Video Article

# Basic *Caenorhabditis elegans* Methods: Synchronization and Observation

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## Abstract

Research into the molecular and developmental biology of the nematode *Caenorhabditis elegans* was begun in the early seventies by Sydney Brenner and it has since been used extensively as a model organism [1]. *C. elegans* possesses key attributes such as simplicity, transparency and short life cycle that have made it a suitable experimental system for fundamental biological studies for many years [2]. Discoveries in this nematode have broad implications because many cellular and molecular processes that control animal development are evolutionary conserved [3].

*C. elegans* life cycle goes through an embryonic stage and four larval stages before animals reach adulthood. Development can take 2 to 4 days depending on the temperature. In each of the stages several characteristic traits can be observed. The knowledge of its complete cell lineage [4,5] together with the deep annotation of its genome turn this nematode into a great model in fields as diverse as the neurobiology [6], aging [7,8], stem cell biology [9] and germ line biology [10].

An additional feature that makes *C. elegans* an attractive model to work with is the possibility of obtaining populations of worms synchronized at a specific stage through a relatively easy protocol. The ease of maintaining and propagating this nematode added to the possibility of synchronization provide a powerful tool to obtain large amounts of worms, which can be used for a wide variety of small or high-throughput experiments such as RNAi screens, microarrays, massive sequencing, immunoblot or *in situ* hybridization, among others.

Because of its transparency, *C. elegans* structures can be distinguished under the microscope using Differential Interference Contrast microscopy, also known as Nomarski microscopy. The use of a fluorescent DNA binder, DAPI (4',6-diamidino-2-phenylindole), for instance, can lead to the specific identification and localization of individual cells, as well as subcellular structures/defects associated to them.

## Video Link

The video component of this article can be found at https://www.jove.com/video/4019/

## Protocol

### 1. Protocol A: Culturing Worms for Bleaching [11]

Large populations of *C. elegans* can be obtained by culturing them either in liquid media or on solid media in plates. They are usually grown on solid NGM (Nematode Growth Media) and fed with *E. coli* bacteria, which is added to the plates either alive or dead (killed by UV[12], by heat[13] or by cold[14]). The most common procedure uses live OP50 *E. coli*, which is defective in the synthesis of uracil and cannot overgrow into a thick layer that would obscure the worms.

1. Mix 3 g of NaCl, 17 g of agar and 2.5 g of peptone and add 975 ml of $H_2O$. Autoclave for 50 min.
2. Cool the flask to 55 °C.
3. Add 1 ml of 1 M $CaCl_2$, 1 ml of 5 mg/ml cholesterol in ethanol, 1 ml of 1 M $MgSO_4$ and 25 ml of 1 M $KPO_4$ buffer (all of them but cholesterol previously autoclaved).
4. Using sterile procedures dispense the NGM solution into Petri plates; fill plates up to 2/3 of their volume.
5. Once dry, it is advisable to leave plates at room temperature for 2-3 days before use for detection of contaminants. Prepare a streak of OP50 *E. coli* from a glycerol stock (OP50 can be obtained from the Caenorhabditis Genetics Center).
6. Pick a single colony and grow it in LB overnight at 37 °C with agitation.
7. Allow excess of moisture to evaporate from the plates by removing the lid in the laminar flow and add OP50 to the center of the plates with a sterile Pasteur pipette.
8. Allow the OP50 *E. coli* lawn to grow overnight at room temperature or at 37 °C for 8 hours.

9. Add the desired amount of worms to the plates (if incubated at 37 °C plates should be cooled at room-temperature before use).

TIPS:

- The pouring of the same amount of media in the plates with a pipette or a pump dispenser ensures the same volume of agar to the plates and facilitates the shifting of plate without need to refocus the stereomicroscope.
- Plates (both seeded and unseeded with bacteria) can be used several weeks after prepared when stored in a container at room temperature or 4 °C.
- Avoid plating the bacteria to the edge of the plate. If the lawn extends to the edges of the plate the worms may crawl up the sides, dry out and die.
- Worms live longer if the bacteria seeded on the plates are already dead [15].

## 2. Protocol B: Treatment with Alkaline Hypochlorite Solution ("Bleaching")[11]

The bleaching technique is used for synchronizing *C. elegans* cultures at the first larval stage (L1). The principle of the method lies in the fact that worms are sensitive to bleach while the egg shell protects embryos from it. After treatment with alkaline hypochlorite solution, embryos are incubated in liquid media without food, which allows hatching but prevents further development.

