar: 5 mm.

(B) Schematic showing the dimensions of each maze in mm; wavy green: the functional corridors of the maze, light green: circular auxiliary areas, allowing for extra room to pipette the food in (left and right maze ends) and placing the worm (maze bottom—starting point). The depth of the actual maze is ∼5 mm.

(C) Actual T-maze in a NGM plate. Scale bar: 2.5 mm.

(D) Key steps followed to fabricate a maze plate. Left to right: The mold is sitting on the rim of a petri dish; The mold is immersed in liquid NGM; Once NGM cools down and solidifies, the mold is pulled out and mazes are shaped, made entirely out of NGM.

(E) Schematics of fabrication steps, modified from Gourgou et al. (2021). Compare to Figure 3.

See also Methods videos S1, S2, and S3.

Note: The current T-maze design includes circular auxiliary areas at the end of every functional corridor, including the stem of the T-maze and the ends of the two maze arms (Figure 1 and Gourgou et al., 2021).

b. Include a background plate (Figure 1), to ensure a "no-trim" design. The background plate serves to prevent liquid NGM from forming a meniscus around the T-shape mold, which would result in protruding, instead of flat, maze edges after solidification (Figure 3). Such edges need to be trimmed manually in an extra post-processing step, in order for maze covers (see below) to fit properly. Trimming, however, might result in removing too much material, therefore creating extra openings for the worms to climb out of the maze (Figure 1). Therefore, including a background plate is advised ("no-trim" design).

Note: In the design used in Gourgou et al. (2021), each individual T-shape mold rises above a square background plate (Figure 1). We opted for a square instead of a circular background plate, with surface area smaller than the one of the petri dish. The reason is that a mold that covers the entire surface of the dish makes removing the mold extremely challenging, as the entire solidified NGM slab tends to stick to the mold. Leaving some uncovered area around the mold makes removing it easier. Moreover, the square shape allows for space between the mold and the dish walls, through which liquid NGM can be poured in the petri dish (see maze fabrication step).
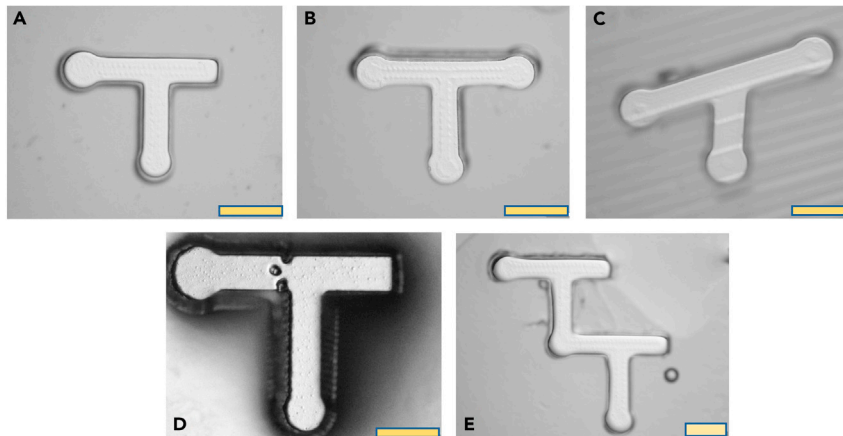
Figure 2. Examples of behavioral arenas generated using the 3D-printed mold fabrication process
(A–E) (A) asymmetrical T-maze, (B) symmetrical T-maze (as used in Gourgou et al., 2021), (C) symmetrical angled maze (as used in Gourgou et al., 2021), (D) asymmetrical T-maze with microfeatures (bumps), (E) double T-maze. Scale bars: 2.5 mm.

Note: Alternatively, each individual T-shape mold could carry its own individual background plate, in a stamp-like manner. We opted for the simpler option of one shared background plate.

Note: In Gourgou et al., 2021, we accommodated on the background plate nine individual T-shaped molds. This is why the mold was named MazEnnea (Ennea: Greek for nine), as seen on the back of the molds in the Methods Videos, in Figure 1, and in the supplementary STL file with the MazEnnea design. The number of mazes can be adjusted to meet the experimental design, maze size, and shape. The distance between the individual T-shape molds is ∼7mm (Figure 1), to prevent the bait from diffusing to a neighboring maze too fast.

c. Design the height of each individual maze mold, which translates into the maze depth, having in mind that i) it helps to confine worms inside the maze by not making the entire arena too shallow, ii) it would not present additional challenges for the experimenter to place the worm inside the maze, and iii) it should allow for a maze floor thick enough for the arenas to be resilient. In consequence of the latter, when petri dishes with different depths are used, the mold height needs to be updated. The dishes used in Gourgou et al. (2021) are provided in the key resources table. Their depth was ∼15 mm and the mold height ∼13 mm.

d. Include a set of supporting arms that should extend out of the main part, i.e., the background plate, in a X-shape or cross-like configuration. These will allow the mold to rest safely on the petri dish rim during the NGM solidification process (see maze fabrication step).

