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**ARTICLE**

# A simple method for long-term vital-staining of ciliated epidermal cells in aquatic larvae

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Abbreviations used: ao, anal opening; ap, apical plate; ASW, artificial sea water; BABB, benzyl-alcohol benzyl-benzoate mixture; bm, body wall muscles; bu, buccal muscles; ch, chaetae; ci, cilia; cLSM, confocal laser scanning microscope; cm, circular muscle; Cy5, cyanine 5; DABCO, 1,4-diazabicyclo[2.2.2]octane; DiD, $DiIC_{18}(5)$; DiI, $DiIC_{18}(3)$; DiO, $DiOC_{18}(3)$; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; EdU, 5-ethynyl-2'-deoxyuridine; ep, primary epidermis cells; FA, formaldehyde; F-actin, fibrillar actin; lm, longitudinal muscle; mc, mitotically active cells; mg, midgut; mo, mouth; NA, numerical aperture; NGS, normal goat serum; oc, ocellus; PBS, phosphate buffer saline; PBTw, PBS with Tween-20; pc, parapodial cirrus; pe, pallial epithelium; pm, prototroch ring-muscle; pt, prototroch; se, secondary epidermis; Tris, tris(hydroxymethyl)aminomethane; ve, velum

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## ABSTRACT

Observing the process of growth and differentiation of tissues and organs is of crucial importance for the understanding of the evolution of organs in animals. Unfortunately, it is notoriously difficult to continuously monitor developmental processes due to the extended time they take. Long-term labeling of the tissues of interest represents a promising alternative to raise these pivotal data. In the case of the prototroch, a band of ciliated cells typical of marine, planktotrophic trochophora larvae, we were able to apply a long-term fluorescent vital-staining to the prototroch cells that remains detectable throughout further larval life. We were able to stain ciliated cells of planktonic larvae from different spiralian clades by using long-chain dialkylcarbocyanine dyes that are detectable in different fluorescent emission spectra in combination with a non-ionic surfactant. The larvae survived and developed normally, their ciliated cells retaining the originally applied fluorescent labels. Combined with additional fluorescent staining of the larvae after fixation, we provide an easy, versatile, and broadly applicable method to investigate the processes of the differentiation of epidermal organs in various aquatic larvae.

**Keywords:** cLSM, DiI, fluorescent staining, prototroch, Spiralia

## INTRODUCTION

The process of organogenesis is of central interest in comparative developmental and evolutionary developmental biology. During the early phase of the developmental process, the majority of animals are confined to an egg case in which they only move sporadically and are thus accessible for examination with modern imaging methods, such as digital time-lapse video recording or 4D-microscopy [1-5]. However, detailed knowledge beyond early, embryological development has been impeded by the fact that further development proceeds on a larger time scale in which animals cannot be continuously monitored. This is especially the case in planktonic, feeding larval stages, in which metamorphosis to the adult body organization can take up to several months. Exactly these types of marine planktonic larvae have always featured prominently in the discussion of the evolution of animal morphologies, the central field of evolutionary developmental biology [6-9]. The trochophora larva observed during the development of many spiralian (= lophotrochozoan) clades can be considered one of the most prominent marine larval types [9]. The eponymous structure of the trochophora is the prototroch, a circumferential ring of ciliated cells dividing the larval body into an anterior episphere and a posterior hyposphere [9]. The prototroch is reported to serve as the main locomotory and feeding structure and is therefore crucial for growth and development of the larva. The prototroch cells of most trochophora larvae have been shown to develop from the same lineage of embryonic cells, termed trochoblasts [10]. Due to an early cleavage arrest and the early formation of cilia in the prototroch cells, the prototroch is the first identifiable differentiated organ of the trochophora larva [11-14]. Although the early formation of the prototroch has been subject to a number of comparative investigations, its subsequent development during larval life and metamorphosis to the juvenile is virtually unknown. Currently, there are no data available regarding the post-embryonic development of the prototroch on a broad comparative scale. Thus, several interesting questions concerning the maintenance and growth of a presumably not mitotically

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active prototroch, and the mechanism of its degenerative fate after settlement of the organism to a benthic existence remain unanswered. As a consequence, the significance of the prototroch and other ciliated bands for hypotheses on evolutionary developmental biology cannot be conclusively evaluated. A way to raise the data needed would be to apply a long-term label to the prototroch cells to be able to monitor them through metamorphosis. With this in mind, we established a very simple vital-staining method, based on a protocol modified from a dissertational thesis on *Platynereis dumerilii* (Audouin & Milne Edwards, 1833) (*P. dumerilii*) [15]. With the lipophilic long-chain dialkylcarbocyanine dyes ($DiIC_{18}(3)$ also known as DiI and $DiIC_{18}(5)$ also known as DiD), we applied a long-term fluorescent label to the prototroch cells to track them after their differentiation through larval development. However, in order to successfully load the prototroch cells with the lipophilic dyes, Pluronic F-127, a non-ionic surfactant that had been reported to aid in loading lipophilic dyes into cells was added [16]. Our modified method has been successfully employed on larvae of different spiralian clades and we can show that the fluorescent label remains within the initially stained cells and does not interfere with the normal development of the larvae. Furthermore, we investigated the compatibility of the new staining method with additional fluorescent and immunohistochemical staining in fixed larvae. If certain preparative steps are modified, the fluorescent label of long-chain dialkylcarbocyanine dyes remains visible even after additional staining in fixed larvae has been performed.