1. Allow worms to grow until adult stage.
2. Recover gravid adults in 15 ml tubes by washing plates with M9 buffer.
3. Pellet worms by centrifuging for 2 minutes at 400xg (~1500 rpm on a standard table centrifuge) at room-temperature and discard supernatant.
4. Perform 1-3 washes until the buffer appears clear of bacteria.
5. Add the desired bleaching solution (**Table I**) and agitate for some minutes (destruction of the adult tissue should be monitored under the dissecting microscope and the reaction stopped when traces of adults are still visible, which typically takes between 3 and 9 minutes depending on several issues, such as the volume of worm pellet, mentioned in the discussion) (**Fig. 3**).
6. Stop reaction by adding M9 buffer to fill the tube.
7. Quickly centrifuge (since treatment may still be active) for 1 minute at 400 x g and discard supernatant.
8. Wash pellet three more times by filling the tube with M9 buffer.
9. Add 1 ml of M9 buffer to the pellet, or place the eggs to unseeded NGM plates, and incubate at the desired temperature with gentle agitation. Proper aeration should be provided (**Fig. 4**).

TIPS:

- There are different bleaching solutions, choose the one that works better in your hands (**Table 1**, **Fig. 1**).
- Eggs already laid on the plates can be recovered by scrapping the surface of the agar with a soft material such as a piece of an X-ray film.
- Too many remains of adult animals may impair synchronization as they constitute a food supply for the recently hatched larvae.
- Higher temperatures slightly speed up the development which is inconvenient if any worm skips synchronization because the difference in development between synchronized and unsynchronized worms will be greater at higher temperatures.
- Bleaching solution must be performed just prior to its use. In addition, bleach loses potency after it has been open for a while, in part due to its photosensitivity. We suggest to aliquot each new bottle into small amber bottles to prevent such loss and minimize exposure to light.

## 3. Protocol C: Worm Plating

1. Wait between 12 and 24 hours (time to complete embryonic development depends on the temperature) after bleaching was performed and recover worms by centrifugation (2 minutes at 400 x g).
2. Discard supernatant, seed worms on the required plates and let remaining liquid dry.
3. Place plates at the required temperature.

TIPS:

- L1 larvae in M9 buffer can be kept at 15 °C rocking at least for one week without obvious alterations.
- be careful when calculating the worms you will seed because too many may exhaust the food faster than expected and ruin your experiment. Approximately 500 L1 can reach adulthood in a 55 mm plate without running out of food.

## 4. Protocol D: *C. elegans* Observation

### D.1 Nomarski observation

Differential interference contrast microscopy is an optical microscopy illumination technique used to enhance the contrast in unstained transparent samples. The word Nomarski refers to the prism used, named after his inventor. By observing animals alive we are able to examine the physiology of the animal with the only alterations derived from immobilization. In addition, as no fixative is added, fluorescent markers can be observed *in vivo*. This fact and the possibility of fusing fluorescent markers to a gene of interest make it feasible to follow processes in which the protein of study may be involved. By using the technique described in this protocol, not only live worms can be observed, but they can also be recovered and plated again.

Agar pad preparation (just before use):

1. Prepare agarose 2% in water and melt. Keep melted at 65 °C.
2. Place two slides with a piece of tape on them at both sides of a third, clean slide.
3. Using a Pasteur pipette place a drop of agar onto the clean surface.
4. Cover the agar with another clean slide placed on top of the three slides perpendicularly.
5. Press gently so the agar drop is flattened to the thickness of the tape spacers.
6. Once the agar solidifies, gently pull out the taped slides and separate the two remaining slides by sliding one relative to the other.

Mounting live animals

7. Place one drop (10 µl) of levamisole 1mM or sodium azide 10-30 mM onto the center of the pad.
8. Transfer animals into the drop using a worm pick.
9. Gently place a coverslip over the animals and fix it at both sides with some nail polish or silicone.