3D printing the maze molds

⏱ Timing: depends on the design complexity, the number of copies printed, and the machine used; est. 3–8 h (including post-printing processing, e.g., rinsing/curing)

2. Set up your 3D printer following the manufacturer's guidelines. Form1+ and Form2 (FormLabs, USA) 3D-printers were used to print the maze molds (Figure 1) in Gourgou et al. (2021), using the FormLabs Black Standard resin. Any 3D printer of acceptable resolution may be employed.

a. The specifications of the 3D printer, especially the *x,y,z* resolution, will affect the smoothness of the maze mold surfaces, therefore of the actual maze surfaces, as well. As shown in Gourgou et al. (2021), this can be crucial for the performance of *C. elegans*. For our experiment with the default, "rough" mazes we used a layer resolution of 200 microns. Consider trying a few different printing resolutions and printing speeds, before concluding which one works better for your experiments.
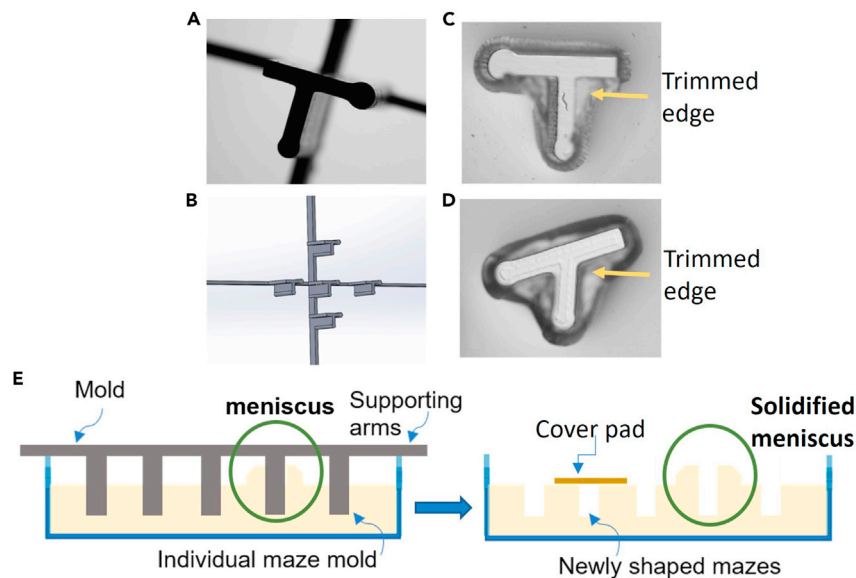
Figure 3. Mazes as a result of a mold that does not include a background plate

(A–E) (A) Individual maze mold, (B) Array of maze molds, (C and D) T-mazes in NGM plates, that had their edges trimmed, (E) Schematic of the maze fabrication process in the absence of a background plate; note the meniscus formed around each individual maze mold, and the resulting peaks after solidification. Compare to Figure 1.

3. After the printing is completed, follow the post-processing guidelines e.g., rinsing, UV-curing) that apply to the specific material and 3D printer used. For the molds used in Gourgou et al. (2021), post-processing included rinsing with 2-propanol (2×12 min) and air drying.

   Note: Rinsing with 2-propanol is a mandatory step suggested by the 3D-printet manufacturer, to ensure that any uncured resin will be thoroughly removed. Airdrying overnight (∼8–12hr) after rinsing results in completely dry and residue-free molds.

4. Store the molds away from direct light, as it might result in reduced shelf life, depending on the material used.

   Note: Molds' shelf-life depends also on how often they are used. After a few dozens of usages (or if they are not used for months) they become fragile or warped. It is advisable to refresh the stock of molds at a regular basis, to avoid undesired diversions from the planned design.

   Note: The molds can be used even if one of the supporting arms is broken, as they can still rest firmly on the dish rim. When more than one arms are missing it is not recommended to used them, as they might be tilted even slightly, resulting in unforeseen deformations of the actual NGM mazes.

Maze fabrication

   ⏲ Timing: 10 min NGM preparation + 30 min NGM solidification + 10 min/overnight (8–12 h) plate drying (all times are estimates, and they depend on ambient humidity and the drying process of choice)

5. Pour freshly prepared liquid NGM (2% agar) according to the standard recipe (Stiernagle, 2006) warm but not boiling hot, in a 60 mm petri dish up to 2/3 of the dish depth.
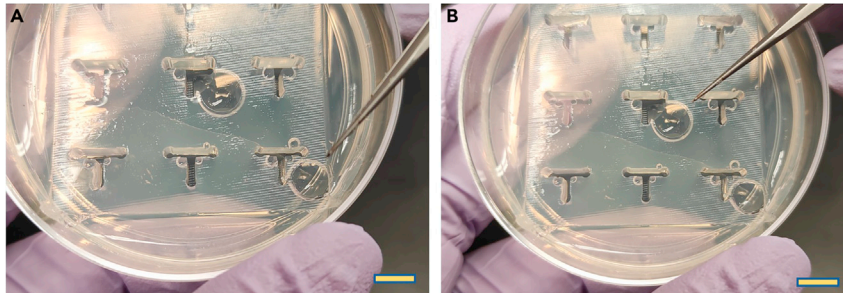
Figure 4. Deformations on the maze plate
Tweezers are pointing to empty areas created by air bubbles trapped between the mold and the liquid NGM.
(A) The bottom right maze is not affected by the bigger air bubble, but it is affected by some smaller ones.
(B) A big air bubble has resulted in a deformed center maze. Scale bars: 5 mm.

Note: Allow enough time for all the NGM materials to dissolve and blend thoroughly. Poorly dissolved components will result in granulated and crumby solid NGM.

Note: 100mL of NGM are enough to prepare 6 maze plates.

6. Place the mold in, with the supporting arms firmly standing on the plate rim (Figure 1 and Methods video S1).

Note: Alternatively, the mold can be placed in the dish first, and then the liquid NGM can be poured in, through the side openings between the mold and the dish walls. This option usually results in less air bubbles trapped between the mold and the NGM (Figure 4 and Methods video S2) .

Note: Do not move the dish/mold complex during the solidification process. Make sure the bench area used is flat and leveled.

7. Allow 20–30 min for the NGM to solidify. This depends on ambient humidity, room temperature, and liquid NGM temperature.

