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TECHNICAL NOTE

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An in situ hybridization protocol for planarian embryos: monitoring myosin heavy chain gene expression

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Abstract The monitoring of gene expression is fundamental for understanding developmental biology. Here we report a successful experimental protocol for in situ hybridization in both whole-mount and sectioned planarian embryos. Conventional in situ hybridization techniques in developmental biology are used on whole-mount preparations. However, given that the inherent lack of external morphological markers in planarian embryos hinders the proper interpretation of gene expression data in whole-mount preparations, here we used sectioned material. We discuss the advantages of sectioned versus whole-mount preparations, namely, better probe penetration, improved tissue preservation, and the possibility to interpret gene expression in relation to internal morphological markers such as the epidermis, the embryonic and definitive pharynges, and the gastrodermis. Optimal fixatives and embedding methods for sectioning are also discussed.

Keywords Planaria · Myosin · Paraffin section · In situ hybridization · Flatworm

Introduction

Planarians are bilateral and relatively simple organisms that belong to the lophotrochozoans (Platyhelminthes, Tricladida), the regenerative capacity of which, in adults, is manifested not only after a traumatic event but also as an integral part of asexual reproduction by fission. Furthermore, planarians grow and shrink depending on temperature conditions and food availability (Romero and Baguñà 1991). This extraordinary plasticity at the cellular level has been linked to the presence of a unique population of totipotent stem cells, the neoblasts, which are responsible for the generation of all body and germ line cells (Baguñà et al. 1989). Because of these properties, the organism has been selected for a genome project (Sánchez Alvarado et al. 2003).

The embryonic development of triclads, however, has been largely neglected and has not yet entered the molecular biology era. Histology-based descriptions of the embryonic development of triclads have provided detailed but incomplete morphological information (Benazzi and Gremigni 1982, and references therein; Cardona et al. 2005). Triclads lay ectolecithal eggs, i.e., yolk-poor egg cells are laid among several thousand helper yolk cells, in an egg capsule. Most remarkably, these animals present an idiosyncratic cleavage aptly named "blastomere anarchy" where blastomeres do not remain attached to each other. In addition, tissues and organs are developed directly in situ without the formation of germ layers. The underlying mechanisms and details of the embryonic development of triclads are unknown.

In an attempt to improve our understanding of triclads, our laboratory has provided detailed descriptions on the formation of tissues and organs in the planarian embryo (Cardona et al. 2005) and redefined previous staging systems to conform with descriptions of the embryonic development of other turbellarians (Younossi-Hartenstein et al. 2000; Hartenstein and Ehlers 2000; Morris et al. 2004). This redefinition resulted in a useful morphological reference for the identification of embryonic developmental stages and associated tissue formation and differentiation.

The time is ripe now for the study of gene expression by means of the in situ hybridization protocol. Historically, this protocol was first performed on sections due to limitations in the signal developing methods, namely, the use of autoradiography of the radiolabeled probes. Whole-mount in situ hybridization using digoxygenin-labeled DNA (and later RNA) probes and enzyme-linked antibodies (Tautz and Pfeifle 1989) revolutionized the field of developmental biology. Whole-mount preparations allow the detection of 3D patterns of gene expression in complete

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structures and organs, and furthermore, the processing of hundreds of samples together. Finer detail can be observed by sectioning hybridized whole-mount preparations. But in some cases, the whole-mount approach is not appropriate. Gene expression in deep layers may not be correctly labeled because of difficulties in probe penetration. Also, tissues may not be properly preserved, and thus, cellular resolution may be lost.

Planarian embryos present these and further difficulties. Consequently, we developed another approach. Here we describe a technique using in situ hybridization on paraffin-embedded sectioned embryos, which allows direct access of the probe to all deep tissues. We adapted available in situ hybridization protocols for adult planarian whole-mount preparations (Agata et al. 1998) and for *Xenopus* embryos sections (Butler et al. 2001). Sections allowed for the positioning and interpretation of gene expression signal relative to internal morphological markers, such as the germ band, the gastrodermis, and the embryonic pharynx (EP), and enhanced probe penetration and tissue preservation.