## MATERIALS AND METHODS

### Animals

The planktonic larvae of four species were stained with different lipophilic long-chain dialkylcarbocyanine dyes: the annelids *P. dumerilii* and *Thalassema thalassema* (Pallas, 1774) (*T. thalassema*), the mollusc *Mytilus edulis* Linnaeus, 1758 (*M. edulis*), and the nemertean *Cephalothrix oestrymnica* (Gibson & Junoy, 1991) (*C. oestrymnica*). *Platynereis dumerilii* larvae were obtained from a culture kept at the Institute of Evolutionary Biology and Ecology of the University of Bonn. *Mytilus edulis*, *T. thalassema* and *C. oestrymnica* larvae were obtained by artificial insemination of animals collected in the intertidal in the vicinity of the Station Marine de Concarneau, France.

### Vital-staining with long-chain dialkylcarbocyanine dyes (DiI, DiD)

Trochophora larvae of *P. dumerilii* were stained according to the original protocol [15]: at 23 h after fertilization, the larvae were incubated for 1 h at 18°C in 1 µg/ml $DiIC_{18}(3)$ (DiI, Invitrogen) in seawater prepared from a stock solution of 1 mg/ml DiI in DMSO (Roth). During the incubation, incubating containers (glass bowls) were gently agitated on a rotary shaker. After the incubation, the animals were rinsed through a sieve with 100 µm mesh size and washed into a clean glass bowl with seawater. Controls were conducted in the same way but the dye was omitted (only DMSO was added to the larvae). Labeling and control experiments were conducted for more than six different batches of larvae; for each batch more than ten larvae were examined. For the larvae of the remaining species, the protocol was modified by mixing the lipophilic long-chain dialkylcarbocyanine dyes (1 mg/ml in DMSO) with the same volume of a 20% (w/v) solution of the non-ionic surfactant Pluronic F-127 (Sigma-Aldrich) in DMSO prior to diluting the mixture 1:1000 in seawater. Thus, a final concentration of 0.5 µg/ml of DiI or DiD, 0.01% (w/v) of Pluronic F-127 and 0.2% (v/v) of DMSO was used. Larvae of *C. oestrymnica* and *M. edulis* were stained when swimming stages were observed, which was 21 h after fertilization in *M. edulis* and 48 h after fertilization in *C. oestrymnica* both at 12°C. Larvae were incubated in the DiI staining solution for one hour at 18°C with gentle agitation of the glass bowls on a rotary shaker. *Thalassema thalassema* trochophora larvae were raised to 43 h after fertilization at 12°C. At that age staining was carried out for 1 h at 18°C in seawater with 0.5 µg/ml $DiIC_{18}(5)$ (DiD, Invitrogen) mixed with Pluronic F-127 in DMSO (DiD/Pluronic F-127) at the same concentrations as described above for DiI/Pluronic F-127-staining of *M. edulis* and *C. oestrymnica*. After staining, the animals were rinsed through sieves with appropriate mesh size to catch the larvae and washed into glass bowls with clean seawater. Controls were run by omitting the dialkylcarbocyanine dye from the mixture with Pluronic F-127, thus only adding the respective volume DMSO. Labeling and control experiments were conducted for more than three different batches of larvae in *M. edulis* and *C. oestrymnica* and three batches in *T. thalassema*; for each batch more than 8–12 larvae were examined. Further rearing, fixation, counterstaining, and mounting were performed in dim light conditions, or if necessary (*e.g.*, when changing media during fixation, staining, and mounting) with the lowest light exposure possible.

### Fixation

Of each species, some larvae were fixed immediately after the stain had been washed out to evaluate the staining success. The remaining larvae were left alive to develop and several subsamples were fixed after several days to record the persistence of the labelling. The larvae were fixed in 4% (v/v) formaldehyde (FA, prepared from a 16% aqueous paraformaldehyde solution, VWR) either in 0.1M phosphate buffer saline (PBS, pH 7.4; *P. dumerilii*) or artificial sea water (ASW, pH 8 approximately; pH-value was not measured after mixing FA with ASW; *C. oestrymnica*, *M. edulis*, and *T. thalassema*). Older larvae, in which musculature had already developed, were immobilized by immersion in a mixture of equal volumes of ASW and 7.5% (w/v) aqueous $MgCl_2$-solution (VWR) prior to fixation for 10–15 min until no muscular contraction was observable. In these cases, EDTA (Fluka) was added to the fixative to a concentration of 3.42 mM to prevent the formation and precipitation of insoluble magnesium-phosphate salts. Depending on the size of the larvae, they were fixed at room temperature for 30 min (*C. oestrymnica*, *M. edulis*, *T. thalassema*, and *P. dumerilii* up to 3 days of age) or 45 min (*P. dumerilii* older than 3 days). To remove the fixative, the larvae were rinsed in three successive changes of fixation buffer (0.1 M PBS, 3.42 mM EDTA, pH 7.4), each for 10 min at room temperature. Larvae were stored in the same buffer with 0.01% w/v $NaN_3$ (Roth) added to prevent microbial growth at 4°C for up to several weeks before further processing them.

### Additional fluorescent staining

Apart from mounting and examining specimens without any additional staining, fluorescent labeling of DNA and F-actin, fluorescent immunolabeling, and fluorescent click-labeling of live-incubated 5-ethynyl-2′-deoxyuridine (EdU, Thermo Fischer Scientific; in *P. dumerilii* at a concentration of 10 µM EdU in seawater for 24 h after 1d of development at 18°C) were tested for their compatibility with the dialkylcarbocyanine dye vital-staining.

Click-labeling with fluorescently conjugated azide of specimens

incubated with EdU was performed according to the following protocol: After a preincubation of the specimens in 100 mM Tris Buffer for 5 min at room temperature, the specimens were permeabilized in PBS with Tween-20 (Roth) added to a final concentration of 0.2% (0.2% PBTw) for 30 min at room temperature. After three subsequent washes in PBS for 10 min each at room temperature, specimens were incubated in the freshly prepared labeling mix (1 mM $CuSO_4$, Merck; 0.8 µM azide/Cy5, Lumiprobe; 10 mg/ml ascorbic acid, Roth; in PBS) for 30 min at room temperature. Finally, the specimens were washed in three changes of PBS for 5 min at room temperature.

