Published in final edited form as:

Methods Mol Biol. 2018 ; 1774: 353–366. doi:10.1007/978-1-4939-7802-1_10.

Fixation, processing, and immunofluorescent labeling of whole mount planarians

David J. Forsthoefer^{1,4,†}, Kelly G. Ross^{2,†}, Phillip A. Newmark³, Ricardo M. Zayas^{2,4}

¹Functional and Chemical Genomics Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA.

²Department of Biology, San Diego State University, San Diego, CA, USA.

³Department of Integrative Biology, Howard Hughes Medical Institute, Morgridge Institute for Research, University of Wisconsin–Madison, Madison, WI, USA.

Summary

Efforts to elucidate mechanisms of regeneration in the planarian *Schmidtea mediterranea* have included the application of immunocytochemical methods to detect specific molecules and label cells and tissues *in situ*. Here we describe methods for immunofluorescent labeling of whole mount planarians. We outline protocols for fixation and steps for processing animals prior to immunolabeling, incorporating commonly utilized reagents for mucus removal, pigment bleaching, tissue permeabilization, and antigen retrieval. Because processing steps can mask or degrade antigens, we also recommend protocol parameters that can be tested simultaneously to optimize sample preparation for novel antibodies.

Keywords

Antibodies; flatworms; immunostaining; histology

1. Introduction

Investigation of the cellular and molecular mechanisms underlying planarian regeneration has been advanced by the application of immunohistochemical techniques to detect proteins, post-translational modifications, and other antigens *in situ*. These efforts have included the development of antibodies that recognize specific proteins (1–5), the identification of commercially and publicly available reagents that cross-react with planarian epitopes (6–10), and larger-scale screens to generate novel monoclonal antibody collections that label a variety of cell types (11–13).

Adaptation of immunocytochemical techniques to planarians has required identification of suitable tissue processing steps to prepare specimens for immunolabeling. Many of the processing steps were initially developed to enable specific, low-background

⁴ To whom correspondence should be addressed (DJF: david-forsthoefer@omrf.org; RMZ: rzayas@mail.sdsu.edu).

[†] these authors contributed equally to this work

immunodetection of modified nucleic acids in whole mount planarians (9,14–17) and subsequently adapted for detection of proteins (6,7,18–20). These efforts have included the implementation of various fixatives (11,13,14,21,22), as well as chemical treatments to compensate for idiosyncracies of planarian tissue such as removal of mucous secretions prior to fixation, bleaching to remove pigmentation and enable visualization of internal structures, and methods for tissue permeabilization and antigen retrieval (14–16,23). Effective sample preparation requires achieving a balance between fixation and permeabilization of tissues to allow antibody access, while simultaneously preserving antigenicity and epitope localization. Therefore, development of novel, planarian-specific antibodies, or evaluation of cross-reactivity of existing antibodies from commercial suppliers or other sources, has entailed efforts to test reagents on samples processed using several tissue preparation procedures (summarized in Fig. 1).

Our laboratories have conducted independent monoclonal antibody screens to raise cell-type-specific markers in uninjured and regenerating planarians (24–25). In the course of these projects, we found that, as in other organisms (26–28), achieving optimal staining necessitated experimenting with multiple tissue processing variables (Fig. 2). Mucus removal, fixative choice, permeabilization methods, and even bleaching in aqueous instead of organic solutions can influence immunolabeling with unique antibodies (see examples in Fig. 2A–C).

In this Chapter, we provide step-by-step protocols for fixation and immunofluorescent labeling of whole mount planarians. We highlight the most critical stages and commonly used sample preparation, blocking, labeling, and detection approaches (Fig. 1), and outline strategies for testing a range of options at each step to optimize processing and labeling for new antibodies.

2. Materials

2.1 Materials for Fixations and Immunolabeling

1. 8 ml borosilicate glass vials.
2. 20 ml borosilicate glass vials with urea poly seal cone lined screw caps.
3. 1.5 ml polypropylene microcentrifuge tubes.
4. 15 ml polypropylene conical centrifuge tubes.
5. Multiwell polystyrene plates (24, 48, or 96 well).

2.2 Mucus Removal/Kill Methods 1 and 2: HCl and NAC

1. 2% HCl: 36.5–38% HCl diluted 1:18 into ultrapure water.
2. 1X phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4.
3. 5% *N*-Acetyl-L-Cysteine (NAC) (w/v) dissolved in 1X PBS.

