Chapter 27

RNAi Screening to Assess Tissue Regeneration in Planarians

Rachel H. Roberts-Galbraith

Abstract

Over the past several decades, planarians have emerged as a powerful model system with which to study the cellular and molecular basis of whole-body regeneration. The best studied planarians belong to freshwater flatworm species that maintain their remarkable regenerative capacity partly through the deployment of a population of adult pluripotent stem cells. Assessment of gene function in planarian regeneration has primarily been achieved through RNA interference (RNAi), either through the feeding or injection of double-stranded RNA (dsRNA). RNAi treatment of planarians has several advantages, including ease of use, which allows for medium-throughput screens of hundreds of genes over the course of a single project. Here, I present methods for dsRNA synthesis and RNAi feeding, as well as strategies for follow-up assessment of both structural and functional regeneration of organ systems of planarians, with a special emphasis on neural regeneration.

Key words Planarian, *Schmidtea mediterranea*, *Dugesia japonica*, RNAi, dsRNA synthesis, Screening, Regeneration, Functional genomics

1 Introduction

Planarian flatworms have grown popular as a study system for regeneration because they can regrow all cell types after nearly any injury. Over one hundred years ago, scientists determined that planarians can achieve whole-body regeneration starting with a small fragment of an adult animal. More recent work, mostly using the species *Schmidtea mediterranea* (Fig. 1a, b) and *Dugesia japonica* (*see* **Note 1**), revealed many important cellular and molecular contributors to planarians' regenerative capacity, including a population of adult, pluripotent stem cells, and constitutive body-wide axial polarity signaling (Fig. 1c, d; [7] and for reviews, see [2, 3, 8]). Planarians also possess diverse tissue types, allowing dissection of the molecular mechanisms that power structural and functional regeneration at the level of organ systems. The planarian body consists of: a tri-lobed intestine and a tube-shaped feeding

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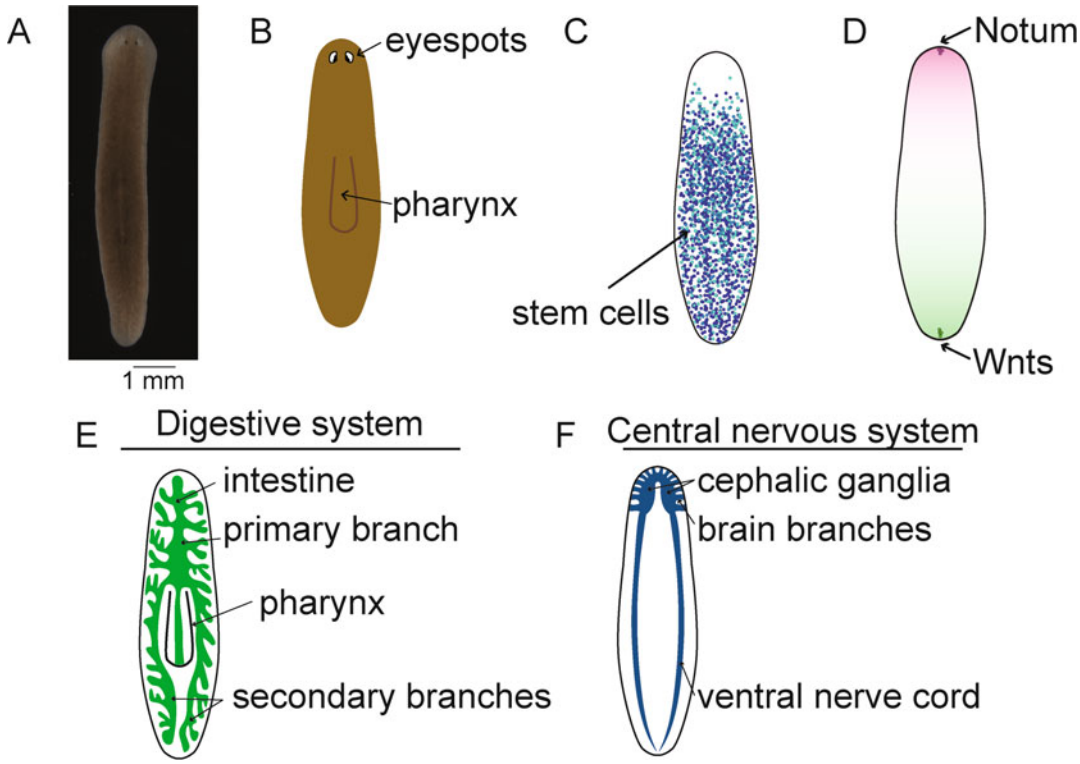


Fig. 1 Introduction to planarians. (**a**) A live planarian is pictured with its anterior (head) toward the top of the page. Eyespots are visible. (**b**) A planarian is diagrammed as pictured in **a**, with eyespots labeled. The pharynx (feeding organ) is tucked inside the body of the planarian when an animal is not feeding, but the outline of the pharynx can be faintly visible from the dorsal side. During feeding, the tube-shaped pharynx emerges from its pouch to extend through an opening on the ventral surface of the planarian body (not shown). (**c**) Planarian stem cells are a heterogeneous population, containing both pluripotent (dark blue) and specialized (light blue, teal) cells (reviewed in [1]). Stem cells are present throughout the planarian body, with two main exceptions. The pharynx has no resident stem cells, and few stem cells exist in the tip of the head (anterior to the eyespots). (**d**) A suite of polarity determinants regulates body patterning in the planarian (for review, see [2, 3]). The anterior/posterior axis of polarity signaling is depicted here. Wnt ligands (e.g., Wnt1, Wnt11-1, and Wnt11-2) are produced in the tail of the planarian. Wnt inhibitors (e.g., Notum, sFRP-1) are produced in the head to oppose Wnt signaling. Additional signaling molecules pattern the trunk of the planarian and pattern additional axes (e.g., dorsoventral) (for review, see [2, 3]). (**e**) The digestive system of the planarian is diagrammed, with the pharynx connecting to the intestine (green), which has one anterior primary branch and two posterior secondary branches. The intestine is composed of multiple cell types and is surrounded by enteric muscle [4, 5]. (**f**) The central nervous system of the planarian is diagrammed (for review, see [6]). Two ventral nerve cords connect with horseshoe-shaped cephalic ganglia which are also referred to as the planarian brain. Brain branches project outward from the cephalic ganglia to the edge of the planarian head