Note: An indication that the NGM is solidified thoroughly and that it is safe to proceed to the next step is its transparency. It should be completely opaque and whitish, and it should feel cool. Alternatively, you can use a pipette tip to check the stiffness of the solid NGM. Removing the mold too early might result in deformed mazes.

8. Pull the mold out gently and in a direction perpendicular to the dish. Mazes should be imprinted in the NGM (Figure 1 and Methods video S3).

Note: To facilitate the separation of the mold and the solidified NGM, you can use a gloved thumb to gently hold the NGM down while pulling the mold out with the other hand. Do this for all sides of the mold (see Methods video S3). Troubleshooting 1

9. Allow the maze plates to dry off excess moisture overnight (8–12hr).

Note: Usually maze plates are not ready to use immediately after removing the mold from the solidified NGM. This is because there is often excess moisture on the maze floor and where the floor meets the walls, generated by the agar polymerization process. Loading the mazes while still wet will result in diffusing the bait all over the maze. Additionally, worms will tend to thrash instead of crawling, resulting in a completely different behavior. To this end, it is advised to allow the maze plates to dry, lid on, overnight (8–12hr).

Note: Alternatively, maze plates can be left to air dry, lid off, in sterile conditions (by the flame or in a sterile chamber) for ~10min, depending on ambient humidity. In this case, caution should be taken not to over dry the plates. Ideally, the same drying process should be used for all maze plates, for consistency.

10. It is advised not to use the maze plates >2 days post fabrication. This is necessary to achieve a consistently moist surface.

    Note: If maze plates need to be maintained for longer, they can be sealed with parafilm to prevent evaporation. This will make them usable for an extra 1–2 days.

    Note: It is not recommended to refrigerate the plates to prolong their lifetime. Refrigerating and then bringing them back to room temperature might result in significant fluctuations in their moisture levels.

11. Inspect the mazes carefully one by one before use. Any deformations or flaws might affect the information registered by the worm (Training maze) or retrieval of learned information (Testing maze).

    Note: If one or two of the individual mazes are deformed while the rest remain usable, then the faulty mazes can be chunked off and you can proceed with the remainders.

    Note: Do not use maze plates with signs of contamination.

Maze cover pads

⏱ Timing: 10 min slide preparation + 10 min NGM preparation + 10 min solidification

To prevent nematodes from escaping, each maze may be covered with a thin agar pad. The process is similar to any standard process followed to create agar pads for other purposes, e.g., microscopy, worm immobilization, etc. Below we provide a description of the steps to follow, with notes that apply to the specific application.

It is recommended that a few trials with the strain under study are conducted in order to determine how often worms escape the mazes. For strains that do not explore a lot, thus do not escape a lot, you might want to consider skipping the use of cover pads. This will simplify the entire process and make it more time efficient.

Note that the agar covers do not result in air-tight sealing of the maze.

12. Set aside NGM from the same batch used to make the maze plates and maintain it warm and liquid using a heat block.

    Note: Alternatively, a magnetic hot plate in medium heat under low mixing speed can be used. Caution should be taken not to overheat NGM, as this might result in smelly material that might interfere with *C. elegans*' chemosensation.

    Note: Cover the containers (glass tubes, beaker) with aluminum foil to prevent excess evaporation that might alter the properties of the material.

    Note: NGM from the batch used to make maze plates may be used the next day to make maze covers. It is not advised to use NGM that is >2days old or that has been solidified and re-melted more than twice, as this might alter the properties and potentially the odor of the material.
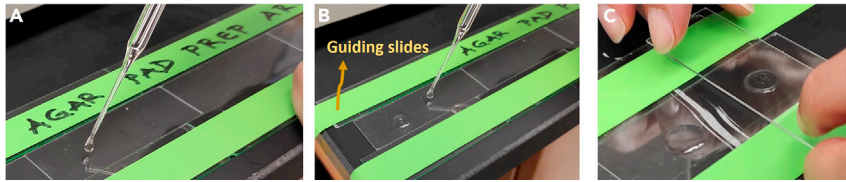
Figure 5. Making the agar cover pads
(A and B) Droplets of liquid NGM are placed on clean glass slides.
(C) A sandwich is created by placing a second slide on top.
See also Methods video S5.

13. On a flat and leveled bench, use labeling tape to create two lines, width of one glass slide apart. These lines are going to serve as guides (Figure 5 and Methods video S4).

    Note: Use two or three layers of labeling tape for each guide, as the thickness of the guides is going to affect the thickness of the agar pads.

14. Place as many glass slides as needed between the guides, one after the other, lengthwise.

    Note: Clean the glass slides before use with 70% ethanol. This will help create smoother agar pads with better optical properties.

15. Using a glass Pasteur pipette, drop a small amount of liquid NGM on a glass slide. Drop 3–4 droplets of NGM. Wait 3 s and then press gently with a second glass slide, making a sandwich. The top slide is positioned perpendicularly to the bottom one, resting on the labeling tape guides (Figure 5 and Methods video S5). Troubleshooting 5

    Note: The 3 sec wait is suggested so that the NGM will slightly cool down before the second slide is placed on top. This results in slightly thicker pads.

    Note: Two sandwiches per bottom slide can be created, to increase efficiency.

    Note: The thickness of the labeling tape guide will practically determine the thickness of the agar pad. The pads need to be not very thin, to not tear easily, and not too thick, to allow imaging the worm when traversing the maze. Some tests are advised prior to the experiment.

    Note: The exact diameter of the agar drops, i.e., of the agar pads is not crucial, as long as the resulting pads are big enough to cover the entire maze and premises. Wider than that is fine, smaller than that is not. For reference, a good size pad has a radius of ~4.5–5mm.

    Note: If the NGM is not homogeneous, this will result in not-so-transparent films, same if it is too cold. If the NGM is too hot, the pad will be too thin. Feel free to experiment to optimize your technique.

