Video Article

Novel Metrics to Characterize Embryonic Elongation of the Nematode *Caenorhabditis elegans*

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Abstract

Dissecting the signaling pathways that control the alteration of morphogenic processes during embryonic development requires robust and sensitive metrics. Embryonic elongation of the nematode *Caenorhabditis elegans* is a late developmental stage consisting of the elongation of the embryo along its longitudinal axis. This developmental stage is controlled by intercellular communication between hypodermal cells and underlying body-wall muscles. These signaling mechanisms control the morphology of hypodermal cells by remodeling the cytoskeleton and the cell-cell junctions. Measurement of embryonic lethality and developmental arrest at larval stages as well as alteration of cytoskeleton and cell-cell adhesion structures in hypodermal and muscle cells are classical phenotypes that have been used for more than 25 years to dissect these signaling pathways. Recent studies required the development of novel metrics specifically targeting either early or late elongation and characterizing morphogenic defects along the antero-posterior axis of the embryo. Here, we provide detailed protocols enabling the accurate measurement of the length and the width of the elongating embryos as well as the length of synchronized larvae. These methods constitute useful tools to identify genes controlling elongation, to assess whether these genes control both early and late phases of this stage and are required evenly along the antero-posterior axis of the embryo.

Video Link

The video component of this article can be found at https://www.jove.com/video/53712/

Introduction

For nearly 50 years the nematode *Caenorhabditis elegans* established itself as a powerful model to study important questions in development, neurobiology, evolution, host-pathogen interactions, *etc.*[1] The strength of this model in the study of development lies in: its short life cycle of 3 days; the ease with which these animals can be genetically altered; its transparency that enables the observation of cell displacement and morphology in living animals and its development that is mostly extra-uterine. The developmental stages of the nematode involve embryogenesis and four larval stages (L1 to L4), followed by adulthood. During embryonic development, epidermal morphogenesis drew considerable attention for its ability to enable a better understanding of how epithelial cells migrate as a group, how they reorganize their junctions and modify their individual morphology as well as their relative positioning within a functional epithelium. Epidermal morphogenesis is divided into four stages: dorsal intercalation consisting in the reorganization of dorsal epidermal cells, referred to as the hypodermis; ventral enclosure, consisting in migration of ventral hypodermal cells towards the ventral midline thus encasing the embryo in an epithelial cell monolayer; early and late elongation transforming the bean-shaped embryo into vermiform larvae. Following morphogenesis, embryo hatch and L1 larvae start feeding using available bacteria in their immediate environment.

Embryonic elongation is therefore a late phase of the embryonic development. It consists of the extension of the embryo along its longitudinal axis and a reduction of its transverse diameter. This involves a dramatic modification of the shape of the hypodermal cells. Elongation is divided into an early and a late phase. The early phase starts at the comma stage and ends when body-wall muscles start contracting at 1.75-fold stage in *wild-type* (*wt*) embryos — corresponding to embryos that are 1.75-fold in length compared to non-elongated embryos. Morphogenic processes occurring at that stage are mainly driven by contraction of filamentous actin bundles (FBs) located at the apical pole of hypodermal cells that drive their elongation along the antero-posterior axis of the embryo[2]. Contraction of FBs is control by phosphorylation of myosin-light chains by three kinases LET-502/ROCK, MRCK-1 and PAK-1 [5]. The late phase of the elongation, starts when body-wall muscles become functional and start contracting. It involves mechanotransduction signaling from the body-wall muscles to the dorsal and ventral hypodermal cells and ends when animals hatch[3].

Elongation defects are generally characterized by the percentage of animals dying as embryos (Embryonic lethality; Emb) and those arresting their development as L1 larvae (Larval arrest phenotype; Lva) and being significantly shorter than *wt*. Identification of the stage of developmental arrest requires microscopic observation of dead embryos and arrested Larvae[3-6].

It was recently shown that several genes, such as the Cdc42/Rac regulator and effector *pix-1* and *pak-1,* control morphogenic processes during both early and late elongation[3,7]. We also recently showed that morphogenic processes differ along the antero-posterior axis of the embryos during early elongation[37]. These findings motivated the development of novel metrics specifically targeting early or late elongation stages and other metrics enabling the characterization of the morphology of embryos along their antero-posterior axis during early elongation.

These novel methods consist in measuring the length of embryos at the beginning and at the end of early elongation as well as the width of their heads and tails.[7] Two protocols were also developed to measure the length of newly hatched larvae, synchronized at L1 stage[7].

The eggshells of the embryos protect them against alkaline hypochlorite treatment while larvae, adults and bacteria present in the culture media are dissolved by the treatment. This treatment is then used to purify embryos from a non-synchronized population containing a majority of well-fed adults[8]. Food restriction is used to synchronize newly hatched larvae. Measuring the length of these larvae is then used to detect elongation defects. This measurement is preferred over the measurement of arrested larvae on culture plates because larvae that hatch from non-fully elongated embryos can recover to "normal length" when feeding but will maintain their reduced size when arrested in the absence of food.

Here, we present detailed protocols enabling the measurement of the length of elongating embryos as well as the width of their head and tail using time-lapse DIC microscopy and image analysis (Protocol 1). We also provide detailed protocols to measure the length of synchronized larvae using image analysis (Protocol 2) and Flow-Cytometry (Protocol 3).

