TECHNIQUES

Formaldehyde-Based Whole-Mount In Situ Hybridization Method for Planarians

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Whole-mount in situ hybridization (WISH) is a powerful tool for visualizing gene expression patterns in specific cell and tissue types. Each model organism presents its own unique set of challenges for achieving robust and reproducible staining with cellular resolution. Here, we describe a formaldehyde-based WISH method for the freshwater planarian *Schmidtea mediterranea* developed by systematically comparing and optimizing techniques for fixation, permeabilization, hybridization, and postprocessing. The new method gives robust, high-resolution labeling in fine anatomical detail, allows co-labeling with fluorescent probes, and is sufficiently sensitive to resolve the expression pattern of a microRNA in planarians. Our WISH methodology not only provides significant advancements over current protocols that make it a valuable asset for the planarian community, but should also find wide applicability in WISH methods used in other systems. *Developmental Dynamics 238:443–450, 2009.* © 2009 Wiley-Liss, Inc.

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INTRODUCTION

Freshwater planarians are well known for their ability to regenerate from injuries considered catastrophic in other animals. When a planarian is decapitated, for example, the resulting trunk regenerates a new head with all the attendant organs (e.g., brain, photoreceptors, sensory neurons) within a week. Even a fragment accounting for roughly 1/279th of the former body size can regenerate a complete animal (Morgan, 1898). The fact that a large population of adult somatic stem cells is required for planarian regeneration has fuelled the recent surge in interest in understanding the molecular mechanisms behind this process (Newmark and Sánchez Alvarado, 2002). A key molecular technique commonly used in planarian and other developmental biology studies is whole-mount in situ hybridization (WISH), which allows for the visualization of gene transcripts within cells and tissues. The central goal of a WISH method is to provide investigators with the technical ability to (a) define specific cell types by their gene expression pattern, (b) analyze the distribution of cell types within the tissue architecture, and (c) study the functional interactions between multiple cell types. However, as the need for more detailed analysis of planarian stem cells and regeneration has proceeded, so has the need for more robust and sensitive WISH methods.

A critical component of successful WISH involves balancing the often contrasting demands between sensitivity and preservation of morphology. While the retention of transcripts and preservation of overall tissue morphology necessitates use of a fixative, permeabilization of tissues and cells is a prerequisite for optimal access of antisense riboprobes to their targets. Any given organism presents its own set of challenges to achieve this balance. For example, in *Drosophila* embryos, the chorion and vitelline membrane must be removed to permeabilize, fix, and vi-

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sualize expression patterns. Zebrafish embryos can be fixed in the chorion, but extensive permeabilization steps must be performed before WISH due to the relatively higher tissue density when compared with many invertebrates. Although WISH is rarely performed in *C. elegans*, WISH protocols commonly use a histological fixative called Bouin's to permeabilize the tough cuticle.

Even though it has been empirically determined in the vast majority of systems that formaldehyde (FA) fixations are optimal for WISH, planarians have not been amenable to this approach. This is mainly due to two major distinguishing characteristics. First, these organisms secrete a dense mucus layer around their entire bodies thought to facilitate osmotic balance, locomotion, and predator avoidance (Hyman, 1951). The mucus layer also presents a considerable barrier to the exchange of fluids and large molecules needed during WISH. Second, experimental animals are often several millimeters in length, posing special challenges with respect to detection efficiencies in deep tissues. Previously, it was found that an alcohol/acid based fixative called Carnoy's could penetrate the mucus barrier and yield WISH signals in planarians (Umesono et al., 1997). This method quickly became the field standard and is now widely used. However, we find that the degree of staining in a batch of Carnoy's-fixed animals can be highly variable. Moreover, Carnoy's is not a cross-linking fixative, and thus the balance between permeability and transcript retention is not optimal (Urieli-Shoval et al., 1992). In contrast, the cross-linking capabilities of FA-based fixations offer higher cellular resolution and thus have emerged over the past 2 decades as the preferred WISH fixative across a wide variety of model systems. The need for cellular resolution and simultaneous labeling with multiple riboprobes has promoted our attempts to develop a more sensitive and robust WISH method for planarians.

Here we report an FA-based, sensitive, and reproducible whole-mount WISH methodology for planarians. Different techniques for fixation, permeabilization, hybridization and post-processing were tested for their general utility and were systematically optimized for maximal synergy. The effects of key innovations in the protocol are demonstrated with a set of markers labeling a range of planarian cell and tissue types. We find that the new methodology is superior to Carnoy's fixation for all tested markers, allowing the visualization of fine anatomical details at cellular resolution, yet at a sensitivity level sufficient even for the detection of microRNA (miR-124a) expression. Finally, we show that double fluorescent WISH along with antibody-staining of protein-epitopes provides robust access to multi-cell-type analysis using this optimized method. Hence, the method described here represents a valuable asset for the planarian community and our optimizations should be widely applicable to WISH methods in other systems.

