

## Video Article

# Visualization of *Caenorhabditis elegans* Cuticular Structures Using the Lipophilic Vital Dye Dil

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## Abstract

The cuticle of *C. elegans* is a highly resistant structure that surrounds the exterior of the animal<sup>1-4</sup>. The cuticle not only protects the animal from the environment, but also determines body shape and plays a role in motility<sup>4-6</sup>. Several layers secreted by epidermal cells comprise the cuticle, including an outermost lipid layer<sup>7</sup>.

Circumferential ridges in the cuticle called annuli pattern the length of the animal and are present during all stages of development<sup>8</sup>. Alae are longitudinal ridges that are present during specific stages of development, including L1, dauer, and adult stages<sup>2,9</sup>. Mutations in genes that affect cuticular collagen organization can alter cuticular structure and animal body morphology<sup>5,6,10,11</sup>. While cuticular imaging using compound microscopy with DIC optics is possible, current methods that highlight cuticular structures include fluorescent transgene expression<sup>12</sup>, antibody staining<sup>13</sup>, and electron microscopy<sup>1</sup>. Labeled wheat germ agglutinin (WGA) has also been used to visualize cuticular glycoproteins, but is limited in resolving finer cuticular structures<sup>14</sup>. Staining of cuticular surface using fluorescent dye has been observed, but never characterized in detail<sup>15</sup>. We present a method to visualize cuticle in live *C. elegans* using the red fluorescent lipophilic dye Dil (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate), which is commonly used in *C. elegans* to visualize environmentally exposed neurons. This optimized protocol for Dil staining is a simple, robust method for high resolution fluorescent visualization of annuli, alae, vulva, male tail, and hermaphrodite tail spike in *C. elegans*.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/3362/>

## Protocol

### 1. Preparation of Dil stain

1. Prepare a stock solution of 20 mg/mL Dil (Biotium, Inc., Hayward, CA) in DMF. Dil is light sensitive, so protect Dil from light by wrapping in foil.
2. Create a working dilution of Dil by adding 0.6  $\mu$ L Dil stock to 399.4  $\mu$ L M9 for each population. This should give a final working dilution of 30  $\mu$ g/mL Dil in M9. This can be scaled up for staining multiple populations simultaneously. Shield Dil from light by wrapping the tube(s) in foil.