To test this protocol, we chose the myosin heavy chain (MHC) gene because probes for this gene are readily available. Detailed descriptions of its expression pattern in both intact and regenerating adult planarians are available for comparison (Cebrià et al. 1997, 1999). MHC protein levels are monitored by the monoclonal antibody TMUS-13 (Cebrià et al. 1997).

Materials and methods

Animals and egg capsules

A *Schmidtea polychroa* population (diploid, 2*n*=8) from Sot de Ferrer (Castelló, Spain) was cultured at 18±1°C in spring water and fed raw liver (sheep) once a week. Egg capsules were collected every other day and stored in Eppendorf tubes containing spring water until reaching the desired stage of development.

Fixation and dissection of embryos

Planarian egg capsules were laid on a glass slide, and the excess water was removed; they were then perforated using a 0.5-mm needle and immediately fixed in 4% paraformaldehyde in PBS (80 g NaCl, 2 g KCl, 2 g KH_2PO_4, 11.5 g Na_2HPO_4) at 4°C for 4 h, and then washed overnight in PBS at 4°C. Individual embryos were dissected over an agar plate under the scope with the help of tweezers and placed in a glass well. At this point embryos can either be stored, after proper dehydration, in 100% ethanol at −20°C, embedded in paraffin, or used directly for in situ hybridization.

Paraffin embedding and sectioning

Batches of embryos were dehydrated by an alcohol–xylene series (5 min in 70, 96, and 100% ethanol, 5 min in 25, 50, and 75% xylene in ethanol, and two rinses for 5 min in 100% xylene). Xylene was replaced by melted paraffin for 1 h at 60°C. Blocks were formed in custom-made paper molds (Fig. 1) to ensure a high density of embryos in a small volume and to completely avoid trimming, which would be problematic due to embryo transparency. Commercially available molds do not fulfill the requirements; first, not enough depth is provided to partially prefill the mold with melted paraffin to prevent embryos from hanging from the edge of the block, which would result in the loss of good sections, and second, the area of the base is too large for an optimal embryo density. The glass well containing the embryos in melted paraffin was kept over a hot plate at 60°C to prevent early paraffin solidification and allow efficient pipetting of embryos into the molds. Best embedding was obtained when the molds were cooled for 1 h at room temperature (RT), and then at 4°C overnight. Ten-micrometer sections were obtained and placed over water in poly-L-lysine-coated glass slides (0.2 μg/ml, Sigma P-1399; 50 μl/slide, extended with a cover glass and

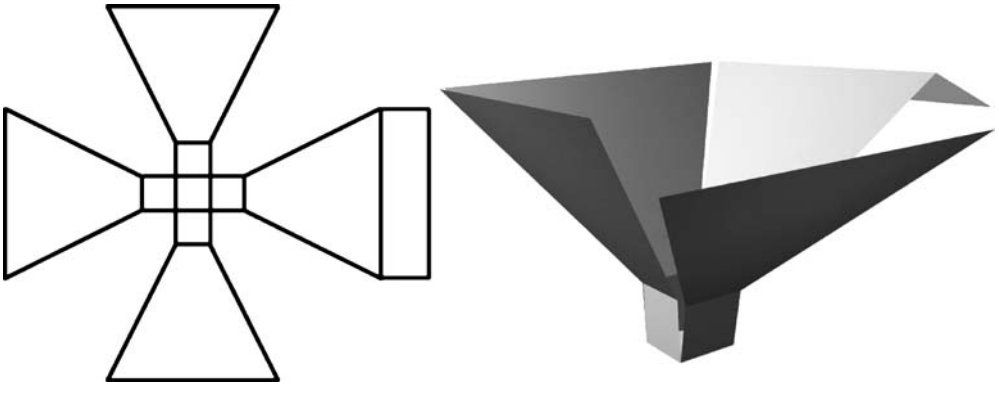

Fig. 1 Paper molds. Custom-made paper molds were designed to concentrate the embryos in the smallest space possible. Any scratch paper can be used. The base measures approximately 0.6×0.6 mm, and the sides and handle can be as long as desired. After folding the model along the creases, the base was wrapped in tape. For block formation, the molds were conveniently kept straight on styrofoam tube racks. When the blocks hardened, the paper and tape were carefully removed with a cutter and stored at 4°C

air dried) and kept over a hot plate (37°C) for 3–12 h. Blocks and sections remained in good conditions for in situ hybridization at 4°C for at least 4 weeks.