RESULTS AND DISCUSSION

Key Optimization Steps for a New Planarian WISH Method

WISH methods contain countless variables that can be subjected to optimization. To develop a robust and sensitive WISH method for the planarian *Schmidtea mediterranea*, we concentrated on the following strategic steps: killing and mucus removal, fixation, permeabilization, dehydration, bleaching, riboprobe hydrolysis, hybridization solution (Hyb) composition, and post-development treatments (refer to the "Detailed Methods" section below). To ensure the universality of our optimizations, these parameters were tested using multiple riboprobes of varying signal intensities, tissue depths, and anatomical locations.

In Figure 1, six conditions using four representative riboprobes depict how key optimizations improved staining compared with Carnoy's. In order of highest to lowest staining intensity, the probes used were the following: (1) *smedwi-1* (Reddien et al., 2005), a stem cell marker in planaria that produces intense staining deep within in the animal; (2) *Smed-wnt-2*, which detects cells in a posterior-to-anterior gradient (Gurley et al., 2008; Petersen and Reddien, 2008); (3) *Smed-slit* (Cebria et al., 2007), a marker that stains both surface and deep cells along the midline; and (4) *Smed-PCNA* (Eisenhoffer et al., 2008), which labels cycling stem cells. While the minimal FA fixation decreased overall signal intensity for all four probes relative to Carnoy's fixation, the signal obtained in FA was less diffused and resolved into a discrete, cell-specific pattern. (Fig. 1B; rows 1–2). A simple treatment with 100% ethanol for ~20 min after development improved signal to noise ratios, which greatly helped determine which aspects of staining were due to nonspecific background (Fig. 1B; row 3). However, *smedwi-1* still stained much darker in Carnoy's fixation than FA fixation, indicating that permeabilization was suboptimal. We, therefore, replaced hydrochloric acid, normally used to kill worms (Umesono et al., 1997), with the mucolytic compound N-acetyl-cysteine (NAC), which both killed the worms and removed the mucus layer surrounding the animals. NAC, along with a short sodium dodecyl sulfate (SDS) -permeabilization step, greatly increased staining intensity and specificity (Fig. 1B; row 4).

Because the dehydration of specimens before hybridization is commonly used in many WISH protocols to increase staining intensity, we tested dehydration conditions. All four probes were intensified by dehydration in methanol or ethanol (Fig. 1B; row 5). Interestingly, dehydration revealed a posterior–anterior gradient of *Smed-wnt-2* expression. Another commonly used method to increase mRNA accessibility for hybridization is treatment with proteinase K. We generally found that proteinase K treatment followed by a short postfixation increased staining intensity (Fig. 1B; row 6). However, these conditions remained sub-optimal because the prepharyngeal region of the animals became more refractory to WISH. To penetrate these tissues, we replaced the SDS permeabilization step with a "reduction" step that uses the reducing agent dithiothreitol (DTT) and an additional detergent (NP40; Fig. 1B, row 7). Not only did this treatment allow staining in impermeable prepharyngeal regions, but it also intensified staining for each probe.

With all conditions combined, it is important to note differences with the current standard Carnoy's used in the majority of planarian publications. First, for strong probes such as *smedwi-1*, our protocol achieves similar staining intensity but with vastly improved cellu-