16. Wait ~10 min for the pads to cool down and solidify.
17. Gently remove the top glass slide to expose the agar pad, which is now ready to use.

    Note: It is suggested to remove the top slide when ready to use the pad, to prevent it from dehydrating and over drying.

    Note: If there are holes in the pad, do not use it.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
C. elegans N2 Bristol	*Caenorhabditis* Genetics Center (CGC), University of Minnesota	N/A
Chemicals, peptides, and recombinant proteins		
NaCl	Fisher	BP358
Agar	Becton Dickinson (BD)	214530
BactoPeptone	Becton Dickinson (BD)	2116677
Cholesterol	Sigma-Aldrich	C3045
KH_2PO_4	Fisher	BP363
K_2HPO_4	Fisher	BP362
$MgSO_4$	Fisher	BP213
$CaCl_2$	Fisher	C79
Software and algorithms		
SolidWorks 3D design software	Dassault Systèmes	https://mysolidworks.com
CellSens Imaging Software	Olympus	https://www.olympus-lifescience.com/en/software/cellsens/
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
Other		
3D printer: Form 2	Formlabs	N/A
3D printer resin: standard black resin	Formlabs	RS-F2-GPBK-04
Glass slides	Gold Seal Products	3010
Petri dishes 60×15 mm	VWR	25384–328
Camera: DP22	Olympus	https://www.olympus-lifescience.com/en/camera/color/dp22/?gclid=Cj0KCQjws ZKJBhC0ARIsAJ96n3UVi_cL4fC1HSmkwfLrt-QYLfhR4bus9arNbw80Ug9v_7c-KQ6a ENIaAnaREALw_wcB

MATERIALS AND EQUIPMENT

Liquid NGM reagent	Final concentration	Amount (for final volume 100 mL)
Agar	2%	2g
NaCl	0.3%	0.3g
BactoPeptone	0.25%	0.25g
5 mg/mL cholesterol in ethanol	0.1%	0.1 mL (to be added last)
1 M KPO_4 buffer pH 6.0 (108.3 g KH_2PO4, 35.6 g K_2HPO_4, H_2O to 1 liter)	0.25%	0.25mL
1M $MgSO_4$	0.1%	0.1mL
1 M $CaCl_2$	0.1%	0.1mL

STEP-BY-STEP METHOD DETAILS

Bait the training mazes

⊙ Timing: 15 min

Each maze plate contains nine mazes, according to the mold design described above (see Figure 1A). Each maze plate is used either as Training or as Testing plate. Training mazes are baited to attract nematodes toward a specific side arm. Each Training plate is used within 1 h from the first baiting or is discarded.

The experimental protocol and the preference indices introduced to assess *C. elegans* behavior in the maze have been developed for use with an attractant, namely OP50 or a mix of OP50 and L-lysine. We have also used successfully isoamyl alcohol 10% in ethanol (data not shown). Other attractants or food sources could be used, or even repellents. In case of the latter, the preference indices (see quantification and statistical analysis) should be adjusted and interpreted accordingly. We have not assessed *C. elegans* learning in the maze driven by a repellent, e.g., negative associative learning.

1. Tilt the Training maze plate so that the target maze arm is lower than the rest of the maze
   a. Perform this step under a dissecting microscope.
   b. Tilt the plate under a ∼45° angle.
   c. Feel free to rotate the plate to a position that allows you to reach the target location with the pipette tip.
2. Pipette carefully 1.2 microliters of liquid *E. coli* OP50 or other attractant onto the floor of the circular auxiliary area (Figure 1B) of the target maze arm.
   a. Use long reach pipette tips, which can be more easily inserted in the narrow maze space.
   b. Hold the pipette perpendicularly to the maze floor.
   c. Aim for the edge between the maze floor and the circular wall of the auxiliary area.
   d. Release the bait slowly but firmly.
   e. The inserted liquid should form a crescent-shape pocket between the maze floor and the circular wall of the auxiliary area.

   ⚠ CRITICAL: This step is critical. Loading the mazes wrongly will heavily affect the results. Refer to Methods video S6.

   Note: Take extra caution not to touch with the loaded pipette tip any other part of the maze or the maze plate. This could result in spreading the scent of the bait at unintended areas, and therefore distract the worms during Training. Mazes and maze plates that are thought to be compromised should not be used.

   Note: The amount (volume) of the bait may vary, depending on the compound concentration and the maze dimensions. It is suggested to run a few trials before deciding on the volume used. Further adjustments can be made, depending on the nematode strain used and the experimental design. As an example, for the aging experiments in Gourgou et al. (2021) (Gourgou et al., 2021) the bait included OP50 mixed in 1:1 ratio with 3M L-lysine, a well-known attractant (Bargmann, 2006), to a final volume of 1.2 microliters, to enhance the mixture's attractiveness to older *C. elegans*.

   Note: If you are baiting the mazes with a bacterial food source, make sure to vortex its container before loading the maze, as bacteria tend to precipitate. If the concentration of bacteria is imperative for the experiment, a spectrometer can be used to measure light absorption.

   Note: It is recommended to practice bait placement by using diluted food color. The process is performed efficiently when bait is placed only in the targeted area. Proceed with experiments only when food placement is mastered satisfactorily (Figure 6).