TIPS:

- Keep aliquots of agarose 2% at 4 °C.
- Melted agarose can be kept at 65 °C for at least one day.
- Note: Levamisole is a nicotinic receptor agonist which elicits spastic muscle paralysis[16].
- **Be cautious, Sodium Azide is extremely toxic!**

**D.2 Ethanol fixation and DAPI staining**

The protocol described here represents a fast way of dyeing worms with DAPI, however because of the dissecation of the worm some structures may present some alteration. There are several other methods to fix worms previous to DAPI staining such as fixation with Carnoy's solution or formaldehyde that preserve better the integrity of the worm [17].

Ethanol fixation (modified from [18])

1. Place ~10 µl of M9 buffer (or water) on a microscope slide.
2. Using a worm pick carefully transfer 10-25 worms to the drop.
3. Using filter paper or a micro-pipette remove as much M9 buffer as possible without removing the worms or letting them dry.
4. Add ~10 µl of 90% ethanol and let it dry.
5. Repeat step 4 once or twice.

4',6-diamidino-2-phenylindole (DAPI) staining

6. Mix DAPI with the desired mounting media to a final concentration of 2 ng/µl.
7. Once the ethanol has evaporated completely, add 7 µl of the DAPI:mounting media mixture.
8. Place a coverslip and fix it at both sides with some nail polish or silicone. Slides will be ready for observation approximately 5 min after the addition of DAPI.

TIPS:

- The mounting media contains glycerol, so a small amount is enough to cover the whole preparation.
- There exists a wide variety of commercial mounting media (Fluoromount or Prolong, for example), their quality and price depend on how long you want to store your sample.
- **Be careful, DAPI is a known mutagen which binds strongly to A-T rich regions in DNA.**

**Recipes**

**Nematode Growth Medium (NGM)**

1.7% (w/v) Agar
50 mM NaCl
0.25% (w/v) Peptone
1 mM $CaCl_2$
5 µg/ml Cholesterol
25 mM $KPO_4$
1 mM $MgSO_4$

**M9 buffer**

22mM $KH_2PO_4$
42 mM $Na_2HPO_4$
86 mM NaCl
1 mM $MgSO_4$

**Bleaching solutions tested**

| | recipe #1 | recipe #2 | recipe #3 | recipe #4 | recipe #5 |
|---|---|---|---|---|---|
| water (ml) | 2.75 | 3.5 | 0.5 | 0.5 | 1.5 |

| | | | | | |
|---|---|---|---|---|---|
| sodium hydroxide (ml) | 1.25 (1M) | 0.5 (5M) | 2.5 (1M) | 2.5 (2M) | 2.5 (1M) |
| sodium hypochlorite ~ 4% (ml) | 1 | 1 | 1 | 2 | 1 |
| total (ml) | 5 | 5 | 4 | 5 | 5 |

**Table I.** Different bleaching solution recipes tested for this article. Recipes #3 and #4 are 2x, and should be added to the same volume of M9. Recipes for #1, #2 and #5 have been previously reported [2, 11, 19]. Final concentrations: #1 NaOH 0.25M, NaOCl ~0.8%, #2 NaOH 0.5M, NaOCl ~0.8%, #3 NaOH 0.625M, NaOCl ~1%, #4 NaOH 1 M, NaOCl ~1.6%, #5 NaOH 0.5M, NaOCl ~0.8%.