For immunostaining a standard protocol was used: Specimens were permeabilized in three changes of 0.2% PBTw each for 10 min at room temperature. Subsequently, specimens were blocked in 10% normal goat serum (NGS, Sigma-Aldrich) in 0.2% PBTw for 2 h at room temperature and afterwards incubated with an antibody against acetylated α-tubulin raised in mouse (Sigma-Aldrich) at a dilution of 1:200 in 0.2% PBTw (antibody from a stock solution of 2 mg/ml in DMSO) overnight at 18°C. Unbound antibody was removed by rinsing the specimens in three changes of 0.2% PBTw for 10 min each at room temperature. As secondary antibody, goat anti mouse, conjugated with Cy5 (Abcam) was used at a dilution of 1:100 in 0.1% PBTw (antibody from a stock solution of 2 mg/ml in glycerol). The specimens were incubated for 2 h at room temperature. Finally, unbound secondary antibody was removed with three washes of PBS for 10 min each at room temperature.

Fluorescent labeling of F-actin was performed by incubation of permeabilized specimens (permeabilization was the same as described for immunostaining) with phalloidin conjugated with Alexa Fluor 488 (Invitrogen) at a dilution of 1:200 from the stock solution of 2 units per 10 µl of DMSO for 45 to 60 min at room temperature. Subsequently, unbound phalloidin was removed with three washes of PBS for 10 min each at room temperature. Nuclei were fluorescently stained with Sytox green (0.5 µM, Invitrogen) for 20 min at room temperature. Excess dye was removed with three washes of PBS for 10 min each at room temperature.

When additional fluorescent labeling techniques were employed, the sequence of staining was: click-labeling of EdU, immunolabelling, labeling of DNA or F-actin (F-actin labeling only if no click labeling was performed). To avoid excessive exposure of the dialkylcarbocyanine dyes to detergents (Tween-20), phalloidin or fluorescent nuclear stains were added at the end of the incubation with the secondary antibody for the respective time period described above.

## Mounting and imaging

To prevent specimens from moving in the viscous mounting media used, they were attached to Poly-L-lysin (Sigma-Aldrich, 0.06% w/v in PBS with 0.01% w/v $NaN_3$) coated coverslips. Early larvae of *P. dumerilii* (1-day old) were mounted in PBS since they would deform in more viscid mounting media), whereas the other specimens were mounted in 90% (v/v) glycerol (Merck) in 0.1 M PBS with 2.5% (w/v) DABCO (Roth) added as antifading agent. To avoid deformation of the specimens in the latter mounting medium, they were transferred to 90% glycerol *via* an ascending series of glycerol in 0.1M PBS (10%, 30%, 60% for 5 min each, followed by 90% for 10 min, all steps at room temperature). The dialkylcarbocyanine dyes were excited with lasers of the wavelengths $\lambda_{ex}$ = 532 nm for DiI and $\lambda_{ex}$ = 635 nm for DiD. For additional fluorescent staining, the appropriate laser lines were used for fluorophore excitation (Sytox green, Alexa Fluor 488: $\lambda_{ex}$ = 488 nm, Cy5: $\lambda_{ex}$ = 635 nm). Confocal stacks were recorded either with 20× dry/0.7NA (*P. dumerilii*) and 40× dry/0.75NA objectives (remaining species) with a Leica TCS SPE confocal microscope system (cLSM). Control specimens were imaged with the same confocal settings as for the respective vital-stained specimens. Images were further processed with the FIJI distribution package of ImageJ by W. S. Rasband, NIH (version 1.52p & 1.52r) [17,18]. Image plates were mounted with Adobe Illustrator CS6.

# RESULTS

## DiI vital-staining of *Platynereis dumerilii* (Annelida)

With the original staining procedure on larvae of *P. dumerilii* (1 µg/ml DiI in seawater, for one hour at room temperature), the previously reported results were largely reproduced (**Fig. 1A**). In larvae incubated at slightly younger age that did not yet have developed the apical ciliary tuft, only the ciliated prototroch cells were stained, whereas larvae with developed ciliary tuft also showed the reported staining in the apical ciliated sensory cells. The control specimens did not show any fluorescence comparable with that of the vital-stained specimens (**Fig. S1A**). During the incubation with DiI, the larvae stayed near the bottom of the glass bowls that they were incubated in. After they had been transferred to clean seawater, the larvae resumed their normal swimming activity. A similar behavior could be seen in larvae of all species incubated in DiI/Pluronic F-127 or DiD/Pluronic F-127.

In the advanced nectochaeta larva of *P. dumerilii* (4 d), in which the prototroch is degenerating, only a few cells of the prototroch show the intense fluorescent signal observed in the young trochophora larvae (**Fig. 2A** and **2B**). Occasionally detected internal DiI signals and an increased fluorescence of the gut epithelium suggest that the lipid fraction of the prototroch cell membrane stained with DiI was internalized and reused in cell membranes of later developing organs, thus distributing but also diluting the dye within the body (**Fig. 2A** and **2B**).

## DiI vital-staining with the addition of Pluronic F-127

When incubated with a mixture of DiI/Pluronic F-127, an uptake of the respective dye into the ciliated epidermal cells of larvae of *M. edulis* and *C. oestrymnica* could be detected (**Fig. 1B** and **1C**). When Pluronic F-127 was omitted and only DiI was used for staining, larvae of *M. edulis* did not show any uptake of DiI in their ciliated cells (**Fig. S1B**). In a few larvae of *C. oestrymnica*, fluorescent signals were observed in a patchy distribution in the epidermal cells (**Fig. S1C**, arrows). However, the pattern was not uniform in the larva so that we assume that it is due to precipitated DiI crystals having contacted the respective epidermis cells during the incubation.