In situ hybridization on paraffin sections

Slides were dewaxed and hydrated through a xylene–ethanol series (2×5 min 100% xylene, 5 min 100, 96, and 70% ethanol) and finally washed for 5 min in PBS. From this step onward, sections were layered in a flat incubation chamber, and small volumes were applied individually to each slide. When incubated at temperatures above RT, bottle caps filled with distilled water were placed in the chamber to avoid evaporation, and the chamber was sealed with tape. Sections were fixed (20 min 4% paraformaldehyde in PBS at RT) and washed (2×5 min PBS), permeabilized (30 min proteinase K 20 μm/ml in PBS at 37°C) and washed (2×5 min PBS), and treated with hydrochloride acid to neutralize the sample (15 min 0.2 M HCl in PBS at RT), followed by washing (2×5 min PBS). Sections were prehybridated with prehybridization buffer (formamide 50%, 5× SSC, yeast tRNA 0.1 mg/ml, Tween 20 0.1% and DTT 10 mM) for 1 h at 50°C, and then hybridization was done at 20 ng/ml of probe in hybridization buffer (prehybridization buffer plus 10% dextran sulfate), 100 μl/slide for 40 h at 50°C, under a coverslip to prevent evaporation. Slides were placed at 4°C for 15 min to cool down, thereby allowing safe coverslip removal in a vertical washing container filled with probe washing buffer (formamide 50%, 5× SSC, 0.01% Tween 20). Next sections were rinsed 2×15 min at RT in probe washing buffer and then washed 4×10 min at RT in maleic buffer (99.4 mM, NaCl 167 mM, Triton X100 0.1%, adjusted to pH 7.5 and filtered immediately before use). Sections were preincubated with blocking solution (maleic buffer plus blocking reagent 10%; Roche 1096176) for 30 min at RT and then incubated with phosphatase-linked antidigoxygenin antibody (Roche 1093274) at 1/2000 in blocking solution. Embryo sections were then rinsed 4×20 min in maleic buffer at RT, and color was developed (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$, 4.5 μl/ml BCIP, 3.5 μl/ml NBT) for 3–6 h until signal was visible; the reaction was arrested by 2×5 min rinses in PBS. Finally, sections were dehydrated through an ethanol–xylene series and mounted in DePeX for long-term storage.

Whole-mount in situ hybridization

Dissected embryos were postfixed for 20 min in 4% paraformaldehyde in PBS at RT, and the protocol from this step onward was as in sections. Hybridization and all washes were done under shaking conditions, and 4×30 min washes in probe washing solution at 50°C were required after hybridization. Labeling was developed as in sections. Stained embryos were preserved in 50% glycerol in PBS at −20°C.

Myosin heavy chain probe

The probe was synthesized from a clone containing a 1,197-bp insert (399 aa) named M71, which corresponded to the end of the head and the start of the S2 region of the *Schmidtea mediterranea* MHC gene (Cebrià 2000). The riboprobe was labeled with UTP–digoxygenin using the in vitro DIG RNA labeling kit (Roche 1175025).

Whole-mount antibody staining

Whole-mount antibody stainings with the monoclonal antibody TMUS-13 (Cebrià et al. 1997) were prepared following the protocol described in Cardona et al. (2005). A cy3-labeled rabbit antimouse IgG (Sigma) was used.

Results and discussion

Sections versus whole mount

The development of whole-mount in situ hybridization by digoxygenin-labeled riboprobes and enzyme-linked antidigoxygenin antibodies (Tautz and Pfeifle 1989) is a milestone of paramount importance in developmental biology. This approach enabled the study of 3D patterns of gene expression, even of several genes simultaneously (Hauptmann and Gerster 2000). Furthermore, the possibility to batch-process hundreds of samples together not only simplifies the protocols but also produces more consistent results. However, for planarian embryos, the whole-mount approach has several drawbacks.