organ called a pharynx (Fig. 1e); muscle cells in many orientations throughout the body, which function to facilitate animal movement and produce signals for body patterning; a cephalized nervous system (Fig. 1f); osmoregulatory protonephridia; an epidermis, much of which is ciliated and promotes movement by gliding;

secretory organs that produce mucus; connective tissue called the parenchyma, within which stem cells are embedded; ovaries, testes, and other reproductive tissues; and other novel cell and tissue types still to be explored [4]. Even within these organ systems, an amazing degree of complexity is present. For example, the planarian nervous system is composed of dozens of neural cell types and glia, all arranged spatially within horseshoe-shaped cephalic ganglia that connect to two ventral nerve cords (Fig. 1f, [6]). Separate peripheral and pharyngeal nerve networks are also present.

As we have learned more about planarian regeneration and physiology, RNA interference has emerged as the most common tool with which to query gene function. Planarian biologists typically produce double-stranded RNA (dsRNA) either in bacteria or in vitro. dsRNA is administered to planarians by feeding, soaking, or injection to trigger RNAi [9–14]. This approach, often repeated several times, depending on the RNAi paradigm, causes a reduction in levels of the target mRNA that can range from nearly a 50% decrease to more than a 95% decrease [15]. The RNAi effect is even stronger in regenerated planarian tissues, which often experience a more penetrant reduction in mRNA [16]. The ease of performing RNAi in planarians allowed several screens of hundreds of genes within a single project (e.g., [15, 17]). Thus, this method is a powerful tool for medium-throughput analysis of gene function during whole-body regeneration and in the context of replacement of specific tissues or cell types after injury. In this chapter, I will outline typical methods for RNAi treatment by feeding of synthetic dsRNA in *Schmidtea mediterranea*. I will also present a range of possible approaches for assessment of regeneration downstream of RNAi.

Taken together, the following approaches can determine the extent to which regeneration occurs normally after gene perturbation by RNAi. dsRNA synthesis and feeding to achieve RNAi in planarians will be an accessible strategy for studies of regeneration, particularly those focused on whole-body regeneration, regeneration of complex organ systems or tissues, and brain regeneration in particular.

2 Materials

All solutions should be prepared with sterile, ultra-pure water, and stored at room temperature (RT) unless otherwise stated.

2.1 Template Preparation

1. RNase-free water (*see* **Note 2**).
2. 1 M Tris base, pH 9.5. Autoclave.
3. 10% Tween-20: 10% (v/v) Tween-20 in RNase-free water.
4. 1 M $MgCl_2$. Autoclave.