Protocol

1. Characterization of Early Elongation Defects in *WT* and Mutant Animals

1. **Mounting Embryos for Normarski DIC Microscopy**
    1. Prepare the following culture media and material:
        1. M9 Buffer, dissolve 12.8 g/L $Na_2HPO_4•7H_2O$, 3 g/L KH_2PO_4, 5 g/L NaCl, 0.25 g/L $MgSO_4•7H_2O$ in distilled water and autoclave to sterilize.
        2. NGM plates, dissolve 3 g/L NaCl, 16 g/L agar, 2.5 g/L bactopeptone in ddH_2O. Autoclave and add 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM phosphate buffer and 5 µg/ml cholesterol. Pour 6 ml of NGM per 60 mm dishes. Allow the medium to solidify for 24 hr at room temperature. Add a few drops of a saturated culture of *E. coli* OP50 bacteria grown in Luria broth (LB). Let the bacteria grow for 48 hr and store the plates at 4 °C.
        3. To generate the worm pick, mount a 0.01" diameter platinum wire on a short Pasteur pipette. Fasten the platinum wire to the extremity of the glass pipet by heating this extremity with a benzene burner. Flatten the extremity of the wire to generate a sort of shovel.
    2. Grow the worm strain on 60 mm NGM plates with OP50 at 20 °C.
    NOTE: Thermosensitive alleles may require growing the animals at non-permissive temperatures at up to 25.5 °C. Plates should contain many young adults and still plenty of food in order to pursue the protocols.
    3. Place a microscope slide between two spacer slides covered by two layers of masking tape (**Figure 1A**).
    4. Prepare a 3% agarose solution (weight/volume) in M9 buffer by dissolving 0.6 g of agarose powder in 20 ml M9 buffer by heating in the microwave during 30 sec. Allow the solution to cool down for 5 min.
    NOTE: The agarose solution can be used for a few days after melting in a microwave. It can also be aliquoted and stored at 4 °C for weeks.
    5. Place a drop of hot 3% agarose solution on the glass slide located between the two spacer-slides. Avoid forming bubbles.
    6. Rapidly cover the agarose with another slide as shown in **Figure 1B** and press down gently. The top slide will flatten the agarose drop and generate a pad with the thickness of two layers of masking tape.
    7. Allow the agarose to solidify for at least 1 min at room temperature or until the embryos are ready to be transferred to the pad.
    8. Using a worm pick, transfer 20 to 30 well fed young adults from the NGM-plate into a micro-centrifuge tube containing 400 µl of M9 buffer.
    9. Allow the worms to sediment by gravity for approximately 5 min and remove the supernatant using a micropipette. This step aims to remove the maximum of bacteria picked with the nematodes.
    10. Add 200 µl of M9 buffer to each tube.
    11. Using a Pasteur pipette, transfer the content of the tube (buffer and nematodes) to a watch glass.
    12. Use one or two 25G 5/8" needles to cut the animals at the mid-body section (between the spermatheca and the vulva).
    NOTE: A scalpel blade can also be used as an alternative to needle(s). Do this on swimming nematodes and may require some practice. The idea is to use needles as scissors or as a knife depending how many needles are used. Once the animals are cut open, hermaphrodites release their embryos into the buffer.
    13. Concentrate embryos at the center of the watch glass through generation of a circular vortex within the liquid.
    14. Remove most of the M9 from the watch glass using a micropipette. Leave approximately 30 µl of M9 with the embryos.
    15. Remove the slide covering the agarose pad (**Figure 1B**) by sliding it off the pad.
    16. Cut the pad with a razor blade in order to be able to completely cover it with a coverslip (**Figure 1C**).
    17. Using a Pasteur pipette, transfer all the embryos (and worm debris) onto the agarose pad.
    18. Group the embryos at the center of the pad using an eyelash glued at the extremity of a tip used for 200 µl micropipettes.
    19. Slowly place a coverslip on the pad avoiding bubble formation and seal it.
    NOTE: Several sealers can be used. We use drawing gum found in art and craft stores (usually used as masking gum for aquarelle painting). This gum is used because it is hydrophilic and solidifies reasonably fast and is not toxic for the worms. Slides can also be sealed with nail polish or VALAP (mixture made of Vaseline, Lanolin and Parafin wax).
    20. Allow the drawing gum dry for approximately 15 min.
2. **Recording Early Elongation using Four-dimensional Normarski DIC Microscopy**

NOTE: The objective of this step is to record early elongation from comma to the beginning of late elongation — defined as the moment when the body-wall muscles start contracting. We also aim to record more than one embryo at the time. Eight hours of recording are usually required to record early elongation for a group of non-synchronized embryos.

1. Place the mounted-slide on the stage of a microscope. Ensure that the microscope is equipped with 10X and 60X objectives as well as DIC lenses, prism, camera and capture software enabling time-lapse microscopy and generation of Z-stacks (automated Z-platform).
   NOTE: ensure that the temperature is constant between 20 and 23 °C in the microscopy room. A heating-cooling chamber may be required on the microscope stage, especially when using thermosensitive mutants.
2. Identify a group of embryos at pre-morphogenesis stages using the 10X objective.
3. Once located, slide off the 10X objective and add a drop of oil immersion on the slide.
4. Using the 60X objective, identify the top and the bottom of embryos to setup the Z-stack imaging parameters.
   NOTE: Do not hesitate to set the Z-stack larger than the thickness of the embryos. Set the distance between two adjacent planes as 0.8 µm. This will enable to cover the total depth of the embryos in 35 to 50 Z-plans.
   NOTE: If the microscope contains an automated xy platform, embryos located at different locations of the pad could be recorded simultaneously. To do so, record xy coordinates of each location on the pad you wish to analyze during the course of the experiment. Make sure to select the xy when setting the recording parameter and follow the rest of the protocol as indicated. Thin Z-sectioning of the embryo during recording is not necessarily required for the measurements detailed in this protocol. Recording embryonic development of *wt* and mutant animals with the maximum resolution is however a good practice in order to build a library of recordings able to support additional analyses.
5. Set up the time-lapse with 2 min intervals between two acquisitions lasting 8 hr.
   NOTE: The exposure time will depend on the light intensity set for the microscope. Cell movement during early elongation is very slow; exposure-time could be approximately one frame per second or less. If the embryos are at early morphogenesis stages (dorsal intercalation, ventral enclosure), early elongation would be recorded within 1 hr. Make sure light shutter is closed between each acquisition.
6. Run the acquisition and save it.