3. Continue with baiting the rest of the mazes.
   a. Bait the mazes in triplets, usually by row or by column, to avoid diffusion to neighboring mazes. Return to bait the next triplet after completing the experiments with the previous triplet.
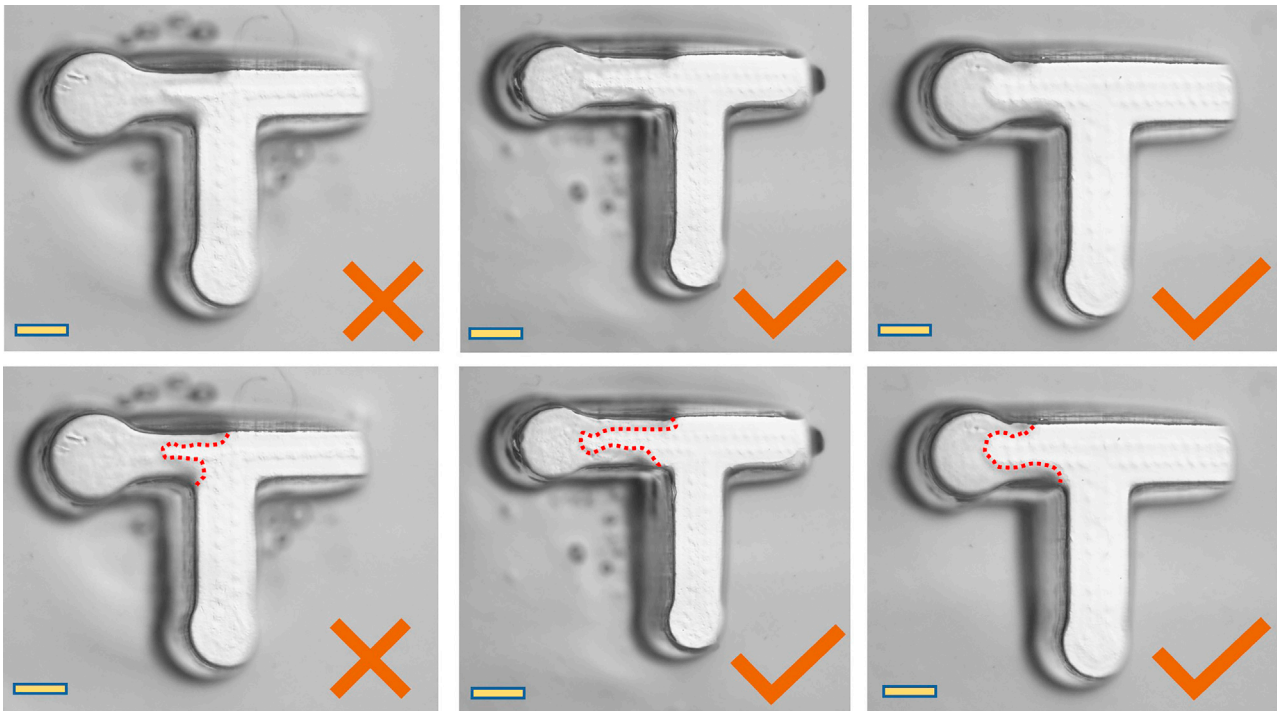
Figure 6. Bait placement in an asymmetrical T-maze
Top row: Three examples of liquid bait placement, the least successful being the left one, and the most successful the right one. Bottom row: the same T-mazes, with highlighted bait borders. Scale bars: 1 mm.

Therefore, in a maze plate with nine individual mazes (Figure 7), there will be three loading sessions.

4. Leave the baited maze plate tilted for about 10 min, until the liquid bait is absorbed.
   a. Bait can be placed on either of the maze arms. It is not advised to switch side arms for baited mazes in the same Training plate.
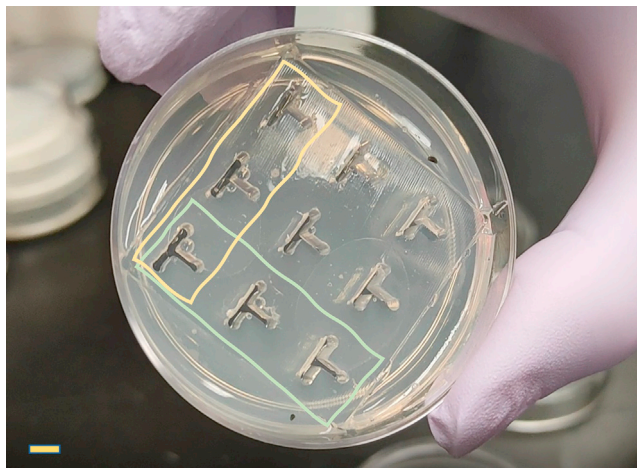

Figure 7. Loading the mazes
In a maze plate with nine mazes, it is advised to load and use them in triplets, either by row (yellow frame) or by column (green frame), to avoid bait diffusion to neighboring mazes. Scale bar: 5 mm.

Note: Bait that is not in liquid form, e.g., scrap of bacterial lawn, small chunk of NGM infused with some odorant, could also be used. The authors have not optimized the protocol for use with non-liquid bait, so preliminary studies would need to be tailored to the process accordingly.

Introduce *C. elegans* nematodes in the maze

⏱ Timing: 5 min for each worm/maze

Each maze is used only once, and only one worm is placed in each maze for each trial. Any *C. elegans* strain with adequate mobility can be tested.

5. Using a platinum wire worm picker, no food attached, pick a worm crawling outside the bacterial lawn on the culture plate.

   Note: This is necessary to minimize transfer of food along with the nematode.

6. Place the worm on the floor of the starting area of the baited Training maze (bottom auxiliary area, Figure 1B).
   a. Orient the plate so that the stem corridor of the T-maze is aligned with the picker to allow for easier placement of the worm. See also Methods video S7.
   b. Tilt the plate slightly toward you to allow for better visual contact.
   c. Lower the picker so that it gently touches the floor of the starting area.
   d. Allow the worm to climb off the picker.