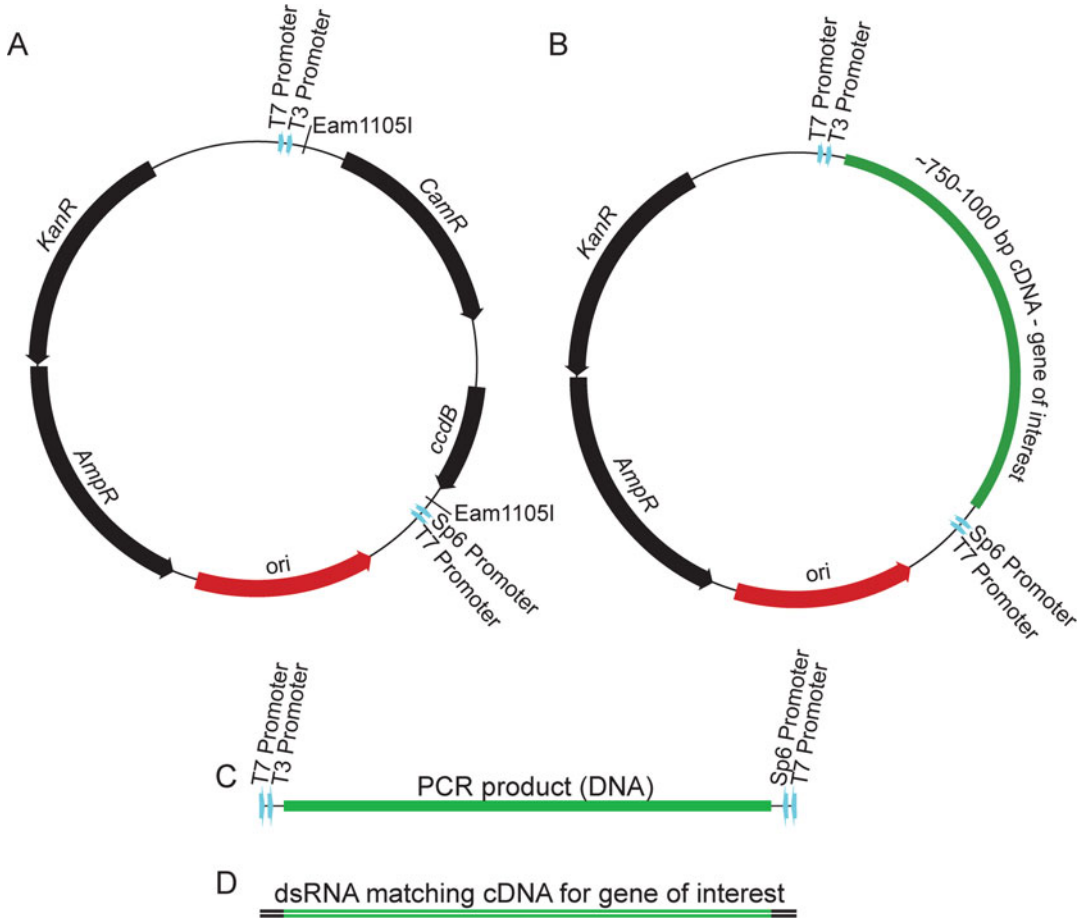


Fig. 2 Molecular strategy for dsRNA synthesis. (**a**) The pJC53.2 plasmid used for cloning upstream of dsRNA synthesis is pictured here [18]. This plasmid is digested with Eam1105I for TA cloning of PCR products generated from cDNA. (**b**) The resulting plasmids contain fragments of each gene of interest. These plasmids are subjected to PCR using a primer that recognizes the T7 promoter sequence to create an amplified product for each target and flanking promoters (**c**). The PCR products are used as a template for in vitro synthesis reactions using T7 RNA polymerase. Each in vitro synthesis reaction generates gene-specific dsRNA (**d**)

5. 35 mM $MgCl_2$. 35 μL 1 M $MgCl_2$ stock in 965 μL sterile water, aliquot 100 μL per tube and store at −20 °C.
6. 1 M ammonium sulfate. Sterile filter, aliquot 100 μL per tube and store at −20 °C.
7. Phosphoric acid (≥85%).
8. Hot start buffer: 500 μL 1 M Tris, pH 9.5, 100 μL 10% Tween-20 in 400 μL RNase-free water. Aliquot 200 μL per tube and store at −20 °C.
9. Hot start mix: 167 μL hot start buffer, 32 μL 1 M ammonium sulfate, 1 μL phosphoric acid. Made fresh or frozen only once.

10. 150–450 ng/μL template plasmid: ~750 bp target gene cloned from cDNA into the pJC53.2 vector. More on this vector can be found in [18]; cloning into this vector positions T7 polymerase sites on each side of the gene fragment (Fig. 2a, b, *see* **Note 3**).
11. dNTP mix: 10 mM dATP, 10 mM dGTP, 10 mM dCTP, 10 mM dTTP. Aliquot 100 μL and store at −20 °C.
12. 10 μM T7 primer: extended sequence GGATCCTAATAC GACTCACTATAGGG . Aliquot in 100 μL and store at −20 °C.
13. Recombinant Taq polymerase.
14. Kit for purification of polymerase chain reaction (PCR) products.
15. Gel electrophoresis setup: 1% (w/v) agarose gel, with ethidium bromide or equivalent, DNA ladder, loading dye, electrophoresis tank.
16. Thermocycler.
17. Gel imager.
18. Spectrophotometer.

2.2 dsRNA Synthesis

1. 1 M Tris base, pH 8.0. Autoclave.
2. 1 M spermidine. Filter sterilize, aliquot 500 μL per tube and store at −20 °C.
3. 1 M dithiothreitol (DTT). Filter sterilize, store at −20 °C.
4. 10× high yield transcription buffer: 4 mL 1 M Tris pH 8.0, 2 mL 1 M $MgCl_2$, 200 μL 1 M spermidine, 1 mL 1 M DTT, in 2.8 mL RNase-free water. Sterile filter, aliquot 200 μL per tube and store at −20 °C.
5. rNTP mix: 25 mM rATP, 25 mM rUTP, 25 mM rCTP, 25 mM rGTP. Aliquot 100 μL per tube and store at −20 °C.
6. T7 RNA polymerase.
7. Thermostable inorganic pyrophosphatase (TIPP) enzyme.
8. Ribonuclease (RNase) inhibitor (e.g., RNasin®).
9. Formaldehyde loading dye.
10. RNase-free DNase.
11. 5 M ammonium acetate. Prepare with RNase-free water. Sterile filter.
12. 100% ethanol.
13. 70% (v/v) ethanol. Prepare with RNase-free water.

2.3 RNAi Treatment and Amputation

1. ≥1 μg/μL purified dsRNA.
2. Planarians—*Schmidtea mediterranea*. 10 animals of 3–5 mm length per gene of interest, plus 10 animals of similar size for negative control.
3. 60–100 mm petri dishes.
4. Cafeteria trays.
5. 1x Montjuïc salts: 1.6 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl, 1.2 mM $NaHCO_3$, pH 7.5 with HCl or NaOH.
6. Large bulb, wide mouth transfer pipettes (e.g., 8.6 mL).
7. Disposable pellet pestles.
8. Planarian food (e.g., liver puree prepared as per [19]).
9. Green food coloring.
10. Filter paper.
11. Low-lint science wipes.
12. Scalpel.
13. Dissecting microscope.
14. 50 mg/mL gentamicin sulfate solution.

3 Methods

3.1 Template Preparation by PCR

The starting material for template preparation is ~750 bp of each target gene cloned from cDNA into pJC53.2 vector (Fig. 2a, b, *see* **Note 3**). Positive and negative controls should also be included (*see* **Note 4**).