The whole-mount approach is most useful when the axes of the sample are easily recognizable by external, visible, morphological landmarks such as the lateral stripes of the developing *Drosophila* embryo. The planarian embryo lacks externally recognizable morphological markers, and its axes are morphologically inconspicuous for more than half of its embryonic development because of its spherical shape and smooth surface. The differentiation of a pair of bilateral eyes and the definitive pharynx does not occur until the last 20% of development, by stages 7 and 8. Thus, even when the expression pattern is highly localized, as in the case of the MHC gene (Fig. 2a–c), the interpretation is meaningless without any morphological references.

The particularities of ectolecithal embryonic development, which imply that planarian embryos are wrapped in thousands of yolk cells, added to the great thickness and opaqueness of the egg capsule shell, require the embryo dissection prior to manipulation. The practical impossibility to scratch away all attached opaque yolk cells is yet another obstacle to the observation of signal in whole-mount preparations, particularly in late embryos that secrete mucus (Fig. 2b). Fluorescent labeling of the probe combined with confocal microscopy may help to address this problem.

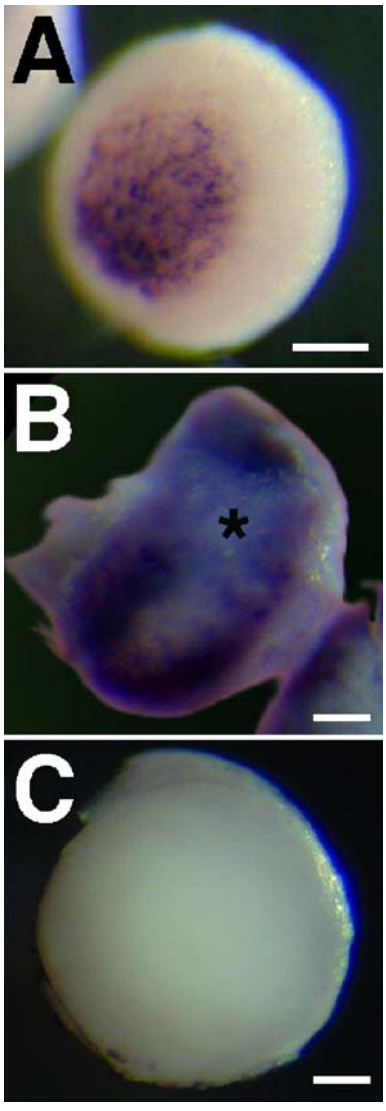

Fig. 2 MHC gene whole-mount in situ hybridization. **A** Late stage 4 embryo. Localized signal on one side of the embryo. Several cells with thin extensions can be seen. In the absence of morphological landmarks, it is impossible to determine the axial positioning of the signal. **B** Stage 7 embryo, ventral view. The body wall muscle stands out particularly on the sides, where it is more concentrated and the signal in transversal muscle fibers adds up. The presence of attached, unstained yolk cells (*asterisk*) masks the signal in large parts of the embryo. Complete removal of yolk cells is impractical and results in embryo breakage. **C** Stage 5 embryo, hybridized with the *sense* probe. The stages are determined visually by size and shape; embryos are spherical in the first half of development and range from 300 μm by stage 2 to 1 mm by late stage 4. By stage 5 the embryo has flattened to a fat disk, which later further flattens and elongates through stages 6 to 8. Scale bar 200 μm.

To overcome problems associated with the visualization of signal deep in the sample, hybridized and color-developed whole-mount preparations can be sectioned. However, genes expressed deep in samples may be difficult to reach because of insufficient permeabilization, as is the case of the yolk-rich planarian embryo, and may thus be poorly or not correctly labeled. Furthermore, existing cavities within the animals, such as the intestinal vaults or the pharyngeal pouch, in the late embryo and the adult planarian accumulate nonhybridized probe, thereby generating background noise, because it cannot be properly rinsed away.

Tissue preservation is also an issue in the whole-mount in situ hybridization protocol because of incubation at high hybridization temperatures in order to reduce unspecific signal (increased stringency) and the use of buffers containing strong solvents and detergents (for washing away nonhybridized probe).

Thus, sectioning whole-mount preparations, the most common approach to investigate gene expression in deep layers, provides no useful results for planarian embryo samples because regardless of labeling and permeabilization issues, cellular resolution is not preserved in the first place (not shown). Sections provide better postfixation and require fewer washes at high temperature and no shaking; therefore, tissue preservation is enhanced.