   ⚠ CRITICAL: Refer to Methods video S7 on how to position the plate during this step.

   Note: The worm should land on the floor of the starting area and not on the wall. A worm that lands on a side wall presents an increased probability of starting its maze trip with a side bias.

   Note: Once the worm lands, immediately bring the plate to the intended orientation and let it rest on the microscope stage.

   Optional: Initiate the recording (see recording C. elegans maze behavior)

7. Cover the maze with an agar pad. See also Methods video S8.
   a. Remove the agar pad from the glass slide using a clean pair of tweezers.
   b. Place the agar pad on top of the maze in a way that covers the maze and its surroundings.
   c. Press it down *very gently* to seal the maze.

   Note: This step must be executed swiftly.

8. Proceed with recording (see recording C. elegans maze behavior)

Recording *C. elegans* maze behavior

⏱ Timing: varying among individual nematodes, estimated range 5–30 min

The process is described for N2 Bristol wild type *C. elegans*, however the same steps can be followed when working with mutant strains. When working with a fluorescent strain, the same protocol can be performed under a fluorescent microscope (see also Gourgou et al., 2021).

9. Switch on the imaging software.

a. Set up the recording and camera settings depending on the imaging software of choice.

b. Make sure that the entire maze can be imaged in the field of view.

Note: In Gourgou et al., 2021, we used CellSens, by Olympus.

Note: It is suggested to turn the software on before you insert the worm in the maze, i.e., before step #6. Step #10 (below) is included in this subsection as part of the recording process.

10. As soon as the worm is placed in the maze and before you put the cover on, hit the recording button.

    a. This is recommended in order to include in the recording as much of the maze trip as possible. However, if you plan to place the maze cover pad during the recording, this action will be also captured. Feel free to adjust the initiation of the recording according to your experimental needs.

    b. If you are not using an agar pad to cover the maze, just start recording as soon as the worm is inserted.

Note: If you plan to use the recordings in the future to track the worms using tracking software, refrain from changing the focus during the maze trip, because this will increase the "noise", from the perspective of the tracking algorithm.

11. Record each nematode until it reaches one of the maze arms (first decision) or until 30 min pass without reaching either end of the maze (see Gourgou et al., 2021, Supplementary Table S1). Troubleshooting 2 and 3

Note: The time limit of 30 min is selected based on the finding that most nematodes reach a maze arm in less than 30min (see Figure 6 in Gourgou et al., 2021).

12. In the Training maze, if the worm reaches the maze side with no bait, it is not processed any further (unless it is required by the experimental design). If the worm reaches the bait-containing maze arm, it is allowed to remain in the area for ~3 min.

13. After 3 min, or as soon as the worm departs from the bait-containing area, whichever happens first, the nematode is picked up using the picker.

14. If the assay is concluded, the worm is discarded.

Note: The same process is followed for worms tested in empty mazes (e.g., control experiments), with the omission of food placement.

15. If a Testing trial is to follow, then immediately after removal from the Training maze, the worm is placed into a new, empty maze (Testing maze).

Note: The Testing maze should be in a different maze plate, made from the same NGM batch.

Note: Step #15 applies also in case a second Training is to follow.

Note: Practice with diligence transferring worms from one maze to another before you run the actual experiment. This is often a time where worms get stressed or injured. Picking a nematode out of a maze should be done swiftly and decisively, as multiple picking attempts might result in stressing the worm, thus jeopardizing learned information retrieval. Troubleshooting 4

16. The second maze (Testing or second Training) is covered with an agar pad as described above (steps #7–9) and the worm is recorded until it reaches one of the maze arms (first decision recorded) or until 30 min pass without reaching either end of the maze (steps #11–16). (see Gourgou et al., 2021, Supplementary Table S1).

17. Make sure to label your recordings to indicate the Training-Testing recording pairs.

> *Note:* In case of multiple Training or Testing sessions, steps 6–18 can be repeated as many times as required. In case of increased time intervals between Training and Testing, the nematodes in study can be transferred to intermediate plates after Training and before Testing, as dictated by the experimental design.

EXPECTED OUTCOMES

A worm was considered to have made a left-side or a right-side decision when it reached the end of the respective functional maze arm (see also Figure 1, and visualization in Supplementary Table S1 of Gourgou et al., 2021). Nematodes that were inconclusive, immobile or lost, as well as worms that escaped, were censored (not scored) and not included in the indices and percentages calculations (Gourgou et al., 2021, Supplementary Table S1).

Overall, the protocol presented here provides a framework for the assessment of *C. elegans* behavior in simple mazes, provide that the strains in question are capable of elementary mobility. The experimental outcomes can provide insight on *C. elegans'* learning (e.g., before and after training). By applying moderate modifications, outcomes can shed light on *C. elegans'* decision making (e.g., use of two different baits-two choice assay) or even on more complex preference/avoidance assessment (e.g., radial maze design combined with multiple baits).

QUANTIFICATION AND STATISTICAL ANALYSIS

Nematodes that were inconclusive, immobile or lost, as well as worms that escaped, were censored (not scored) and not included in the indices and percentages calculations (Supplementary Table S1, in Gourgou et al., 2021). The time required to reach one of the maze arms can be an insightful metric, when evaluating nematodes' maze behavior (Gourgou et al., 2021), in addition to the preference indices presented below.

Decision, chemotaxis, and learning indices

To quantify *C. elegans'* behavioral outcome in simple T-mazes, we introduce the Decision Index (DI). Thus, for each maze experiment, the DI is

$$(DI) = (n_L - n_R)/(n_L + n_R)$$

where n_L = worms that reached the left side of the maze, n_R = worms that reached the right side of the maze. Note that $n_L + n_R = n$=scored worms = $N - n_{censored}$, where N = total number of worms processed, and $n_{censored}$ = worms that were censored, namely worms categorized as inconclusive, immobile, lost, or escaped (Gourgou et al., 2021, Supplementary Table S1). The DI for the control experiment is $(DI)_{Control}$, for the Training Maze experiments is $(DI)_{Train}$, and for the Testing Maze experiments is $(DI)_{Test}$.

