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TECHNICAL NOTE

Use of freeze-cracking in ontogenetic research in *Macrostomum lignano* (Macrostomida, Rhabditophora)

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Abstract A method for studying whole mount flatworm embryos based on freeze-cracking of the eggs is described. This method allows successful immunohistological and immunocytological studies of whole mount embryos. It does not require the use of sharpened needles or a microinjection system to puncture the eggshell. Moreover, this method is more practical and less time-consuming than classical puncturing and much cheaper than the use of a microinjection system. The advantages of this method are illustrated by results of several immunolocalisation experiments in the macrostomid flatworm *Macrostomum lignano*. The optimal procedure and crucial steps for this method are discussed.

Keywords Platyhelminthes · Freeze-cracking · Embryonic development · Eggshell · Immunohistochemistry

Introduction

Since molecular studies of flatworms have suggested that the “phylum” Platyhelminthes might not be monophyletic but could actually be a polyphyletic group consisting of basal Bilateria (Acoela, Nemertodermatida) and some taxa within Spiralia (Catenulida, Rhabditophora), research on several aspects of flatworm biology has known a great revival. Recently, one area of research that has received attention is the study of the ontogeny of flatworms and related evo-devo themes. For this type of studies, the most suitable animals are those which can be kept in culture under laboratory conditions. Preferably, these model animals should have features that provide independent elucidation of evo-devo’s conceptual themes (see arguments in Jenner and Wills 2007 for a deliberate choice of model organisms).

Ladurner et al. (2005a) introduced *Macrostomum lignano*, a free-living macrostomid flatworm, as a new model organism to answer evolutionary and developmental questions. *M. lignano* has been successfully used in several different research areas: analysis of stem cell dynamics, regeneration, behavioral ecology, ecotoxicology, gene expression based on an EST library, neurobiology, germ line development, production and characterisation of cell and tissue monoclonal antibodies, and embryonic development (see references in Ladurner et al. 2008).

A major drawback to the use of *M. lignano*, and rhabditophoran flatworms in general, in ontogenetic research is the fact that the eggs have an impermeable eggshell. Cytochemical research pointed out that eggshell granules in macrostomids include polyphenols (Gremigni and Falleni 1992), which are the precursors for sclerotin, a stable and resistant molecule probably responsible for the impermeability of the eggshell (Domenici and Gremigni 1977).

Until now, the impermeability of the flatworm eggshell hampers in situ hybridization and immunohistochemistry of whole mount embryos. Recently, Cardona et al. (2005)

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have tried an in situ hybridization protocol for the embryos of the triclad species *Schmidtea polychroa*. This protocol is extremely useful for monitoring gene expression in whole mounts and sections of the larger egg capsules of triclads, but is difficult to apply to smaller eggs with flexible eggshells, like those of *M. lignano*. For the same species, Martín-Durán et al. (2008) developed a method to deliver exogenous material into embryos using nanosecond laser pulses. This new approach, albeit very promising, necessitates access to elaborate equipment, which is not always at hand. Moreover, this method remains to be tested for various markers.

In this study, we present a freeze-cracking protocol based on a technique used for eggs of the nematode *Caenorhabditis elegans* (Albertson 1984) in order to study whole mount embryos of *M. lignano* and, possibly, of various other flatworm species. Cytological and immunohistochemical markers as well as monoclonal antibodies were used to test the effectiveness of this protocol.

This technique will provide new means of following the embryonic origin and differentiation of tissues and organs in *M. lignano*. We also discuss the new opportunities, which this technique provides, to address particular developmental questions in this species.

Materials and methods

Animals and eggs

M. lignano is a marine flatworm from the Mediterranean (Ladurner et al. 2005a). The animals were cultured as described by Rieger et al. (1988). Adult worms lay one egg a day and it takes approximately 120 h at 20°C for an embryo to complete embryonic development. For this study, adults were put together in embryo dishes at 20°C. These were checked regularly for freshly laid eggs. When a sufficient number of eggs were reached within a timeframe of 12 h, adults were removed from the dishes and the embryos developed until the stage of interest was reached. These stages are in accordance to the staging system described in Morris et al. (2004).