An apparent strong signal in whole-mount preparations caused by the visual addition of several layers of stained tissue does not necessarily mean a strong signal in the cells composing these layers when visualized individually in sections. An increase in developing time does not fully address this problem and always results in greater background noise.

Therefore, sections allowed the localization of single cells expressing the MHC gene relative to morphologically distinctive tissues such as the gastrodermis, the epidermis, and the EP (Fig. 3). The seriated nature of the sections obtained allowed us to track structures and axes through consecutive sections.

Crucial steps in the in situ protocol

The inherent lack of pigmentation in the planarian embryos poses severe difficulties to the manipulation and orientation of samples. Embedded embryos are barely visible within the paraffin block. This difficulty was overcome by embedding a large number of embryos (30–40) together in the smallest possible space, for which custom-made paper molds were used (Fig. 1). This approach removed the need for block trimming, which would have resulted in a loss of samples, and conveniently concentrated distinct stages of development in the same section.

The standard adult planarian whole-mount in situ hybridization protocol (Agata et al. 1998) uses Carnoy's as the fixative of choice. For planarian embryos, Carnoy's fixative does not prove optimal. First, external yolk cells are fixed together in large packed clumps that impede a proper dissection. Second, yolk-rich tissues such as the germ band and gastrodermal cells are not properly fixed and appear disrupted in sections. Here we used paraformaldehyde as a fixative, which improves results in both respects and has been used previously for a similar protocol in *Drosophila* embryos (Tautz 2000).

Poly-L-lysine-coated slides, which are inexpensive and easy to prepare, were crucial to keep the tissue sections attached to the glass slides during the many washes in-