2.3 Fixation Method 1 and 2: Formaldehyde Reagents

1. PBSTx: 1X PBS, 0.3% Triton X-100.
2. 4% Formaldehyde Solution: dilute to 4% from commercial 37% stabilized formaldehyde stock in PBSTx.

2.4 Fixation Method 3: Methacarn Reagents

1. Methacarn: 6 volumes methanol, 3 volumes chloroform, 1 volume glacial acetic acid.

2.5 Fixation Method 4: Carnoy's Fixation Reagents

1. Carnoy's Fixative: 6 volumes ethanol, 3 volumes chloroform, 1 volume glacial acetic acid.

2.6 Permeabilization/Reduction Reagents

1. 1 M Dithiothreitol (DTT) Solution: 1 M DTT, 10 mM sodium acetate (pH 5.2). Store aliquots at -20°C .
2. 10% (w/v) Sodium Dodecyl Sulfate (SDS): Weigh out appropriate amount and dissolve in ultrapure water. Careful: SDS is a fine powder and strong irritant when inhaled, thus take appropriate protective measures.
3. Reduction Solution: 50 mM DTT diluted from a 1 M stock, 1% NP-40, and 0.5% SDS diluted from a 10% (w/v) stock in 1X PBS.

2.7 Bleaching Methods 1 and 2 (Aqueous or Dehydrated) Reagents

1. 6% H_2O_2 -PBSTx: Dilute commercial 30% H_2O_2 stock solution in PBSTx.
2. 6% H_2O_2 -MeOH: Dilute commercial 30% H_2O_2 stock solution in methanol.

2.8 Proteinase K Treatment Reagents

1. Proteinase K (ProK): Dilute to a 10 mg/ml stock according to the manufacturer's instructions. Store aliquots at -20°C .
2. ProK Solution: 2 $\mu\text{g}/\text{ml}$ Proteinase K, 0.1% SDS diluted in PBSTx.

2.9 Reagents for Immunolabeling Whole Planarians

1. Blocking Solution A: 1% BSA (IgG-free), diluted in PBSTx.
2. Blocking Solution B: 0.3% BSA (IgG-free), 0.45% Fish Gelatin diluted in PBSTx.
3. 1% (w/v) sodium azide diluted in ultrapure water.
4. HRP-conjugated secondary antibody (e.g., Goat Anti-Mouse IgG+IgM, Horseradish Peroxidase Conjugate).
5. Fluorophore-conjugated secondary antibody.

6. Mounting Media (e.g., 50–80% glycerol in PBS, Vectashield [Vector Laboratories] or similar).

2.10 Tyramide Signal Amplification (TSA) Development Reagents

1. Fluorophore-conjugated Tyramide (Fluor-tyramide), (commercially available or prepared as described in (29,30)).
2. TSA Buffer: 10 mM Imidazole, 1:250–1:1000 Fluor-Tyramide, diluted in PBSTx.
3. Mounting Media (e.g., 50–80% glycerol in PBS, Vectashield [Vector Laboratories] or similar).

3. Methods

Below we list steps optimized for processing and immunolabeling 3–5 mm long asexual *Schmidtea mediterranea* planarians or regenerating fragments. Animals are usually starved 5–7 days prior to processing or amputation (*see Note 1*). A flowchart of the complete process, with optional steps indicated, is illustrated in Fig. 1.

3.1 Dispensing Planarians into Vials for Fixation

1. Transfer animals in planarian salts [*see Chapter 5*] to appropriate tubes or vials (*see Note 1*).
 - 1.5 ml polypropylene microcentrifuge tube: 1–10 animals
 - 8 ml glass scintillation vial: 20–50 animals
 - 20 ml glass scintillation vial: 50–100 animals
 - 15 ml polypropylene conical: 10–100 animals
2. Use the following guide to determine the volume of reagents to use for all steps in the following methods (until Section 3.10, Immunolabeling of Whole Planarians). Use at least 1 ml of fluid per 10 planarians (*see Note 1*).
 - 1.5 ml polypropylene microcentrifuge tube: 1 ml volume
 - 8 ml glass scintillation vial: 2–5 ml volume
 - 20 ml glass scintillation vial: 5–10 ml volume
 - 15 ml polypropylene conical: 5–10 ml volume

3.2 Mucus Removal/Kill Method 1: Hydrochloric Acid

1. Incubate animals on ice 30 seconds – 2 minutes, until movement slows considerably. Quickly remove salt solution (e.g., by decanting or using a plastic Pasteur pipette).
2. Replace planarian salts with pre-chilled, ice-cold 2% HCl.