3. **Measurement of Early Elongation Defects using Image Analysis**
   1. Open Fiji-ImageJ software (v1.48o – http://fiji.sc/Fiji).
   2. In the menu select Analyze/Set Measurements, select Perimeter. For each embryo, adjust the Z-scale bar to focus on the pharynx of the embryo as shown in **Figure 2A**. This will ensure that you focus on the center of the embryo.
   3. To measure the length of the embryo, adjust the time scale bar to have the embryo of interest at the start of the early elongation (comma stage; **Figure 2A**). Note the time ("Time-beginning").
   4. Choose the segmented line tool. Draw a segmented line from the tip of the mouth of the embryo up to the tip of its tail following the midline of the embryo (**Figure 2A**). Using the menu tab Analyze/Measure, obtain the length of the drawn line ("Length beginning").
   5. Repeat this measurement for the same embryo at the end of early elongation (moment when muscles start contracting). Note the time ("Time-end") and measure the length of the embryo ("Length-end") as detailed above (**Figure 2B**).
   6. Calculate the duration (D) of early elongation and the length increase (L) during this stage as follows:
      D = "Time-end"-"Time-beginning"
      L = "Length-end" -"Length-beginning"
   7. To measure the head width, adjust the time scale bar to have an embryo at the stage of interest (1.2-fold stage or end of early elongation). Choose the straight-line tool. Draw the transversal (dorso-ventral axis) midline of the head. This section is the thickest part of the embryo (**Figure 2C**). Using the menu Analyze/Measure, obtain the length of the line corresponding to the head width.
   8. At the same time point, repeat this step by drawing the transversal midline of the tail (Mid-line between the intestinal valve and the tip of the tail; **Figure 2C**) and measure it to obtain the tail width.
      NOTE: Use this measurement to compare embryos at the same stage across different phenotypes. To ensure the reproducibility of this measurement, make sure to find a location located around the mid-line of the tail of the animal that can easily be recognized from one animal to another.
   9. To calculate the head to tail width ratio, divide the head width by the tail width.

2. Characterization of Late Elongation Defects Using Image Analysis

1. **Synchronization of L1 Larvae using Alkaline Hypochlorite Treatment**
   1. From a non-starved 60 mm plate containing many gravid adults, collect all worms in a micro-centrifuge tube by washing the nematodes off the plate with 1 ml of M9 buffer using a Pasteur pipette.
   2. Sediment nematodes at 3,500 x g for 3 min at room-temperature and remove the supernatant using a micropipette without disturbing the worm pellet.
   3. Add 1 ml of hypochlorite solution to each tube (0.4 M hypochlorite, 0.5 M NaOH) and shake for 3 min.
      NOTE: Alkaline hypochlorite solution should be freshly prepared and embryos in this solution should be agitated gently but continuously to optimize dissolution of the adults and oxygenation of the embryos. After 3 min agitation, most adults should release their eggs into the solution.
   4. Spin at 3,500 x g for 3 min at room-temperature and quickly remove the supernatant using a micropipette without disturbing the pellet.
   5. Wash four times with 1 ml of M9 buffer followed by centrifugation at 3,500 x g for 3 min.
   6. After the fourth wash, remove the supernatant and resuspend eggs in 700 µl of M9 buffer without pipetting up the eggs (as the eggs would stick to the plastic of the tip).
   7. Tape the tube on a 3-mm orbital shaker placed in an incubator at the appropriate growth temperature and shake at 600 rpm overnight.

2. **Length Measurement of Synchronized Larvae**

1. Centrifuge arrested L1 larvae at 3,500 x g for 3 min at room-temperature and remove the supernatant. Resuspend the larvae in 100 µl of M9 buffer and transfer them onto an agarose pad using a Pasteur pipette (agarose pads should be prepared as described at section 1.1.2 to 1.1.8). Place a coverslip on the pad.
   NOTE: The pad does not need to be sealed with gum for this experiment that involves short acquisition.
2. Use a 10X objective and phase contrast illumination to measure the length of the larvae if a high resolution camera is used. Increase the intensity of the light to be able to capture images within a few milliseconds and consequently obtain a clear picture of the larvae (**Figure 2D**).
   NOTE: While the swimming trashes of larvae are reduced by the agarose pad, they are still moving. Therefore, fast recording is essential to obtain a clear image.
3. Open Fiji-ImageJ and repeat step 1.3.1. To measure the length of larvae, choose the segmented line tool. Draw a segmented line from the tip of the head up to the tip of the tail following the midline of the larvae (**Figure 2D**, right panel).
4. Using the menu Analyze/Measure, obtain the length of the drawn line corresponding to the length of the larvae in micrometer.

3. Characterization of Late Elongation Defects Using Flow Cytometry

1. **Synchronize Larvae**
   1. Purify embryos using the alkaline hypochlorite treatment as described in section 2.1 from full 100 mm plates of well-fed young adults and let the embryo hatch and the L1 arrest in M9 buffer for 16 to 24 hr at 20 or 25.5 °C depending on the strain analyzed.
      NOTE: One full plate of well-fed adults per strain is sufficient for the analysis described below.

2. **Calibration of the Worm Sorter**
   NOTE: The flow cytometer used here is a large particle flow cytometer (hereafter referred to as the worm sorter). The worm sorter includes a 670 nm red diode laser, which is located in front of an extinction detector. The worm sorter also contains a multi-line argon laser for fluorescent excitation. The standard instruments have three photomultiplier tubes (PMT) fluorescence detectors used to detect fluorescence emissions in the green, yellow, and red regions of the spectrum. In the protocol described here, TOF will be used to measure the size of the larvae, the red channel will be used to identify dead worms that will be stained with Propidium Iodide (PI). GP (General Purpose) High Fluorescence Control Particles used to calibrate the instrument and as an internal control in our experiment display high fluorescence in green, yellow and red. They will be the only objects in the sample analyzed with high emission detected in the green channel and will subsequently be identified based on this characteristic.
   NOTE: Living animals are identified based on the absence of fluorescence in the Green and Red channel as well as on the TOF. Autofluorescent emission from the gut of the larvae is not detectable at L1 stage.
   1. Switch on the laser block, the computer, the compressor and the worm sorter instrument. Press the START button on the opened worm sorter software as indicated in the instruction manual of the instrument.
   2. Observe the argon laser control pop-up window. Select RUN mode and wait as the laser powers reach 10 ± 1 mW. Select DONE on the laser control window.
   3. Set the sheath and the sample pressures to 5.10 ± 0.02 and 5.51 ± 0.01 respectively. Allow the pressure to equilibrate for at least 15 min and click the PRESSURE OK check box.
      NOTE: The flow rate depends on the sheath and the sample pressures.
   4. Make sure to eliminate all bubbles present in the flow channel tubules between the sample cup and the analyzing chamber. To do so, click ACQUIRE and gently flick the tubule until no bubble are acquired (as seen in the acquisition window).
   5. Setup of all parameters for fluorescent and TOF measurement and detection as follows:
      1. Set the scales for TOF, Ext, Green, Yellow and Red to 256. Set gain as described in **Table 1**. Set PMT Control at 700 for Green and Yellow and to 900 for Red.
         NOTE: Two excitation filters are available (488 nm and 514 nm) and can be used to excite either Green fluorescent protein (GFP) or Yellow fluorescent protein (YFP) or any fluorochromes excited at these wavelengths with the multiline argon laser. Appropriate filters need to be inserted in the filter chamber of the equipment. We used the 488 nm filter for the following experiment.