1. Prepare one PCR per template to be amplified (*see* **Note 5**).
2. Combine 5 μL of the hot start mix solution and 5 μL of 35 mM $MgCl_2$ in each tube.
3. Incubate for 15 min at RT to precipitate the $MgCl_2$.
4. Add to each tube: 32.5 μL sterile water; 1 μL template plasmid diluted 1:30 in water; 4 μL 10 μM T7 primer, which will serve as both forward and reverse primers in this reaction; 1.5 μL 10 mM dNTPs; 1 μL Taq polymerase.
5. Incubate the reactions in the thermocycler for the following program: incubation for 5 min at 95 °C; 35 cycles of the following three steps—30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C; incubation for 5 min at 72 °C; and hold at 4 °C.
6. Load 1.5 μL of each reaction into an agarose gel.
7. Run the gel electrophoresis setup on 120 V for 45 min.

8. Image the gel on the gel imager following the manufacturer's instructions to check for amplification.
9. Identify PCR products as single strong bands of 750–800 bp (insert plus promoter sequences) (*see* Fig. 2c).
10. Purify PCR products using a DNA clean-up kit, as per the manufacturer's protocol, except elute in 20 μL RNase-free water (*see* **Note 6**).
11. Determine the concentration of PCR products using a spectrophotometer.
12. Dilute with sterile water to a concentration of 50–150 ng/μL for in vitro dsRNA synthesis.

3.2 In Vitro dsRNA Synthesis

The starting material for dsRNA synthesis is template DNA with T7 promoter sequences on each side generated in Subheading 3.1 (*see* **Note 7**). For all steps in this section of the protocol, use RNase-free materials, including RNase-free filter tips and RNase-free tubes.

1. For each template, combine in a 1.5-mL tube: 10.5 μL template DNA; 2 μL 10× high yield transcription buffer; 5 μL rNTP mix (25 mM each); 1 μL T7 RNA polymerase; 1 μL TIPP; 0.5 μL RNase inhibitor. Total volume will be 20 μL.
2. Incubate at 37 °C overnight (>5 h).
3. Add the following to each tube: 8 μL RNase-free water; 1 μL 10× high yield transcription buffer; and 1 μL RNase-free DNase.
4. Mix well.
5. Incubate at 37 °C for 15 min.
6. To precipitate each reaction, add the following to each tube: 70 μL RNase-free water; 100 μL 5 M ammonium acetate; 400 μL 100% ethanol.
7. Mix well by inverting.
8. Incubate the mixture at −20 °C for >1 h.
9. Centrifuge samples for 15 min at 4 °C and at maximum speed (~20,000 rcf). A pellet should appear at the bottom of each tube. The pellets should be large and glassy or white in appearance.
10. Remove the supernatant carefully by pipetting with RNase-free filter tips. Be careful not to disturb the pellet.
11. Discard supernatant.
12. Add 500 μL 70% ethanol to each tube (containing precipitated dsRNA). This wash will help to remove any additional materials from the synthesis reaction.
13. Cap and invert tubes two times to mix.

14. Centrifuge samples to pellet for 5 min at 4 °C and at maximum speed.
15. Remove and discard *all* supernatant from tubes by pipetting.
16. Allow pellets to air dry on a bench-top for 5 min, with tubes uncapped and covered with a science wipe.
17. Resuspend pellets in 30 μL RNase-free water.
18. Cap and flick tubes with a finger to dislodge the pellets into the water.
19. Allow resuspension of pellets at RT until they are dissolved completely.
20. Anneal dsRNA with the following sequence of incubations: 95 °C for 5 min; 75 °C for 5 min; 50 °C for 5 min.
21. Allow to cool to RT for 5 min. The product will be well-annealed dsRNA (Fig. 2d).
22. Dilute each sample 1:10 by combining 1 μL of dsRNA with 9 μL RNase-free water.
23. Mix 1 μL of each 1:10 dilution with 8 μL RNase-free water and 1 μL of loading dye.
24. Run out these mixtures on a 1% agarose gel with a known volume of DNA ladder.
25. Image with a gel imager. Each dsRNA reaction should run as a clear band at approximately the expected molecular weight, with no degradation products smaller than the band. Occasionally, higher molecular-weight bands will be present; these are dsRNA with more complex secondary structure.
26. Using the ladder as a reference, estimate the concentration for each dsRNA sample (*see* **Note 8**).
27. Once purified, dsRNA can be stored at −20 °C for up to several months.

3.3 dsRNA Feeding for RNA Interference (RNAi)

Determine the design for the RNAi experiment. The time course frequently used in my laboratory is to complete three feedings every 5 days with 3–5 μg dsRNA per feeding for 10 planarians (Fig. 3a). We wait for 7 days after the last feeding and amputate animals pre-pharyngeally. After amputation, we wait for 7 days until observing or fixing animals for assessment of regeneration as detailed below. Other feeding paradigms, injection paradigms, dsRNA doses, and amputation strategies may be used (Fig. 3b–e, *see* **Note 9**).

1. Line a cafeteria tray with paper towels to absorb spills.
2. For each gene of interest, half-fill one Petri dish with 1x Montjuïc salts.