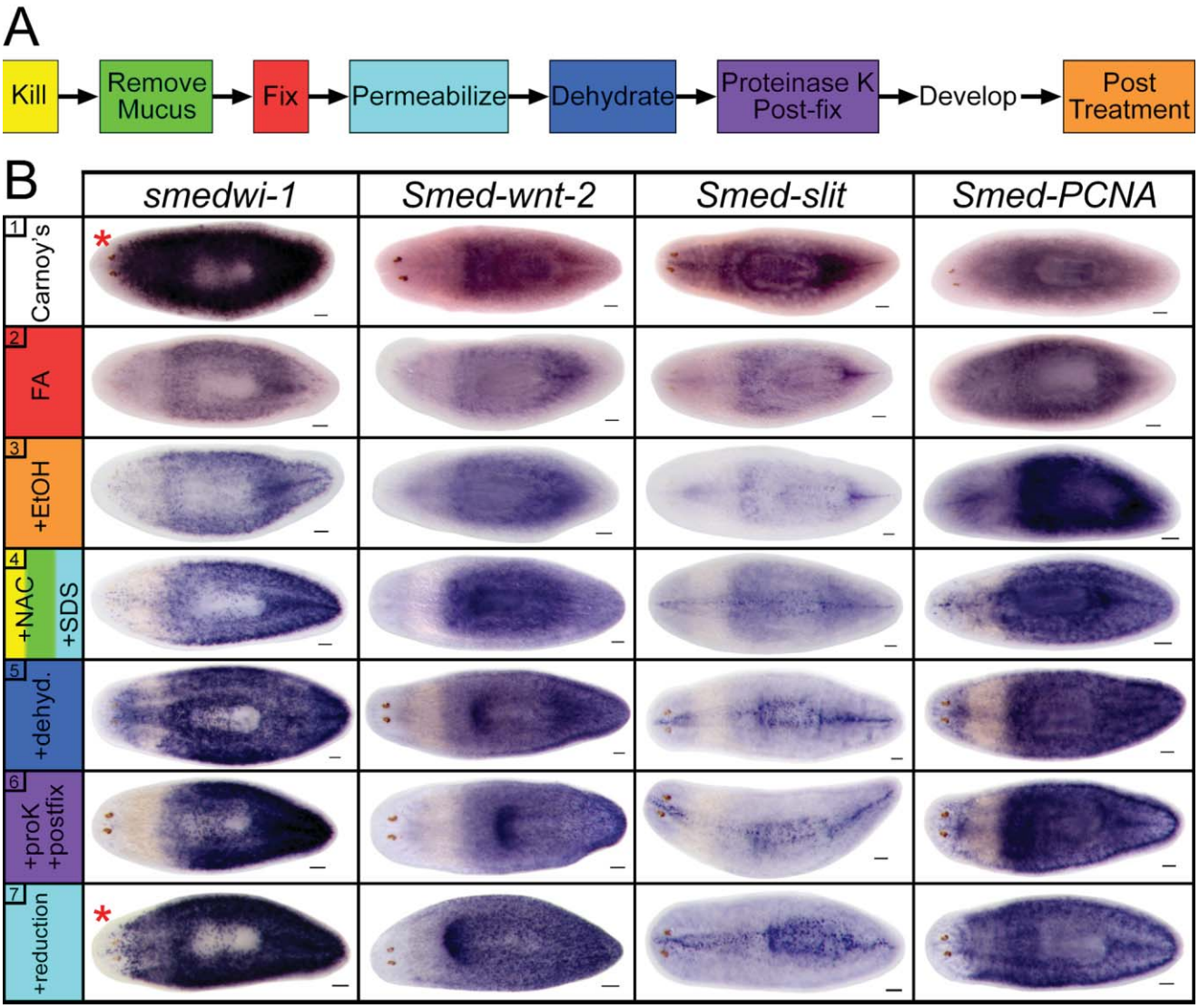

Fig. 1. Key steps to an optimized formaldehyde (FA) -based whole-mount in situ hybridization (WISH) method for planarians. **A:** Protocol timeline with color-coded steps of WISH optimizations. Note that development is the only step with no changes. **A:** Conditions tested in optimization. Row numbers are boxed, upper left hand corner. The top condition is the Carnoy's protocol typically used in planarian publications. Every subsequent row illustrates the same conditions as the row above it, with results obtained when a single variable is optimized (e.g., row 2 shows the same procedure as row 1, but with the fixation changed to FA (minimal FA); Row 3 shows the same as row 2, but with EtOH posttreatment, etc.). Red asterisks in the *smedwi-1* column show that Carnoy's and the best FA condition are overdeveloped, because all panels had to be developed the same length of time for every condition to make them directly comparable to each other (Fig. 2 shows *smedwi-1* for these conditions developed at optimized time lengths). Pictures were taken from the dorsal side. Scale bars = 100 μm.

lar resolution and animal morphology. For slightly weaker probes like *Smed-wnt-2* and *Smed-slit*, our WISH method improves on all metrics of a WISH stain: improved intensity, minimal background, increased cellular resolution, and complete and thorough staining penetration. Finally, the weak probe for *Smed-PCNA* shows the greatest improvements with FA vs. Carnoy's. Whereas there is high background and very little specific staining observed in Carnoy's, we achieved cellular resolution and appreciably better staining intensity with minimal background using the *Smed-PCNA* riboprobe in our optimized FA method.

Optimizations Also Improved Carnoy's Fixations, Yet FA Fixations Increased Cellular Resolution and Higher Signal to Noise Ratios

To test whether our optimizations also improved staining for Carnoy's fixation, we used NAC treatment, ethanol postprocessing, and optimized hybridization buffer on Carnoy's-fixed animals. We assumed that the proteinase K and reduction steps would not help because Carnoy's-treated animals are already highly permeable. In addition, Carnoy's does not covalently cross-link molecules and proteinase K could disrupt associations between protein and RNA molecules in the fixed specimens that could ultimately lead to diffuse or weaker staining. Figure 2 shows that these few optimizations improved the Carnoy's fixations in

terms of increased signal to noise ratios and cell specific staining. For example, *Smed-slit* and *Smed-wnt-2* showed discernible staining, whereas they did not in the standard Carnoy's condition (Fig. 1, row 1). Similar to Figure 1, Figure 2 shows that FA had superior signal over background and well-defined cellular resolution. For example, *smedbmp4-1* (Reddien et al., 2007), *smedwi-1*, *Smed-slit*, and *Smed-wnt-2* hybridizations resolved sharply in much more detailed staining patterns in FA-fixed animals. For the *H94.12e* probe, delicate and unknown cells which stain in the dorsal epithelia (as well as the lack of midline staining) were not readily observed using Carnoy's.