   6. To calibrate the worm sorter, select "run control particles" in tool menu. Put 20 ml of 1x GP (General Purpose) High Fluorescence Control Particles in the cup (these particles are fluorescent particles of precise size, sold by the cytometry manufacturer to calibrate their instrument).
   7. Click on ACQUIRE button to begin sheath and sample flow.
      NOTE: Expect a mean related to the control particle distribution of 21 ± 6 and a coefficient of variation around that mean (C.V.) ≤11. If the C.V. is >11 and the mean is >27 try to clean the tubules by clicking several times on the clean button or by flicking the flow channel.

3. **Acquisition of Animal TOF**
   NOTE: Light scatters when an object passes in the flow cell between the laser source and the extinction detector. The time it takes for the light to be scattered by the flying object (time of flight, TOF) is used to measure the axial length of the object. The extent of light masked by the object (extinction, EXT) is used as a measure of its opacity/optical density.
   1. Place 10 µl of synchronized wild-type (*wt)* L1 in a watch glass and estimate the percentage of dead-eggs using a dissecting microscope.
      NOTE: Synchronized L1 displaying high Emb (higher than 20%) in *wt* should not be used for further analysis.
   2. Transfer synchronized L1 from the microcentrifuge (approximately 700 µl of M9 containing L1) to a 15 ml conical tube. Add propidium iodide (PI) at a final concentration of 10 µg/ml (dilution 1/100th from a 1 mg/ml stock solution) and incubate for 30 min at room temperature.
      NOTE: Dead eggs and larvae will be stained using PI and detected as highly fluorescent objects using the red channel.
   3. Add 10 ml of M9 buffer to dilute stained populations. Take 5 ml of the diluted populations and dilute them four times by adding 15 ml of M9. Place this dilution in the sample cup.
   4. Run the sample flow by clicking ACQUIRE and observe the flow rate.

NOTE: In order to have accurate measurement, the flow rate should stay between 15 and 25 objects/sec.

5. If necessary, adjust the amount of animals in the cup to reach the desired flow rate.
   NOTE: The flow rate will depends on the pressures (sheath and sample) that are constant as indicated in 3.2.3 and on the concentration of larvae in the cup. If the flow rate is higher than 25 objects/sec add some buffer in the cup. If it is lower than 15 objects/sec add concentrated L1 (from step 3.3.3) in the cup.
6. Once the appropriate concentration of object is reached, evaluate the volume in the cup and add 1/4th of this volume in High Fluorescent GP Control Particles 4x solution.
7. Adjust the gating and sorting parameter. To do so, click 'Gating' on the menu and select ' TOF vs.' and 'Green'. Click on 'Sorting' on the menu and select 'TOF vs. ' and 'Red'. Gating and sorting graphics will then appear as shown in **Figure 3A**.
   NOTE: This will allow the visualization of the control particles (emission in Green and Red), dead animals stained by PI and bubbles (emission in Red and not Green) and living animals (no emission in neither Green nor Red channels) (**Figure 3A**).
8. Run the experiment by clicking ACQUIRE.
   NOTE: To identify small size differences between larvae, analyze around 10,000 objects, so approximately 8,000 animals. Stop the experiment and export the data as .txt format by clicking on STORE.

4. **Measurement of the Relative Size of Living Animals in Synchronized Populations**
   1. Open the .txt file using a software able to manage large data tables.
   2. From the analyzed objects extract the TOF values associated with control particles that are characterized by emission in the green channel higher than 100 arbitrary units (this value depends on the parameters set in section 3.2.5). Create a new column and call it 'control particles'. Create a column containing all the other objects except the control particles called 'sample'. NOTE: the fluorescence emission of new particle batch needs to be verified before starting the acquisitions. This will set the fluorescent threshold enabling the identification of control particles over L1s.
   3. For each analyzed strain identify the portion of the experiment where the flow rate has been altered (due to the presence of a plug of eggs for example). To do so:
      1. Plot the TOF of 'control particles' for each sample as a factor of time (**Figure 3B**).
      2. Draw the linear trendline using the trendline tool and display the equation on the chart.
         NOTE: The TOF of control particles should be constant over time (horizontal line, **Figure 3B**). Considering the equation of the linear trendline drawn on the data y = ax+b, ensure that the 'a' value is lower that 10^{-4}. All measurements made within time sections displaying a rupture in the alignment of control particle should be removed from the analysis.
   4. Remove the dead embryos and bubbles (emission higher than 15 in the red) as well as small debris and large egg plugs (TOF lower than 10 and higher than 70 respectively) from the 'sample' measurement.
   5. Plot the 'control particles' distribution for each strain analyzed. As seen in **Figure 3C** these distributions should overlay almost perfectly. This ensures that TOF measurements obtained for different strains can be compared. NOTE: normalization of sample elements TOF over control particles can be used if the distributions of control particles in a sample do not overlay with that of control samples. Normalized TOF are computed as followed:
      1. Normalize TOF for genotype G = (sample TOF for G) / (average 'control particle' TOF for G) x (average 'control particle' TOF for *wt*) where *wt* is the control sample.
   6. Plot Larvae TOF distribution for each strain including control sample, in our case *wt* animals (**Figure 3D**). Measure the mean and the standard error of the mean (SEM) for each population (**Figure 5A** and **B**).