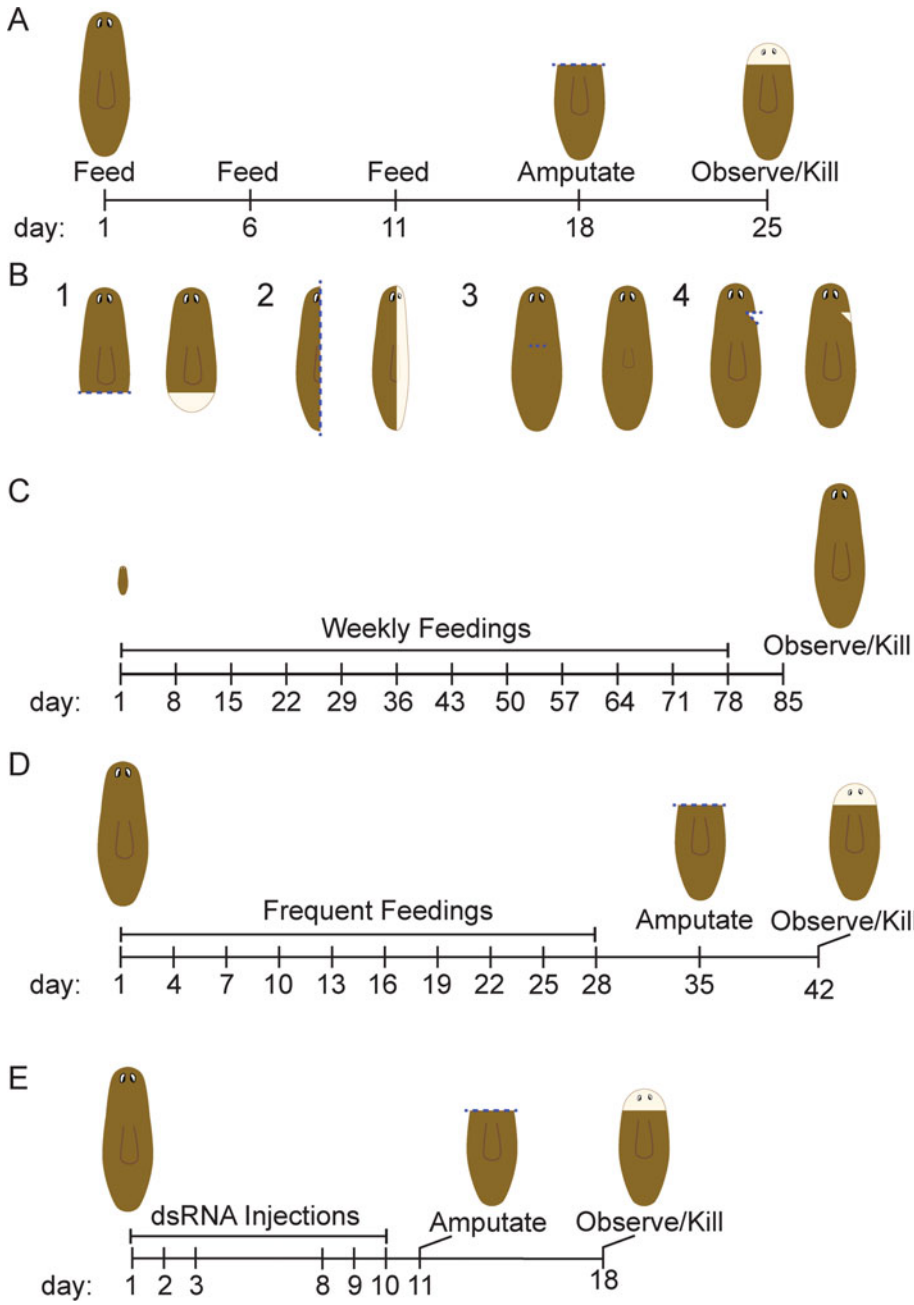


Fig. 3 Paradigms for RNAi. (**a**) The typical strategy used in my laboratory for dsRNA feedings and amputation is shown here. Planarians receive three feedings of dsRNA over the course of ~11 days. Pre-pharyngeal amputation occurs approximately 1 week after the final feeding and animals are observed for head or brain regeneration 1 week after amputation. (**b**) Additional amputation strategies are presented. Amputation is indicated with a dashed line on the left image of each pair and regeneration is diagrammed in the right image of each pair, with blastema tissue shown in a lighter color. (1) Animals can be amputated post-pharyngeally to observe tail regeneration. (2) Sagittal amputation can be used to observe regeneration of lateral structures and reestablishment of mediolateral patterning. (3) Chemical amputation can be used to remove the pharynx to observe pharyngeal regeneration [20]. (4) Other excisions can be made to determine local wound responses after minor injuries. (**c**) A long-term RNAi strategy with weekly dsRNA feedings can be used to determine