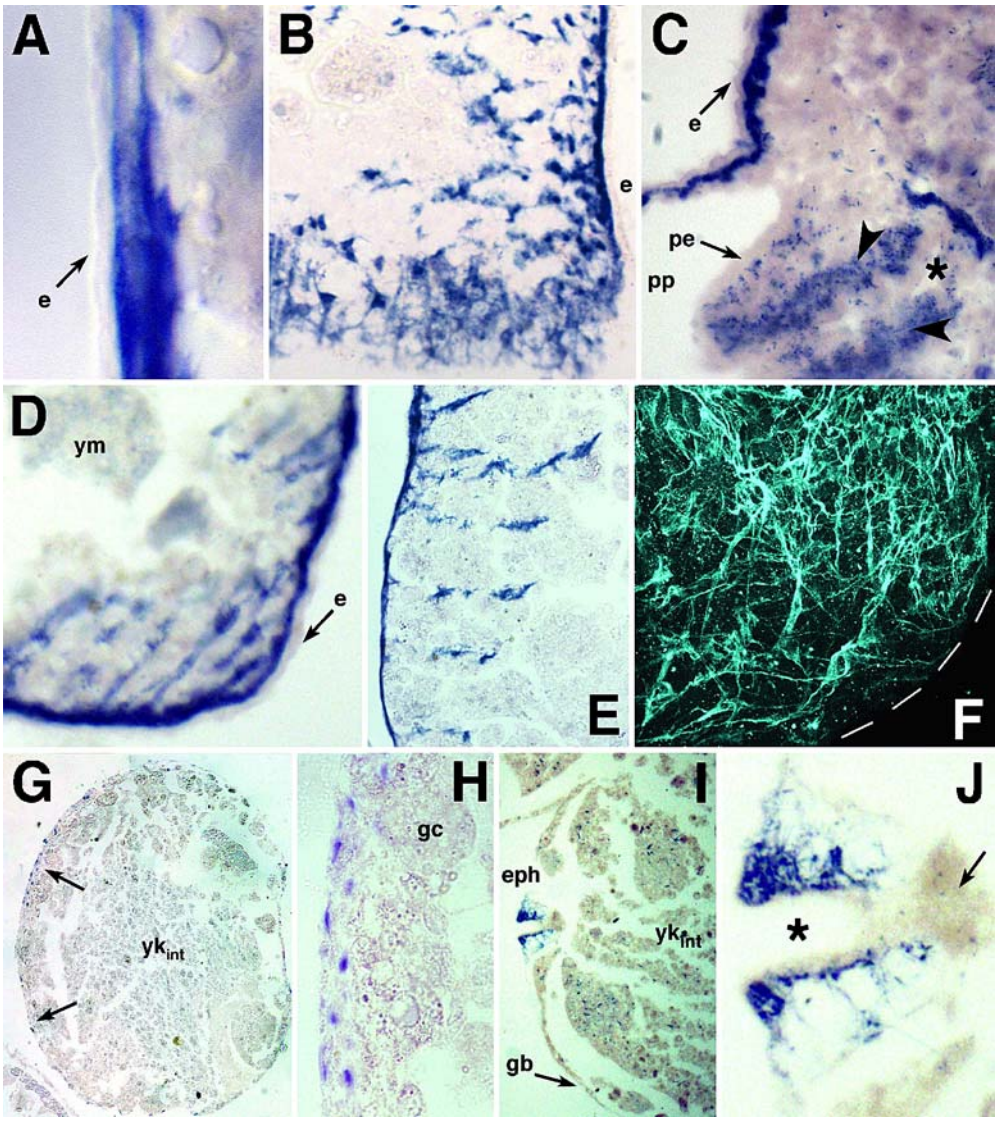

Fig. 3 MHC gene expression in sections. All panels show MHC gene in situ hybridization on sections, except **F**, which shows a whole-mount TMUS-13 monoclonal antibody staining (cy3 rabbit secondary antibody). **A** Body wall myocytes underneath the unstained epidermis (*white band*, *e*) of a stage 7 embryo. The staining is present along the elongated processes of the myocyte. **B** Stage 8 embryo. Lateral, sagital section. *Right*, strong staining of the body wall myocytes (*vertical blue band*) underneath the unstained epidermis (*e*). Longitudinal body wall fibers were sectioned along their long axis (*bottom third of the panel*). Sections of transversal muscle fibers are visible in deeper layers (*center*). **C** Body wall muscle by the pharynx pouch of a stage 8 embryo. Pharyngeal muscle is shown strongest (*arrowheads*) by the inner epithelium. The pharyngeal epidermis is unstained. *Asterisk*, pharyngeal lumen. **D** Strong staining of the body wall muscle of a stage 7 embryo, underneath the unstained epidermis (*e*). Transversal muscle fibers extend from dorsal (*top right*) to ventral (*bottom left*). *ym*, internal mass of lysated yolk cells within the gut lumen, contracted because of paraformaldehyde fixation. **E** Flat longitudinal section of a stage 7 embryo, showing lateral parenchymatic branches rich in myocytes extending inward from the body wall (*vertical blue band*, *left*). **F** Stage 7. Z-projection of a confocal stack, showing superficial, fine circular (*horizontal*), and deep, thicker (*vertical*) muscle fibers stained with TMUS-13 mAb. *Broken line*, limit of the embryo. **G** Early stage 5 embryo. Only the ventral (*left*) side contains stained myocytes (*arrows*). yk_{int}, internal yolk cells. **H** Higher magnification of **G**. Stained myocytes appear as round, slightly elongated cells within the germ band. Gastrodermal cells (*gc*) line the inner side of the germ band. **I** Stage 4 embryo showing MHC gene expression in the embryonic epidermis (*eph*). The germ band (*gb*), still syncytial, is totally devoid of stained cells. The embryonic epidermis, which is very thin and not visible, is attached to the germ band. **J** Higher magnification of **I**. The embryonic pharynx (EP) shows a lumen (*asterisk*) lined by unstained epithelial cells, wrapped by circular muscle fibers (transversally sectioned; blue profiles by the epithelium). Radial muscle fibers (centripetal blue fibers) are visible. The innermost part of the EP contains four unstained cells (two visible; *arrow*) which may be part of the inner epithelium or represent a separate class of cells

cluded in the protocol. The cooling of the slides after hybridization and before removal of the cover glass also increased section preservation. Gelatin-coated slides are contraindicated because gelatin melts at hybridization temperatures, and thus sections detach from the glass slide during washing.

Proteinase K concentration and treatment time was fundamental for the proper permeabilization of the embryos.

