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Phalloidin Staining of Actin Filaments for Visualization of Muscle Fibers in *Caenorhabditis elegans*

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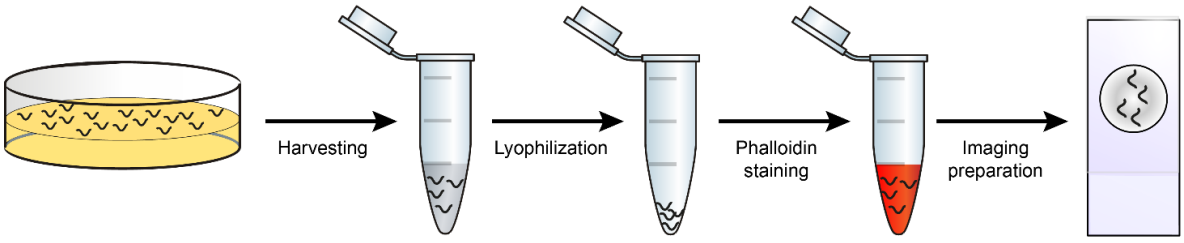
[Abstract] Advances in *C. elegans* research have allowed scientists to recapitulate different human disorders, from neurodegenerative diseases to muscle dysfunction, in these nematodes. Concomitantly, the interest in visualizing organs affected by these conditions has grown, leading to the establishment of different antibody- and dye-based staining protocols to verify tissue morphology. In particular, the quality of muscle tissue has been largely used in nematodes as a readout for fitness and healthspan. Phalloidin derivatives, which are commonly used to stain actin filaments in cells and tissues, have been implemented in the context of *C. elegans* research for visualization of muscle fibers. However, the majority of the phalloidin-based protocols depend on fixation steps using harmful compounds, preparation of specific buffers, and large amounts of worms. Herein, we implemented a safer and more flexible experimental procedure to stain actin filaments in *C. elegans* using phalloidin-based dyes. Lyophilization of the worms followed by their acetone permeabilization allows bypassing the fixation process while also providing the opportunity to suspend the experiment at different steps. Moreover, by using conventional buffers throughout our protocol, we avoid the additional preparation of solutions. Finally, our protocol requires a limited number of worms, making it suitable for slow-growing *C. elegans* strains. Overall, this protocol provides an efficient, fast, and safer method to stain actin filaments and visualize muscle fibers in *C. elegans*.

Graphic abstract:



Schematic overview of phalloidin staining in *C. elegans* for assessing muscle fiber morphology.

Keywords: Phalloidin, *C. elegans*, Actin, Muscle fibers, Cytoskeleton, Nucleus, Muscle disease, Aging

[Background] In the last decades, the nematode *Caenorhabditis elegans* has emerged as a powerful model organism and has provided an unprecedented opportunity to expand our knowledge in different

fields of research, from developmental biology (Mello *et al*., 1992; Bowerman *et al*., 1993; Hubbard and Greenstein, 2000), to neuromuscular degeneration (Braungart *et al*., 2004; McColl *et al*., 2012; Sorrentino *et al*., 2017; Romani *et al*., 2021) and aging (Kimura *et al*., 1997; Herndon *et al*., 2002). One of the main reasons that promoted the usage of *C. elegans* as a laboratory model is that despite its simplicity, it has defined tissues. In particular, it consists of five main tissues: epidermis, reproductive tissues, digestive system, nervous system, and muscle tissue (Spencer *et al*., 2011). Among these, muscle tissue morphology and function have been widely used to typify *C. elegans* fitness and healthspan (Fire *et al*., 1991; Ryu *et al*., 2016; Fang *et al*., 2017; D'Amico *et al*., 2019; Romani *et al*., 2021). In recent years, numerous protocols have emerged to facilitate the visualization of *C. elegans* muscles, taking advantage of the transparent nature of this nematode. Phalloidin is a mushroom-derived molecule that specifically binds filamentous actin (F-actin); hence, phalloidin derivatives tagged with fluorescent molecules are commonly used in microscopy to visualize F-actin in different fields of research (Chazotte, 2010). Body wall muscles of the nematode *C. elegans* possess obliquely striated myofibrils composed of highly organized filaments of actin, whose disposition is altered in pathological conditions (Gieseler *et al*., 2018). For this reason, phalloidin-based staining of actin filaments has been successfully implemented in nematodes for the detection of cytoskeleton and muscle structure, with important applications in muscle disease and degeneration (Gieseler *et al*., 2018). However, phalloidin-based protocols typically rely on tedious fixation steps using harmful compounds, such as PFA, and require the preparation of specific, complicated buffers (Costa *et al*., 1997; Ono, 2001; Bansal *et al*., 2015; Geisler *et al*., 2020). We present here a method for *C. elegans* research that allows phalloidin staining of actin filaments, bypassing fixation, and using conventional buffers. Moreover, by integrating 4',6-diamidino-2-phenylindole (DAPI) staining in our protocol, we allow the visualization of muscle cell nuclei, whose morphology can be used to interpret cellular homeostasis. Finally, our protocol requires a limited amount of worms and can be suspended at different steps, ensuring a versatile experimental procedure. Overall, our methodology provides a robust, simple, and safer pipeline for the visualization of muscle fibers and nuclei in *C. elegans* with applications in multiple fields of biomedical research. We predict that this protocol could be coupled with additional dyes specific for other tissues or cellular components, such as endoplasmic reticulum and mitochondria, allowing an even broader overview of worm physiology and homeostasis.