Finally, Carnoy's-fixed animals consistently displayed lack of cellular transcript retention. When using the strong probe *H1.3b*, for example, we observed diffuse signal near robust expression domains (red arrows, Fig. 2). Such diffuse staining appeared real, but comparison with FA-fixed animals (green arrows, Fig. 2) suggested instead that it likely represented transcript that had leeched out of expressing cells. To support this conclusion, we attempted to detect a ~22-nucleotide-long microRNA (miRNA). We hypothesized that miRNAs should readily leech out of cells in Carnoy's-fixed animals. Previous studies have identified numerous planarian miRNAs (Palakodeti et al., 2006), yet their expression patterns are unknown. We examined miR-124a because it represents a conserved miRNA that is expressed in the brain and nervous system in diverse organisms such as zebrafish, flies, and mice (Kloosterman and Plasterk, 2006; Kapsimali et al., 2007). The miRNA was detected using a short DNA-labeled locked nucleic acid oligonucleotide obtained commercially (Exiqon, probes hybed at 50°C, used at a 1:500 dilution). We observed strong staining for miR-124a in the brain of FA-fixed animals, but little to no staining was detected in Carnoy's-treated animals. This is the first example of whole-mount detection of a miRNA in planaria, and also shows that our optimized FA fixation achieves a fine balance of permeabilization and detection.

Optimized FA WISH Protocol Was Used to Detect Anatomical Structures at Unprecedented Resolution by Colorimetric and Fluorescent Development in Planarians

To verify that our new high-resolution WISH methodology was suitable to detect specific planarian anatomical structures, we selected previously published genes with various anatomical expression patterns. On the basis of cell morphology in macerates, it has been estimated that planarians contain roughly 40 cell types (Sánchez Alvarado and Kang, 2005). However, using several markers expressed in unknown cell types, our data suggest that this may be a low estimate. These markers demonstrate a new level of cell and molecular complexity within the planarian body plan, and define specific anatomies by means of gene expression (Fig. 3). For example, unknown cell types were revealed at various levels along the anterior–posterior axis (Fig. 3A–D), and in structures associated with the pharynx, which is a major organ system in planarians (Fig. 3E–H). The excretory, gastrovascular, and nervous systems were also readily visualized with our optimized WISH protocol (Fig. 3I–K). Finally, this protocol is amenable to double fluorescent in situ hybridization (FISH) and to antibody staining using 3 different fluorophores, thus allowing the simultaneous visualization of multiple cell types and organ systems within the same animal (Fig. 3L). This result illustrates that our protocol does not overtly destroy antibody epitopes, even after the FISH detection of two separate riboprobes (Fig. 3L). Combined, these data show that the optimized protocol described here can be used to assay coexpression and/or multiple cell types using combinations of riboprobes with antibodies, all in whole-mount animals and at cellular resolution—a feat that has yet to become a reality for most other adult model organisms.

In a broader context, we have shown that WISH in planarians using a cross-linking based fixative is not only possible, but more robust, sensitive, and consistent than current Carnoy's-based methods. The optimizations described here are likely to have widespread applicability to other systems, especially in animals that are aquatic and produce large amounts of mucus. For example, the use of NAC to chemically remove mucus provides avenues to explore gene expression in organisms not traditionally amenable to WISH analysis. Additionally, we found that the permeabilization steps using reduction and proteinase K do not appear to adversely affect many epitopes commonly detected with primary antibodies. The antibodies against alpha-tubulin and H3ser10p (which label pharynx/central nervous system and Histone H3 phosphorylated on Serine 10, respectively) are also easily detected after the WISH procedure (not shown). In addition, our data highlight that planarian stem cells must generate a wide array of differentiated cell types during both physiological turnover and regeneration of new tissue after injury. Essentially, any cell type that can be detected with a robust and sensitive WISH protocol can now be assayed in RNAi screens to find genes required for its patterning and regeneration. Furthermore, promoters from genes that label specific cells are potential candidates to drive tissue specific green fluorescent protein (GFP) in transgenic animals. Overall, our WISH optimizations are part of the common effort in the planarian community to advance the establishment of this organism as a powerful model system in biomedical research.

DETAILED METHODS

Animal Preparation

Note: Unless noted at particular steps, intact planarians may be nutated/rocked, whereas early regenerating fragments (up to day 2) should be slowly rocked.

