Chapter 2

Methods for Studying the Germline of the Human Parasite *Schistosoma mansoni*

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Abstract

Schistosomes are flatworm parasites that claim the lives of more than 200,000 people in poverty-stricken regions every year. Much of the pathology due to infection is the direct result of injury spurred by the parasite's eggs becoming lodged in host tissues. Thus, asking basic questions about germ cell biology may not only identify novel therapeutic approaches, but could also uncover conserved mechanisms that regulate the germline in diverse metazoa. Here, we detail useful methods for studying the schistosome germline including EdU labeling, whole-mount *in situ* hybridization, and RNA interference. These methods will hopefully lead to new insights about germline development in the schistosome and facilitate new investigators to begin asking questions about these important and fascinating parasites.

Key words Schistosome, Germ cell, Reproduction

1 Introduction

When one considers the schistosome's remarkably complicated life cycle (Fig. 1), it is astounding that these parasites are successful enough to infect more than 200 million people worldwide [1, 2]. Their life cycle begins when eggs expelled via the urine or feces of the parasite's mammalian host reach freshwater. Inside these eggs are ciliated larvae, called miracidia, which are induced by the reduced osmotic concentration of freshwater to escape from the egg and identify a snail intermediate host. Once a suitable host is located the miracidia burrow through the snail's epidermis and transform into sporocysts, which undergo a clonal expansion making new generations of sporocysts and ultimately infective cercariae. Cercariae are triggered by sunlight to escape from the snail and seek out and penetrate the skin of their mammalian definitive host (e.g., a human). These larvae then enter the bloodstream, develop as either male or female worms, mate, and begin laying eggs. Although the goal of the parasite is to have its eggs excreted from the host, a large fraction of the parasite's eggs are washed away

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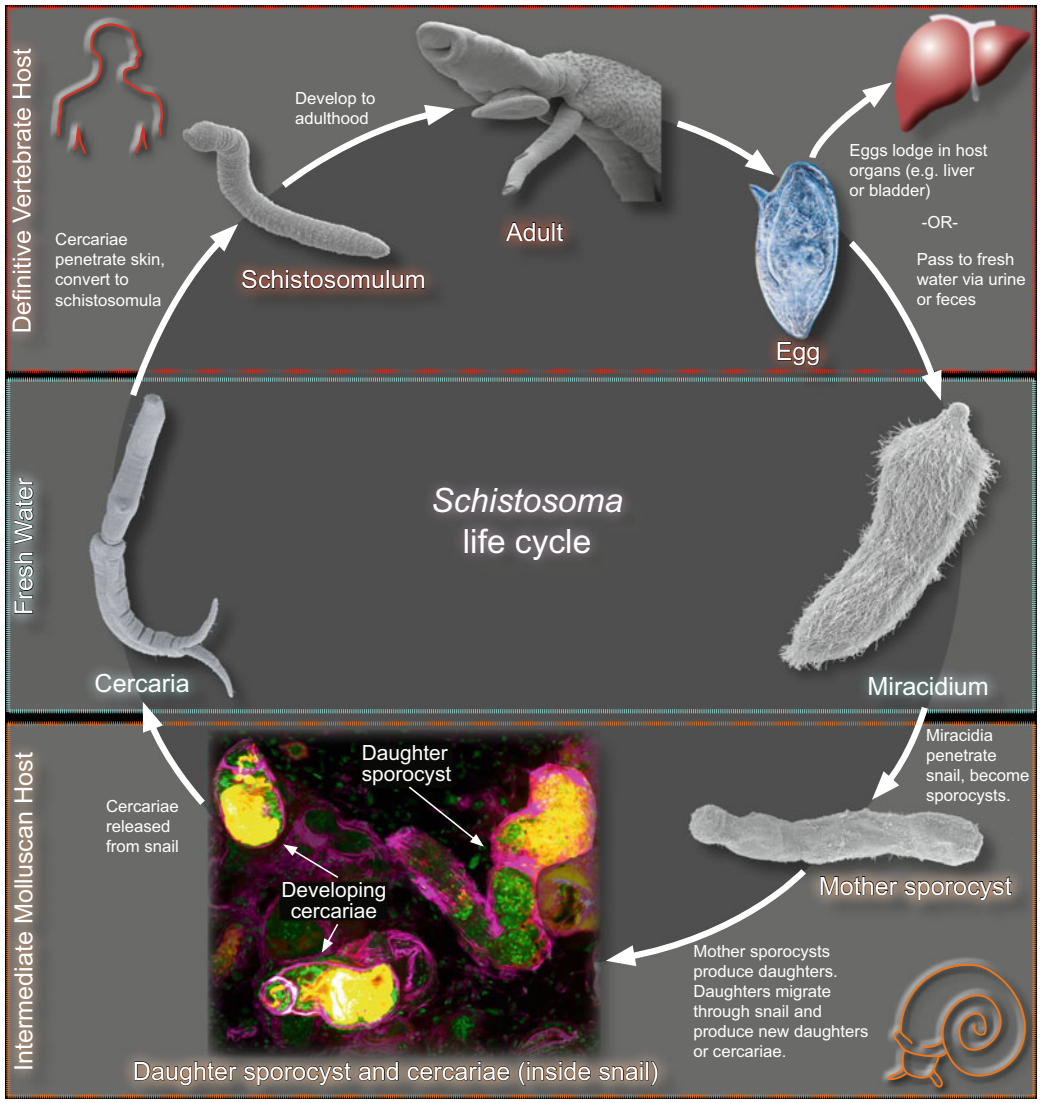