Materials and Reagents

1. 1.5 ml Eppendorf tube (Eppendorf, catalog number: 11.3817.01)
2. Glass pipette
3. Microscope slides (Thermo Scientific, catalog number: 11950657)
4. Cover slips (VWR, catalog number: 43211.KG)
5. Plastic dropper (VWR, catalog number: AMP-30)
6. Aluminum foil
7. Liquid nitrogen

8. Acetone (Millipore, catalog number: 1.00014.1011)
9. Rhodamine Phalloidin (Invitrogen, catalog number: 219920-04-4)
10. Methanol (Sigma-Aldrich, catalog number: 646377-1L)
11. BSA (Sigma-Aldrich, catalog number: A7906-100G)
12. Tween-20 (Sigma-Aldrich, catalog number: P1379)
13. DAPI (Invitrogen, catalog number: D1306)
14. Agarose (Promega, catalog number: V3125)
15. M9 buffer (see Recipes)
16. Washing buffer (see Recipes)
17. 2 µg/ml DAPI in M9 (see Recipes)
18. 2% (w/v) agarose (see Recipes)

Equipment

1. Standard equipment for worm culture (see Brenner, 1974)
2. Centrifuge (Eppendorf, Centrifuge 5424)
3. SpeedVac Vacuum Concentrator (Labogene, Speed Scan 40)
4. Laboratory fume hood
5. Leica SP8 confocal microscope (Stand: Upright Leica DM6 CS; Illumination: Lumencor Sola SM II LED, Laser; Software: LAS-X; Camera: DFC 7000 GT (B/W); Objective: HC PL APO, 63×/1.40 oil, DIC POL0.14)

Software

1. Fiji (Schindelin *et al*., 2012)

Procedure

A. Culture 50 worms on a NGM agar plate. Each plate of 50 worms serves as a replicate
Note: The protocol will work with any number of worms between 50 and 100. We suggest using two replicates for each experimental condition.

B. Worm retrieval from agar plates
1. Wash worms off the plate using 1 ml of M9 buffer and place the solution in a 1.5 ml Eppendorf tube.
Note: We suggest retrieving the M9 solution from the plates using a glass pipette, which reduces the adhesion of worms to the pipette's walls.
2. Spin briefly in a centrifuge to pellet the worms but not the bacteria (do not exceed 400 × *g*); 10 s is sufficient to pellet adult worms.

3. Remove as much supernatant as possible using a micro-pipette (1,000 µl and 200 µl).
   Note: C. elegans tend to swim, so the pellet is not stable; avoid bringing the tip of the pipette too close to the pellet to prevent sucking in any worms.
4. Repeat steps from 1 to 3 one more time to ensure that all the worms are retrieved from the plate.
5. Snap freeze the worms by immerging the Eppendorf tube in liquid nitrogen.
   Note: At this stage, worms can be stored at -80°C for months.

C. Worm permeabilization

1. Open the Eppendorf tube and place it in a SpeedVac Vacuum Concentrator to dry the worm pellet at 20°C. This step typically takes ~10 min but can vary according to the amount of supernatant that is left from Step B3.
2. Add three drops of ice-cold acetone to the dry worms. Gently tap the Eppendorf tube five times.
   Note: It is important to cover all the worms; additional drops of acetone could be used if necessary. If worms are not correctly permeabilized, the protocol will fail.
3. Incubate 5 min at room temperature.
4. Remove as much of the acetone as possible using a micro-pipette (1,000 µl and 200 µl) and air dry the remaining acetone in a laboratory fume hood.
5. After complete evaporation of the acetone, worms can be stored at -20°C for months.