3. Incubate in 2% HCl for 3 minutes total, alternating between gently inverting by hand for 1 minute and incubating on ice for 1 minute (*see* Note 2).
4. Rinse once with 1X PBS.
5. Proceed immediately to fixation.

3.3 Mucus Removal/Kill Method 2: *N*-Acetyl Cysteine

1. Replace planarian salts with 5% NAC solution (*see* Note 3).
2. Incubate 5–10 minutes (*see* Note 4) at room temperature, with gentle rocking.
3. Remove 5% NAC.
4. Proceed immediately to fixation.

3.4 Fixation Method 1: Formaldehyde at Room Temperature

1. Add 4% Formaldehyde Solution (*see* Note 5).
2. Incubate 15–20 minutes at room temperature, with gentle rocking.
3. Remove 4% Formaldehyde Solution and rinse 2–3 times with PBSTx.
4. Proceed to reduction step, bleaching, or storage (*see* Note 6).

3.5 Fixation Method 2: Formaldehyde at 4°C

1. Add 4% Formaldehyde Solution.
2. Incubate for 6 hours at 4°C, with gentle rocking.
3. Remove fixative and rinse 2–3 times with PBSTx.
4. Proceed to reduction step, bleaching, or storage (*see* Note 6).

3.6 Fixation Method 3: Methacarn

1. Add pre-chilled, ice-cold Methacarn and rock gently for 20 minutes at 4°C.
2. Remove Methacarn, add pre-chilled –20°C methanol, and rock gently for 15–30 minutes at 4°C.
3. Rinse animals 2 more times in methanol (room temperature).
4. Proceed to Bleaching Method 2 (recommended, Section 3.10, **step 5**), Bleaching Method 1 (optional, *see* Note 7), or storage (*see* Note 8).

3.7 Fixation Method 4: Carnoy's

1. Add pre-chilled, ice-cold Carnoy's Fixative and rock gently for 2 hours at 4°C.
2. Remove Carnoy's, add pre-chilled –20°C methanol, and rock gently for 1 hour at 4°C.
3. Rinse animals 2 more times in methanol (room temperature).

4. Proceed to Bleaching Method 2 (recommended, Section 3.10, **step 5**), Bleaching Method 1 (optional, *see* Note 7), or storage (*see* Note 8).

3.8 (Optional) Permeabilization/Reduction

1. Add Reduction Solution (pre-heated to 37°C) (*see* Note 9).
2. Incubate at 37°C (in water bath) for 5–10 minutes, with gentle inversion every 2–3 minutes.
3. Remove solution and rinse 1–3 times with PBSTx.
4. Proceed immediately to bleaching or storage (*see* Note 6).

3.9 Bleaching Method 1: Aqueous in PBSTx

1. Add 6% H₂O₂-PBSTx (*see* Note 10).
2. Place tubes in an open, foil-lined box, 10–15 cm under a fluorescent lamp, for 8–24 hours (*see* Note 11 and Note 12).
3. Remove bleaching solution and rinse 3 times in PBSTx.
4. Proceed immediately to Proteinase K treatment, immunolabeling, or storage (*see* Note 6).

3.10 Bleaching Method 2: Dehydrated into MeOH

1. Add 1:1 methanol:PBSTx.
2. Incubate 5 minutes at room temperature, with gentle rocking.
3. Replace solution with methanol and incubate 5 minutes at room temperature, with gentle rocking.
4. Replace with fresh methanol and incubate at –20°C for at least one hour.
5. Replace methanol with 6% H₂O₂-MeOH or store animals at –20°C (*see* Note 8).
6. Place tubes in an open, aluminum foil-lined box, 10–15 cm under a fluorescent lamp, for 8–24 hours (*see* Note 11 and Note 12).
7. Remove bleaching solution and rinse 2–3 times in methanol. Animals can be stored at –20°C (*see* Note 8).
8. Replace with 1:1 methanol:PBSTx and incubate for 5–10 minutes at room temperature, with gentle rocking.
9. Replace with PBSTx and incubate for 5–10 minutes at room temperature, with gentle rocking.
10. Rinse 1–2 times with PBSTx.
11. Proceed immediately to Proteinase K treatment, or blocking.