Fig. 1 The schistosome life cycle. Originally published in [27]

in the vasculature becoming lodged in organs such as the liver and bladder. Since these parasites can lay 300–3000 eggs per day over the course of several decades [3], schistosome infection often leads to chronic and debilitating symptoms [2]. Importantly, since eggs are the primary driver of pathology [4], parasites incapable of egg production cause fewer complications in their host. Thus, understanding the mechanisms that coordinate egg production could have therapeutic applications.

Unlike most other flatworms, which are cross-fertilizing hermaphrodites, schistosomes are diecious, having separate male and female sexes (Fig. 2). In the blood female worms are held within a specialized grove on the ventral surface of the male, called the

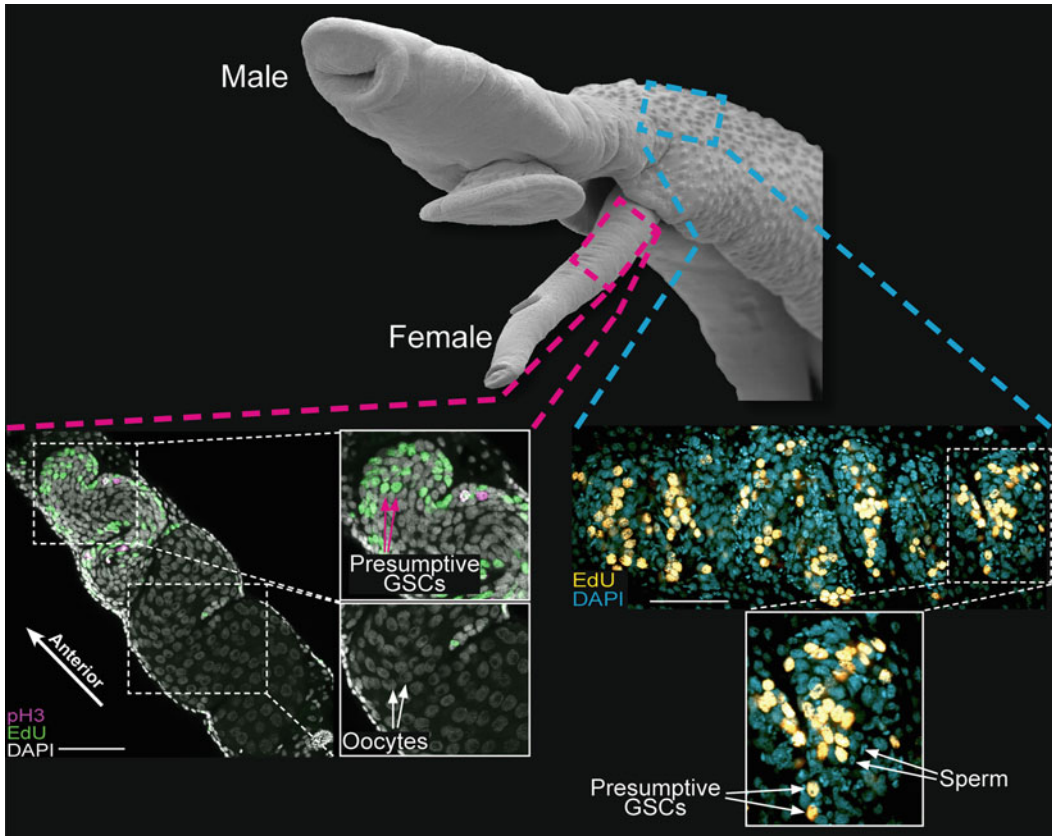

Fig. 2 The gonads of female and male *S. mansoni*. *Top*: Scanning electron micrograph of a male and female worm pair. *Bottom left*: Female ovary one day following an EdU pulse. EdU labeling is restricted to the anterior proliferative compartment. As the stem cells differentiate EdU labeling will be chased into oocytes and eggs. Labeling for DAPI (nuclei, *white*) and anti-phosphohistone H3 (pH3, *magenta*) to detect cells in M-phase are also shown. Image originally published in [11]. *Bottom right*: Male testes one day after an EdU pulse. Eventually, EdU labeling will be seen in other more differentiated cell types of the testes such as spermatids and sperm. DAPI labeling is shown in *cyan*. Scale bars, 50 μm