## 5. Representative results

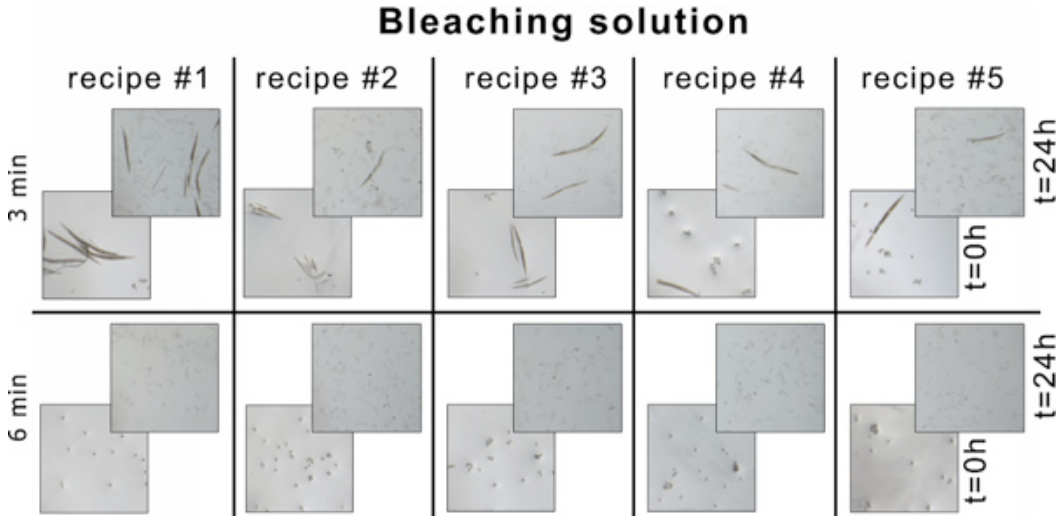


**Figure 1. Comparison of five different bleaching solutions at two different incubation times.** N2 worms washed twice with M9 were split into five 15 ml conical tubes containing each bleaching solution. Tubes were shaken vigorously and 1 ml transferred to a new tube with M9 to stop the reaction after the time specified. After bleaching procedure worms were incubated with 1 ml of M9 at 20 °C for 24 hours. In each case, lower picture was taken just after bleaching, upper picture 24 hours later.

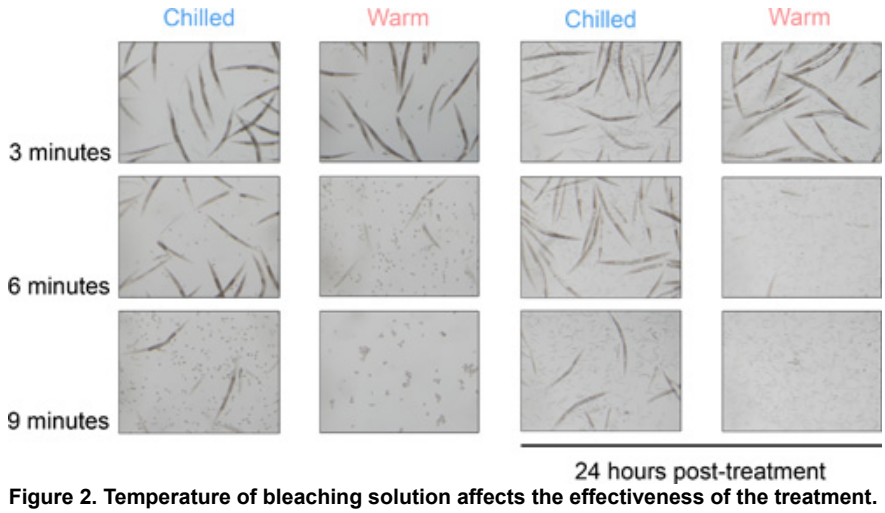


**Figure 2. Temperature of bleaching solution affects the effectiveness of the treatment.** Equal volumes of N2 worms were bleached with the same bleaching solution either previously chilled on ice for 20 minutes or kept at 25 °C for the same time. The two columns on the left show pictures just after bleaching. After treatment worms were incubated in 15 ml conical tubes with 1 ml of M9 at 20 °C for 24 hours. Columns on the right display pictures 24 hours later.

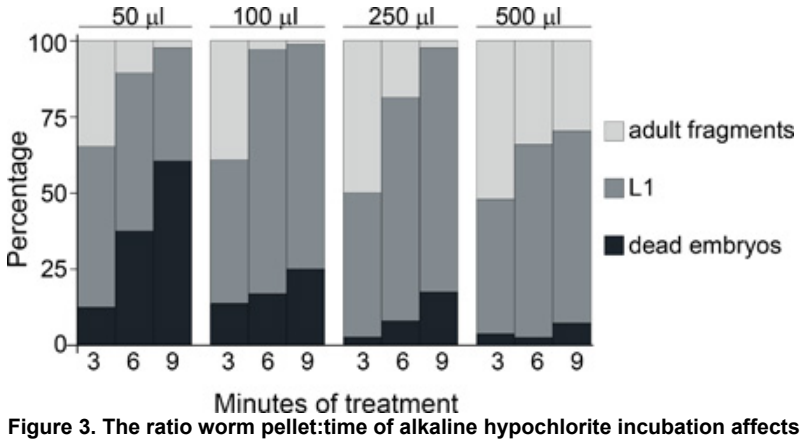


**Figure 3. The ratio worm pellet:time of alkaline hypochlorite incubation affects the effectiveness of the treatment.** 50, 100, 250 and 500 µl of worm pellet were incubated with 2 ml of bleaching solution #3 for 3, 6 and 9 minutes. Hatched L1, dead embryos and remains of adult fragments were quantified after incubation at 20 °C for 24 hours in 15 ml conical tubes with 1 ml of M9 buffer. Approximately three confluent 55 mm plates with adult worms are needed to get a 100 µl worm pellet.