Freeze-cracking

Eggs of specific developmental stages were collected and washed (three times) in phosphate-buffered saline (PBS). This was followed by fixation for 1 h in 4% paraformaldehyde (PFA, pH 7.4; Sigma, St. Louis, MO, USA) at room temperature. In comparison to the original *C. elegans* protocol (Albertson 1984), where embryos and worms were fixed in subsequent steps of cooled (−20°C) methanol and acetone, we use PFA as a fixative because we obtained satisfactory tissue preservation with it. Then, the eggs were again washed three times in PBS before the freeze-cracking procedure (see the "Results and discussion" section for a detailed description) was performed. Subsequent steps were done using a Gene Frame® (11.40 cm^3 or 300 µL; Westburg, The Netherlands), i.e., a small plastic frame around the sample premounted on the slide to work clean and with appropriate reagent volume. The polyester cover of the Gene Frame® prevents reagent loss due to evaporation. The same effect may be achieved with a hydrophobic pen and a carefully laid cover slip. The subsequent procedure was staining-dependent and is described below.

Staining techniques

After freeze-cracking, the embryos were used in several different staining techniques that are commonly used in developmental studies in order to test for the applicability of the freeze-cracking procedure.

Histochemical stainings

Phalloidin Freeze-cracking was followed by short washing steps (3×5 min) in PBS and permeabilization with PBS +0.1% Triton X-100 (PBS-T). Embryos were incubated in 1:100 phalloidin (tetramethylrhodamine isothiocyanate- or fluorescein isothiocyanate (FITC)-conjugated; Dako) followed by washing (3×5 min PBS-T and then 3×5 min PBS).

Immunohistochemical stainings using M. lignano monoclonal antibodies (mAb) and antiacetylated and tyrosinated tubulin We tested two monoclonal antibodies from the *Macrostomum* library (described in Ladurner et al. 2005b). The freeze-cracking procedure was followed by a 3×5-min washing series with PBS followed by PBS-T. To prevent nonspecific staining, embryos were blocked for 30 min in bovine serum albumin+0,1% Triton X-100 (BSA-T) and incubated in primary antibody overnight (4°C). MDa-1 and Mmu-2 stain, respectively, gut cells and muscles fibers in the adult *M. lignano*. After washing series (3×5 PBS-T, three times PBS), the embryos were incubated in secondary antibody (FITC-labeled goat antimouse; Dako).

Primary antibody concentrations were 1/50 for MDa-1 and 1/10 for Mmu-2. Secondary antibody concentrations were 1/150. Additionally, for Mab MDa-1 stainings, nuclei were counterstained with propidium iodide (PI; 1/2,500; Sigma-Aldrich, St. Louis, MO, USA).

Embryos were finally mounted in PBS instead of, for instance, a viscous mounting medium as this resulted in a better preservation of the overall shape of the embryo.

Antiacetylated tubulin (actub) and antityrosinated tubulin antibodies (tyrtub; both from Sigma-Aldrich, St. Louis,

MO, USA) were used to label microtubular skeleton. Primary antibody concentrations were 1/100. Secondary antibody concentrations were 1/150.

Controls Controls were done by performing simultaneous labeling of hatchlings to test the probe or antibody, omitting the freeze-cracking procedure to test the method, and finally, checking for unspecific staining by omitting the primary antibody.

Microscopy

Slides were examined using a Zeiss Axiovert 135 or Reichert-Jung Polyvar microscope equipped with epifluorescence or with a Nikon C1 confocal microscope. Pictures were taken using an Olympus Camedia C 5050 camera or a Zeiss Axiocam MrC.

Digital images were assembled in montages using Adobe Photoshop CS3 (Adobe). Drawings of freeze-cracking schemes were done using Adobe Illustrator CS (Adobe).

Results and discussion

Procedure

In the freeze-crack procedure for embryos, eggshells of embryos affixed to subbed slides are physically broken through the force of quickly popping off a frozen cover slip from the slide (Albertson 1984; Miller and Shakes 1995). The samples on the slide can then be subjected to subsequent staining steps. Fixed eggs are pipetted onto a microscopic slide with a droplet of PBS and covered with a cover slip, which is positioned transversally with respect to the slide (Fig. 1). The slide is then gently lowered on a concrete brick (or any solid material) which was previously submerged into a bath of liquid nitrogen. Depositing the slide directly on a brick of dry ice or immerging it directly into liquid nitrogen using forceps yielded similar results. After freezing, which should take only a few seconds, the tip of a scalpel blade is wedged under one corner of the cover slip and it is "popped" off with a quick arm movement. This step must be done while the slide is still frozen. Once the cover slip is removed, the slide is immediately rehydrated with PBS.