3.11 (Optional) Proteinase K Treatment

1. Add ProK Solution (pre-heated to 37° C) (*see* Notes 13 and 14).
2. Incubate at room temperature for 10 minutes, with gentle rocking.
3. Replace ProK Solution with 4% Formaldehyde Solution and incubate for 10 minutes, with gentle rocking.
4. Remove fixative and rinse animals 2–3 times with PBSTx.
5. Proceed to Immunolabeling.

3.12 Immunolabeling of Whole Planarians

1. Transfer fixed and processed animals to a 96-, 48-, or 24-well plate, or a 1.5 ml polypropylene microcentrifuge tube (*see* Note 15).
2. Replace PBSTx with Blocking Solution (*see* Note 16), then incubate 4 hours at room temperature with gentle rocking (*see* Note 17).
3. Replace with primary antibody diluted into Blocking Solution (*see* Note 18).
4. Incubate overnight (12–16 hours) at 4°C with gentle rocking.
5. Remove antibody solution (*see* Note 19).
6. Remove antibody and rinse 3 times with PBSTx, then wash 6–8 hours, replacing PBSTx at least 6 times.
7. Re-block for 1 hour at room temperature on rocker in Blocking Solution.
8. Replace with secondary antibody (*see* Note 20 and 21) diluted in Blocking Solution (at a dilution of 1:250–1:1000) plus DAPI at 1 µg/ml (*see* Note 22). Keep plate or tubes wrapped in aluminum foil or in a light-protective container from this point forward if using fluorophore-conjugated secondary or DAPI.
9. Incubate overnight (12–16 hours) at 4°C with gentle rocking.
10. Remove antibody. Rinse 3 times with PBSTx, then wash 6–8 hours, replacing PBSTx at least 6 times. If HRP-conjugated secondary antibody was used, proceed to Tyramide Signal Amplification Development (Section 3.13).
11. Mount animals in mounting medium for imaging.

3.13 Tyramide Signal Amplification Development

1. Pre-incubate in TSA Buffer for 30 minutes at room temperature.
2. Replace with TSA Buffer plus 0.015% H₂O₂ (*see* Note 23), then incubate for 5–10 minutes at room temperature (*see* Note 24).
3. Remove TSA Buffer and rinse animals in PBSTx.
4. Wash 3 times in PBSTx (10 minutes each), then overnight at room temperature or 4°C. Keep plate or tubes wrapped in aluminum foil or in a light-protective container.

5. Mount animals in mounting medium for imaging.

4. Notes

4.1 Dispensing Planarians into Vials for Fixation

1. Processing steps for larger animals or different species might require additional testing. For example, *S. mediterranea* hermaphrodites are considerably larger and require higher reagent concentrations and longer incubation times during some processing steps (31). In addition, it has been reported that species such as *Dendrocoelum lacteum* require adjustments for successful immunolabeling (32). Animal number per tube will also need to be adjusted for larger animals.

4.2 HCl Mucus Removal/Kill Step

2. Mucus removal may be more effective with a longer, 5 minute HCl treatment on animals larger than 3–5 mm, when more animals are fixed in the same vial, or for some antibodies. Consider optimizing the duration of this step if you notice uneven or poor antibody labeling of the specimens.

4.3 N-Acetyl Cysteine Mucus Removal/Kill Step

3. NAC concentrations of up to 7.5% and treatment times up to 10 minutes may be required for larger animals and should be optimized for individual antibodies.
4. NAC-treated, Methacarn-fixed animals may disintegrate during bleaching. NAC treatment can be reduced to 3 minutes and/or H₂O₂ concentration can be reduced to 1%. We have not tested NAC treatment with Carnoy's fixation.

4.4 Formaldehyde-based Fixations

5. We have compared the labeling of multiple antibodies using either PBSTx or PBS (detergent-free) during all steps in formaldehyde-based fixation (Sections 3.4 and 3.5). Labeling quality was unaffected by the presence of detergent, but may need to be tested for new antibodies. Additionally, animals fixed and washed in PBSTx are less likely to stick together. Further measures that can reduce stickiness include: increasing tube size and fluid volumes, minimizing buffer exchange time to 45 seconds or less, and processing only 3–4 tubes at once to avoid delays during buffer exchanges.
6. For many antibodies, labeling is unaffected by animal storage in PBSTx at 4°C for up to one week. We advise testing of storage effects for new antibodies.