In *C. oestrymnica* stained with DiI/Pluronic F-127, the signal is outlining not only the apical cell surface but the complete cell membrane. In this species, there is also a well visible DiI signal in the cilia of the epidermis (**Fig. 1B**, arrows). In deeper tissue layers, such as the gut epithelium, no DiI signal is detected (**Fig. 1B**). The older larva of *C. oestrymnica* (8 d) shows unstained patches in the epidermis mainly in its frontal part (**Fig. 2C** and **2D**). This is due to the intercalation of smaller but more numerous secondary epidermal cells between the primary epidermis cells, which is typical of nemertean larvae. Since the secondary epidermis cells have been intercalated between the primary epidermis cells from underneath the latter after the incubation with

DiI/Pluronic F-127, neither their cellular nor their ciliary membranes are stained with DiI/Pluronic F-127 (**Fig. 3C** and **3D**, arrowheads).

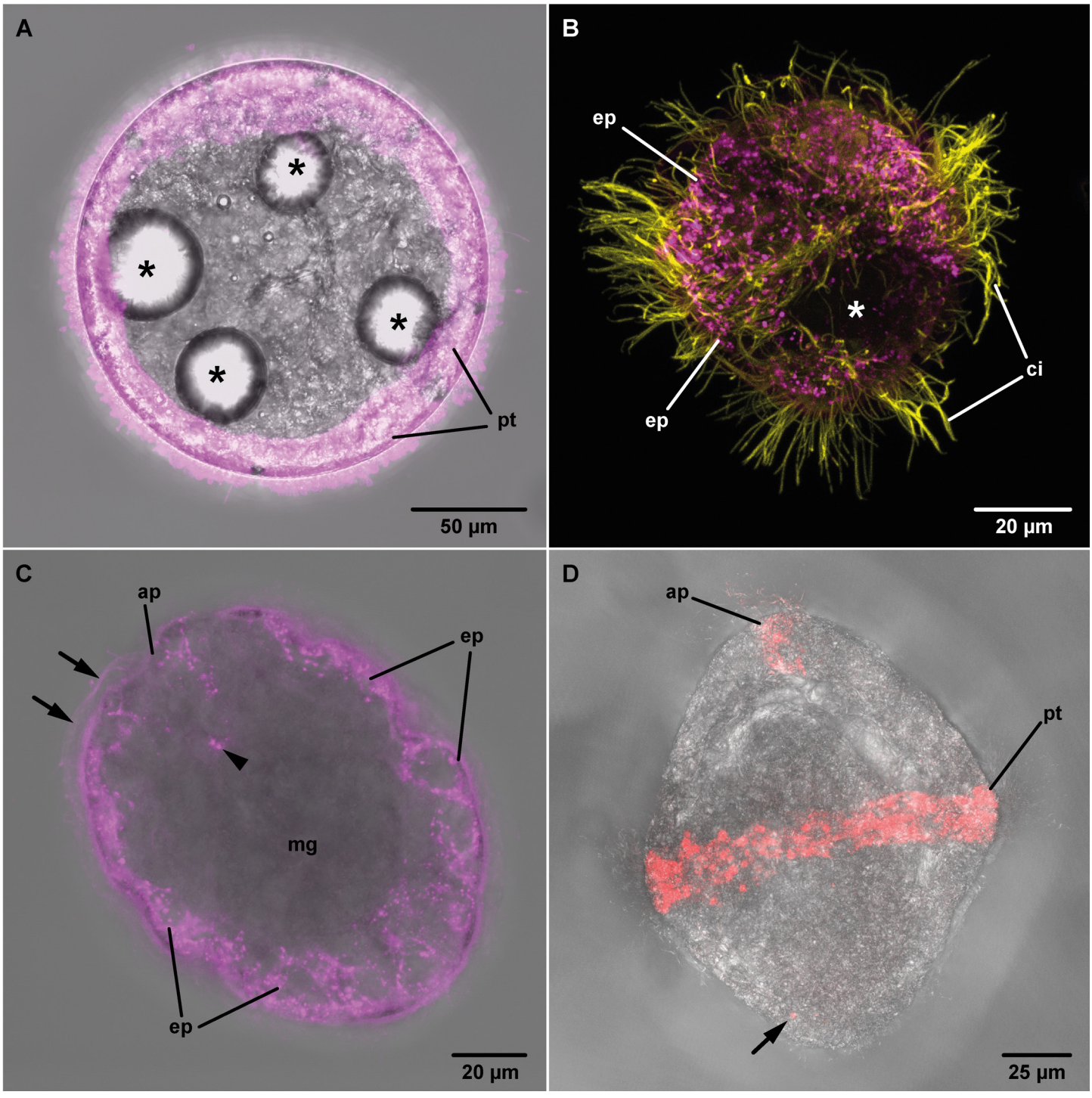


**Figure 1. Young larvae after staining with DiI (A) or DiI/Pluronic F-127 (B and C) or DiD/Pluronic F-127 (D), cLSM (magenta: DiI, yellow: anti α-tubulin, red: DiD). A.** *Platynereis dumerilii*, 24-hours old larva, maximum projection (apical view): the ciliated prototroch cells (pt) are stained. Note the large yolk vesicles in the four gastrulated macromeres (asterisks). **B.** *Mytilus edulis*, 21-hours old larva, maximum projection (anterior to upper left): only epidermal cell (ep) with cilia (ci) show DiI signal, unciliated regions of the epidermis are unstained (asterisk). **C.** *Cephalothrix oestrymnica*, 2-day old larva, maximum projection of 5 middle sections (4.4 µm; anterior to upper left): only the epidermal cell (ep) layer including the apical tuft (arrows) is stained; cells of the midgut epithelium (mg) are unstained. Cells of the apical plate (ap) extend deeply into the larva (arrowhead). **D.** *Thalassema thalassema*, 44-hours old larva, maximum projection (anterior is up): fluorescent DiD signals detectable in the ciliated prototroch (pt) and in some ciliated cells of the apical plate (ap). Note the small, dot-shaped signal visible in an unciliated part of the larva (arrow).