Day 1 (kill, remove mucus, fix, reduce/permeabilize, dehydrate, bleach).

1. Asexual planarians of a length between 2 and 4 mm were starved for 1 week and transferred either into 1.5-ml Eppendorf tubes (for processing up to 20 worms) or in 15-ml Falcon tubes (for processing up to 200 worms).

2. Planarian water was replaced

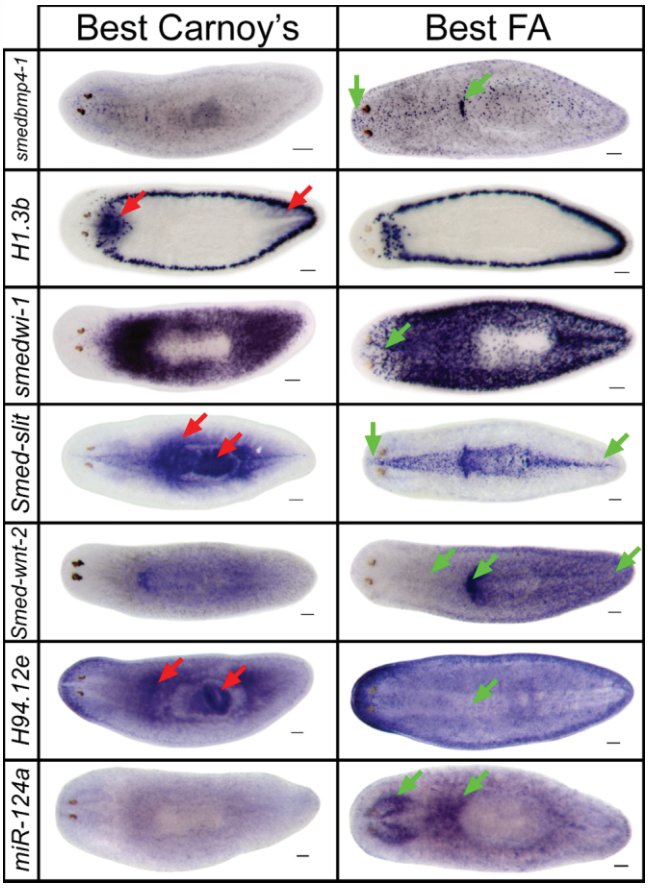

Fig. 2. Formaldehyde (FA) fixation is superior to optimized Carnoy's fixation. The left column shows whole-mount in situ hybridization (WISH) staining for various cellular markers in an optimized Carnoy's fixation and staining method. The right column shows the same markers in an optimized FA fixation and staining method. Both types of WISH were run in the same experiment, side by side, with the same staining lengths, postprocessing, and image processing. Red arrows in the Carnoy's column show background staining and/or real signal that leeches out of cells. For example, the red arrows for marker *H1.3b* show staining that appears to have diffused out of the cells. Green arrows in the FA column show real staining that is not detected in the Carnoy's staining, for example, the amount and extent of staining in *smedbmp4-1*, the anterior cells in *smedwi-1*, the posterior- and anterior-most staining in *Smed-wnt-2*, the lack of midline staining in *H94.12e*, and the brain and parapharyngeal staining in miR-124a. All images are dorsal views. Scale bars = 100 μm.