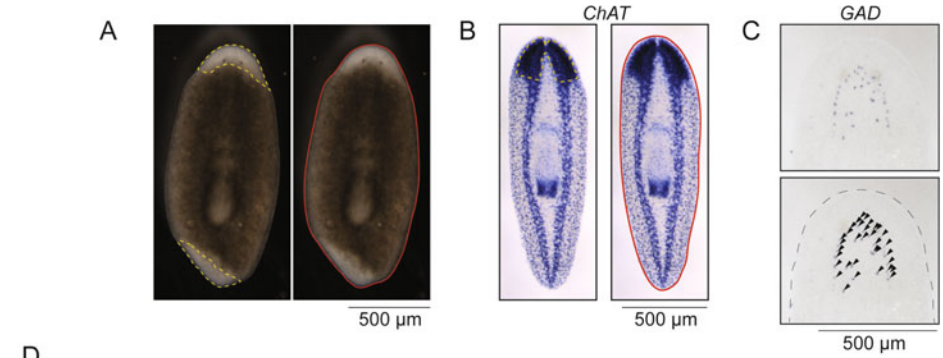
3. Include dishes for positive and negative controls (*see* **Notes 4** and **10**).
4. Label both the tray and each petri dish with experimental (tray) and sample (dish) information.
5. Add planarians to each dish. A 60-mm petri dish will fit 10 asexual planarians or a 100-mm deep-well petri dish can be used for 30 asexual planarians. Choose planarians that are ~5 mm long and that have been starved for ~1 week before the experiment. Details that follow are appropriate for experiments using 10 planarians.
6. In a 1.5-mL tube, mix 100 μL 1× Montjuïc salts per 400 μL planarian food.
7. Using a disposable pestle, mix the food and salts until the combination reaches a uniform consistency.
8. Pulse the tube in a centrifuge to pellet large pieces of tissue.
9. For each target of interest, aliquot 3–5 μg of purified dsRNA into a separate, labeled tube.
10. Add 1 μL green food coloring to each sample of dsRNA (*see* **Note 11**).
11. Pipet 30 μL of food mixture into each dsRNA-containing tube using a 200-μL pipette tip with the end cut off.
12. Using the same tip, stir until the food and coloring are evenly mixed.
13. Using the same tip, pipet the colored food and dsRNA mixture onto the bottom of a petri dish. The food mix should stay together well with a paste-like consistency and should not diffuse.
14. Repeat **steps 9–13** for each subsequent dsRNA sample.
15. Cover petri dishes with lids and cover the tray with a second tray or aluminum foil.
16. Allow planarians to eat for 1.5–2 h in near darkness. Planarians should appear green under a microscope after feeding, due to the food dye within their intestines.
17. Remove excess liver to a waste container using a transfer pipette.

Fig. 3 (continued) whether genes are required for growth or tissue maintenance under homeostatic (non-injury) conditions. (**d**) Frequent feeding strategies can be used to increase the efficiency of gene knockdown by RNAi and to improve phenotype penetrance. (**e**) Injection strategies can be used instead of or in addition to dsRNA feeding [13]. Though this strategy is more time-consuming, it can be especially valuable when gene knockdown interferes with proper feeding of the planarians

18. Using Montjuïc salts in a squirt bottle and a fresh transfer pipette for each plate, wash animals gently but thoroughly three times in the bottom of the petri dish.
19. Using the squirt bottle, add salts and pour to transfer the worms to the lid of the petri dish for three more washes.
20. After washes, transfer the planarians to fresh, labeled petri dishes half-filled with Montjuïc salts.
21. Incubate planarians in the dark at a constant, cool temperature (recommended incubation at 18 °C).
22. Wait for 5 days before feeding planarians.
23. Repeat **steps 6–22** two more times to induce a robust RNAi phenotype.
24. For paradigms that involve regeneration, amputation should occur 7 days after the last feeding.
25. Place two-folded science wipes on a solid metal block and then place one piece of filter paper on top of the science wipes.
26. Wet this setup until damp with Montjuïc salts.
27. Place this setup under a dissecting microscope to more precisely amputate the planarians.
28. Using a transfer pipette with a wide tip, transfer planarians from the petri dish onto the filter paper.
29. During the time in which planarians are on the filter paper, gently spray with Montjuïc salts as needed to keep planarians damp.
30. For head regeneration studies, use a clean scalpel to amputate planarians approximately 1/3 of the length of the body away from the tip of the head. This will remove the entire brain of the planarian (Fig. 3a).
31. Once all planarians from this dish are amputated, use a spray bottle of Montjuïc salts to rinse amputated pieces from the filter paper into a fresh petri dish.
32. Remove any unwanted pieces.
33. Fill the new petri dish half-full with 1x Montjuïc salts.
34. Repeat **steps 28–33** for each petri dish in the experiment.
35. Add gentamicin solution 1:1000 to prevent any bacterial growth.
36. Incubate planarians in the dark at a constant, cool temperature (18 °C) for regeneration.

3.4 Strategies for Assessing Regeneration After RNAi

Performing RNAi for 10 or more animals per sample is usually sufficient to assess whether significant differences exist between control and experimental RNAi-treated animals. After RNAi treatment and amputation, one or more of the following approaches may be used to assess and quantify regeneration: blastema



D

Cell type	Immunofluorescence	*in situ* hybridization
Stem cells	α-SMEDWI-1 ***(25)***, α-H3P(S10) ***(26)***	*smedwi-1* ***(30)***
Neurons (broad)	α-synapsin ***(22)***	*ChAT* ***(20)***, *PC2* ***(21)***, *nAChR*
Neural subsets	α-beta arrestin ***(40)***, α-5-HT ***(22)***	*gpas* ***(43)***, *TH* ***(44)***, *GAD* ***(35)***, *cintillo* ***(45)***, *CNG1* ***(13)***, *ppp-2* ***(16)***
Intestine	3G9 ***(46)***, 3F11 ***(24)***	*npc2*, *ctsla*, *slc22a6* ***(47)***, *dd2841*
Muscle	6G10 ***(23)***, 2G3 ***(23)***, 2H3 ***(24)***	*mhc* ***(48)***, *collagen* ***(49)***
Protonephridia	α-acetylated tubulin ***(50)***, 5B1 ***(23)***	*smedinx-10* ***(51)***, *cavII-1* ***(52)***, *rootletin* ***(53)***
Germ cells, etc.		*nanos* ***(54)***, *gH4* ***(54)***, *dmd-1* ***(55)***
Epidermis	3H3, 1E12 ***(24)***	*vim-1*, *laminB* ***(56)***, *rootletin* ***(57)***
Other cell types	α-IF-1 (glia) ***(58)***	*laminin* (pharynx) ***(31)***, *estrella* (glia) ***(13)***, *F-spondin* (parench) ***(13)***, many novel types ***(59-60)***