Excessive squash and explosion of the eggs due to the procedure results in destruction of some of the embryos, but this does not exceed 10% of the total initial amount of eggs. Moreover, this assures that the cover slip effectively contacts the remaining eggshells, as this is crucial for successful permeabilization.

Crucial steps of freeze-cracking protocol

It is crucial that as many eggshells as possible are in contact with the cover slip as this contact will provide the permeabilization site. Therefore, care should be taken with the embryos when placing the cover slip transversally on the slide before the cracking procedure. We use pure white Vaseline spacers between the slide and the cover slip (Fig. 1a), which enables us to adapt the distance between the eggshells and the cover slip if necessary. As additional adjustment, one can decrease the liquid volume either in the initial drop or by wicking liquid from the edges of the cover slip.

When "popping" the cover slip off the slide after freezing, make sure that the movement is quick, fluent, and away from the slide. This prevents the cover slip from falling back on the slide or breaking off.

As a protocol can include many washing steps, the use of poly-L-lysine-coated slides, which are inexpensive and easy to prepare, is crucial to keep the embryos attached to the glass slides. This will also be required for more elaborate protocols; for example, the adaptation of the in situ hybridization protocols described by Morris et al. (2006) and Pfister et al. (2007) for *M. lignano* embryos.

The trick during all of the solution changes is to minimize both solution carryover and the wetting surface without ever letting the sample dry out completely. The use of a 'Gene Frame' was necessary so that the small drops of

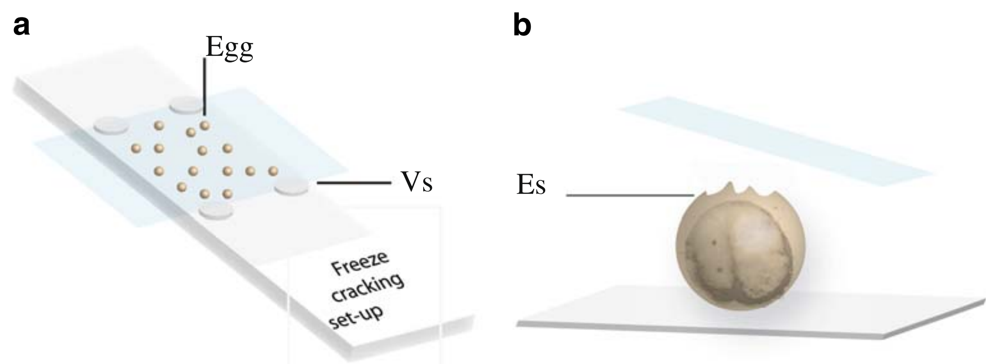

Fig. 1 Schematic drawing of freeze-cracking set-up. **a** Set-up before freeze-cracking procedure. Eggs are placed between slide and cover slip (*light blue*). The Vaseline "feet" (*Vs*) allow adapting the space between cover slip and slide so the cover slip touches the top of the eggshell (*Es*). **b** Detail of one embryo after the freeze-cracking procedure when the cover slip has been popped off

solution saturate the specimen directly and are not diluted by spreading over a large area of the slide.

Advantages of the freeze-cracking procedure over other techniques

In the past, dissection of the eggs with electronically sharpened tungsten needles was commonly used to free the embryos in order to study the embryonic development of rhabditophoran flatworms (see Younossi-Hartenstein and Hartenstein 2000; Morris et al. 2004). This technique was sufficient when specimens were performed for histochemical stainings, semithin serial sections, tubulin stainings, and electron microscopy. Unfortunately, puncturing comes with some difficulties.

First, the number of individual embryos one can handle is limited, so it is impractical for immunohistochemistry and in situ hybridization where a large number of samples have to be processed together. Secondly, puncturing necessitates a certain form of dexterity, which is only acquired after practice. Moreover, the handling time needed for puncturing can be an impediment for specific developmental studies. Finally, puncturing of the eggshell of developmental stages before 60% of the developmental time can possibly damage the embryo and it is more difficult to puncture early stages as they tend to fall apart more easily than later stages. This can be due to an undeveloped epidermis as junctional complexes between epidermis cells were only observed around 50–60% of the developmental time (Rieger et al. 1991a, b; Morris et al.