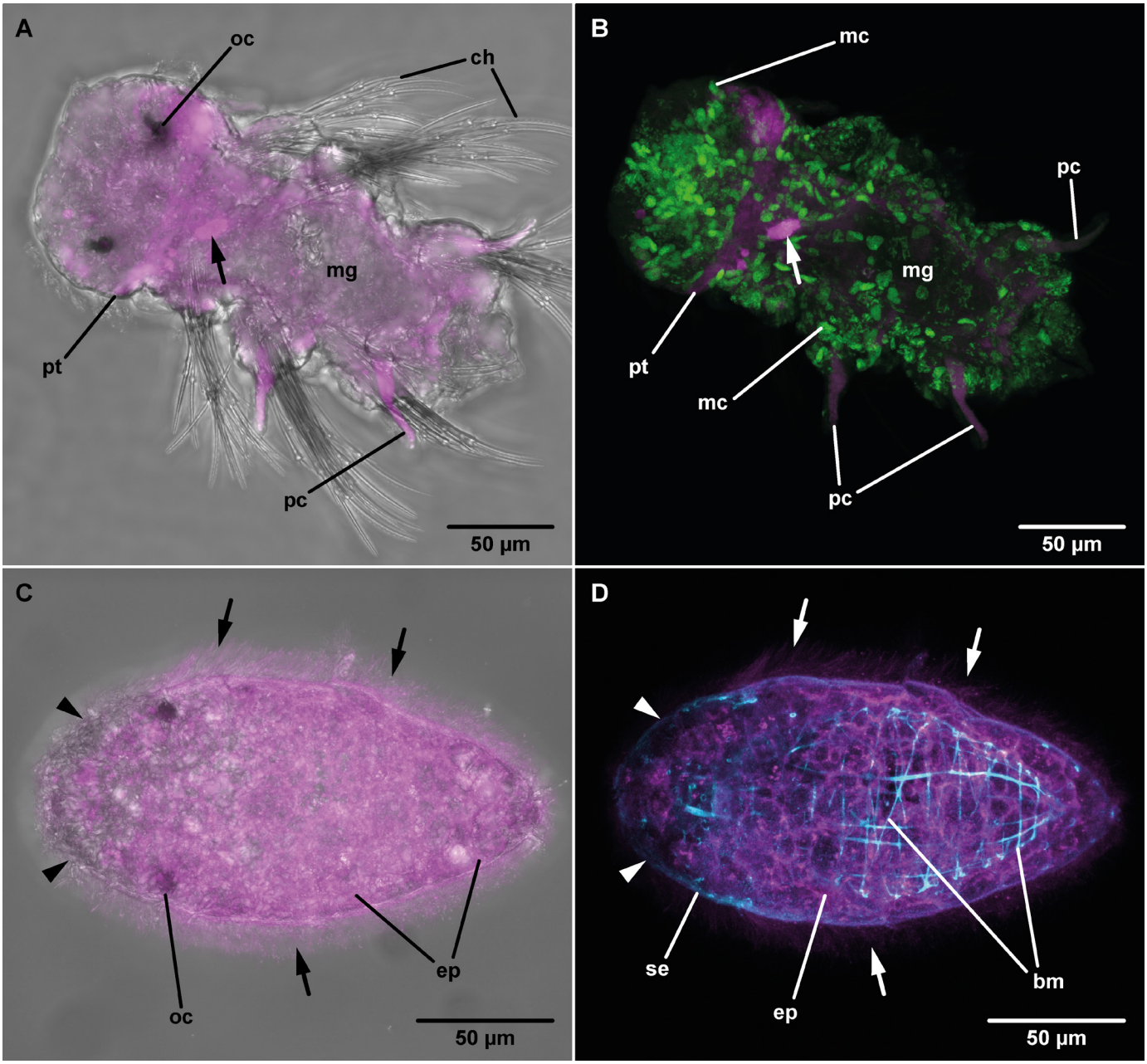


**Figure 2. Advanced larvae, some days after staining with DiI (A and B) or DiI/Pluronic F-127 (C and D), cLSM (magenta: DiI, green: EdU, cyan: phalloidin). A.** *Platynereis dumerilii*, 4-day old larva, maximum projection (anterior to upper left corner): DiI signal is most prominent in the prototroch (pt), although some weak fluorescence is seen in the midgut (mg); the parapodial cirri (pc) next to the chaetae (ch) show autofluorescence due to glandular tissue. Note the strong, cell-shaped, internalized DiI signal (arrow). **B.** Same as in (A): Mitotically active cells (mc) are seen throughout the larval body, indicating ongoing development after DiI incubation. Note the strong, cell-shaped, internalized DiI signal (arrow). **C.** *Cephalothrix oestrymnica*, 8-day old larva (anterior to left side): The posterior part of the epidermis, up to the level of the larval ocelli (oc) show DiI signal, whereas the anterior-most part is unstained (arrowheads). Note the DiI signal visible in the cilia (arrows) of the primary epidermal cells (ep). **D.** Same as in (C): Phalloidin staining of F-actin reveals the body wall muscles (bm) and the outlines of the primary (ep) and secondary epidermis (se) cells. The primary epidermis shows DiI labelling, whereas the secondary epidermis is unstained (arrowheads). Note the DiI signal visible in the cilia (arrows) of the primary epidermis (ep).