gynecophoral canal. Although this "pairing" between the male and female worm is essential for copulation, it is also required for female worms to initiate and maintain their reproductive maturation. Indeed, the ovaries of female worms in single-sex infections fail to mature and ovaries of mature females deprived of male contact regress to an immature state [3]. Since egg production is required for pathology, hosts harboring either male or female worms fail to develop any observable signs of infection.

Aside from the potential therapeutic applications, the biology of these organisms raises a number of important basic questions relevant to germ cell biology, including the following: At what point during the life cycle is the germline specified? Is it during embryonic development or later as the parasite develops inside its human host? Since many broadly conserved germline regulators

(e.g., PIWIs, Vasa, Tudor) have been lost in parasitic flatworms [5–7], what roles do the remaining canonical germline regulators (e.g., *nanos*) play in the germ cell development? Because a vast majority of studies of invertebrate germ cell development have taken place in Ecdysozoan model systems (i.e., *C. elegans* and *Drosophila*), can we discover new conserved regulators of the germline in this Lophotrochozoan organism? Planarian flatworms have emerged as an important model to understand germ cell specification, development, and regeneration [8, 9]. How similar are germline development among parasitic schistosomes and free-living planarians? What is the nature of signals between male and female parasites that induce female sexual maturation? Clearly studies of these parasites have the potential to enhance our understanding of germ cell biology while simultaneously addressing a major public health challenge.