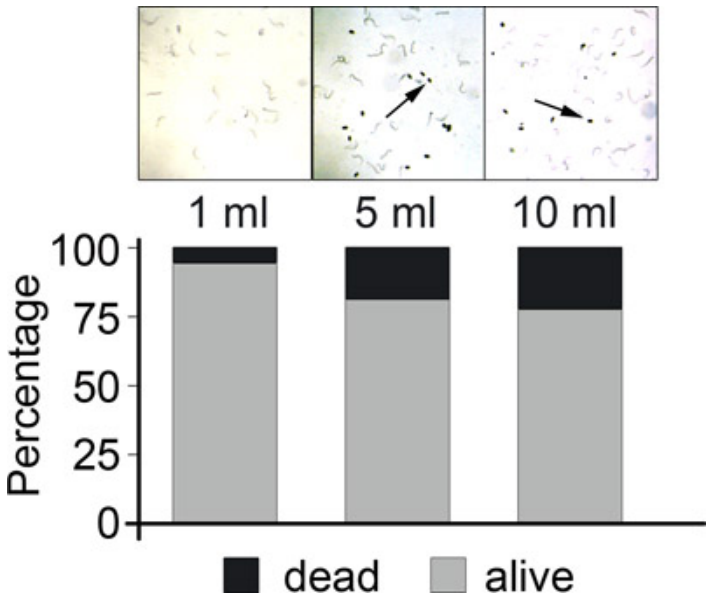


**Figure 4. Proper aeration is required for hatching and survival of *C. elegans* embryos.** A 100 µl pellet of N2 worms were bleached for 6 minutes and incubated in 15 ml conical tubes with 1, 5 or 10 ml, as specified, of M9 at 20 °C for 24 hours. The upper part of the figure displays pictures of the cultures after 24 hours, where arrows indicate eggs that did not hatch. At the bottom, there is a graph depicting the amount of larvae (light grey) and dead embryos (dark grey) 48 hours after bleaching at the stated conditions.