In the ciliated epidermis cells of the early trochophore larva of *M. edulis*, DiI aggregates in a dot-shaped pattern (**Fig. 1B**). In the subsequently developing veliger larva of *M. edulis* (6 d), the DiI signals have the same shape and distribution within the stained cells as in the younger trochophora larva of this species (**Fig. 1B**, **Fig. 3A-3C**). As in the trochophora larva, only ciliated epidermis cells show DiI signal (**Fig. 3A** and **3B**). In the veliger larvae these cells constitute the velum, and ciliated regions around the mouth and the anal opening (**Fig. 3B**). Comparably larger unciliated epidermal regions constituting the pallial epithelium have developed after the incubation with DiI/Pluronic F-127 from unciliated and therefore unstained regions in the trochophora larva (**Fig. 3A** and **3C**).

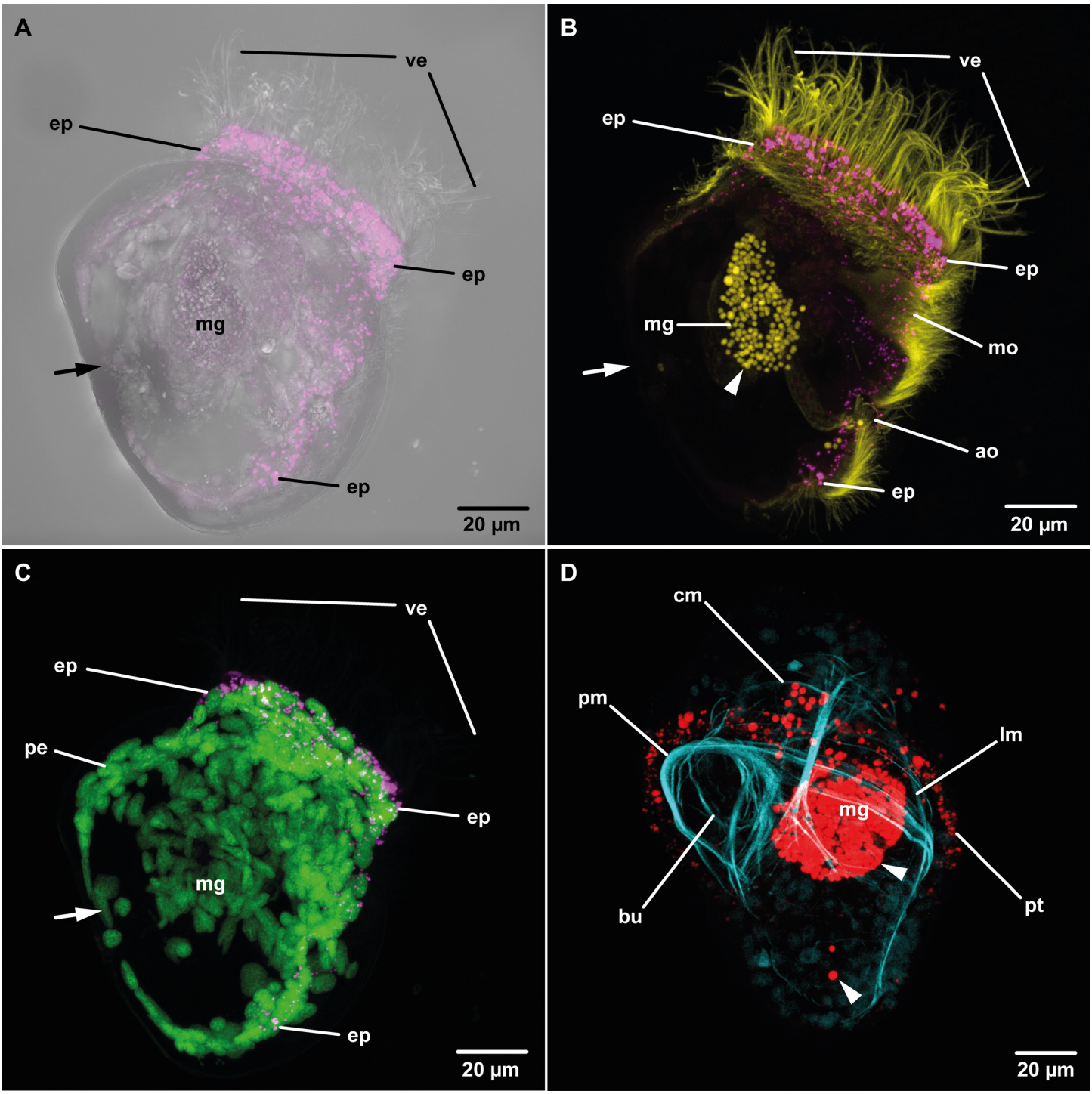


**Figure 3. Advanced larvae some days after staining with DiI/Pluronic (A-C, anterior to top right corner) or DiD/Pluronic (D), cLSM (magenta: DiI, yellow: anti α-tubulin, green: nuclei, red: DiD, cyan: phalloidin). A.** *Mytilus edulis*, 6-day old larva: The most prominent DiI signals are seen in epidermis (ep) of the velum (ve) and along the ventral side, no signal is detectable on the dorsal side (arrow). The midgut (mg) is filled with unicellular planktonic algae. **B.** Same as in (A): DiI signal is prominent in the ciliated epidermis (ep) of the velum (ve) and along the ventral side encompassing the mouth (mo) and the anal opening (ao). No DiI signal is seen in the unciliated epidermis (arrow). The midgut (mg) is filled with algae that show intense chlorophyll autofluorescence (arrowhead). **C.** Same as in (A): The primary epidermis cells (ep) of the velum (ve) and on the ventral side show DiI signal. No DiI signal is detected in the midgut (mg) and the dorsal pallial epithelium (pe, arrow). **D.** *Thalassema thalassema*, 4-day old larva (anterior is up): The ciliated prototroch (pt) shows prominent DiD signal. Labeling of F-actin with phalloidin shows the prominent ring-muscle underneath the prototroch (pm), the buccal muscles (bu) and some additional circular (cm) and longitudinal muscle (lm) strands. Ingested unicellular planktonic algae in the midgut (mg) show an intense chlorophyll autofluorescence (arrowheads).