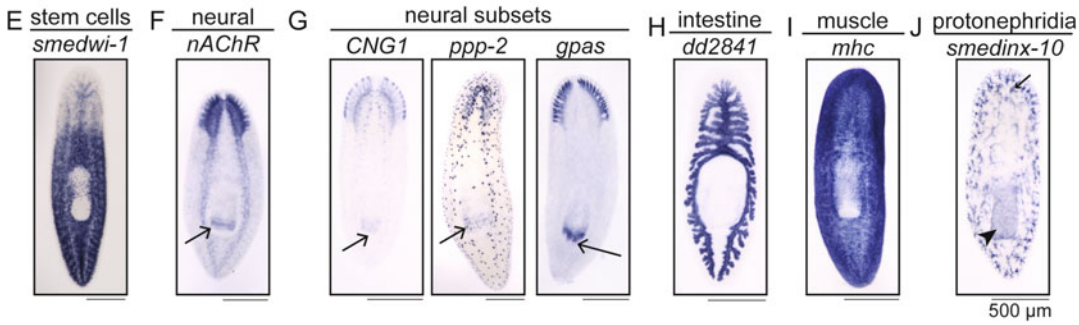


Fig. 4 Assessing regeneration phenotypes. (**a**) Blastema size can be measured by outlining the blastema (yellow dashed line) and measuring blastema area in ImageJ [30]. The body size can similarly be measured (red line). By dividing blastema size by body size to normalize for variable animal size, the resulting value can be compared across populations and RNAi treatments. (**b**) Similarly, I use in situ hybridization with a *choline acetyltransferase* (*ChAT*) riboprobe [23] to stain the central nervous system for measurement of brain size after regeneration (yellow dashed line). Brain size can also be normalized to body size (red line) for comparison of brain regeneration across RNAi treatments. (**c**) Some cell types are present in numbers low enough that they can be accurately counted, like cells expressing *glutamic acid decarboxylase* (*GAD*) [31]. Counting these cell types for regenerated animals following RNAi treatment would allow determination of genes that affect regeneration of GAD^+ cells. In the image shown, 34 cells are labeled (arrowheads). (**d**) This table lists some available antibodies and examples of riboprobes that can be used for staining diverse cell types or organ

measurement (*see* **Note 12**); in situ hybridization to examine specific organs or cell types ([21, 22], *see* **Note 13**); immunofluorescence to detect cell types or tissue regeneration ([23–29], *see* **Note 14**); reverse transcription and quantitative PCR (RT-qPCR) to examine gene expression (*see* **Notes 15** and **16**); functional assessment including behavioral assays (*see* **Note 17**). Potential data from these types of experiments are shown in Fig. 4.

4 Notes

1. This methods chapter describes the approach used in my laboratory to perform RNAi using the species *Schmidtea mediterranea*. RNAi has also been used to query gene function in other freshwater planarians including *Dugesia japonica* [11], *Procotyla fluviatilis* [52], *Phagocata kawakatsui* [53], and *Dendrocoelem lacteum* [54].
2. I use diethyl pyrocarbonate (DEPC) treatment as per the manufacturer's instructions to generate RNase-free water, but it can also be purchased directly.
3. The pJC53.2 vector was designed for TA cloning of cDNA fragments [18] (Fig. 2a, b). Cloning is performed to ligate PCR products amplified from cDNA into pJC53.2 which has been cut by Eam1105I. This strategy positions inserts so that they have T7 promoter sites on 5′ and 3′ ends to facilitate dsRNA synthesis. This vector also has an Sp6 promoter on one side of the insert and a T3 promoter on the other side of the insert. By cloning into this vector, one can use the same cloned fragment for dsRNA synthesis and for synthesis of antisense riboprobe for in situ hybridization. This vector is available from Addgene (plasmid #26536).
4. Prior to cloning, the pJC53.2 vector has two bacterial genes in the insert region, *ccdB* and *camR*. Thus, undigested pJC53.2 can be used to synthesize a dsRNA product that does not match planarian genes and can be used as a negative control. In my laboratory, we also routinely use *Aequoria green fluorescent protein* (*GFP*) cloned into pJC53.2 as a template for a

Fig. 4 (continued) systems [23–29, 32–51]. These approaches may be used to determine whether regeneration proceeds normally after RNAi, including the shape of organs and the renewed expression of key markers. (**e–j**) In situ hybridization examples are presented. These expression patterns could be used to explore regeneration of the following cell types, structures, and organs: stem cells (*smedwi-1* [32]); neurons (*nAChR*, *dd_Smed_v6_8058_0_1*); subsets of neurons (*CNG1* [15], *ppp-2* [18]) including brain branches (*gpas* [34]); the intestine (*dd_Smed_v6_2841_0_1*); muscle (*mhc* [39]); and protonephridia (*smedinx-10* [42]). Note some neural staining in the pharynx (arrows in **f** and **g**). *smedinx-10* also stains cells in the pharynx (arrowhead in **j**) and pigment cups of the eyespots (small arrow in **j**)

negative control. In parallel, for a positive control, I recommend that dsRNA be generated from a fragment of *Smedwi-2* or another gene for which RNAi produces a known phenotype. *Smedwi-2(RNAi)* causes lysis and death of the planarians in a short period of time [32]. Observation of this phenotype can help ensure that synthesis and dosage are appropriate and consistent.