Representative Results

Head-, Tail- and Head/Tail-width Ratio are Robust Metrics.

The protocols described here have been successfully used to characterize the function of regulators and effectors of Rho GTPases *pix-1, pak-1* and *let-502* during early elongation[7]. *pix-1* and *pak-1* code respectively for a guanine-exchange factor (GEF) and an effector specific for Rac/Cdc42 GTPases and *let-502* codes for an effector for RHO-1 [7]. In this study,*pix-1* and *pak-1* were shown to control epidermal morphogenesis during early elongation[7]. This study used head and tail width measurement as metrics demonstrate for the first time, that hypodermal cells located in the anterior embryo follow different morphogenic programs than those located in its posterior [7]. To establish the reproducibility and robustness of these metrics, head width was measured independently five times on 12 wild-type (*wt)* embryos at 1.2-fold stage. Variances among the five different groups of measurement were compared then assessed using the Brown-Forsythe test (using R statistical package) revealing no significant differences amongst the measurements (*F*-test *p*-value > 0.5; **Figure 4A**) suggesting that measurements for a group of embryos are reproducible. Assessment of reproducibility and batch effects associated with these measurements was done through measurement of the head width of *wt* embryos at 1.2-fold stage from 4D-DIC imaging done on three different days (n = 12 embryos). Across these three measurement groups, the variance was not significantly different and no significant batch effects were found to impact the head-width measurement results (F-test *p*-value > 0.5; **Figure 4B**).

Similar results were obtained for tail width measurements and for measurements done at different stages of early elongation (data not shown). These data established head-width, tail-width and head/tail width ratio as robust metrics to characterize early elongation defects.

Measuring Head and Tail Width as well as Length of the Embryos Allows for the Characterization of Genes Controlling Early Elongation along the Antero-posterior Axis of the Embryo.

Measurement of the length of the embryos, as well as the width of their head and tail was done on *wt* and mutant embryos carrying null or thermosensitive strong loss-of function alleles for genes controlling early elongation: *pix-1(gk416)*; *pak-1(ok448)* and *let-502(sb118ts)*[7]. We measured the head/tail (H/T) width ratio of *wt* and mutant embryos at the beginning (1.2-fold stage) and at the end of the early elongation at non-permissive temperatures (**Figure 4C** left and right panel respectively). While at the beginning of early elongation there was no change- or a reduced H/T ratio was observed in *pix-1*, *pak-1* and *let-502* mutants when compared to *wt* animals (1.2-fold stage, **Figure 2C**, left panel), at the end of early elongation all three mutants showed a significantly higher H/T ratio than *wt* embryos (*t*-test *p*-values < 0.006; **Figure 4C**; right panel). This demonstrated that *pix-1*, *pak-1* and *let-502* mutant embryos display abnormal antero-posterior morphology at the end of early elongation. Further analysis comparing head width and tail width between 1.2-fold stage and the end of early elongation, using the same measurement parameters revealed that the head width is less reduced in *pix-1*, *pak-1* and *let-502* mutants while the tail width reduces significantly less in *let-502* mutants only [7]. This revealed that *let-502* controls morphogenic processes similarly along the antero-posterior axis of the embryo, while *pix-1* and *pak-1* control morphogenic processes occurring mainly at the anterior part of the embryo[7]. The length difference between the embryos at the end versus the beginning of early elongation (**Figure 4D**) was also measured. We found that early elongation was significantly reduced in *pix-1*, *pak-1* and *let-502* mutants when compared to *wt* suggesting that alteration of the anterior morphogenesis in *pix-1* and *pak-1* mutants alone is sufficient to significantly reduce the elongation of the embryo.

Protocol 2 and 3 were used to assess the length of arrested larvae in *wt* and mutant backgrounds. The length of these larvae was assessed using both image analysis (Protocol 2)[7] and flow-cytometry (Protocol 3; unpublished data). Measurements of larvae length using image analysis results in absolute measurements of animals' length in micrometers in a robust and highly reproducible manner (**Figure 5A**). This analysis revealed that synchronized mutant larvae display significantly reduced length when compared to *wt* (**Figure 5A**)[7]. Measurement of these larvae using the flow-cytometry based protocol (Protocol 3) gave comparable results (**Figure 5B**). However, it should be noted that the large number of larvae measured using the latter approach significantly increased the statistical robustness of genotype comparison (*t*-test *p*-values $<10^{-24}$). Based on these findings, the flow-cytometry approach may be a better choice over image analysis in order to characterize mutant animals displaying very subtle elongation defects.

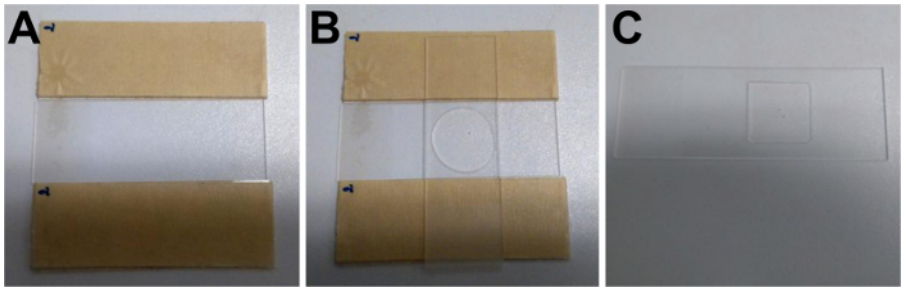

Figure 1. Preparation of an Agarose Pad. A, Microscope slide placed between two spacer-slides covered by two layers of masking tape. **B,** The agarose pad is covered with another slide supported by the two spacer-slides. **C,** The final shape and size of the agarose pad after cutting with a razor blade to fit the coverslip. Please click here to view a larger version of this figure.