### DiD vital-staining with the addition of Pluronic F-127 in *Thalassema thalassema* (Annelida)

DiD without the addition of Pluronic F-127 is virtually insoluble in sea water resulting in a crystalline precipitate. Only with the addition of Pluronic F-127 prior to diluting in seawater a suspension of dispersed DiD could be produced. In the young larvae of *T. thalassema* that were stained with DiD mixed with Pluronic F-127 (DiD/Pluronic F-127) the detected fluorescent signal is dot-shaped and thus reminiscent of the DiI signal detected in *M. edulis* larvae (**Fig. 1D**). The ciliated cells of the prototroch and the apical plate show intense DiD staining. In some larvae, there are signals detected in parts of the epidermis that are not evidently ciliated (**Fig. 1D**, arrow).

Advanced trochophora larvae of *T. thalassema* (4 d) show the most prominent staining in the ciliated prototroch cells (**Fig. 3D**). It should be noted that the larvae of *T. thalassema* are planktotrophic and had to be feed with unicellular planktonic algae to further develop. The algal chlorophyll possesses a fluorescence spectrum that completely overlaps with that of DiD, both with respect to excitation and emission wavelengths. Therefore, the algae ingested by the larvae show up as equally intense, but slightly larger, spherical fluorescent signal in the midgut lumen of the more advanced trochophore larva of *T. thalassema* (**Fig. 3D**, arrowheads).

### Additional fluorescent staining in fixed specimens

In all additional fluorescent staining protocols performed on fixed larvae the dialkylcarbocyanine dye signals were preserved (**Fig. 1B**, **Fig. 2B** and **2D**, and **Fig. 3B-3D**). Fluorescent click-labeling of EdU incubated nectochaeta larvae of *P. dumerilii* show the distribution of cells that have undergone mitosis during the EdU incubation (**Fig. 2B**). As would be expected, the ciliated prototroch cells that are cleavage arrested after the sixth round of cleavage divisions and thus well before incubation with either DiI or EdU, do not show any EdU incorporation in the DNA of their nuclei (**Fig. 2B**).

Phalloidin labeling of larvae of *C. oestrymnica* show the F-actin in the developing body wall muscle cells and in the apical F-actin network (belt desmosome) of the epidermis cells (**Fig. 2D**). In the larvae of *T. thalassema*, the prototroch muscle under the ciliated prototroch cells is prominently labeled with F-actin. Longitudinal muscles, several additional strands of circular muscles, and the prominent buccal musculature are clearly visible (**Fig. 3D**).

Immunohistochemical staining of acetylated α-tubulin in *M. edulis* reveals that the DiI signal is exclusively found in ciliated epidermal cells (**Fig. 1B** and **Fig. 3B**). It should be noted that acetylated α-tubulin is massively located in the cilia outside of the cell soma and therefore the comparably little acetylated α-tubulin present in other compartments of the cytoskeleton could not be detected with the confocal settings used herein. Like the trochophora larvae of *T. thalassema*, the veliger larvae of *M. edulis* are planktotrophic and ingested unicellular planktonic algae are visible in the larval midgut due to their algal chlorophyll fluorescence in the red emission spectrum (**Fig. 3B**, arrowhead).

Fluorescent labeling of DNA with Sytox green stains the nuclei not only of the ciliated epidermis cells, but also all other non-ciliated cells of the larvae, thus providing a good counterstain in the otherwise almost transparent veliger larva of *M. edulis* (**Fig. 3C**).

## DISCUSSION

### Staining of ciliated cells with DiI

The original protocol used for the vital-staining of *P. dumerilii* larvae was taken from the doctoral thesis of Tessmar-Raible [15] and had originally been used to stain apical neurons and ciliated prototroch cells of the developing larva. Already therein, several problems of staining with DiI are reported and the potential staining mechanism is discussed. It has been assumed that the cells taking up DiI in *P. dumerilii* larvae are ciliated cells directly in contact with the surrounding medium and with a chemosensory function. Problems of the protocol reported by Tessmar-Raible [15] include the tendency of DiI to precipitate from the suspension as well as the inability to stain the ciliated cells of the prototroch after the 5$^{th}$ day of development in *P. dumerilii* larvae. The reason why DiI is not incorporated into cells of older larvae is not clear, but it might be dependent on the constitution of the cellular membranes that might be different in advanced as opposed to young larvae.

### DiI/DiD applied together with Pluronic F-127

The application of the original protocol did not yield any labeling of ciliary band cells on any of the tested species besides *P. dumerilii*. The reason we suspected was the low dispersibility of lipophilic long-chain dialkylcarbocyanine dyes, especially in sea-water. Therefore, we looked for substances that enhance the dispersibility of the staining substances and thus facilitate uptake of lipophilic markers into cells or cellular membranes. We found that the non-ionic surfactant Pluronic F-127 is commonly used to load other lipophilic stains into cells without affecting their viability [16]. Applying the lipophilic dye mixed with the non-ionic surfactant Pluronic F-127 not only increased the dispersibility of DiI in seawater and thus reduced precipitation, it also resulted in labeling of virtually all epidermal cells with cilia projecting to the exterior. The application of Pluronic F-127 was not seen to affect larval viability in the species examined. Whereas DiD alone is insoluble in sea water, mixing it with Pluronic F-127 resulted in fluorescent staining of the ciliated epidermal cells, although it seems that there was still some precipitation of the dye from the suspension in seawater shown by irregularly stained non-ciliated cells. Whether the precipitation can be avoided by using higher concentrations of Pluronic F-127 and whether there are detrimental effects of the increased surfactant concentrations on the viability of the larvae needs further testing.