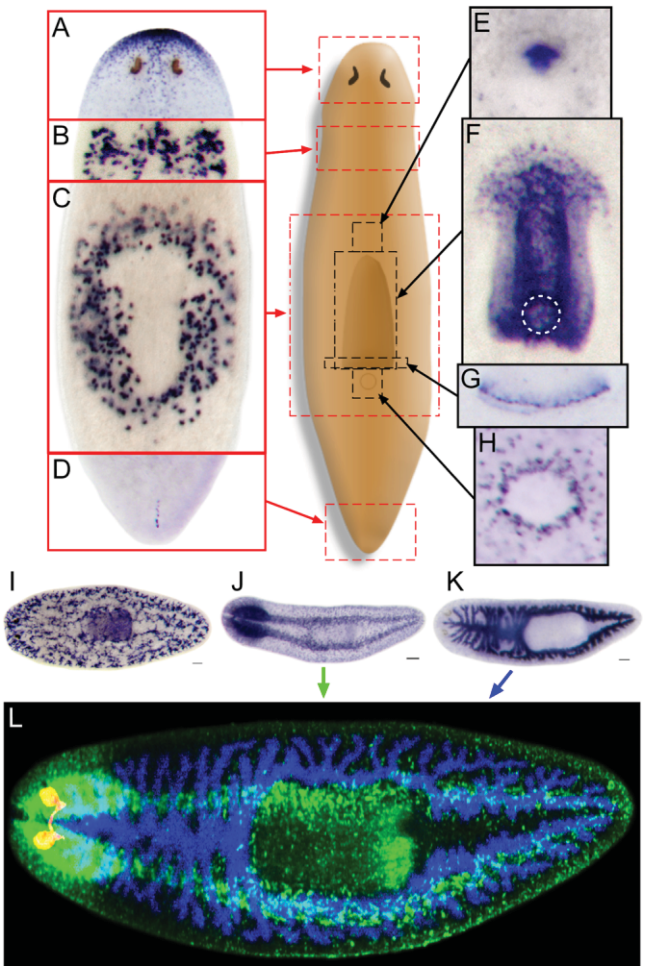

Fig. 3. Fine anatomical structures detected at cellular resolution and by fluorescence. A–D: The left side of the cartoon shows several markers that stain unknown cell types along the anterior–posterior axis. **A:** *Smed-sfrp-1* stains the anterior edge of the body (body edge). **B:** *H69.2a* stains the prepharyngeal region. **C:** *Smed-spondin-1* stains cells in tissues that surround the pharynx. **D:** *Smed-wnt-1* stains a dorsal row of cells in the tail. E–H: The right side of the cartoon shows cell types of the pharynx and body opening. **E:** *Smed-wnt-3* stains a group of cells just anterior to the pharynx. **F:** *Laminin* stains the pharynx itself, as well as the anterior end of the pharyngeal cavity. **G:** *Smed-sfrp-1* also stains the opening of the pharynx. **H:** *Smed-frz-8* stains the exterior body opening to the pharyngeal cavity. **I:** *smedinx10* labels the excretory system. **J:** *Smed-porcn-1* labels the gastrovascular system. **K:** *Smed-PC2* labels the central nervous system. **L:** Our protocol also works well for double fluorescent in situ hybridizations combined with immunofluroescence. Shown is a triple label with *Smed-porcn-1* (blue), *Smed-PC2* (green) and the monoclonal antibody to β-arrestin (red; VC-1, kind gift from Kiyokazu Agata), which labels photoreceptor neurons. Scale bars = 100 μm.

with 5% NAC solution, 5–10 min, room temperature (RT). *Notes:* 1 ml in 1.5-ml Eppendorf tubes or 10 ml in 15-ml Falcon tubes; NAC is a mucolytic reagent that both kills the worms and removes their mucus.

3. NAC was replaced with 4% Fixative, 15–20 min, RT.

4. Fixative was removed and worms were rinsed 1× with PBSTx.

5. PBSTx was replaced with preheated Reduction solution, 5–10 min, 37°C. *Notes:* Reduction was carried out in a water bath with intermittent gentle agitation (specimens are fragile at this step); reduction aids with permeabilization to allow probe penetration.

6. After removal of Reduction solution, worms were rinsed 1× with PBSTx.

7. PBSTx was replaced with 50% Methanol solution, 5–10 min, RT.

8. 50% Methanol solution was replaced with 100% Methanol, 5–10 min, RT. *Notes:* Specimens were then stored at −20°C for at least 1 hr (or up to several months); Ethanol can be used in place of Methanol in steps 7–12, which seems better for long-term storage at −20°C, but may cause slightly weaker staining overall. Proceed to step 9 when ready to bleach.

9. Methanol was replaced with 6% Bleach solution, under direct light, overnight, RT. *Note:* This step removes pigment from the animal to help with visualization of the signal.

10. 6% Bleach solution was removed and specimens were rinsed twice with 100% Methanol. *Note:* After this, specimens were then returned to −20°C or used immediately.

Day 2 (rehydrate, proteinase K, post-fix, hybridization).

11. 100% Methanol was replaced with 50% Methanol solution, 5–10 min, RT.

12. 50% Methanol solution was replaced with PBSTx, 5–10 min, RT.

13. PBSTx was replaced with Proteinase K solution, 10 min, RT.

14. Proteinase K solution was replaced with 4% fixative, 10 min, RT.

15. Fixative was removed and specimens were rinsed 2× with PBSTx.

Hybridization.

Note: When using an in situ robot (Intavis), specimens were added to robot for the hybridization steps.

16. Wash in 1:1 (PBSTx:PreHyb), 10 min, RT.

17. 1:1 mix was replaced with Prehyb, 2 hr, 56°C.

18. Prehyb was replaced with Riboprobe mix, >16 hr, 56°C. *Note:* Digoxigenin (DIG) -labeled riboprobes were used for all specimens developed with NBT/BCIP

Day 3 (washing and antibody incubation).

Note: You do not need to take precautions against RNAse contamination after hybridization, because RNAses from your hands and bacteria etc. are generally single-stranded RNAses and will not cleave dsRNA. In fact, some WISH protocols treat with RNAse posthybridization to cleave unbound probe.

19. Riboprobe mix was removed and stored at −20°C and specimens were washed with the following times and *preheated (to 56°C)* solutions: (1) 2 × 30 min 1:1 [Wash hyb: (2×SSC + 0.1% Triton-X)]; (2) 2 × 30 min 2×SSC + 0.1% Triton-X; (3) 2 × 30 min 0.2×SSC + 0.1% Triton-X.