The Chemotaxis Index (CI), which refers to *C. elegans* performance in the baited, Training Maze is

$$(CI) = (DI)_{Train} - (DI)_{Control}$$

The Learning Index (LI), which refers to *C. elegans* performance in the Testing Maze is

$$(LI) = (DI)_{Test} - (DI)_{Control}$$

Note that we calculate all percentages and indices over the number of worms that reached a maze arm, because we calculate a conditional probability: the probability of a worm reaching the same maze arm, given it has reached an arm in the first maze.

Statistical analyses

It is advised for key experiments to be replicated by different lab members, in different lab locations, if possible. Comparisons between experiments are made by applying the binomial distribution probability

test. For example, in Gourgou et al. (2021), the test was performed in MATLAB R2016b (Mathworks, USA) using the binomial cumulative distribution function of the Statistics and Machine Learning Toolbox. In all cases, comparisons are considered statistically significant when p-value<0.05.

As already explained, each worm is tested only once, and only one single worm is placed in the maze for each trial. Therefore, each worm corresponds to one single experiment. The percentages and indices reported are the percentages and indices that illustrate the behavior of the entire population in study (e.g., 85% of the worms that were scored reached the left arm of the maze, with an index value equal to 0.68). We report the standard deviation of the preference indices, along with the total number of worms that were scored (n) and the p-value of the binomial distribution test. For examples on reporting data, see Gourgou et al. (2021).

On the standard deviation of the binomial probability and the preference indices

The binomial distribution, used to model our experimental results, has both a mean and a variance. The standard deviation of the binomial distribution is calculated using the formula

$$m = n * p$$

where m=actual number of worms that reached the left (food-containing) maze arm, and n=number of worms that were scored, and p= percentage of worms that reached the left (food-containing) maze arm, and then we define

$$m' = p$$

This leads to defining the variance as

$$\sigma^2 = \frac{p(1-p)}{n}$$

And therefore, standard deviation (STD) is defined as

$$\sigma = \sqrt{\frac{p(1-p)}{n}}$$

We report the standard deviation of the calculated index as follows:

For the DI of each experiment, it is (see methods)

$$(DI) = (n_L - n_R)/(n_L + n_R)$$

where n_L = worms that reached the left side of the maze, n_R = worms that reached the right side of the maze, and $n_L + n_R = n$ = scored worms .

Therefore, it is

$$DI = \frac{2n_L - n}{n} = 2\,\frac{n_L}{n} - 1$$

Therefore, the mean is $\mu = 2p_{ctrl}-1$, and the STD is $\sigma_{DI} = 2\sqrt{\frac{p_{ctrl}(1-p_{ctrl})}{n}} = 2\sigma$

Consequently, for the CI of each experiment, it is

$$(CI) = (DI)_{Train} - (DI)_{Ctrl} = 2\left(\frac{n_{LTrain}}{n_{Train}} - \frac{n_{Lctrl}}{n_{ctrl}}\right) = 2(p_{Train} - p_{ctrl})$$

Therefore, the mean is $\mu_{CI} = 2(p_{Train}-p_{ctrl})$

And the STD of the CI is $\sigma_{CI} = 2\sqrt{\frac{p_{ctrl}(1-p_{ctrl})}{n_{ctrl}} + \frac{p_{Train}(1-p_{Train})}{n_{Train}}}$

Similarly, the STD of the LI is $\sigma_{LI} = 2\sqrt{\frac{p_{ctrl}(1-p_{ctrl})}{n_{ctrl}} + \frac{p_{Test}(1-p_{Test})}{n_{Test}}}$

On the binomial distribution probability test in MATLAB using the binocdf function

In MATLAB, the binomial cumulative distribution function binocdf(x,n,p) computes a binomial cumulative distribution function at each of the values in x using the corresponding number of trials in n and the probability of success for each trial in p, where

x: the number of worms that reached the target location (i.e., left maze side),

n: the number of worms scored, and

p: the number of worms that reached the target location over the number of worms scored, in the reference (e.g., control) experiment.

The binomial cumulative distribution function binocdf(x,n,p) allows the user to obtain the probability of observing less than or equal to x successes in n trials, with the probability p of success on a single trial. The function 1-binocdf($x-1,n,p$) allows the user to obtain the probability of observing greater than or equal to x successes in n trials, with the probability p of success on a single trial. These two functions were used to calculate the p-value under the null hypothesis in each comparison.

We provide below the MatLab code used in Gourgou et al. (2021), for reference. Numbers (n, n1, n1control) are indicative examples of real data.

```
n=80; %number of scored worms

% treatment experiment

n1=43; % actual number of worms who reached target location (e.g., left arm)

n1control=0.52*n; % expected number of worms that would have reached the target location

if the control ratio applied

t1=min([n-n1,n1]); % lower critical threshold for H0 (exclusive)

t2=max([n-n1,n1]); % upper critical threshold for H0 (exclusive)

pvalue = 1-binocdf(t2-1,n,n1control/n)+binocdf(t1,n,n1control/n)
```

LIMITATIONS

Successful food location in the Training maze requires the contribution of chemical and tactile input (Gourgou et al., 2021). In the current assay it is not possible to distinguish between food detection and subsequent food location. The current assay cannot distinguish between learning formation and learning expression (retrieval) processes. Moreover, the current experimental setting does not allow tracking of the nematodes throughout their journey, especially when they crawl on the maze walls. This limits our ability to extract information about locomotion features that are affected by learning and possibly change after successful training. Since the protocol presented here does not depend on worms' actions during the maze trip but takes into account only the destination maze arm, this does not interfere with preference indices calculations.