### 2. Preparation of nematodes

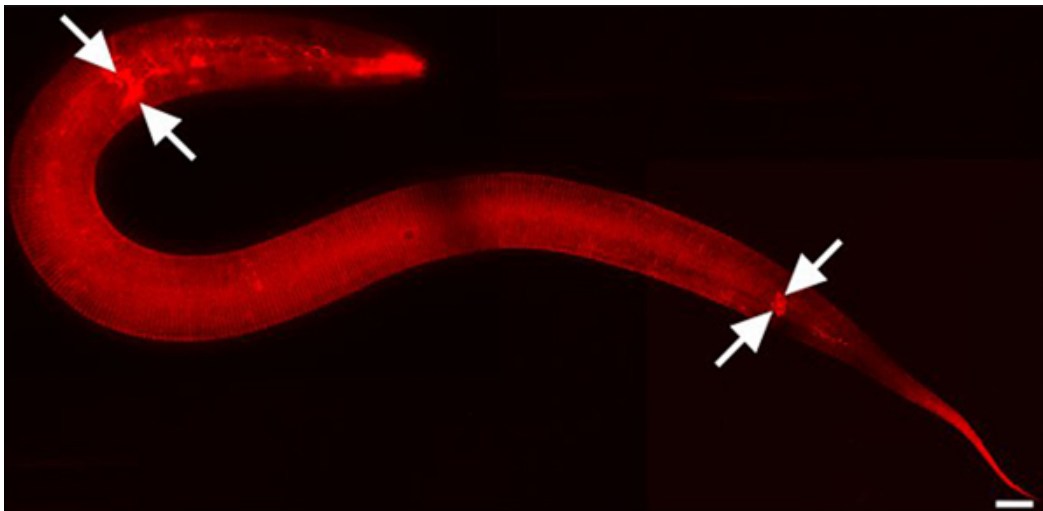
1. Use a 60 mm plate containing a population of uncontaminated nematodes. Wash animals from plate using a solution of 0.5% Triton X-100 in M9 buffer by gently swirling liquid in a circular motion across the surface of the plate to loosen all larval and adult animals. Transfer wash into a sterile 1.5 mL tube.
2. Immediately spin down the animals at 2000 rpm for 30 sec. Remove and discard as much supernatant as possible without disturbing the mass of animals at the bottom of the tube.
3. To reduce residual Triton X-100, rinse animals using M9 buffer, spin, and remove supernatant. Repeat this step once.
4. Add 400  $\mu$ L of working Dil solution in M9 to the tube and vortex briefly to resuspend animals in the solution.
5. Shake tube at 20°C horizontally for 3 hours at 350 rpm in a light-protected environment. If desired, animals can be incubated up to 16 hours for staining.
6. To reduce the amount of unbound dye, spin down the animals at 2000 rpm for 20 sec. Remove and discard as much supernatant as possible without disturbing the mass of animals.
7. Resuspend animals in 400  $\mu$ L M9 buffer and pour liquid onto a bacteria-free portion of a NGM agar plate seeded with OP50 *E. coli*. Allow animals to recover at least 30 minutes. During the recovery time the animals should crawl away from the Dil staining liquid and onto the food. This step reduces background fluorescence from free Dil.

### 3. Mounting and observing specimens

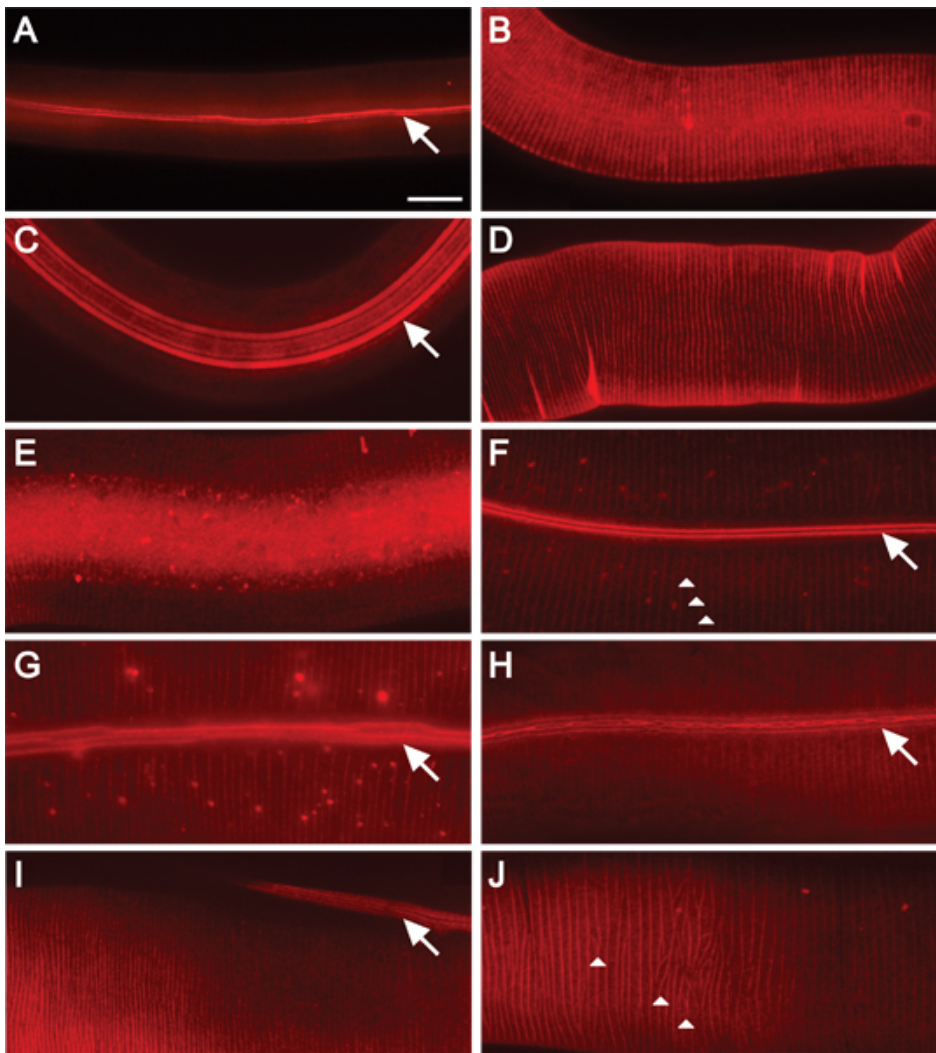
1. Melt 4% agar in water using an autoclave or a microwave.
2. Create reusable spacers, which can be used to ensure uniform thickness of the agar pad, by layering two pieces of lab tape on a glass slide. Make two spacer slides total.
3. Arrange a clean glass slide between two spacer slides. Pipette about 150  $\mu$ L (four drops) of molten 4% agar onto the center of the clean glass slide. Quickly cover the molten agar using an additional slide to form an agar pad. Carefully remove the cover slide, keeping the pad centered on the top of the mounting slide.
4. Pipette about 5  $\mu$ L of nematode anesthetic (100  $\mu$ M - 1 mM levamisole, for example) onto the pad.
5. Mount 8 - 12 animals in the anesthetic and cover with a microscope coverslip.
6. Observe animals using a compound or confocal microscope fitted with at least a 40x objective and a DSRed/TRITC (or other compatible) filter. The fluorescence excitation maximum of Dil is 549 nm and its emission maximum is 565 nm for bound dye (Biotium, Inc., Hayward, CA).