4.5 Methacarn and Carnoy's Fixations

7. Animals must be rehydrated prior to bleaching in PBSTx. Incubate animals in 1:1 methanol:PBSTx for 5 minutes, then 2 times in PBSTx (5 minutes each).
8. Animals can be stored in Methanol at –20°C for up to one month. Further storage could potentially decrease labeling efficiency. Storage effects should be tested for new antibodies.

4.6 Reduction/Permeabilization

9. Carnoy's- or Methacarn-fixed animals should be rehydrated first (*see* Note 7). We have no examples of this treatment improving labeling on Methacarn and Carnoy's fixed animals. However, this combination has not been tested extensively and is an option for antibodies that only label alcohol-fixed specimens.

4.7 Bleaching

10. We have compared labeling by multiple antibodies after bleaching in PBSTx or PBS, and found no difference in labeling quality. PBSTx is recommended because animals in PBSTx were less likely to stick together, but PBS may be more appropriate for antibodies with detergent sensitivity. The shelf life of commercial peroxide varies and the effective concentration can decrease over time as H₂O₂ decays. We recommend storing the stock solution at 4°C and replacing it regularly.
11. Bleach times of 12–16 hours are appropriate for asexual planarians 3–5 mm in length and for many antibodies. However, smaller animals may require shorter bleaching times, while larger animals may require longer times. In addition, some antibodies are more sensitive to hydrogen peroxide treatment. Optimal bleaching time should therefore be determined empirically for individual antibody and animal size combinations.
12. Animals may retain a light yellow color, especially after shorter bleaching times. Although rehydration and Proteinase K treatment will further reduce pigment levels, fresh H₂O₂ solution can be added after 8–12 hours to accelerate bleaching if animals retain dark yellow pigment. Effects on immunolabeling should be determined on an antibody-to-antibody basis.

4.8 Proteinase K Treatment

13. We have no examples of this treatment improving labeling on Methacarn and Carnoy's fixed animals, but this step is an option for antibodies that only label alcohol-fixed specimens.
14. ProK concentration (2–20 µg/ml) and/or treatment time (5–20 minutes) may need to be empirically determined for smaller or larger animals, or for regenerates.

4.9 Immunolabeling of Whole Planarians

15. Recommendations for appropriate animal numbers and volumes per well/tube for blocking and labeling. For animals/fragments larger than 3–5 mm, animal numbers should be reduced. Use twice the volumes listed for washing steps.
 - 96-well plate: 3–5 animals, 75–100 µl
 - 48-well plate: 3–10 animals, 200–400 µl
 - 24-well plate: 10–20 animals, 300–600 µl

- 1.5 ml microcentrifuge tube: 10–20 animals, 250–500 μ l
16. We have successfully used both Blocking Solution “A” and “B” for antibodies and hybridoma supernatants. Blocking Solution “B” with fish gelatin may be more suitable for antibodies/supernatants with higher background labeling. Optimal blocking solution can be determined empirically for new antibodies.
 17. Four hour blocking time is standard. However, blocking can be extended overnight (12–16 hours) to improve signal-to-noise for antibodies with higher non-specific labeling.
 18. Optimal dilution should be determined for each lot of antibody. Some examples of antibodies commonly used to label whole planarians and recommended dilutions are listed in Table 1.
 19. Primary antibodies can be stored at 4° C after adding 2 μ l per ml of 10% Sodium Azide (for a final concentration of 0.02%). Sodium Azide inactivates HRP and should not be added to HRP-conjugated secondary antibodies. Diluted, stored antibodies can often be used multiple times, but storage effects should be determined empirically for each antibody.
 20. 1:250 dilution is optimal for HRP-conjugated Anti-mouse IgG+IgM (Jackson) and 1:1000 is optimal for Alexa Fluor 488 Anti-Mouse IgG (Life Technologies).
 21. Additional secondary antibodies that we have successfully used to label whole planarians and their recommended dilutions:
 - Alexa Fluor 568 Goat Anti-Mouse IgG (Life Technologies), 1:400.
 - Alexa Fluor 488 Goat Anti-Rabbit IgG (Life Technologies), 1:1000.
 - Alexa Fluor 568 Goat Anti-Rabbit IgG (Life Technologies), 1:1000.
 - Goat Anti-Rabbit IgG, Horseradish Peroxidase Conjugate (Life Technologies), 1:250.
 - Goat Anti-Mouse IgG, IgM, Horseradish Peroxidase Conjugate (Life Technologies), 1:1000.
 22. DAPI incubation can be performed later, after TSA, but will add time to the protocol.