The protocol provides semi-quantitative assessment of how a genetic or other manipulation affects maze performance. For example, if a mutation results in a drop of the percentage of animals that reach the food containing arm by 20% compared to the control experiment (e.g., from 80% to 60%), this does not mean that the gene in question contributes to the food location ability by 20%.

The protocol, as most behavioral assays, is sensitive to environmental factors that can interfere with nematodes physiology, sensory perception, and overall behavior. Examples include ambient

temperature (protocol tested in temperatures varying from 18°C–22°C) and air humidity (protocol not tested outside a range of 36%–55% air humidity). Experimenters are also advised to refrain from using odorants (perfume, body or hand lotion, etc.) on the day of the experiment, as their scent might interfere with *C. elegans* olfaction.

TROUBLESHOOTING

Experience has shown that there is a steep learning curve and adequate time is required for most experimenters to become familiar with the numerous steps of the protocol and the factors that might interfere with nematodes performance in the maze. The steps that are more sensitive and require additional caution are i) transferring worms from one maze to the next, ii) loading the maze with food/attractant, and iii) using non-fresh NGM for cover pads.

Abort the experiment and start over, if:

the worm gets poked/injured

the destination maze is destroyed in any way

the worm is pushed into the NGM when introduced into the maze

the worm gets lost during the transfer from one maze to the next

the worm is dropped on some other place in the maze rather than the intended starting area

In all these cases, use a new worm and a new maze.

Problem 1

When removing the mold from the solidified NGM to reveal the imprinted mazes, some mazes are destroyed or there are spherical pockets not filled with NGM. Maze fabrication (step 8 in "before you begin").

Potential solution

Chunks of NGM are stuck to the mold: Allow for additional time for the NGM to cool down completely and solidify thoroughly.

Air bubbles were formed between the mold and the liquid NGM during maze fabrication: Try the alternative path of pouring the NGM after placing the mold in the petri dish, as described in step #6 of the protocol. See Figure 4.

Problem 2

Nematodes reach either of the maze arms with equal probability (this does not apply to control experiments) and/or they are not reaching the food/attractant containing arm, although they are expected to do so. Recording *C. elegans* maze behavior (step 11)

Potential solution

The food/attractant has not been loaded with precision and it has leaked significantly out of the intended baited area: Practice loading the bait using food color. Return to experimenting after you have mastered the loading technique sufficiently. This is the most common cause of non-biased behavior in the Training maze. See Figure 6.

The volume of bait solution was too large, resulting in significant leakage to other parts of the maze: Adjust the volume of bait solution.

Droplets of the bait are retained on the outer surface of the loading tip and they touch other maze surfaces besides the intended baited area: Use some tissue to wipe the tip and opt for low retention pipette tips.

The pipette tip is not thin or long enough to reach safely the target location and the bait is not released with precision: Switch to elongated pipette tips, engineered for long reach.

OP50 or other bacterial food source had precipitated in the stock container, resulting in not aspirating enough bacteria to attract the worms: Vortex the container before use.

The nematode in study was picked while crawling on the bacterial lawn in the stock plate and food was attached on its body, thus carrying food odor around: Aim for worms that crawl outside of the food lawn in the stock/culture plate.

When working with adult Day 1 (L4+1) worms, make sure that they have indeed reached Day 1, i.e., have started laying eggs, as larvae might respond differently to attractants.

When working with older animals, e.g., adult Day 5 and older, consider enhancing the food bait by adding some attractant, e.g., L-lysine, isoamyl alcohol, or other.

Problem 3
Worms escape the maze in large numbers, i.e., more than 10–20% of scored animals or more than usual. Recording C. elegans maze behavior (step 11)

Potential solution
If using cover pads, make sure that the NGM used is not >2days old, was not burnt during preparation, and that here are no holes in the pads themselves.

Make sure that there is no unintended odor source around, including perfume or other scented product on the experimenter's hands.

Check the stock/culture plate and the maze plate for any contamination.

Problem 4
When worms are placed in the second maze, they are not moving or they stop moving shortly after they land on the maze floor. Recording C. elegans maze behavior (step 15)

Potential solution
Make sure that transferring the worms from the first to the second maze is done swiftly and without touching the worm multiple times. If you cannot pick up the worm by the first or at most the second attempt, abort the experiment and start over with a new worm. *C. elegans* nematodes can be stressed by multiple picking attempts. This in turn might result in unmotivated animals or in "overwriting" what they have learned during Training, thus affecting experimental outcome.

Problem 5
The agar maze covers are too thin, or they have holes. Maze cover pads (step 15 in "before you begin")

Potential solution
When NGM is too hot, the droplets tend to spread more on the glass slide. To avoid agar pads that are too thin, as a rule of thumb, make sure that the beaker containing the NGM is tolerable to touch for a few seconds.

When dropping liquid NGM on the glass slides with a pipette (Figure 5), if there are air bubbles trapped in the droplet, they will result in holes in the solidified pad. To avoid trapped air bubbles, work to aspirate and release liquid NGM gently.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ao-Lin Hsu, aolinhsu@umich.edu.

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate/analyze any datasets/code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100829.

AUTHOR CONTRIBUTIONS

E.G. contributed in conceptualization, ideation, design, and manufacturing of molds; testing, troubleshooting, and development of the protocol; and wrote the manuscript. A.-L.H. contributed in conceptualization and ideation of molds; consultation and troubleshooting of the protocol; and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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