Table 1 Conditions tested for permeabilization with proteinase K

Concentration (μm/ml)	Time (min)			
	5	15	30	45
5	–	–	–	–
10	–	–	+	+
20	–	+	+++	++

Plus signs mean good hybridization signal, and negative signs mean lack of signal. Note that application for 45 min at the maximum concentration resulted in lower signal, most likely due to excessive embryo destruction

Low concentrations such as 3 μg/ml (used in *Xenopus* embryo sections, Butler et al. 2001) or a shorter time as in planarian adults whole mount protocol (Agata et al. 1998) rendered faintly labeled embryos. A summary of all tested conditions is shown in Table 1.

Hybridization temperatures below 50°C resulted in increased background noise and decreased signal. At 55°C, the standard temperature in the adult planarian in situ protocol (Agata et al. 1998), the signal was significantly reduced; this may be explained because the M71 probe is from *S. mediterranea* and not *S. polychroa*, the species used in this study, and thus it was necessary to lower stringency conditions slightly. A 12-h hybridization was enough for regions in which the gene was heavily expressed or expressed in a great number of adjacent cells, such as the body wall (Fig. 3a, b), but a 40-h procedure (2 nights and 1 day) proved optimal for maximal signal versus tissue destruction and minimal background noise.

Fluorescent nuclei DAPI staining in sections was not useful because the blue precipitate completely cloaked the nucleus. The requirement of a minimum section thickness of 10 μm to obtain sufficiently contrasted labeling, which surpasses the nuclear diameter, prevents the circumvention of this cloaking problem by staining only every other section.

MHC gene expression

The in situ hybridization of the MHC gene in planarian embryos detected expression at the cellular level in myocytes at several stages of cell and tissue differentiation. Expression was observed as deep, uniform staining throughout the cytoplasm of the myocyte (Fig. 3a) as reported for adult planarian myocytes (Cebrià et al. 1997).

In stages 6 to 8, the formation and consolidation of the typical orthogonal pattern of muscle fibers in the body wall were observed (Fig. 3a–f). Transversal muscle fibers first formed at the sides of the stage 6 embryo (Fig. 3d), bringing together the dorsal and ventral surfaces, thus contributing to the morphing of the formerly spherical embryo to the flatworm body plan of the hatchling. These transversal muscle fibers extended inward, pinching the central gut chamber at regular intervals, thus delineating the lateral branches of the three gut caeca (Fig. 3e). Other tissues have been reported to be related to these lateral ingrowths, such as the protonephridal system (Cardona et al. 2005).

The TMUS-13 antibody failed to label embryonic cells by stage 5, although the in situ hybridization revealed MHC gene expression by this stage (Fig. 3g). The TMUS-13 antibody started labeling embryonic cells by early stage 6 (Fig. 3f), when the body wall completed its wrap and showed signs of orthogonal organization of fibers (Cardona et al. 2005). The detection of MHC gene transcripts by in situ hybridization before the detection of the MHC protein by the TMUS-13 antibody indicates a posttranscriptional regulation of the MHC gene transcript during early myocyte differentiation. A similar finding has been reported for the regeneration of the adult planarian pharynx muscle (Kobayashi et al. 1999).

From stages 2 to 4, MHC gene expression was restricted to the EP (Fig. 3i, j). By early stage 5, when the syncytial rind containing the proliferating embryonic cells was disrupted and replaced by several cellular layers, MHC gene expression is detected in one half of the embryo (Figs. 2a and 3g, h). By following seriated sections, we located the EP (a posterior–ventral structure; Cardona et al.2005) in this side and thus determined it as ventral. This assertion would be impossible to make using the whole-mount technique because the EP, the only landmark available at this stage, is inconspicuous in these preparations. This finding indicates that muscle differentiation starts ventrally and then spreads throughout the entire embryo, as reported for the epidermis (Cardona et al. 2005). This process indicates that although triclads possess unique structures such as the syncytial rind that sets them apart from less-derived flatworms, general differentiation waves within the germ band may be conserved as in more primitive flatworms like Macrostomida (Morris et al. 2004).