To date, few robust molecular markers for the schistosome germ cells have been described. Thus, examination of these cell types has required the use of relatively nonspecific approaches including nuclear morphology, histology, anatomical position, and EdU pulse-chase approaches [10–12]. The female ovary is a large tubular organ where the germline stem cells (GSCs) are located exclusively in an anterior proliferative compartment (Fig. 2). As these cells differentiate to oocytes they migrate towards the posterior (Fig. 2) before being released from the ovary for fertilization. Unlike the well-defined organization of the ovary, the male testes have no clear spatial organization. The testes are comprised of 4–9 lobes of cells in which the GSCs are more or less randomly packed in the lobes among cells at various states of differentiation (i.e., spermatocytes, spermatids, and sperm). Not surprisingly given the complicated life cycle of the parasite, only a handful of studies have explored factors that regulate the germline [12, 13]. However, recent breakthroughs in the isolation of whole testes and ovaries are certain to yield important insights into the molecular characteristics of the schistosome germline [14]. Combination of these data, with the approaches described in this chapter, is certain to make important contributions towards understanding the schistosome germline.

Here, we integrate previously published techniques, and our own experimental experiences, to describe approaches that can be applied for studying germ cells in adult schistosomes, including whole-mount *in situ* hybridization [11, 15–17] to monitor gene expression, *in vivo* EdU labeling [11] to follow germ cell proliferation and differentiation, and RNA interference [11, 18–21] to examine gene function. When used singly or together, these approaches should represent a powerful tool kit for beginning to understand schistosome germline development. Hopefully, the implementation of new techniques to address these fascinating problems will attract new interest for studying these amazing parasites.

2 Materials

2.1 Parasite Separation, Fixation, and Permeabilization Components

1. Dulbecco's modified Eagle medium (DMEM) + 5 % fetal bovine serum (FBS).
2. Anesthetic solution: 2.5 % Ethyl 3-aminobenzoate methanesulfonate (Sigma Aldrich) in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Make fresh before use.
3. 0.6 M $MgCl_2$.
4. PBSTx: PBS + 0.3 % Triton X-100.
5. Fixation solution: 4 % Formaldehyde in PBSTx. Make fresh before use.
6. Methanol.
7. Bleaching solution: 9 ml H_2O, 500 µl formamide, 250 µl 20× SSC, 400 µl 30 % H_2O_2. Make fresh before use.
8. Proteinase K solution: PBSTx + 5 µg/ml proteinase K (*see* **Note 1**). Make fresh before use.

2.2 EdU Labeling and Detection Components

1. 5 mg/ml 5-Ethynyl-2′-deoxyuridine (EdU) (Molecular Probes) in PBS. Filter sterilize and store at −20 °C.
2. 1 ml Syringe and 23 G needle.
3. 10 mM Alexa Fluor® 488 Azide in Dimethyl Sulfoxide. Store at −20 °C.
4. Click chemistry development solution: For 100 µl, add 78.8 µl PBS, 1 µl 100 mM $CuSO_4$, 0.25 µl 10 mM Alexa Fluor® 488 Azide, 20 µl 500 mM ascorbic acid (*see* **Note 2**).

2.3 Riboprobe Synthesis Components

1. 10× *In vitro* transcription buffer: 0.3 M HEPES pH 7.5, 1 M potassium acetate, 0.15 M magnesium acetate, 2.5 mM EDTA, 10 mM DTT, 0.5 % Tween-20. Store at −20 °C.
2. 100–500 ng Purified PCR-generated DNA template (*see* **Note 3**).
3. 10 mM Digoxigenin-11-UTP (Roche).
4. 10/6 rNTP solution: 10 mM rATP, rCTP, and rGTP with 6.0 mM rUTP (Promega).
5. T7, T3, or SP6 RNA polymerase depending on the promoters present on DNA template.
6. RNase inhibitor (e.g., RNasin).
7. RNase-free DNase (e.g., RQ1 RNase-free DNase).
8. Ethanol (EtOH).
9. 4 M LiCl.