4.10 Tyramide Signal Amplification:

23. To preserve H₂O₂ activity, aliquot and store 30% stock at –20°C.
24. Tyramide-only pre-incubation and/or longer development times may improve labeling in animals over 5 mm in length.

Acknowledgments

We thank Roma Munday, Matthew Taylor, and Amy Hubert for testing parameters to optimize whole mount immunostaining in the Zayas Laboratory; Carlo Quintanilla for helping to process the anti-Synapsin samples presented here; Forrest Waters for optimization efforts in the Newmark Laboratory; our colleagues in the planarian research community whose contributions led to protocol improvements discussed in this Chapter. DJF was

supported by National Institutes of Health Ruth L. Kirschstein National Research Service Award F32-DK077469. Work in the Zayas Laboratory was supported by California Institute for Regenerative Medicine Grant RN2-00940-1 to RMZ. PAN is an investigator of the Howard Hughes Medical Institute.

References

- Guo T, Peters AH, Newmark PA (2006) A Bruno-like gene is required for stem cell maintenance in planarians. *Dev Cell* 11 (2):159–169. doi:10.1016/j.devcel.2006.06.004 [PubMed: 16890156]
- Orii H, Ito H, Watanabe K (2002) Anatomy of the planarian *Dugesia japonica* I. The muscular system revealed by antisera against myosin heavy chains. *Zoolog Sci* 19 (10):1123–1131 [PubMed: 12426474]
- Orii H, Sakurai T, Watanabe K (2005) Distribution of the stem cells (neoblasts) in the planarian *Dugesia japonica*. *Dev Genes Evol* 215 (3):143–157. doi:10.1007/s00427-004-0460-y [PubMed: 15657737]
- Nishimura K, Yamamoto H, Kitamura Y, Agata K (2008) Brain and neural networks. In: Raffa RB, Rawls SM (eds) *Planaria: A Model for Drug Action and Abuse*. Landes Bioscience, pp 4–12
- Ito H, Saito Y, Watanabe K, Orii H (2001) Epimorphic regeneration of the distal part of the planarian pharynx. *Dev Genes Evol* 211 (1):2–9 [PubMed: 11277401]
- Cebrià F (2008) Organization of the nervous system in the model planarian *Schmidtea mediterranea*: an immunocytochemical study. *Neurosci Res* 61 (4):375–384. doi:10.1016/j.neures.2008.04.005 [PubMed: 18499291]
- Robb SM, Sánchez Alvarado A (2002) Identification of immunological reagents for use in the study of freshwater planarians by means of whole-mount immunofluorescence and confocal microscopy. *Genesis* 32 (4):293–298 [PubMed: 11948917]
- Fraguas S, Barberán S, Ibarra B, Stöger L, Cebrià F (2012) Regeneration of neuronal cell types in *Schmidtea mediterranea*: an immunohistochemical and expression study. *Int J Dev Biol* 56 (1–3):143–153. doi:10.1387/ijdb.113428sf [PubMed: 22451002]
- Newmark PA, Sánchez Alvarado A (2000) Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. *Dev Biol* 220 (2):142–153 [PubMed: 10753506]
- Zayas RM, Cebrià F, Guo T, Feng J, Newmark PA (2010) The use of lectins as markers for differentiated secretory cells in planarians. *Dev Dyn* 239 (11):2888–2897. doi:10.1002/dvdy.22427 [PubMed: 20865784]
- Bueno D, Bagnà J, Romero R (1997) Cell-, tissue-, and position-specific monoclonal antibodies against the planarian *Dugesia (Girardia) tigrina*. *Histochem Cell Biol* 107 (2):139–149 [PubMed: 9062799]
- Moritz S, Stockle F, Ortmeier C, Schmitz H, Rodriguez-Esteban G, Key G, Gentile L (2012) Heterogeneity of planarian stem cells in the S/G2/M phase. *Int J Dev Biol* 56 (1–3):117–125. doi:10.1387/ijdb.113440sm [PubMed: 22450999]
- Romero R, Fibla J, Bueno D, Sumoy L, Soriano MA, Baguna J (1991) Monoclonal-Antibodies as Markers of Specific Cell-Types and Regional Antigens in the Fresh-Water Planarian *Dugesia (G) Tigrina*. *Hydrobiologia* 227:73–79. doi:10.1007/Bf00027585
- Umesono Y, Watanabe K, Agata K (1997) A planarian *orthopedia* homolog is specifically expressed in the branch region of both the mature and regenerating brain. *Dev Growth Differ* 39 (6):723–727 [PubMed: 9493832]
- Pearson BJ, Eisenhoffer GT, Gurley KA, Rink JC, Miller DE, Sánchez Alvarado A (2009) Formaldehyde-based whole-mount in situ hybridization method for planarians. *Dev Dyn* 238 (2):443–450. doi:10.1002/dvdy.21849 [PubMed: 19161223]
- Tazaki A, Kato K, Orii H, Agata K, Watanabe K (2002) The body margin of the planarian *Dugesia japonica*: characterization by the expression of an intermediate filament gene. *Dev Genes Evol* 212 (8):365–373. doi:10.1007/s00427-002-0253-0 [PubMed: 12203092]
- Pellettieri J, Fitzgerald P, Watanabe S, Mancuso J, Green DR, Sánchez Alvarado A (2010) Cell death and tissue remodeling in planarian regeneration. *Dev Biol* 338 (1):76–85. doi:10.1016/j.ydbio.2009.09.015 [PubMed: 19766622]