### 4. Representative Results

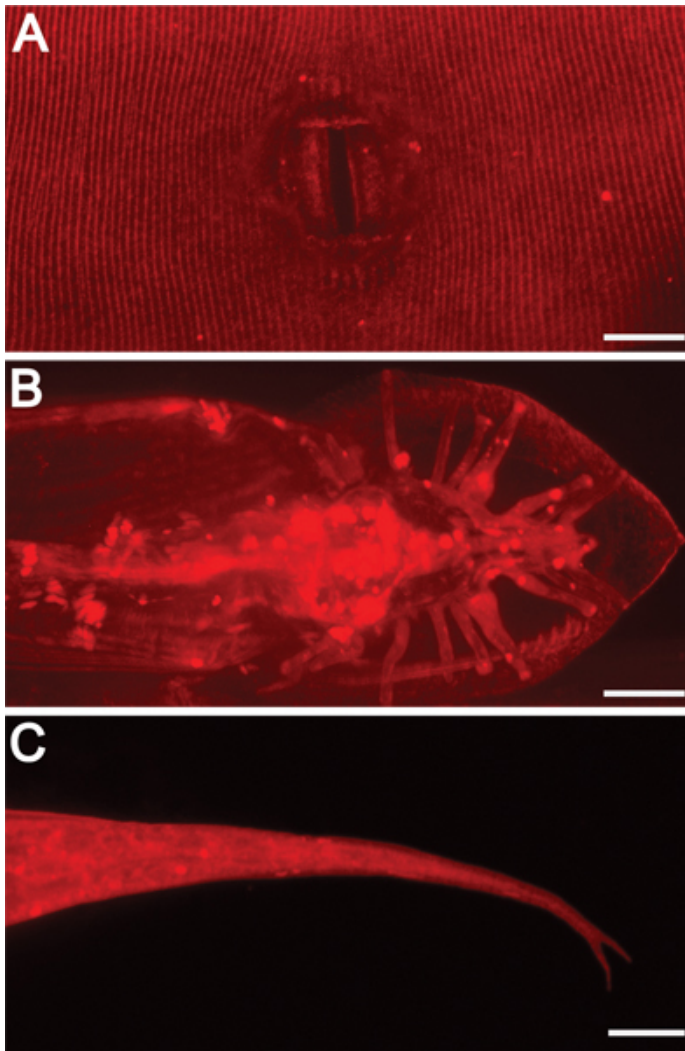
Dil stains the cuticle of wild-type and mutant *C. elegans*. The cuticular surface contains annuli separated by circumferential furrows and, in some stages, longitudinal ridges called alae. Each developmental stage has cuticular structures with distinct compositions<sup>2</sup>. Ridges or furrows of both alae and annuli fluorescently stain, depending on surface composition, throughout larval and adult stages and remain visible up to a day after recovery using this method. Background fluorescent speckles are sometimes observed (Figures 2F, G), but not routinely (Figure 1, Figure 2A-E, H, I). All images were taken with spinning disk confocal or, when noted, widefield (wf) compound microscopy.