5. I use a Hot Start PCR protocol for template amplification, but these steps could be exchanged for many other PCR protocols.
6. My laboratory uses Zymo Clean and Concentrator kits, but other kits or methods for DNA purification can be substituted. Take care to remove all ethanol during the purification of DNA products, as it can inhibit in vitro synthesis of RNA. For some kits, an extra drying spin after the wash steps can help to remove extra ethanol that is present in the wash buffer.
7. This protocol has been adapted from a previously published protocol for in vitro synthesis of dsRNA [12]. In this work, the authors also present helpful data on the effective timing and concentration for RNAi administered by dsRNA feeding.
8. Though a spectrophotometer can be used to estimate dsRNA concentration, I find that this approach typically overestimates the concentration of dsRNA. A falsely high spectrophotometer reading could be caused by residual rNTPs that are precipitated or retained during the purification steps. Determining dsRNA concentration based on the intensity of the band leads to more reproducible gene knockdown. For an example of this approach, if one compares a 1:10 dsRNA band and find that it is similar in intensity to 100 ng of the 1 kb band in the ladder, then one can estimate that our dsRNA concentration (undiluted) is ~1 μg/μL.
9. Here, I provide notes on experimental design for RNAi experiments. Three main variables must be chosen for RNAi experiments: dosage, frequency/duration of feeding, and method of dsRNA administration (injection vs. feeding). Our main experimental design is presented in the Methods section of this chapter (Fig. 3a). This paradigm is appropriate for most screening and assessment of gene effects on regeneration. Alternative amputation strategies to examine tail regeneration, lateral regeneration, pharyngeal regeneration [20], or regeneration after minor injuries are diagrammed (Fig. 3b). To observe the effects of long-term RNAi without amputation, I typically use a weekly feeding paradigm (Fig. 3c). Other approaches, including frequent feedings and injection (Fig. 3d, e), have been used for successful knockdown of genes of interest (e.g., [55] for frequent feedings, [56] for injections). Injection might be a preferred strategy in situations where a phenotype occurs

quickly or when the phenotype prevents feeding (e.g., paralysis, loss of pharynx).

10. Here, I provide notes for setup of RNAi experiments. Though cafeteria trays are convenient for organization, dropping a tray can be devastating. Be careful when transporting RNAi experiments and be cautious around other lab members when they are moving trays. I prefer deep-well 100-mm petri dishes for our bigger (30 worms) or long-term RNAi experiments *or* when I expect to use larger (1 cm) asexual planarians or sexual planarians. For especially large worms or large RNAi experiments, small Ziploc® containers may be used instead of petri dishes. The number of planarians per dish as well as animal size can be optimized for atypical RNAi paradigms. For example, I start with very small (<2 mm) worms for long-term experiments to avoid fissioning.
11. Though 3–5 μg per feeding (final concentration in liver mix of ~0.1–0.2 μg/μL) is sufficient for most of our experiments, the range of concentrations used in the planarian field is *very* broad. Concentration, as well as animal size and feeding paradigm, can impact the penetrance and speed of RNAi phenotypes [12]. In some experiments, I have combined knockdown of several genes to investigate whether genes work together or oppose one another (e.g., *activin* and *follistatin* [57]). In these experiments, I standardize both concentration and total mass of dsRNA across control, single knockdown, and double knockdown conditions.
12. Animals may be killed and fixed for blastema measurement (Fig. 4a) or observation of eyespots. Initially, regenerating tissue is unpigmented, which allows for the newly regenerated tissue within the blastema to be clearly observed and measured.
13. Animals may be killed and fixed for in situ hybridization using a riboprobe to mark specific organs or cell types [21, 22]. This allows one to assess the regeneration of a specific cell or tissue type (Fig. 4b–j). For example, in situ hybridization with *choline acetyltransferase* (*ChAT*) [23] marks cholinergic neurons and can be used to broadly mark the brain to measure brain size after regeneration [15] (Fig. 4b). Alternatively, with some in situ hybridization staining (e.g., *glutamic acid decarboxylase*, *GAD* [31]), cells can be counted (Fig. 4c).
14. Animals may be killed and fixed for immunofluorescence experiments to assess organ regeneration (Fig. 4d) [23–27]. Immunofluorescence is also useful for examining proteins that localize differently than their mRNA; for example, *smedwi-1* is present in dividing stem cells, but SMEDWI-1 protein is present in differentiating stem cells as well [28]. Immunofluorescence can also be used to detect protein

modifications. For example, histone H3 phosphorylated at Serine 10 is a marker of mitotic stem cells [29].

15. Animals may be killed and processed to purify mRNAs for downstream reverse transcription and quantitative PCR (RT-qPCR). This approach can be used to determine the effectiveness of RNAi knockdown or the effect of knockdown on other tissues using cell type-specific target genes (*see* **Note 12**, and for one example [15]).
16. When choosing primers for assessment of mRNA knockdown by RT-qPCR, it is best to choose a pair of primers that will recognize the target mRNA but not the dsRNA that is administered for RNAi. In some instances, the knockdown efficiency has seemed poor despite a strong phenotype, potentially due to a primer pair binding to and amplifying fragments of cDNA generated from both mRNA and dsRNA.
17. Animals may be subjected to functional analyses. For example, behavior could be assessed in a feeding assay [5, 15, 20, 58], which could give an indication of whether neural, intestinal, and/or pharyngeal regeneration has been achieved. Live imaging to assess movement or response to stimuli (touch, light, temperature) can indicate whether neural function and muscle function are normal after regeneration [33, 59, 60].

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