18. Cebrià F, Newmark PA (2005) Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* 132 (16):3691–3703 [PubMed: 16033796]
19. Sánchez Alvarado A, Newmark PA (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc Natl Acad Sci U S A* 96 (9):5049–5054 [PubMed: 10220416]
20. Forsthoefel DJ, James NP, Escobar DJ, Stary JM, Vieira AP, Waters FA, Newmark PA (2012) An RNAi screen reveals intestinal regulators of branching morphogenesis, differentiation, and stem cell proliferation in planarians. *Dev Cell* 23 (4):691–704. doi:10.1016/j.devcel.2012.09.008 [PubMed: 23079596]
21. Willier BH, Hyman LH, Rifenburgh SA (1925) A histochemical study of intracellular digestion in triclad flatworms. *Journal of Morphology and Physiology* 40 (2):299–340. doi:10.1002/Jmor.1050400205
22. Kobayashi C, Kobayashi S, Orii H, Watanabe K, Agata K (1998) Identification of two distinct muscles in the planarian *Dugesia japonica* by their expression of myosin heavy chain genes. *Zoological Science* 15 (6):861–869. doi:10.2108/Zsj.15.861
23. Bueno D, Espinosa L, Baguña J, Romero R (1997) Planarian pharynx regeneration in regenerating tail fragments monitored with cell-specific monoclonal antibodies. *Dev Genes Evol* 206:425–434 [PubMed: 27747385]
24. Forsthoefel DJ, Waters FA, Newmark PA (2014) Generation of cell type-specific monoclonal antibodies for the planarian and optimization of sample processing for immunolabeling. *BMC Dev Biol* 14:45. doi: 10.1186/s12861-014-0045-6 [PubMed: 25528559]
25. Ross KG, Omuro KC, Taylor MR, Munday RK, Hubert A, King RS, Zayas RM (2015) Novel monoclonal antibodies to study tissue regeneration in planarians. *BMC Dev Biol* 15:2. doi: 10.1186/s12861-014-0050-9 [PubMed: 25604901]
26. Ramos-Vara JA, Beissenherz ME (2000) Optimization of immunohistochemical methods using two different antigen retrieval methods on formalin-fixed paraffin-embedded tissues: experience with 63 markers. *J Vet Diagn Invest* 12 (4):307–311 [PubMed: 10907858]
27. Stadler C, Skogs M, Brismar H, Uhlen M, Lundberg E (2010) A single fixation protocol for proteome-wide immunofluorescence localization studies. *J Proteomics* 73 (6):1067–1078. doi:10.1016/j.jprot.2009.10.012 [PubMed: 19896565]
28. Duerr JS (2006) Immunohistochemistry (June 19, 2006). In: The *C. elegans* Research Community (ed) WormBook. doi:10.1895/wormbook.1.105.1, <http://www.wormbook.org>
29. Hopman AH, Ramaekers FC, Speel EJ (1998) Rapid synthesis of biotin-, digoxigenin-, trinitrophenyl-, and fluorochrome-labeled tyramides and their application for In situ hybridization using CARD amplification. *J Histochem Cytochem* 46 (6):771–777 [PubMed: 9603790]
30. King RS, Newmark PA (2013) In situ hybridization protocol for enhanced detection of gene expression in the planarian *Schmidtea mediterranea*. *BMC Dev Biol* 13:8. doi:10.1186/1471-213X-13-8 [PubMed: 23497040]
31. Wang Y, Stary JM, Wilhelm JE, Newmark PA (2010) A functional genomic screen in planarians identifies novel regulators of germ cell development. *Genes Dev* 24 (18):2081–2092. doi:10.1101/gad.1951010 [PubMed: 20844018]
32. Liu SY, Selck C, Friedrich B, Lutz R, Vila-Farre M, Dahl A, Brandl H, Lakshmanaperumal N, Henry I, Rink JC (2013) Reactivating head regrowth in a regeneration-deficient planarian species. *Nature* 500 (7460):81–84. doi:10.1038/nature12414 [PubMed: 23883932]