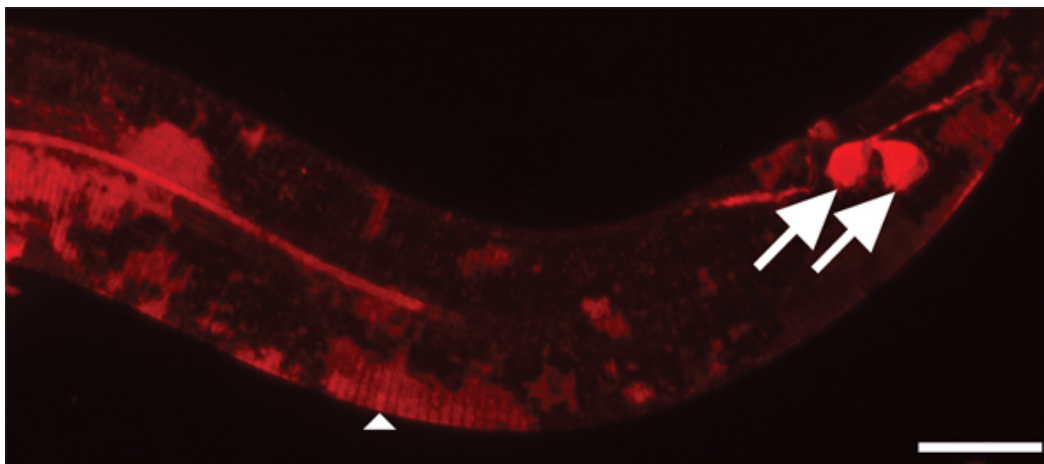
**Figure 1. Dil stains cuticle and environmentally exposed neurons.** L2 stage animal. 630x magnification. Mosaic image parts were captured using iVision-Mac software (BioVision Technologies, Exton, PA). Images were joined using Adobe Photoshop CS3 (Adobe Systems, Inc., San Jose, CA). Scale bar = 10  $\mu$ m. Dil also fluorescently stains amphid and phasmid sensory neurons in the head and tail respectively (arrows mark some).