2.4 In Situ Hybridization Components

1. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, at pH 7.0. Make solution and treat with 0.1 % diethylpyrocarbonate (DEPC) overnight and autoclave.
2. DEPC water: Treat water with 0.1 % DEPC overnight and autoclave.
3. Deionized formamide: Treat 500 ml of formamide with 25 g AG501-X8(D) Resin (BioRad) while stirring for 1 h. Use a filter sterilization device to separate beads from formamide. Freeze 25 ml aliquots at −80 °C.
4. Yeast RNA: Dissolve yeast RNA at 1 g in 100 ml of DEPC water (*see* **Note 4**). Once dissolved, add 1 volume of phenol, mix, and centrifuge at 4000 × *g* for 10 min at 4 °C. Retain aqueous (top) phase. Extract again with an equal volume of phenol–chloroform–isoamyl alcohol and then with an equal volume of chloroform. To precipitate RNA, add 2.5 volumes of EtOH and 1/10 volume of 3 M sodium acetate and centrifuge at >10,000 × *g*. Wash pellet once with 70 % EtOH and resuspend in formamide. Use spectrophotometer to determine RNA concentration and adjust to 50 mg/ml with formamide. Freeze 0.5 ml aliquots at −80 °C.
5. Prehybridization solution: 50 % Deionized formamide, 5× SSC, 1 mg/ml yeast RNA, 1 % Tween-20. Store at −20 °C.
6. Hybridization solution: 50 % Deionized formamide, 5 % dextran sulfate (sodium salt, average molecular weight >500 kD; Sigma), 5× SSC, 1 mg/ml yeast RNA, 1 % Tween-20. Store at −20 °C.
7. Wash hybridization solution: 25 % Formamide (non-deionized works), 3.5× SSC, 0.5 % Tween-20, 0.05 % Triton-X100. Store at −20 °C.
8. TNT: 0.1 M Tris pH 7.5, 150 mM NaCl, 0.1 % Tween-20.
9. Blocking solution: 7.5 % Heat-inactivated horse serum in TNT. Store at −20 °C.
10. Antibody solution: Blocking solution with anti-digoxigenin-AP, Fab fragments (Roche) diluted 1:2000 (*see* **Note 5**).
11. Alkaline phosphatase buffer: 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1 % Tween-20 brought up to volume with 10 % filter-sterilized polyvinyl alcohol (average molecular weight 30–70 kD; Sigma) solution.
12. In situ hybridization development solution: Alkaline phosphatase buffer plus 4.5 μl/ml NBT (4-nitro blue tetrazolium chloride) and 3.5 μl/ml BCIP (4-toluidine salt).
13. Glycerol.
14. Microscope slides and cover slips.
15. Stereomicroscope.

2.5 Components for dsRNA Synthesis

1. 10× High-yield transcription buffer: 0.4 M Tris pH 8.0, 0.1 M $MgCl_2$, 20 mM spermidine, 0.1 M DTT.
2. ~1 μg of PCR product with inverted T7 promoter sites (*see* **Note 3**).
3. 100 mM rNTPs (i.e., 25 mM rCTP, rGTP, rUTP, and rATP).
4. Inorganic pyrophosphatase (New England Biolabs).
5. T7 RNA polymerase (*see* **Note 6**).

3 Methods

Important: Investigators considering schistosome work should contact the Schistosome Resource Center (http://www.niaid.nih.gov/labsandresources/resources/dmid/schisto/pages/default.aspx). This center provides training for safely working with schistosomes and provides stages of the parasite's life cycle to investigators. These services are provided free of charge and are supported by the National Institutes of Allergy and Infectious Disease (NIAID). For more information see Lewis et al. [22].