D. Phalloidin staining

Note: We suggest working in a dark environment to prevent phalloidin degradation and consequent failure of the protocol.

1. Place 2.5 U (for each replicate) of Rhodamine Phalloidin in an Eppendorf tube.
2. Use the SpeedVac Vacuum Concentrator to remove the methanol in which the Rhodamine Phalloidin is dissolved.
   Note: It takes ~5-10 min to dry the Rhodamine Phalloidin, but this varies according to the volume that is used.
3. Resuspend the dried Rhodamine Phalloidin in 20 µl (for each replicate) of M9.
4. Add 20 µl of the M9-resuspended Rhodamine Phalloidin to the dried worms. Gently tap the Eppendorf tube five times.
5. Incubate in the dark at room temperature for 30 min.
6. Wash the worms twice in 1 ml of washing buffer.
7. If nuclei staining is not required, proceed to Procedure F.

E. DAPI staining

Note: We suggest working in a dark environment to prevent DAPI degradation.

1. Resuspend the worms in 20 µl of 2 µg/ml DAPI in M9. Gently tap the Eppendorf tube five times.
2. Incubate in the dark at room temperature for 5 min.
3. Wash the worms twice in 1 ml of washing buffer.

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F. Preparation of the worms for imaging

Note: We suggest working in a dark environment to prevent degradation of the fluorescent dyes and consequent failure of the protocol.

1. Resuspend the worms in 10 µl of M9.
2. Prepare a 2% (w/v) agarose pad on a microscope slide for each replicate. To prepare the agarose pad, apply lab tape on two microscope slides and place a third one in between them (Figure 1a). Apply 2-3 drops of agarose in the center of the microscope slide not wrapped in tape using a micro-pipette (1,000 µl). Place another microscope slide on the top of the agarose (Figure 1b). Wait 1-2 min for the agarose to solidify and remove the microscope slide on the top. Store the agarose pads in a humid place before usage to prevent dryness.

   Note: One layer of tape is sufficient to create the space needed for the formation of an agarose pad. Cut the micro-pipette tip to facilitate the dispensation of agarose. Gently put the last microscope slide on the liquid agarose to avoid the formation of bubbles in the agarose pad.

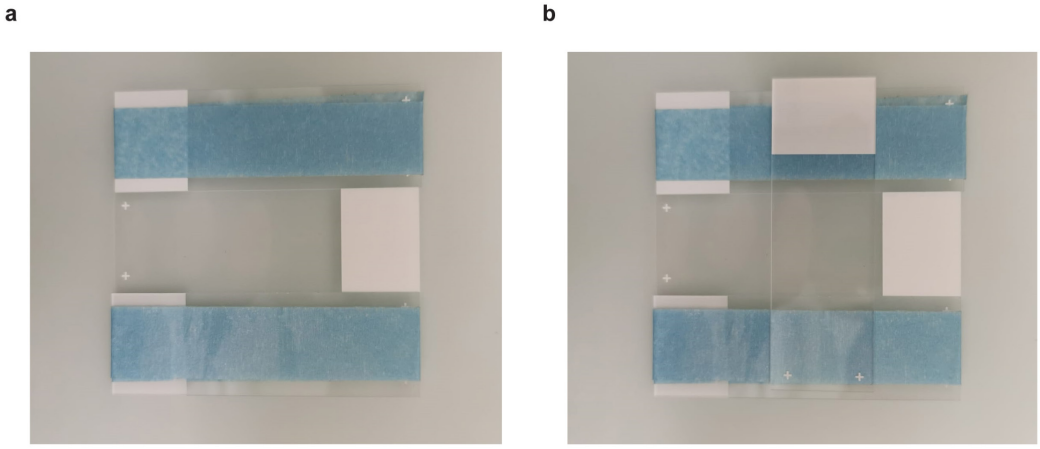

Figure 1. Agarose pads preparation. First (a) and second (b) steps for the preparation of agarose pads described in Step F2.