**Figure 2. Dil fluorescently stains *C. elegans* cuticle at all stages of post-embryonic development.** 630x magnification. Scale bar = 10  $\mu$ m. Staining of wild-type animals in A) L1; B) L2; C) dauer; D) L3; E) L4; and F) adult stages. The alae and annular ridges are fluorescently stained in L1 and dauer animals (A, C). Dil stains annular ridges in L2 animals (B). Annular furrows stain in L3 and L4 animals (D, E). The furrows of the alae and annuli are stained in adult animals (F-H). Alae are composed of two, five, or three ridges (in L1, dauer, or adult animals, respectively) that run the length of the animal (arrows)<sup>16</sup>. Annuli create circumferential ridges around the animal (arrowheads, F and J). The cuticle of adult mutant animals display moderate cuticular organization defects (G-H). G) Ridges of alae are discontinuous (wf). H) Supernumerary alae ridges are fused and branched or bifurcated (wf). Collagen gene mutants exhibit alae and annular organization defects (I-J). I) In transgenic animals overexpressing pRF4 (*rol-6(su1006)*), ridges of alae lie at an angle to the length of the animal. J) Annuli in transgenic animals overexpressing pRF4 (*rol-6(su1006)*) display an irregular pattern.



**Figure 3. External morphological structures are illuminated by Dil staining.** 630x magnification. Scale bars = 10  $\mu$ m. Dil also highlights other exterior features, including A) adult hermaphrodite vulva, B) adult male tail rays and fan, and C) hermaphrodite tail spike (forked in this mutant background).



**Figure 4.** Cuticle takes longer to stain with Dil than environmentally exposed neurons. 630x magnification. Scale bar = 10  $\mu$ m. After two hours of staining, amphid (not shown) and phasmid sensory neurons (arrows) are sufficiently stained. In contrast, the cuticle of younger animals is only partially stained in patches (arrowhead).

Wash	Stain solution (+ Dil)	Incubation time	Cuticle stained
M9 + 0.5% Triton X-100	M9 + 0.5% Triton X-100	2 hrs	no
M9 + 0.5% Triton X-100	M9 + 0.5% Triton X-100	3 hrs	no
M9 + 0.5% Triton X-100	M9	2 hrs	partial
M9 + 0.5% Triton X-100	M9	3 hrs	yes

M9 + 0.5% Triton X-100	H <sub>2</sub> O	2 hrs	partial
M9 + 0.5% Triton X-100	H <sub>2</sub> O	3 hrs	yes

**Table 1. Cuticular staining under different conditions.** Various incubation solutions and times were tested to optimize cuticular staining in animals. H<sub>2</sub>O, sterile distilled water. Partial staining indicates patchy staining of larval cuticle (Figure 4), though adult cuticle stains consistently.

## Discussion

The Dil staining method presented here allows for a relatively quick and convenient way to visualize the cuticle in *C. elegans*. By repurposing and optimizing a method commonly used to image environmentally exposed sensory neurons<sup>15,17</sup>, Dil can be used to fluorescently stain both alae and annular structures (Figures 1 and 2), as well as the vulva, male tail, and hermaphrodite tail spike (Figure 3). We have found that the incubation solution and time influence the ability of Dil to consistently stain the cuticle (Table 1, Figure 4). The method for Dil staining environmentally exposed neurons uses a two-hour incubation time in M9 with Triton X-100<sup>15</sup>. An initial wash of M9 with the surfactant Triton X-100 helps remove contamination from the cuticular surface and prevents the animals from clumping. However, incubating the animals three hours in a staining solution in the same buffer prevents the dye from staining the cuticle (Table 1). This may be caused by lipids on the nematode surface being stripped off by the longer treatment in the detergent solution. Incubation of animals in water with Dil stains the cuticle, indicating that the M9 salt solution is not required for Dil staining of cuticle. Although a water-based protocol is recommended for neuronal Dil staining<sup>15</sup>, we find that animals treated this way often appear unhealthy. Washing animals briefly in 0.5% Triton X-100 in M9, followed by a three-hour incubation in staining solution made with M9, provides consistent staining of cuticular structures and maintains the well-being of the animals.

Animals can be rescued after observation and maintained, permitting direct downstream analyses. This method is a convenient tool that can be used in many studies, including, but not limited to, cuticular secretion and organization, epidermal cell development, heterochronic gene pathways, and nematode evolution.

## Disclosures

We have nothing to disclose.

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