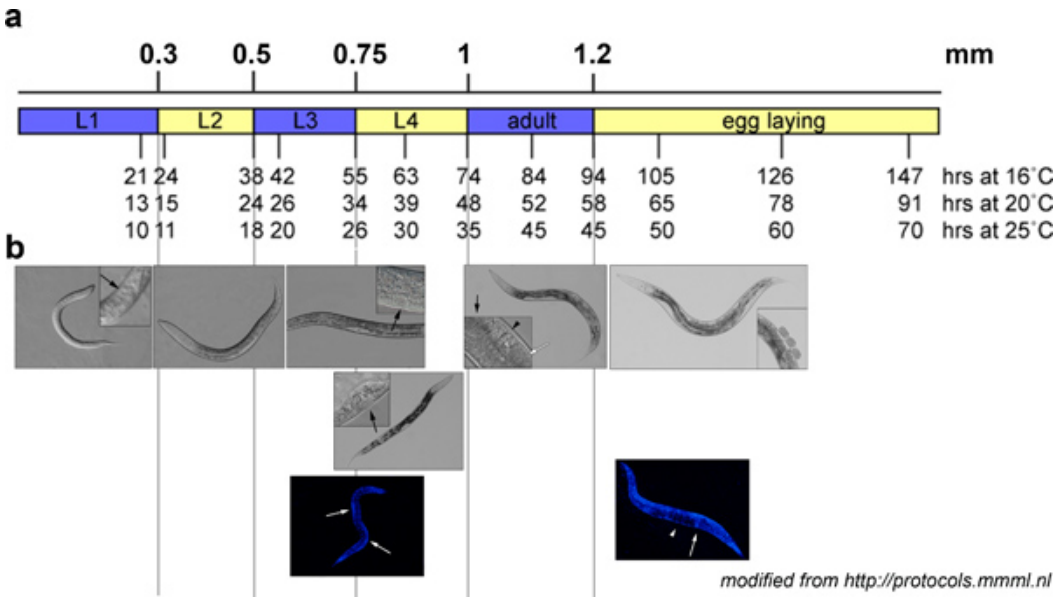


**Figure 5. Life cycle of *C. elegans*. a** . Approximate length of the worms at different stages. Hours required to reach each stage depending on the temperature (modified from [20]). **b.** Nomarski (up) and DAPI (down) pictures of different worms at the indicated developmental stages. Most significant features in each phase are magnified. **L1:** arrow indicates the precursors of the somatic gonad and the germ line. **Early L4:** black arrow (Nomarski) indicates the developing vulva; white arrows (DAPI) indicate the two gonadal arms. **Mid-late L4:** arrow indicates the developing vulva at the so-called Christmas tree stage. **Young Adult:** black arrow indicates an embryo inside the uterus, arrowhead points to the spermatheca, white arrow indicates an oocyte. **Gravid adult:** arrowhead (DAPI) points out fertilized embryos. Arrow in DAPI image indicates spermatheca.

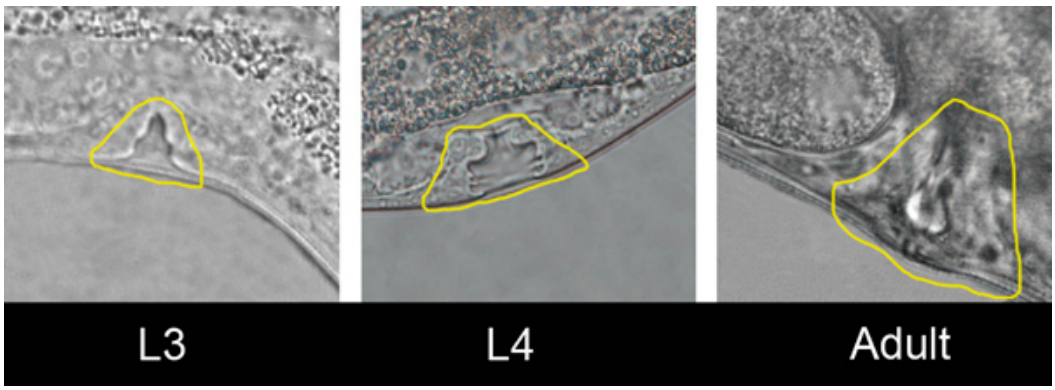


**Figure 6. Vulva morphology at L3, L4 and Adult stages.** At the L3 stage only a small lumen where the vulva is formed can be observed. At L4, this lumen expands forming the so-called "Christmas tree". In the adult the vulva is already closed. Yellow lines indicate the location of the vulva at these three stages.

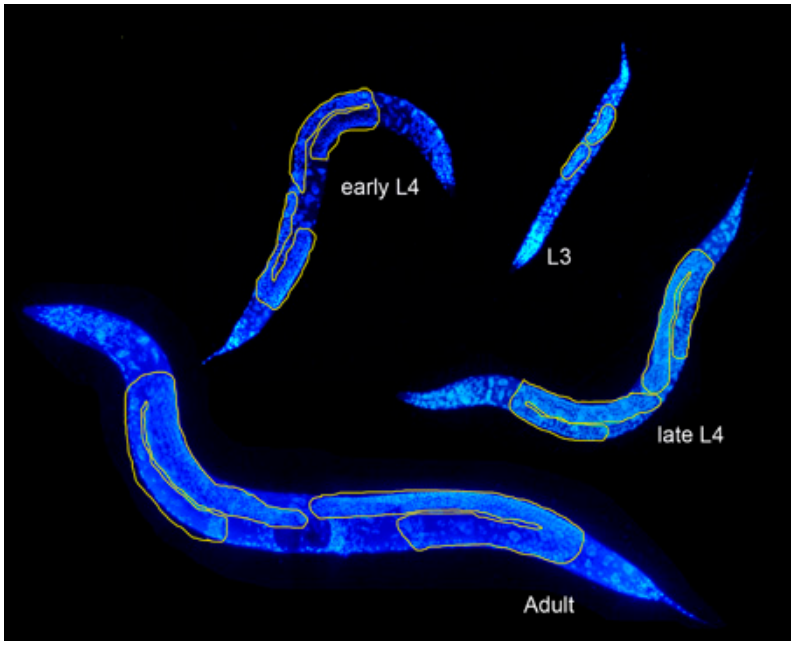


**Figure 7. DAPI staining at L3, early L4, late L4 and Adult stages.** At L3, germ line is elongated. At L4, gonad arms present U-shape morphology. At late-L4 stage sperm can be observed in the distal part of the gonad. Young Adults present oocytes. The Adult germ line presents oocytes and embryos. Yellow lines delimitate germ lines at the different stated stages.