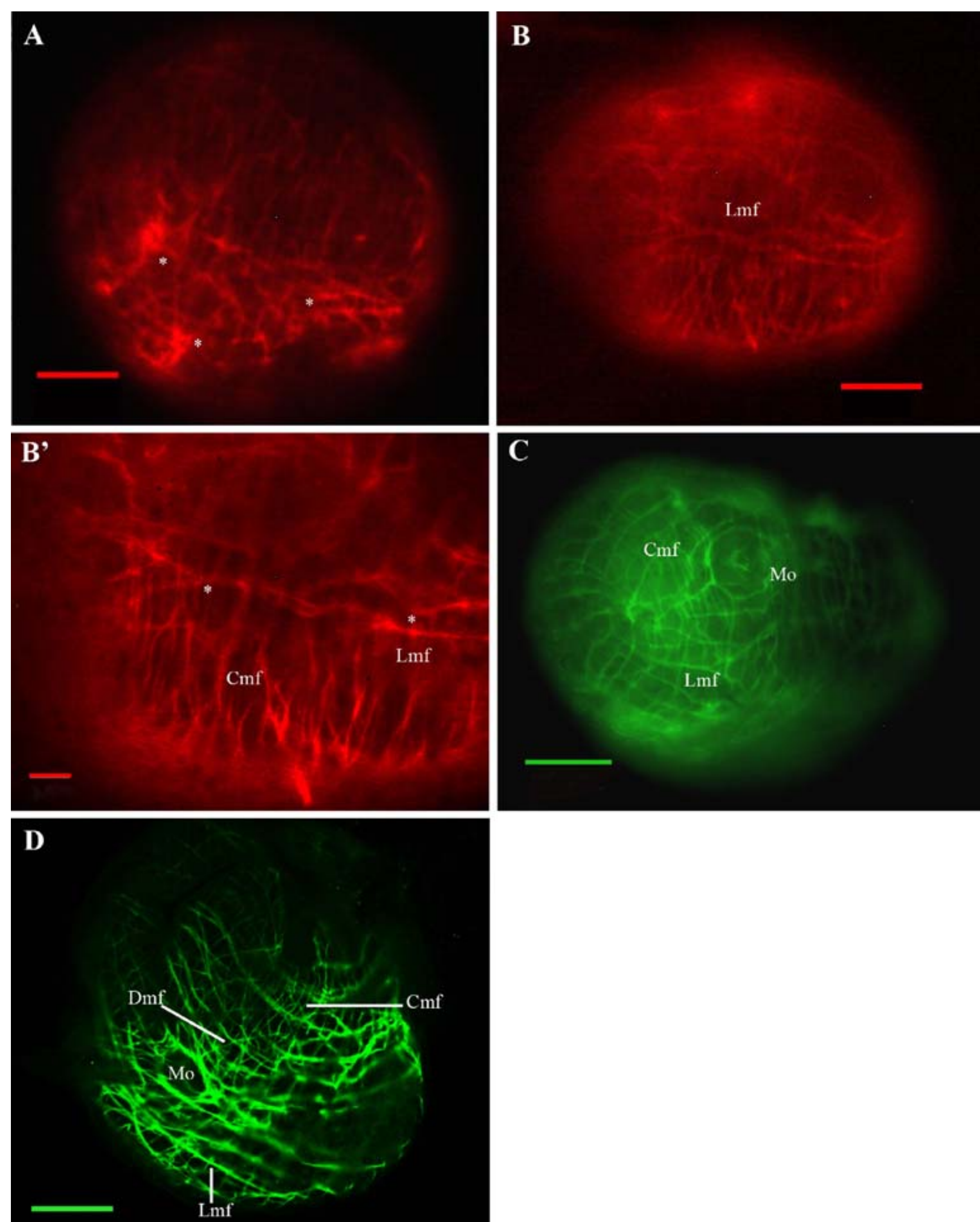

Fig. 2 Phalloidin (**a**–**c**) and monoclonal Mmu-2 (**d**) muscle fiber staining at different stages during development. **a** Fifty percent of developmental time. A network of undefined muscle fibers originates at one side of the embryo. A few connections between fibers are also visible (*asterisks*). **b** Sixty-five percent of developmental time. Longitudinal (*Lmf*) and circular muscle fibers (*Cmf*) can be identified. Circular muscle fibers appear to be more abundant. **b′** Details of **b**. Clear connections between circular and longitudinal muscle fibers are observed. **c** Eighty percent of developmental time (FITC-labeled). The embryo has already elongated and is shaped like a hatchling. The orthogonal pattern as observed in hatchlings can be observed. The orthogonal grid is interrupted at the level of the mouth (*Mo*). **d** Mmu-2 mAb staining at 80% of development time when the embryo is not yet elongated. Diagonal muscle fibers (*Dmf*) can also be observed. **a**–**c** Epifluorescence images, **d** confocal image. Scale bar **a**–**d** 20 µm, **b′** 5 µm