3.1 Fixation and Permeabilization of Parasites for In Situ Hybridization and EdU Detection

1. Euthanize schistosome-infected mice with pentobarbital plus heparin. Perfuse parasites from mice using 37 °C DMEM + 5 % FBS. Collect parasites in 50 ml conical tubes and use DMEM + 5 % FBS to rinse bloody perfusate. Transfer ten to several hundred worms to a 15 ml conical tube in 9 ml media.
2. Separate male and female parasites by adding 1 ml anesthetic solution to 9 ml of parasites in DMEM + 5%FBS. Rock samples by hand gently for 1–2 min or until parasites are relaxed and separated.
3. Remove anesthetic solution and kill the parasites in 1 ml of 0.6 M $MgCl_2$ for ~1 min.
4. Replace $MgCl_2$ with 4 % formaldehyde in PBSTx, and incubate for 4 h at room temperature (RT) on a rocker.
5. Rinse once with PBSTx.
6. Dehydrate in methanol and store at −20 °C. Samples can be stored for weeks, if not months or years, at −20 °C.
7. Rehydrate samples in 50 % methanol solution in PBSTx, for 5–10 min at RT.
8. Incubate in PBSTx, for 5–10 min at RT.
9. Add bleaching solution, incubate for 1 h at RT under bright light.
10. Rinse twice in PBSTx and then incubate in proteinase K solution for 45 min at RT (*see* **Note 1**).
11. Post-fix in 10 ml 4 % formaldehyde in PBSTx, for 10 min at RT.
12. Rinse in PBSTx.

3.2 *In Vivo EdU Labeling in* S. mansoni

1. Inject schistosome-infected mice intraperitoneally with 100–200 mg/kg EdU in PBS. To monitor cell proliferation in adult parasites, inject mice more than 6 weeks following infection.
2. To monitor the differentiation of germ cells, sacrifice mice and recover parasites at various times following the EdU pulse. At day 1 EdU is largely restricted to the germline stem cells; however at days 2–7 EdU is chased into various differentiated progeny in the testes and ovaries.
3. Fix and permeabilize parasites as described in Subheading 3.1.
4. Remove PBSTx and incubate 10–15 parasites in 25 μl of click chemistry development solution for 30 min at RT in the dark (*see* **Note 7**).
5. Wash parasites with constant agitation for 20 min in 1 ml PBSTx. Repeat wash step five times.
6. Stain parasites with 1 μg/ml DAPI in PBSTx overnight at 4 °C.
7. Clear parasites in 80 % glycerol for 4 h at RT.
8. Mount parasites on slides, seal cover slips with nail polish, and visualize by fluorescence microscopy.

3.3 *Riboprobe Synthesis*

1. In an RNase-free 1.7 ml tube combine:
   - 2 μl 10× *in vitro* transcription buffer.
   - 1 μl 10/6 rNTP solution.
   - 0.4 μl 10 mM digoxigenin-11-UTP.
   - 0.6 μl RNase inhibitor.
   - 2.0 μl T3, T7, SP6 RNA polymerase.
   - 1 μl of unpurified PCR product (100–500 ng) (*see* **Note 3**).
   - Bring to 20 μl with DEPC water.
2. Incubate at 37 °C for 4 h or 27 °C overnight.
3. Add 1 μl DNase, and incubate for 20 min at 37 °C.
4. Add 5 μl 4 M LiCl and 50 μl EtOH prechilled to −20 °C. Incubate at −20 °C for 10 min.
5. Centrifuge at >10,000 × *g* for 10 min at 4 °C.
6. Decant EtOH and resuspend pellet in 20 μl DEPC water.
7. Quantify RNA concentration on a spectrophotometer using 1 μl riboprobe.
8. Run 2 μl on a 1 % agarose gel.
9. Add 17 μl of hybridization solution and store at −20 °C.