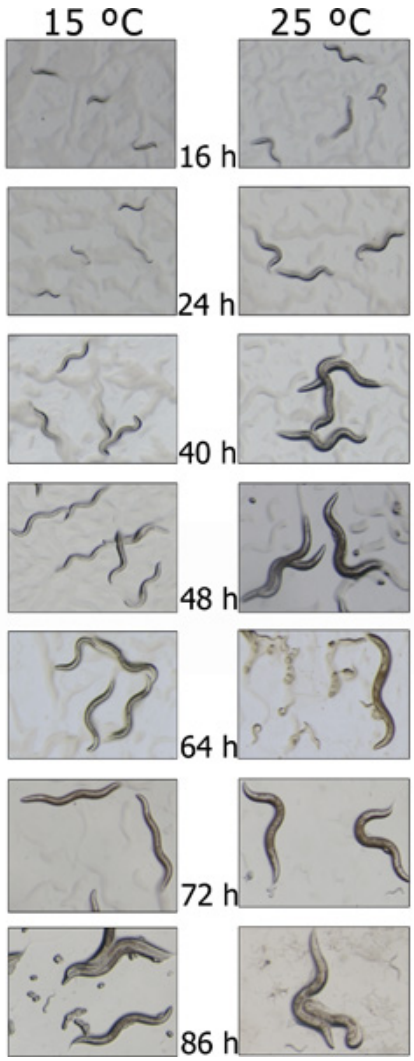

**Figure 8. *C. elegans* development at 15 and 25 °C.** N2 worms were bleached, incubated overnight in M9 and agitation at 15 °C, transferred to plates and grown the indicated times at the stated temperatures.

## Discussion

### Nematode Synchronization

Several bleaching solutions have been described. We tried five different recipes (**Table I**) and, in our hands, they did not show significant differences in the synchronization of worm populations (**Fig. 1**). However, our experiments did show that parameters such as temperature (**Fig. 2**), the ratio bleaching solution:volume of worms (**Fig. 3**) and the volume of M9 with which the embryos are incubated for hatching (**Fig. 4**) do affect the survival of the worms, being related to proper aeration of the culture. In our shaking conditions, while in a tube of 15 ml a volume of 1 ml allows survival of all worms, a volume of 5 ml is already too much to allow proper egg hatching and comparable to the maximum volume of 15 ml (not shown).

### *C. elegans* Development

During its development, *C. elegans* goes through four larval stages (**Fig. 5**) prior to the adult stage. The germ line is a good indicator of the developmental stage of *C. elegans*. The easiest feature of *C. elegans* development that can be observed under Nomarski optics is the development of the vulva, which starts to form at early L4 stage. At first, only a small lumen is observed, which later expands to the so called "Christmas tree" shape, by mid-late L4. Finally, by the end of L4 the vulva closes (**Fig. 6**). On the other hand, DAPI staining allows the observation of the development of the gonad. From the four cells in L1 to the dividing cells and elongating gonad in L2 and L3. At L3 the distal tip cells can be observed, starting to migrate dorsally. Meiosis also starts by the end of L3. At L4 distal tip cells reach their definitive position and germ cells differentiate to sperm. By the end of L4 sperm production ends and oocyte production starts. In adult worms embryos can be observed inside the uterus (**Fig. 7**).

### Development and Temperature

*C. elegans* develops at a different rate depending on the temperature: while it takes about 90 hours from the moment the egg is laid until the new worm starts to lay its own eggs at 15 °C, 45 hours are enough when grown at 25 °C (**Fig. 6**). The study of the differential developing rate at diverse temperatures leads to relative flexibility in setting up conditions and performing experiments. Additionally, it offers the possibility not only to monitor the effects of a particular treatment or alteration (for example temperature sensitive alleles), but also to establish the best conditions in which carrying out a particular experiment.

## Disclosures

We have nothing to disclose.

## Acknowledgements

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