2004). Moreover, the epidermis only surrounds the embryo from 60% of the developmental time onwards, making the embryo more vulnerable for handling. Because in the freeze-cracking procedure the damage is restricted to the eggshell, the morphology of the embryo can be preserved.

The freeze-cracking protocol described in this study is quick, easy to perform, and allows to batch process a large number of embryos in the contained area of the slide, producing more consistent results.

Other techniques often used in developmental studies show several disadvantages. For example, a microinjection system can be used to puncture the eggshell or even inject solutions directly into the embryonic tissues. However, this technique is very time-consuming and expensive. Moreover, it is a rather difficult technique in practice, requiring some experience before it can be applied properly. Puncturing the eggshell with needles is suited for histology.

Dissolving the eggshell by enzymatic treatment only makes the protocol more elaborate, does not yield constant results, and results in the deterioration of the morphology of the specimen.

In contrast, the freeze-cracking procedure as presented in this study is very cheap and only takes a small amount of time to be carried out. Indeed, with the freeze-cracking procedure, it is possible to batch process different samples simultaneously and relatively quickly, therefore, yielding more consistent results.

Use for developmental studies

We tested this method using cytological, histochemical, and immunohistochemical markers. We also discuss how this method bears the potential to enlighten us on specific developmental questions regarding *M. lignano*.

In flatworms, the muscle system has recently become an important character for evo-devo studies. The organization of the muscle system during embryonic development was studied using fluorescent-conjugated phalloidin in several flatworm species and one acoel species (Reiter et al. 1996; Ladurner and Rieger 2000). In this study, the development and organization of muscles was studied using phalloidin at different percentages of total development time (according to Morris et al. 2004), each time with an interval of 15% starting from 50% of the total developmental time (Fig. 2). Similar results were yielded using the monoclonal antibody Mmu-2, which confirmed the results of our phalloidin labeling (Fig. 2). After 50% of development time, irregularly distributed muscles fibers were visible as a diffuse network (Fig. 2c). At that specific developmental stage, the differences between longitudinal, circular, and diagonal muscle could not be defined. We conclude that muscle fiber differentiation starts at 50–55% of total developmental time as observed in the acoel *Isodiametra pulchra* and in the macrostomid *Macrostomum hystericinum marinum* (Reiter et al. 1996; Ladurner and Rieger 2000). At 65% of the developmental time, muscle fibers were distributed in an orthogonal pattern. Circular muscle fibers were more abundant than longitudinal fibers. The first diagonal muscles were also clearly observed. Our observations corroborates the results of previous studies done in *M. hystericinum marinum* and the triclad *S. polychroa* stating that circular muscle fibers appear earlier and are more numerous than longitudinal muscles at the onset of differentiation (Reiter et al. 1996; Ladurner and Rieger 2000; Cardona et al. 2005). At 80% of developmental time, the muscle organization as described in hatchlings is found. With the current mAbs and the freeze-cracking procedure, it will be possible to combine Ab stainings (double labeling) or