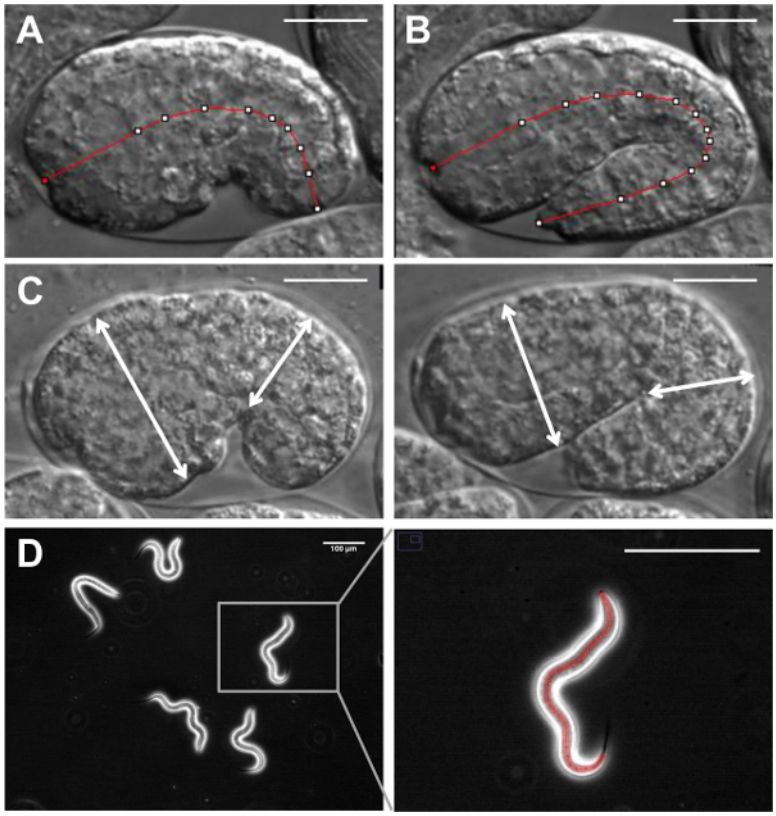

Figure 2. Measurement of Early and Late Elongation Defects. A - B, Measurement of the length of an embryo at comma stage (A) and at the end of early elongation (B). The red line, used to measure the embryos was drawn using the segmented line tool of ImageJ. **C,** Head and tail width are measured in embryos at 1.2-fold stage (left) and at the end of early elongation (right). Arrows represent the localization of measured areas (modified from Martin *et al.*, 2014). Scale bar: 20 µm. **D,** Length of Larvae is measured for synchronized L1-larvae. Right panel is an enlarged view of the captured image (left). The red line was drawn using the segmented line tool of ImageJ. It is used to measure the length of the larva. Scale bar: 100 µm. Please click here to view a larger version of this figure.

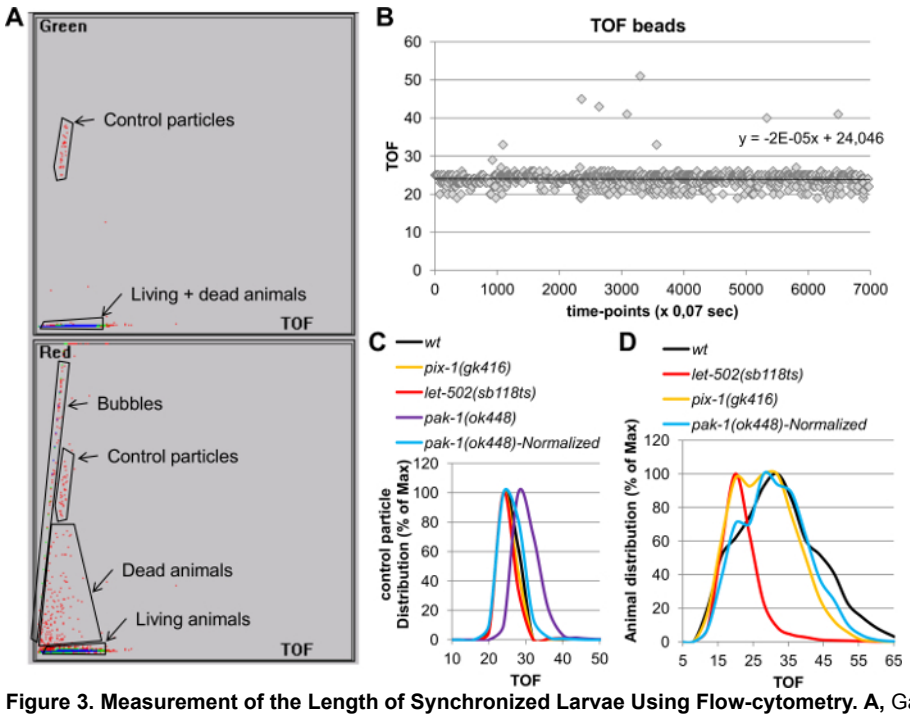

Figure 3. Measurement of the Length of Synchronized Larvae Using Flow-cytometry. A, Gating and sorting window of COPAS Biosort showing Green and Red emission of control particles, bubbles, dead and living animals with respect to their Time-Of-Flight (TOF). **B,** TOF of control particles at different time points for a representative experiment. The slope of the linear function of TOF versus time is around 10^{-5}, indicating that TOF of control particles is constant over time throughout the experiment. **C,** Distribution of control particle TOFs expressed as a percentage of maximal value of distributions. Non-normalized and normalized control particle distributions are represented for *pak-1(ok448)*. Other distributions are not normalized. **D**, distribution of TOFs for living animals expressed as a percentage of the maximal value of the distributions. TOFs are not normalized on control particles except for *pak-1(ok448)* as indicated. Please click here to view a larger version of this figure.