20. Specimens were then allowed to return to RT and washed with MABT 2× 10 min RT.

Antibody incubation and development.

Note: If using an in situ robot, specimens were transferred to 24-well plates, each step was carried out with nutating or rocking.

21. Specimens were transferred to 24-well plastic plates, and solution was replaced with Block solution, 1–2 hr at RT, or overnight at 4°C.

22. Block solution was replaced with Antibody solution, 4 hr at RT, or overnight at 4°C.

Day 4 (antibody washes and development).

23. Antibody solution was removed and specimens were rinsed with MABT. *Note:* Antibody solution may be recovered and used at least 2 more times over the course of a couple weeks.

24. Specimens were then rinsed at least 6 more times with MABT, 20 min each.

25. MABT was replaced with AP buffer, 10 min, RT.

26. AP buffer was replaced with Development buffer and placed in the dark.

27. Rate and extent of probe development was monitored under a dissection microscope and stopped once an optimal signal-to-background ratio was reached. *Notes:* Time of development ranges from ~20 min to >8 hr on a probe-by-probe basis; Development taking longer than 3–5 hr was continued overnight at 4°C.

28. Development was stopped by replacing Development buffer with PBSTx.

29. Specimens were post-fixed with 4% Fixative, 10 min, RT.

30. 4% Fixative was removed and specimens were rinsed with PBSTx.

31. PBSTx was replaced with 100% Ethanol, ~20 min, RT. *Notes:* This step removes nonspecific background staining; Specimens remained in ethanol until optimal signal to background ratio was reached and the NBT/BCIP precipitate had turned dark blue.

32. 100% Ethanol was replaced with 50% Ethanol Solution, 5 min, RT. *Note:* Wait until specimens sink before continuing. This can be aided by adding drops of 50% Ethanol Solution directly on top of floating specimens.

33. 50% Ethanol Solution was replaced with PBSTx.

34. PBSTx was replaced by 80% Glycerol solution and stored at 4°C.

35. Cleared specimens (i.e., no longer floating) were transferred to a slide and mounted under a #1-weight coverslip.

36. Slides were stored at 4°C until viewing or imaging on a Lumar dissecting scope (Zeiss), equipped with an Axiocam digital camera (Zeiss).

Solutions

Animal preparation.

5% NAC solution: 5% N-acetyl cysteine (NAC; Sigma) dissolved in 1× PBS. *Note:* 5% NAC solution is best when made fresh, but may be stored at 4°C and appears to be stable > 6 months, although the activity declines somewhat during this period.

PBSTx: 1× PBS + 0.1–0.5% Triton X-100.

4% Fixative: prepared fresh for each experiment by dilution of a 36.5% formaldehyde stock solution (Formalin; Sigma) into PBSTx.

Reduction solution: 50 mM DTT, 1% NP-40, 0.5% SDS, in 1× PBS.

50% Methanol solution: equal volumes of 1× PBSTx and 100% Methanol.

50% Ethanol solution: equal volumes of 1× PBSTx and 100% Ethanol.

6% Bleach solution: 6% H_2O_2 (30% stock; Sigma) in Methanol.

Proteinase K solution: 2μg/ml Proteinase-K (Invitrogen), 0.1% SDS, in 1× PBSTx.

Hybridization.

Hyb:50–55% De-ionized Formamide (Roche): 5–10% Dextran Sulfate (Sigma, from 50% stock); 5× SSC; 1 mg/ml yeast torula RNA (Sigma); 1% Tween-20 (Sigma, from 10% stock). *Notes:* SDS may be used instead of Tween-20, but tends to precipitate during storage at −20°C; Fresh deionization of formamide appears to affect staining differently in different organisms, but is critical for planarians. We de-ionize 1 L of formamide (Roche) with 50 g of Bio-Rad AG 501-X8 (D) Resin for 1 hr at RT, then filter, aliquot, and store at −80°C.

Prehyb / Wash Hyb: Hyb without the dextran sulfate.

Riboprobe mix: 400 μl of Hyb plus ~400 ng (~4 μl) of riboprobe. *Note:* Riboprobe mix was denatured at 72–90°C for 5 min, then placed at 56°C before use.

MABT: 100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20, pH to 7.5 with NaOH.

SSC: 20× SSC stock (Sigma).

Antibody incubation and development.

Blocking solution: 5–10% horse serum in MABT. *Note:* Head-to-head comparisons indicate that bovine serum albumin (BSA) in the blocking solution is detrimental to signal detection.

Antibody solution: Antibody diluted into Blocking solution. *Note:* anti-DIG-AP (Roche) was used at 1:4,000 for all NBT/BCIP experiments.