The monoclonal antibody TMUS-13, which labels planarian MHC protein in the body wall, pharynx, and transversal muscle fibers of the adult (Cebrià et al. 1997) and the late embryo (Fig. 3f), did not label the EP. This observation indicates that the EP, a transient embryonic structure, uses a myosin protein that is not present in the body wall or in the definitive pharynx muscle (which expresses a distinct myosin gene; Kobayashi et al. 1999; Cebrià 2000). The M71 probe used, containing the end of the head and the start of the S2 region (Cebrià 2000), thus labels all known muscle structures of the planarian.

Concluding remarks

Here we set up an in situ hybridization technique for planarian sections and whole mounts. The sections were most useful in identifying individual cells and, given the lack of conspicuous morphological landmarks, in determining their relative location to other tissues. This technique has been tested successfully with several other genes with significantly fewer transcripts per cell, such as myoD and a putative mef2 sequence (both from Cebrià 2000; in preparation) and a gastrodermal genetic marker (Fernández-Rodríguez et al., unpublished data).

The protocol gives virtually no background signal. The very small amount of probe used (20 ng/ml versus 1 μg/ml in *Xenopus* sections; Butler et al. 2001) makes the protocol affordable, although it may be necessary to increase this amount for genes with lower levels of expression.

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References

Agata K, Soejima Y, Kato K, Kobayashi C, Umesono Y, Watanabe K (1998) Structure of the planarian central nervous system (CNS) revealed by neuronal cell markers. Zool Sci 15:433–440

Baguñà J, Saló E, Auladell C (1989) Regeneration and pattern formation in planarians. III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells. Development 107: 77–86

Benazzi M, Gremigni V (1982) Developmental biology of triclad turbellarians (planaria). In: Harrison FW, Cowden RR (eds) Developmental biology of freshwater invertebrates. Alan R. Liss, Inc. New York, pp 151–211

Butler K, Zorn AM, Gurdon JB (2001) Nonradioactive in situ hybridization to *Xenopus* tissue sections. Methods 23:303–312

Cardona A, Hartenstein V, Romero R (2005) The embryonic development of the triclad *Schmidtea polychroa*. Dev Genes Evol 215:109–131

Cebrià F (2000) Ph.D. Thesis. University of Barcelona

Cebrià F, Vispo M, Newmark P, Bueno D, Romero R (1997) Myocyte differentiation and body wall muscle regeneration in the planarian *Girardia tigrina*. Dev Genes Evol 207:306–316

Cebrià F, Bueno D, Reigada S, Romero R (1999) Intercalary muscle cell renewal in planarian pharynx. Dev Genes Evol 209(4): 249–253

Hartenstein V, Ehlers U (2000) The embryonic development of the rhabdocoel flatworm *Mesostoma lingua* (Abildgaard, 1789). Dev Genes Evol 210:399–415

Hauptmann G, Gerster T (2000) Multicolor whole-mount in situ hybridization. Methods Mol Biol 137:139–148

Kobayashi C, Watanabe K, Agata K (1999) The process of pharynx regeneration in planarians. Dev Biol 211:27–38

Morris J, Ramachandra N, Ladurner P, Egger B, Rieger R, Hartenstein V (2004) The embryonic development of the flatworm *Macrostomum sp*. Dev Genes Evol 214:220–239

Romero R, Baguñà J (1991) Quantitative cellular analysis of growth and reproduction in freshwater planarians (Turbellaria; Tricladida). I. A cellular description of the intact organism. Invert Reprod Dev 19:157–165

Sánchez Alvarado A, Reddien PW, Newmark PA, Nusbaum C (2003) Proposal for the sequencing of a new target genome: white paper for a planarian genome project. The *Schmidtea mediterranea* Sequencing Consortium

Tautz D (2000) Whole mount in situ hybridization for the detection of mRNA in *Drosophila* embryos. In: Kessler C (ed) Nonradioactive analysis of biomolecules, 2nd edn. Springer, Berlin Heidelberg New York, pp 573–80

Tautz D, Pfeifle C (1989) A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98(2):81–85

Younossi-Hartenstein A, Ehlers U, Hartenstein V (2000) Embryonic development of the nervous system of the rhabdocoel flatworm *Mesostoma lingua* (Abilgaard, 1789). J Comp Neurol 416 (4):461–474