3. Transfer all the M9 containing the worms to the agarose pad using a glass pipette.
4. Apply a cover slip, avoiding the formation of bubbles. Surface tension of M9 will keep the cover slip steady during the imaging process but avoid touching it.

G. Imaging using a confocal microscope

Note: We suggest not using inverted microscopes to keep the cover slip steady during the imaging process.

H. Images analysis

1. Use Fiji to open the images recorded with the confocal microscope.
2. Add scale bar: “Analyse” -> “Tools” -> “Scale bar” (Figure 2).

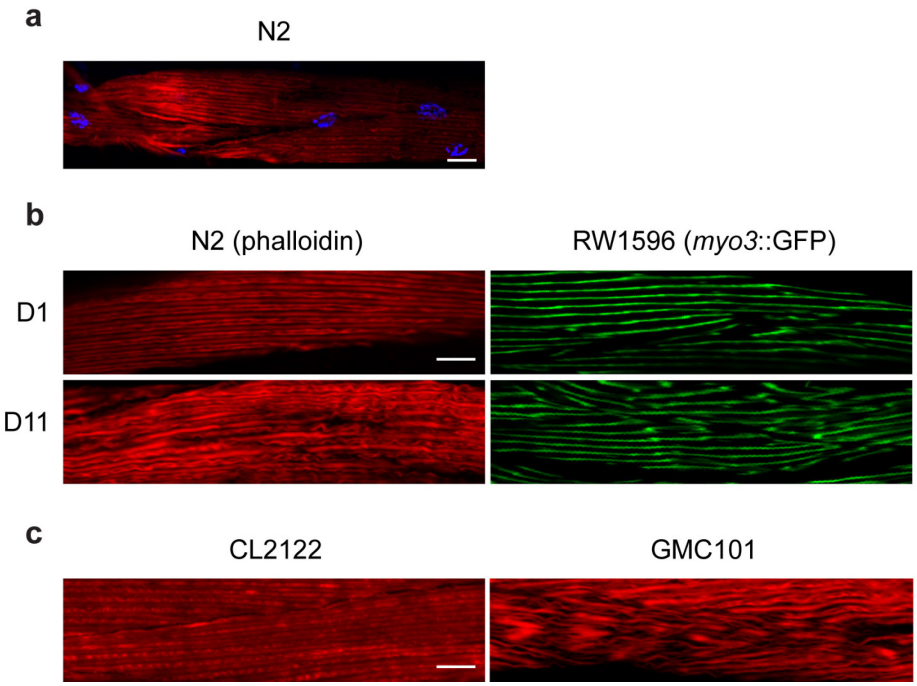

Figure 2. Phalloidin staining applications and comparison with GFP-tagged muscle fibers. (a) Representative image of day 1 adult N2 worms; in red, actin filaments stained with Rhodamine-Phalloidin; in blue, nuclei stained with DAPI. (b) Day 11 adult N2 worms present disorganized muscle fibers, visualized here through Rhodamine-Phalloidin staining (in red), when compared to young (day 1) worms as described before (Romani *et al*., 2021). These results are comparable to those obtained using a transgenic worm strain (RW1596) in which the muscle fibers are labeled with GFP (in green). (c) Rhodamine-Phalloidin staining of the inducible muscle proteotoxicity model, GMC101, reveals altered muscle fiber morphology compared to the control strain CL2122, consistent with the data obtained in young and old worms and as described before (McColl *et al*., 2012). Scale bar, 10 μm; magnification, 63×.

Recipes

1. NGM agar plates (see Brenner, 1974)
2. M9 buffer (Table 1)

Table 1. M9 buffer composition. List of components and volumes for 1 L of working stock.

Components	Quantity
Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	5 g
$MgSO_4 \cdot 7H_2O$	250 mg
Water	1 L

Note: Filter before using.

3. Washing buffer
   M9 1 ml
   BSA 50 mg
   Tween-20 50 µl
4. 2 µg/ml DAPI in M9
   To make a 5 mg/ml DAPI stock solution, dissolve the content of one vial (10 mg) in 2 ml of water. For long-term storage the stock solution can be aliquoted and stored at ≤ -20°C. For short-term storage the solution can be kept at 2-6°C, protected from light.
5. 2% (w/v) agarose
   Dissolve 500 mg of agarose in 25 ml of water.
   Note: Warm up the solution to near-boiling temperature to dissolve the agarose. We suggest using a microwave for this step.

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Competing interests

The authors have no competing interests to declare.

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