Figure 1.
Flowchart of steps used to prepare and immunolabel whole mount planarians.

Figure 2. Variations in tissue processing protocols affect immunolabeling.

Planarians were killed in either hydrochloric acid (HCl) or *N*-Acetyl-L-Cysteine (NAC), fixed with Carnoy's (CA), Formaldehyde (FA) or Methacarn (MC) and bleached in either aqueous (Aq-Bleach) or Methanol (MeOH-Bleach). **(A)** Anti-Synapsin labeling of the cephalic ganglia. Labeling was reduced by mucus removal with NAC and abolished by Proteinase K (ProK) treatment. Aq-Bleach was prepared in PBSTx. **(B)** Planarian intestine labeled with monoclonal antibody 3F11 (DJF, unpublished). The addition of a reduction step (Red) post-fixation enhanced labeling, whereas bleaching in methanol inhibited labeling. Aq-Bleach was prepared in PBS. **(C)** Planarians labeled with the mitotic marker anti-H3-PS10. Fixation in either Carnoy's or Methacarn produced the clearest labeling. Signal was reduced in Formaldehyde-fixed animals and further diminished by NAC mucus removal. Anterior is to the left in all panels. Scale bars in A = 100 μ m; B-C = 500 μ m.

Table 1.

Recommended immunolabeling protocol steps for antibodies commonly used to label whole mount planarians¹.

Antibody, Cat #, Supplier	1° Dilution, 2° Antibody Detection	Recommended Preparations for Whole Mount Labeling			
		Kill Step Method	Fixation Method	Optional Steps	Bleaching Method
Mouse anti-Synapsin, 3C11, DSHB	1:400, Fluor-conjugated	HCl	Formaldehyde	None	Aqueous
		HCl	Carnoy's	None	Aqueous
Mouse anti-β-Tubulin, E7, DSHB	1:500, Fluor-conjugated	HCl	Carnoy's	None	Dehydrated
		NAC	Formaldehyde	ProK	Dehydrated
Mouse anti-Acetylated-Tubulin, T6793, Sigma	1:1000, Fluor-conjugated	HCl	Carnoy's	None	Dehydrated
		HCl	Formaldehyde at 4°C	ProK	Aqueous
Rabbit anti-Histone-H3-PS10, 3377S, Cell Signaling	1:2000, HRP-conjugated	HCl	Carnoy's / Methacarn	None	Dehydrated
Mouse anti-Phospho-Tyrosine, 9411S, Cell Signaling	1:500, Fluor-conjugated	HCl	Carnoy's / Methacarn	None	Dehydrated
		HCl	Formaldehyde at 4°C	None	Aqueous

¹The table lists parameters the authors have identified that enable specific and robust labeling with individual antibodies. However, we have not tested all possible combinations of protocol variables and double-labeling with any of them could require additional adjustments. Antibodies from other suppliers can be substituted, but optimal sample preparation and antibody dilution may need to be empirically determined.