AP buffer: 100 mM Tris, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$; 0.1% Tween-20 brought up to volume with 10% polyvinylalcohol solution (PVA; Sigma P8136). This was prepared fresh before every experiment from stocks of 1 M Tris, pH 9.5; 5M NaCl; 1 M $MgCl_2$; 10% Tween-20. The PVA solution is a 10% w/v stock in H_2O stored at RT.

Development buffer: Freshly made AP buffer with 4.5 μl/ml NBT (Roche) and 3.5 μl/ml BCIP (Roche). *Note:* PVA greatly increases AP activity and helps the most for weak probes.

80% Glycerol solution: 80% Glycerol; 10 mM Tris, pH 7.5; 1 mM EDTA.

Riboprobe Synthesis

Antisense riboprobes were synthesized from polymerase chain reaction templates using either T7 or T3 RNA-polymerase (Promega). Probe synthesis was carried out for 2 hr at 37°C using digoxigenin (DIG; Tautz and Pfeifle, 1989), fluorescein (Hauptmann and Gerster, 1996), biotin (Hauptmann and Gerster, 1996), or dinitrophenol (DNP; Denkers et al**.,** 2004) -labeling mix and 400 ng of DNA template. DNA was then degraded with DNAse (Promega) for 15 min at 37°C. Probes were hydrolyzed by adding 60 μl of Carbonate Buffer (80 mM Na_2CO_3, 53.3 mM $NaHCO_3$; pH 10.2) and incubating at 65°C for 2–3 min. The hydrolysis reaction was quenched with 80 μl of Stop solution (200 mM NaAc, pH to 6.0; adjusted with Acetic Acid). Hydrolyzed probes were precipitated by adding 40 μg Glycogen (20 mg/ml stock solution, Roche) and 400 μl ice-cold 100% ethanol, and then centrifuged at high speed at 4°C for 40 min. The resulting pellet was dried briefly after complete aspiration of the supernatant and dissolved in 100 μl of Hyb solution. Riboprobe stocks were stored at −80°C. For use, riboprobes stocks were generally diluted 1:100 or 1:200 into 400–500 μl of Hyb solution.

Fluorescent Multicolor Probe Development With Antibody Epitope Detection

Animal preparation and hybridization was carried out as detailed above in the appropriate sections. The protocol only differs during the *Antibody incubation and development* steps and was adapted from the protocols on the Web links below. Sequential rounds of Tyramide signal amplification were used for fluorescent probe development. FITC-Tyramide and Cy3-Tyramide were synthesized according to the protocols of Lance Davidson and Peter Vize, respectively (FITC-Tyramide: http://www.engr.pitt.edu/ldavidson/fluor_insitu/fluorescent_in_situ.html Cy3-Tyramide: http://www.xenbase.org/other/static/methods/FISH/Cy3_tyramide.jsp). Riboprobe antibodies included anti-DIG-POD (1:500, Roche), anti-fluorescein-POD (1:300, Roche), streptavidin-POD (Roche), or anti-DNP-HRP (1:100, Perkin-Elmer). After the initial riboprobe antibody incubation, specimens were washed 6× for 20 min in MABT and subsequently 1× 30 min in PBSTI (PBSTx + 10 mM Imidazole). Specimens were preincubated for 30 min in 400 μl of the appropriate Tyramide dilution (FITC-Tyramide: ~1:1,000; Cy3-Tyramide: ~1:500; dilutions in PBSTI). Development was initiated by adding H_2O_2 to a final concentration of 0.002–0.015% (H_2O_2 should be freshly diluted in PBSTI from a 30% stock) and allowed to proceed for 45 min under constant agitation on a shaker in the dark. Specimens were rinsed 2× 5 min in PBSTx, remaining peroxidase activity was quenched by incubating for 45 min in 1% H_2O_2 in PBSTx, followed by 4 rinses with MABT. Incubation with the second riboprobe-antibody (directed against the second riboprobe) and subsequent detection were carried out exactly as above, except that the blocking step was omitted.

Nonriboprobe antibody staining was performed during the riboprobe antibody steps above. Specifically, after incubating specimens with the first riboprobe antibody (anti-DIG-POD) for 4 hr at RT, washes were performed with Blocking solution. Specimens were incubated with VC-1 antibody (diluted 1:10,000 in Antibody solution) overnight at RT. Following washes with MABT, the first riboprobe was developed and the reaction quenched. After incubation with the second riboprobe-antibody for 4 hr at RT, specimens were washed in Blocking solution and then incubated overnight at RT with secondary antibody (anti-mouse-Alexa Fluor 647) for VC-1. After the appropriate washes, the second riboprobe was developed with tyramide signal amplification. Following at least 6× 20 min washes with MABT, specimens were mounted in 80% Glycerol solution under #1-weight coverslips and imaged on a Zeiss 510 Live confocal microscope.

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