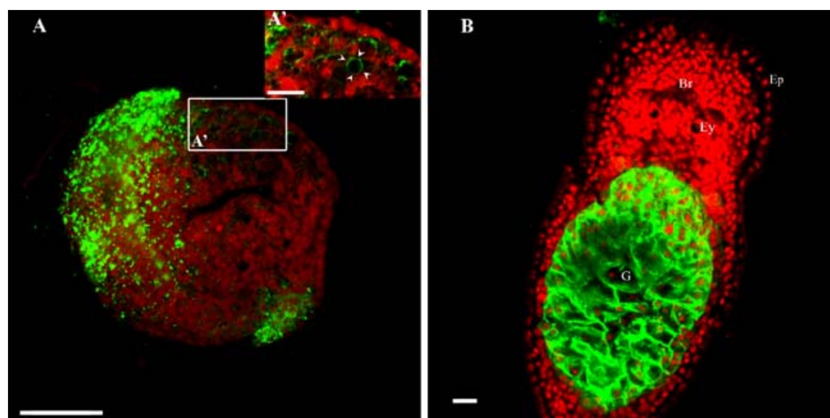

Fig. 3 Monoclonal MDa-1 (Mab MDa-1) gastrodermal cells staining. Embryos were counterstained with PI to visualize nuclei. **a** Embryo at 50% of developmental time. Merged image of Mab MDa-1 (*green*) and PI (*red*). Inset **a′** shows detail of **a**. A number of cells show clear cytoplasmic staining pattern (*arrowhead*) possibly representing gastrodermal cell precursors. **b** One-hour-old hatchling. Merged image of Mab MDa-1 (*green*) and PI (*red*). Clear gut (*G*) pattern. Anterior is to the top. *Ey* eye, *Ep* epidermal nuclei, *Br* brain Scale bar **a**, **b** 20 μm, **a′** 10 μm

perform in situ hybridizations. For example, mesoderm specification in embryos of *M. lignano* could be investigated.

We performed MDa-1 mAB stainings in order to label gastrodermal cells and the gut precursor cells. We did not observe any MDa-1 labeling prior to 40% of total developmental time (<48 h), although the eggshells were clearly open and the secondary antibody did penetrate the egg (Fig. 3). This is consistent with the study of the embryonic development of *M. lignano* (Morris et al. 2004) where the authors found that the formation of the gut primordium initiates at stages 4–5 (48–76 h, i.e., 40–63% time after egg laying).

Stage 4 and stage 5 embryos (±50–60% of developmental time; Morris et al. 2004) were also stained with acetylated tubulin (actub) and tyrosinated tubulin (tyrtub) to label microtubular skeleton (Fig. 4; Electronic supplementary material S1 and S2). These stages are characterized by the appearance of organ primordia (e.g., brain, pharynx) and the onset of cellular differentiation (e.g., epidermal cells; Morris et al. 2004). Cells located at the external