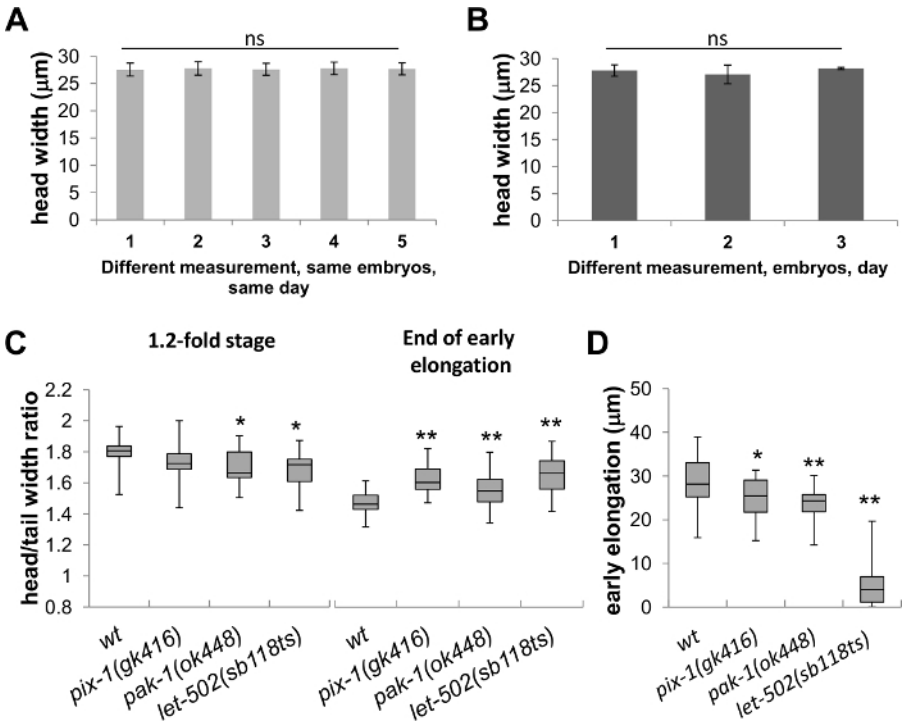

Figure 4. *Pix-1, Pak-1* and *Let-502* Mutants Present Early Elongation Defects. A - B, Reproducibility and robustness assessment of head width measurements. **A,** Five independent measurements of the head width for *wt* embryos at 1.2-fold stage (n = 12 embryos). Means and standard deviations (error bars) are indicated. Non significant (ns) differences between measurements were computed using the Brown-Forsythe test (using R statistical package) (F-test *p-value* > 0.5). **B,** Head width measurement for *wt* embryos at three different days (n = 12 embryos). Means and standard deviations are indicated as well; there was no significant difference in variance across the measurements (ns; Brown-Forsythe F-test *p*-value > 0.5). **C,** Distributions of head/tail width ratio at 1.2-fold stage (left panel), at the end of early elongation (right panel) in *wt*, *pix-1(gk416)*, *pak-1(ok448)* and *let-502(sb118ts)* mutants at 23 - 24 °C. Note that mothers of *let-502ts* embryos used for this study were grown at 25.5 °C. **D,** Distribution of the elongation in *wt*, *pix-1(gk416)*, *pak-1(ok448)* and *let-502(sb118ts)* mutants between comma stage and the start of late elongation. The box-plots represent the min, max, 25th, 50th (median) and 75th percentiles of the populations. * *t*-test *p*-value < 0.05, ** *t*-test *p*-value < 0.006 (modified from Martin *et al.*, 2014). Please click here to view a larger version of this figure.

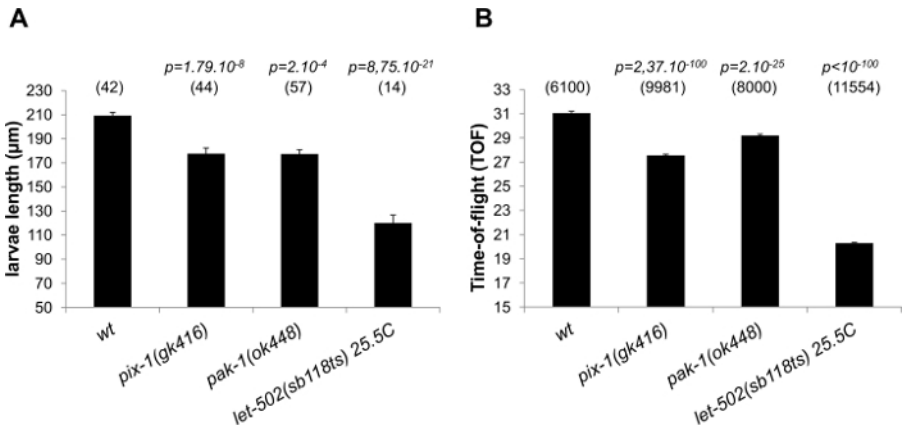

Figure 5. *Pix-1(gk416), Pak-1(ok448)* and *Let-502(sb118ts)* Larvae Present Length Defects. A, Length of larvae measured in *wt*, *pix-1(gk416)*, *pak-1(ok448)* and *let-502(sb118ts)* animals measured using image analysis (Protocol 2). **B,** Relative larvae length measured using flow-cytometer (Protocol 3). Numbers in brackets correspond to the number of animals used for the measurements. Means of lengths and standard error of the mean (SEM; error bars) are represented. Student's *t*-test *p*-values are indicated (p). Please click here to view a larger version of this figure.

Discussion

This protocol describes novel metrics to characterize early and late phases of embryonic elongation.

In section 1, the critical step is the potential presence of bacteria on the pad. The embryos are hermetically enclosed between the pad and the coverslip during image acquisition. Sealing the slide is required to avoid desiccation of the animals during acquisition that lasts more than two hours. To our knowledge, none of the sealers used to mount agarose pads between slide and coverslip are air-permeable. Consequently, when a large amount of bacteria (or embryos) is present on the pad, they may be deprived of oxygen after a few hours leading to their premature death.

Having three-fold embryos — corresponding to embryos that are 3-fold in length compared to non-elongated embryos — recorded along with the embryos of interest will be a good indicator of potential hypoxic conditions, since those embryos will stop moving in their eggshells in the absence of oxygen. No morphological measurements should be done on hypoxic conditions or on dead embryos.