3.4 Whole-Mount In Situ Hybridization

1. Transfer parasites fixed and permeabilized as in Subheading 3.1 to a 1.7 ml tube or a 24-well tissue culture plate (*see* **Note 8**).
2. Wash in 1:1 (PBSTx:prehybridization solution) for 10 min at RT.
3. Replace with prehybridization solution and incubate for at least 2 h at 52 °C.
4. Preheat hybridization solution + riboprobe (~100–500 ng/ml) at 72 °C for 5 min and hold at 52 °C.
5. Replace prehybridization solution with hybridization solution + riboprobe (~100–500 ng/ml). Hybridize overnight (>16 h) at 52 °C.
6. Preheat wash hybridization solution, 2× SSC + 0.1 % Triton-X, and 0.2× SSC + 0.1 % Triton-X at 52 °C.
7. Remove hybridization solution and wash twice at 52 °C for 30 min in wash hybridization solution.
8. Wash twice at 52 °C for 30 min with 2× SSC + 0.1 % Triton-X.
9. Wash twice at 52 °C for 30 min with 0.2× SSC + 0.1 % Triton-X.
10. Wash twice with TNT for 10 min at RT.
11. Incubate in blocking solution for 2 h at RT.
12. Incubate in antibody solution overnight at 4 °C.
13. Wash six times for 20 min each with TNT.
14. Develop in in situ hybridization development solution until desired signal to noise is reached, usually 1–4 h at RT.
15. Development can be stopped by removing development solution and adding PBSTx.
16. Rinse with PBSTx several times.
17. Incubate in 100 % EtOH for 10–20 min at RT.
18. Remove EtOH and add a few drops of PBSTx. Allow parasite to rehydrate for about 5 min.
19. Remove PBSTx and clear parasites in 80 % glycerol in 1× PBS. Incubate at 4 °C overnight.
20. Mount on slides and image by bright-field microscopy.

3.5 Generation of dsRNA for RNAi

1. In an RNase free 1.7 ml tube combine:
   - 10 μl 10× high-yield transcription buffer.
   - 20 μl 100 mM rNTPs
   - 5.0 μl T7 RNA polymerase.
   - 1.0 μl Inorganic pyrophosphatase.
   - 5 μl of unpurified PCR product with inverted T7 promoters (~1 μg) (*see* **Note 3**).
   - 59 μl DEPC water.

2. Incubate at 37 °C for 4 h to overnight (*see* **Note 9**).
3. Treat with 5 μl of DNase for 20 min at 37 °C.
4. Precipitate at RT for 10 min by adding 50 μl of 7.5 M ammonium acetate and 750 μl of EtOH.
5. Centrifuge at >10,000 × *g* for 10 min at 4 °C.
6. Decant EtOH and resuspend the pellet in 300 μl DEPC water.
7. Anneal dsRNA by successive 3-min incubations at 95, 75, and 55 °C. Allow to cool at RT for 5 min.
8. Dilute 1:10 and determine concentration on a spectrophotometer. Expect ~0.5 mg of dsRNA per 100 μl prep.
9. Run remainder of sample dilution on 1 % agarose gel (*see* **Note 10**).
10. Dilute the dsRNA to 1 μg/μl using DEPC water. Store at −20 °C.

3.6 RNAi in Adult *S. mansoni*

1. Euthanize mice with pentobarbital plus heparin. Perfuse parasites from mice using 37 °C DMEM + 5 % FBS. Collect parasites in 15 cm sterile Petri dishes.
2. In biosafety cabinet, pool parasites in 50 ml conical tubes and allow them to settle. Replace bloody media with fresh DMEM + 5 % FBS until blood is removed.
3. Replace DMEM + 5 % FBS with warmed tissue culture media suitable for maintaining schistosomes (*see* **Note 11**).
4. Transfer 5–10 healthy male/female worm pairs in 5 ml of media in a 6 cm Petri dish. This is day 0.
5. Add dsRNA to a final concentration of 20–30 μg/ml.
6. Replace media and dsRNA on days 1 and 2.
7. Change media every other day and provide new dsRNA every 5–8 days until desired experimental endpoint (*see* **Note 12**).