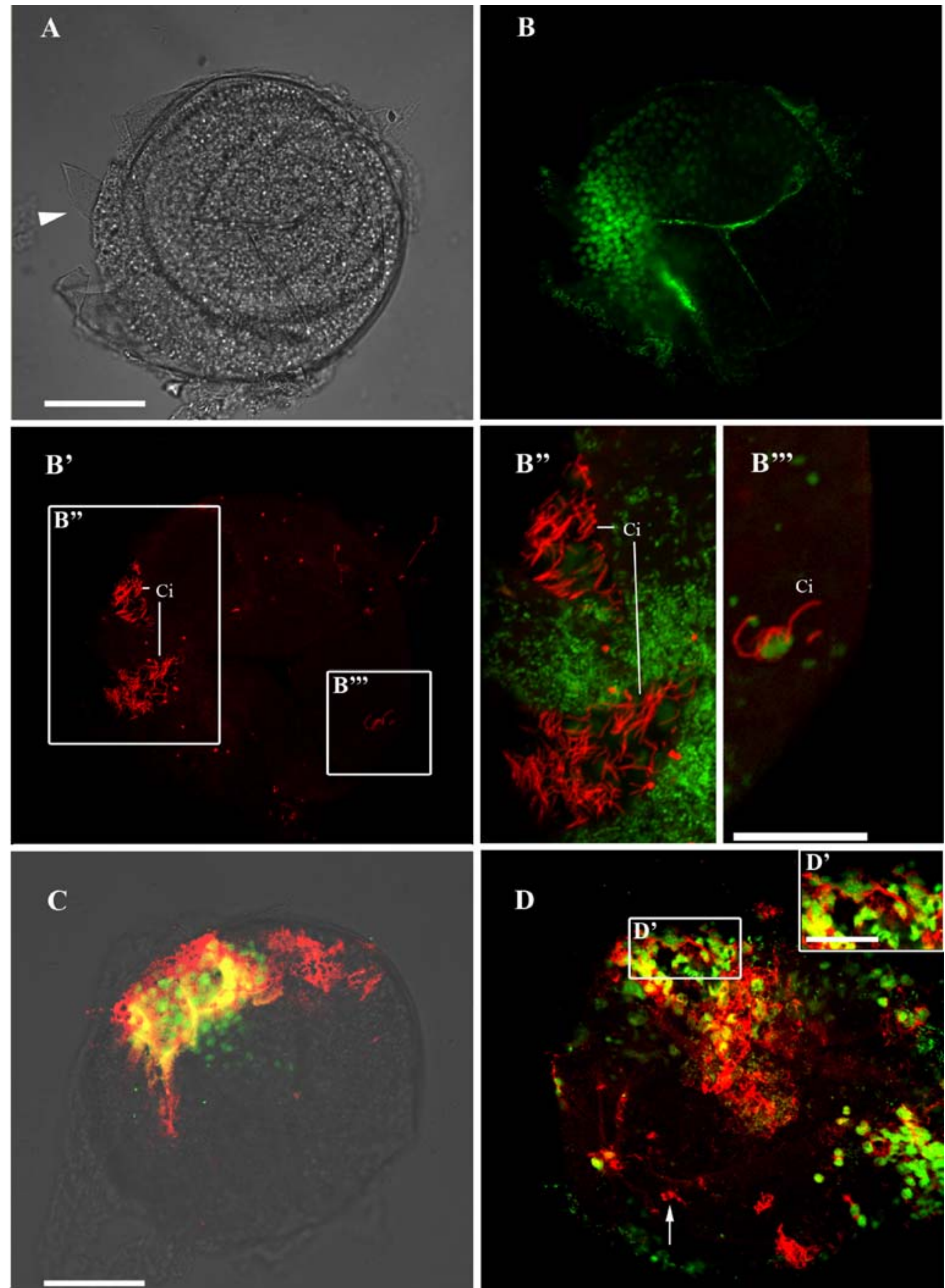

Fig. 4 Wholemount of stage 4–5 embryos (±50% of development time) labeled with acetylated tubulin (actub, *red*; **a**–**b**‴) and tyrosinated tubulin (tyrtub, *red*; **c**–**d**′) antibody staining. Anterior is to the *top*. Interference contrast image (**a**), sytox (**b**), and actub (**b**′ and digitally magnified details **b**″, **b**‴) labeling of the same embryo. *Arrowhead* indicates the large opening in the eggshell due to the freeze-cracking procedure. Note the singular ciliated cell at the posterior of the embryo in **b**‴ (compare with **d**). **c** and **d** are two different embryos, **d**′ is a magnified detail of **d**. Only the anterior region is strongly labeled even when the eggshell is permeabilized at different sites ruling out the possibility that the embryo is only successfully labeled at the permeabilization site. Note also the ciliated singular cell at the posterior of the embryo (*arrow*; cf. **b**‴). *Ci* cilia. Scale bar **a**, **b**, **b**′, **c**, **d** 20 µm, **b**″, **b**‴, **d**′ 10 µm

surface of the embryonic primordium transform into a ciliated epithelial epidermal layer that displaces the external yolk mantle. The onset of ciliation of the multiciliated cells has been described in detail by Tyler (1981) and Morris et al. (2004). During stage 5, the epidermis covers only the flanks of the embryo. This is confirmed by our actub labeling of a stage 5 embryo (Fig. 4a, b) where cilia of epidermal cells were labeled, although in only one flank of the embryo. This could be explained by the fact that those were early stage 5 embryos. We also stained singular actub cells. These could be single epidermal cells or may be developing cyrtocytes of the flame bulbs of the protonephridia (Fig. 4b, b‴). As for the tyrtub labeling, a clear anterior staining pattern can be distinguished at stages 5 and 6 (Fig. 4c, d). Based on Morris et al. (2004), we think that this anterior region represents ciliated epidermal cells and the developing pharynx and brain. We could also distinguish singular ciliated cells in more posterior parts of the embryo comparable to the actub stainings (Fig. 4d). In our opinion, these are the precursors of the protonephridia.

Conclusion

In this study, we describe a freeze-cracking procedure to permeabilize embryos of *M. lignano* so that they can still be easily studied using several labeling techniques. Therefore, freeze-cracking facilitates the study of the embryonic development of this model species and will be particularly helpful for subsequent immunohistochemical, molecular, and in situ hybridization studies.

This will further strengthen the position of *M. lignano* as a suitable model organism for comparative evolutionary and developmental studies both within Platyhelminthes and to other established model taxa like *C. elegans*, *Drosophila melanogaster*, *Platynereis dumerilii*, and *Danio rerio*. In addition, the freeze-cracking technique for permeabilization of *M. lignano* embryos may be easily adapted to other flatworm species.

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