Another critical step when using time-lapse imaging is temperature at which development of the embryo occurs. Thermosensitive mutants are currently used in *C. elegans*. The biological effect of temperature shift may be immediate or delayed depending on the half-life of the protein and the nature of the mutation its carries. Consequently, the temperature at which the embryo is exposed should be constant over time and should be controlled by appropriate ventilation of the microscopy room or a heating-cooling chamber on the microscope stage.

Section 2 is dependent on larvae synchronization using alkaline hypochlorite treatment. Under certain circumstances, this treatment may lead to embryonic lethality (Emb). Emb above 20% in *wt* population suggests an elevated toxicity during hypochlorite treatment that may negatively impact morphogenesis. Synchronized L1 displaying high Emb in *wt* background should not be used for further analysis. This restriction also applies to protocol 3.

We do not recommend the use of anesthetic drugs to immobilize larvae. Levamisol in particular, immobilizes the nematode through induction of muscle tetany that tends to shrink the larvae introducing experimental bias. If exposure time is not quick enough as a result of the limitations of the microscope, we recommend reducing the motility of larvae by increasing the concentration of the agarose in the pad and reducing the amount of liquid between the pad and the coverslip. Care should be taken however, not to desiccate the larvae, since desiccation will reduce their size.

In section 3, measurement of the length of larvae used comparison of flow rates between analyzed samples. To do so, the distribution of control particles needs to be compared. If the distributions fully overlay, the TOF (time-of-flight) values obtained for corresponding samples can be compared, if not, these values need to be normalized. Normalization of sample-TOF over control particles-TOF (as detailed in 3.4.5.1) has been used successfully as shown for *pak-1(ok448)* (**Figure 3C** and **Figure 5B**). Relative length of *pak-1(ok448)* vs *wt* was found to be similar in at least 3 independent experiments with or without normalization (data not shown). However, we recommend, confirming the results obtained with normalization with those obtained without it, especially when comparing larvae with small size differences. It should be noted that measurement of the larvae length using flow-cytometry provides a length relative to a control sample, in this case, *wt* larvae rather than an absolute length in micrometer as for image analysis. This implies that measurements from independent experiments cannot be combined unless computing size ratio over *wt*.

The buffer used for dilution in the sample cup will have an impact on the flow rate. We observed that the sheath buffer recommended by the manufacturer contains detergent that increases the amount of bubbles generated during acquisition. Using M9 buffer, which does not contain detergent, significantly reduced the formation of bubbles but was less efficient in avoiding the plugging of eggs in the tubules of the sorter, which affects the flow rate of samples. Egg plugging is easily detected during the acquisition by a marked decrease of the observable TOF of control particles for a few seconds followed by an elevated TOF- also for a few seconds. Egg plugging may also lead to the obstruction of the channel and the complete arrest of the particle flow (less that 5 objects per second). If this should occur, click on the CLEAN button until flow rate is restored. Any measurements occurring during these events should be excluded from the data analysis. Sheath buffer may be recommended for experiments involving strains characterized by high rates of dead eggs.

The sample and sheath pressure (set at the step 3.2.3), may change (slightly) over time and should be adjusted manually throughout the experiment in order to ensure a very constant flow rate. Reduction or increase of the low rate will be observable when analyzing the results and plotting the TOFs of control particles over time (**Figure 3C**). Reduction of the flow rate will result in the increase of the average TOFs of particles over time, while an increase of the flow rate will result in the opposite. Alteration of the flow rate will negatively impact the sensitivity of the method in detecting small size differences between populations of larvae.

Methods aiming to identify genes controlling elongation and requiring time-lapse microscopy recording are generally highly time-consuming and tedious when phenotyping several genotypes. The flow-cytometer-based approach, while requiring equipment not available to all laboratories, is less time-consuming and consequently more efficient when several strains need to be characterized. This method is also more statistically robust compared to measurements using image analysis (assessed by the student's *t*-test comparing mutant and *wt* TOF distributions; **Figure 5A and B**). This method may then be highly suitable for strains expressing elongation defects with low expressivity/penetrance.

Several methods using flow-cytometry have been developed in the past to measure fitness of nematodes[9-12]. These methods use animals dispensed in a 96-well plates and the Reflex module of the worm sorter'. The Reflex module enables direct analysis of nematode population dispensed within 96-well plates. Consequently, these methods are able to characterize hundreds of conditions per day and constitute a robust manner in which to measure the fitness of a non-synchronized population. They are however, not well suited to identify small size differences between synchronized L1. Measurement of small differences between L1 larvae requires the measurement across a large number of animals, which is incompatible with the use of 96-well plates and of the Reflex module that may efficiently characterize 100 objects at the most per well. The method described here is designed for this purpose at the expense of the throughput, which is significantly reduced. It enables the characterization of 3 to 4 conditions per hour once the instrument is calibrated, which is a marked improvement over using image analysis in protocol 2.

Measurement of head and tail width ratio is the first method that was developed to characterize morphogenic processes occurring unevenly along the antero-posterior axis of the embryo[7]. When applied to genes shown to control early elongation, these methods will clarify the spatial distribution of signaling pathways controlling morphogenesis at that stage. Measurement of the length of arrested larvae using either image analysis or flow cytometry in combination with measurement of the length of the embryos at the end of early elongation will enable the identification of genes controlling either early or late elongation or both with high sensitivity and precision. These procedures may then contribute significantly to future understanding of the spatial and temporal regulation of signaling pathways controlling embryonic elongation in *C. elegans*. Furthermore, these approaches can also be adapted to study signaling pathways controlling body length such as the insulin and TGF-beta pathways [13,14] and chronic exposure to environmental contaminants [15,16]. These measurements can be done at different larval stages or in adults

using minor variations of Protocols 2 and 3. Measuring size differences in L1 is more challenging with the worm sorter than larger objects such as L3, L4 larvae or adults. Protocol 3 can then easily be adapted to do these measurements.

Disclosures

The authors declare that they have no competing financial interests.

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