### Stability of cellular labeling in fixed specimens

Lipophilic long-chain dialkylcarbocyanine dyes, especially DiI have been used repeatedly for cellular labeling of neuronal tracts in both living and, more commonly, fixed material [19-21]. Another field of application is long term labeling of blastomeres for cell lineage studies by injection of DiI dissolved in soybean oil [22-25]. Applied to living cells (blastomeres or neurons), it has been shown that the dye is not noticeably transferred from the labeled cell to neighboring ones [19,26]. In neuronal tract labeling experiments with fixed tissue, transfer of DiI from the labeled cells to the adjacent tissue has been reported to increasingly occur at higher incubation temperatures [21]. To inhibit dye transfer between cells, addition of EDTA to the incubation medium has been recommended [21]. Following this recommendation, we added EDTA to the buffer when fixed larvae were stored over an extended time period, up to several weeks. However, the low storing temperatures employed in this study (4°C) might decrease the fluidity

of the membrane and thus also inhibit potential dye transfer. Therefore, storage at low temperatures might make the addition of EDTA unnecessary. The option of omitting EDTA from fixation, washing, and storing buffers might be of potential importance to experimental setups in which calcium detection is planned.

## Influence of staining on development

The results reported herein furthermore show that, at the concentrations tested, the dialkylcarbocyanine label stays within the initially labeled cells without affecting viability or further development of the larva. However, the observations made for the development of *P. dumerilii* suggest that dialkylcarbocyanine dyes are contained in the lipid fraction of the membrane and may be distributed along with it to other tissues during metabolic membrane turnover. These results however do not exclude dialkylcarbocyanine dyes from being used as long-term markers of cell fates since, in our experiments, the secondary signal of the dye presumably produced by recycling of stained lipid components has not been shown to reach the intensity of the primary signal resulting from the initial incubation with the respective dye. Moreover, some inhibitory effect of dialkylcarbocyanine dyes to the ciliary activity during the dye incubation seems possible but, with the concentrations we tested, we have not seen this effect to persist after the end of the incubation.

## Compatibility with additional fluorescent staining and mounting for fluorescence microscopy

The possibility of labeling ciliated epidermal cells by mixing Pluronic F-127 with long-chain dialkylcarbocyanine dyes with different fluorescence emission spectra (DiI, DiD, and potentially also DiO, although the latter was not extensively tested by us, but see **Fig. S1D** and [15]) makes multi-color labeling with additional fluorescent staining techniques possible. Labeling techniques shown to be compatible with dialkylcarbocyanine dye vital-staining include fluorescent labeling of nuclei and F-actin, cell proliferation by click-labeling of EdU incorporated into replicating DNA, and immunostaining, exemplified herein with antibodies against acetylated α-tubulin. As seen in the uniformly ciliated larvae of *C. oestrymnica*, staining of DiI mixed with Pluronic F-127 labels all ciliated epidermal cells uniformly. However, the possibility of applying additional fluorescent labeling techniques may allow for distinguishing between different types of ciliated epidermal cells (*e.g.*, serotonergic sensory cells) by counterstaining with specific antibodies (*e.g.*, against serotonin). Care should be taken if developmental stages of animals stained with dialkylcarbocyanine dyes have to be fed with auto-fluorescent food items, such as green algae, since fluorescence spectra might overlap, as is the case in chlorophyll and DiD. Due to the lipophilic nature of dialkylcarbocyanine dyes, the use of lipophilic organic solvents for lipid extraction steps during fixation and permeabilization, as well as lipophilic organic mounting agents, such as BABB (also known as Murray's Clear), are incompatible with dialkylcarbocyanine dye labeling [27]. For permeabilization, Tween-20 is reported to perform well in preserving the dialkylcarbocyanine dye signals [28]. Contrary to accounts stating the incompatibility of glycerol mounting with dialkylcarbocyanine dyes [29], we have not experienced any significant quenching of the DiI/Pluronic F-127 or DiD/Pluronic F-127 label in our glycerol-mounted preparations within several days of storage at low temperatures (4°C) after mounting.

In conclusion, detailed data on the development of the ciliated prototroch, the iconic feature of trochophora larvae, have been impossible to obtain due to the inability to reliably identify individual prototroch cells and to follow them through further development. Although fluorescent staining with the lipophilic, long-chain dialkylcarbocyanine dye DiI has been demonstrated in the annelid species *P. dumerilii*, the same protocol fails in other planktonic spiralian larvae. With the addition of the non-ionic surfactant Pluronic F-127, we were able to modify the protocol so that ciliated cells in other spiralian larvae could be stained without affecting the viability of these larvae. This modification thus makes comparative investigations on a wide spectrum of different species possible. Additionally, the modification of the original protocol facilitates the use of other long-chain dialkylcarbocyanine dyes, such as DiD, opening up the possibility of multi-label experiments. When certain precautions are made regarding fixation, permeabilization, and mounting, the staining with dialkylcarbocyanine dyes is stable and can be used with a wide range of additional fluorescent staining procedures, including immunohistochemistry, fluorescent labeling of F-actin and of nuclei, and labeling of EdU incorporating, mitotically active cells. Thus, the developmental fate of larval ciliated structures can be analyzed in the context of several other developmental processes.

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## Supplementary information

**Figure S1**. Young larvae from control experiments (A, only DMSO incubation; B and C, DiI without Pluronic F-127) and DiO stained larva (D), cLSM (magenta: DiI, cyan: DiO).

Supplementary information of this article can be found online at http://www.jbmethods.org/jbm/rt/suppFiles/320.