4 Notes

1. Proteinase K activity can decrease over time and vary depending on the vendor or manufacture lot. In our experience proteinase K treatment is essential and can greatly enhance signal. However, overdigestion can degrade tissue integrity and signal intensity. Thus, optimal proteinase K concentration and incubation times should be empirically determined for the particular set of genes being examined. Furthermore, since schistosomes can vary greatly in size (e.g., male worms are larger than female worms) proteinase K treatment regimes should be tailored to the specimen of interest.

2. Components of the click chemistry development solution should be mixed together fresh for every experiment. The ascorbic acid solution should be made immediately before the experiment and added to the development solution last.
3. We have found that the most convenient way to generate templates for riboprobes and dsRNA is to clone PCR products into plasmid pJC53.2 [23]. This vector allows for convenient TA-based cloning and includes T3 and SP6 promoters flanked by inverted T7 promoters. Thus, a single PCR product generated with T7 primers can be used to produce sense, antisense, and double-stranded RNA depending on the RNA polymerase used. This plasmid is available from addgene (https://www.addgene.org/26536/). For generation of PCR products for in vitro transcription we use Platinum *Taq* (Invitrogen) to amplify DNA, confirm by agarose gel electrophoresis that a single band of the correct size is amplified, and add specified amount of the PCR to the in vitro transcription reaction.
4. At this concentration, yeast RNA often takes several days to go into solution. Thus, we will start the dissolving process a few days before we wish to begin the RNA purification (e.g., start on Friday if you want to begin on Monday). Gentle heat can also be applied; we typically use a setting of 2–3 on our stirring hot plate. For the purification, it is often convenient to purify enough yeast RNA for several months of experiments; thus we typically start with 6 g of yeast RNA in 600 ml of water. Since these volumes necessitate the use of a large-capacity centrifuge, and typical centrifuge bottles are not compatible with solvents such as chloroform, we use Nalgene FEP 250 ml centrifuge bottles (Fisher Scientific) for all steps with phenol and chloroform. Alternatively, steps with organic solvents can be performed using appropriate 50 ml conical tubes.
5. For fluorescence in situ hybridization, as an alternative to the anti-digoxigenin-AP antibody the anti-digoxigenin-POD antibody (1:1000) (Roche) can be used. Following antibody incubation and washes, signals can be detected by Tyramide Signal Amplification (TSA Plus, Perkin Elmer) following the manufacturer's instructions. These samples can be labeled with DAPI and observed by fluorescence microscopy.
6. As a cost-saving measure, consider purifying your own HIS-tagged T7 RNA polymerase [24].
7. Alternatively, EdU detection can be performed in combination with fluorescence in situ hybridization. For these approaches perform fluorescence in situ hybridization on EdU-labeled parasites using the modifications detailed in **Note 5** and detect EdU after TSA. Make sure to use fluorophores that can be distinguished by fluorescence microscopy (e.g., FITC and Cy3).

8. Due to the large number of wash steps involved in the in situ hybridization procedure a great deal of time can be saved by making or purchasing mesh baskets for washing. These baskets can be easily fabricated with a small plastic tube, nylon mesh, and a hotplate [25]. After pipetting the parasites in these baskets, washes can be performed by simply moving the baskets to separate wells on a multiwell plate. Alternatively, Intavis Bioanalytical Instruments sells an instrument that can automate the entire in situ hybridization process.
9. As the dsRNA is synthesized the reaction will often become viscous.
10. Under these non-denaturing conditions we find that dsRNA often migrates roughly similar to dsDNA. However, we occasionally note additional high-molecular-weight bands.
11. Many types of commercially available cell culture media have been used to culture *S. mansoni* including DMEM, RPMI, and M199 [18]. For adult *S. mansoni* we have had success with Basch Media 169 that was specifically developed for culturing larval schistosomes [26].
12. We have seen phenotypic consequences of dsRNA treatment as early as day 5. But some phenotypes take 2–3 weeks to manifest. Thus, verification of mRNA and/or protein depletion is key to determining the length of the dsRNA treatment regime.

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