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Establishing and Maintaining a Colony of Planarians

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INTRODUCTION

To provide sufficient material for experimentation, a laboratory needs to expand and maintain a colony of planarians. It is crucial to keep a stable, healthy population of animals in a consistent environment to avoid inter-animal variability and modifier effects that can mask true phenotypes from experimental perturbation. In this protocol, we describe basic procedures for establishing and maintaining healthy colonies of *Dugesia japonica*, *Schmidtea mediterranea*, and *Girardia tigrina* (commonly found in the wild and commercially available in the United States). Although the recommendations are based on our optimization of conditions for *G. tigrina*, many of the procedures (such as food preparation and feeding strategy) can be applied to other species. For best results, the culture water must be carefully monitored and adjusted for each species.

RELATED INFORMATION

For an introduction to planarians as a model system, see **Planarians: A Versatile and Powerful Model System for Molecular Studies of Regeneration, Adult Stem Cell Regulation, Aging, and Behavior** (Oviedo et al. 2008a). Protocols for **Gene Knockdown in Planarians Using RNA Interference** (Oviedo et al. 2008b) and **Live Imaging of Planarian Membrane Potential Using DiBAC$_4$(3)** (Oviedo et al. 2008c) are also available.

METHOD

Obtaining Planarians

Planarians may be found in the wild—in ponds and streams (usually under rocks or attached to other surfaces)—or ordered from commercial sources (Table 1). There are no commercial suppliers for either *S. mediterranea* or *D. japonica* at this time; stocks may be obtained from research labs working with the desired species (Table 1). Currently, we know of no commercial supplier that maintains its own stock of *G. tigrina*, so acquiring these may mean obtaining planarians that were recently in the wild.

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Planarians may be sent by overnight mail when the temperature is mild enough not to harm the worms; above freezing and below 25°C is recommended. A cold pack and insulation may also help to keep the temperature down in the shipping container, if necessary. For shipping, it is very important that the worms be placed in containers completely filled with water, leaving no air bubbles; this will prevent sloshing, which can kill planarians. The oxygen already dissolved in the water should be sufficient for 1–2 d. Upon receipt of planarians from a commercial source, immediately replace at least half of the water in which the animals were transported (be careful not to shock them with too great a temperature change), and completely change the water by no later than the next day. Water that arrives with worms is typically quite toxic with ammonia and other metabolic byproducts and is extremely low in oxygen. In our experience, a gradual change of water upon arrival renders better results for clonal lines of *S. mediterranea* and *D. japonica* (e.g., reduce to 75%, 50%, and 25% every 2 d, until worms are in 100% fresh appropriate medium).

Culture Conditions

Media—The aqueous medium in which each species lives differs slightly. In each case, the medium should be freshly prepared (e.g., weekly) because the pH tends to drift and salts may precipitate with time. The conductivity of our water after buffering is ~450 μS. Water is typically changed every 2 to 5 d when the planarian containers are cleaned (see Steps 13–20). Planarian cultures should be virtually odorless (if they are not, see Troubleshooting).

For *S. mediterranea*, use the recipe for 1X Montjuïch salts published by Cebrià and Newmark (2005). For *G. tigrina*, adapt the recipe for 1X Montjuïch salts by omitting the $NaHCO_3$, and, instead, use aquarium buffers to bring the water to a pH of 7.5. Alternatively, use Poland Spring water (which gives better results than several other types of spring water) and adjust the pH in the same way. If Poland Spring is unavailable, another brand of bottled water may be tried, although some trial and error may be necessary to locate one with adequate characteristics. In either case, use an alkaline buffer:acid buffer ratio of 12.5:1 (~2.5 g of alkaline buffer and 0.2 g of acid buffer in 11 L of water) to adjust the pH. These numbers are only approximate because water from different sources and even from the same source in different seasons varies slightly in ion composition. The pH will drift upward over time but will not exceed the upper limit of *G. tigrina*'s tolerance, even if the water is not changed for 1 wk. A pH range between 7.5 and 9.5 is tolerated by *G. tigrina*. For *D. japonica*, Poland Spring water may be used alone, or the pH may be raised slightly, to as high as 7.5, using the above buffers.

Containers—Worms thrive in plastic containers (e.g., ~2000-mL capacity with ~1500 mL of culture water). To remove the manufacturing residue from new containers, soak them overnight in pure water (without soap), and then thoroughly wipe them clean. The containers should be covered, but significant air circulation should be ensured. It is important to control the number of animals per container (e.g., keep between 400 and 700 worms in each 2000-mL container). Too many worms can induce stressful situations, leading to infections by opportunistic pathogens (see "Troubleshooting").

Temperature—Planarian colonies are kept in an incubator or in a temperature-controlled light cabinet at a temperature of between 17°C and 20°C. However, all three species discussed here can tolerate a variety of temperatures. *G. tigrina* have been found at water temperatures ranging from 3°C to 31°C in the wild (Stokely et al. 1965). Higher temperatures (~25°C) are acceptable to planarians, but this warmer environment encourages bacterial growth, and infections are more likely. Infections are particularly a concern in experimental animals that have been cut; regenerating worms may have higher mortality rates when kept in warmer environments.

Dissolved Oxygen—Dissolved oxygen (DO) is an important factor in worm health, but as long as planarians are kept at an appropriate temperature in boxes that have a broad water surface and reasonable air circulation, sufficient oxygen should be incorporated into the water to avoid the need for manual circulation or aeration. Our DO for *G. tigrina* normally reads between 7.6 and 8.4 ppm using a LaMotte DO test kit; fluctuation of ±0.5 ppm during the course of a day is typical. DO may be checked sporadically or when abnormalities in the colony are observed.

Light/Dark Cycle—Planarians are nocturnal, and *S. mediterranea* and *D. japonica* are best maintained in dark environments (although they are exposed to light during feeding and cleaning). Worms (of any of the above-mentioned species) used in behavioral experiments are typically kept on a 12-h light/12-h dark cycle that helps to synchronize their peak activity time, thereby benefiting many types of training trials. If possible, learning trials on *G. tigrina* should be conducted during the worms' night cycle. The worms can be sluggish during the day, and this creates problems in obtaining consistent responses for behavioral work. We have compared the behavior of *G. tigrina* at the beginning of their night cycle versus midday, and a definite difference was observed (C. Nicolas, unpubl.).

Worms used in behavioral studies have also been kept in dark cabinets, exposed to light only for feeding and cleaning, with no apparent ill effects. These worms have an increased aversion to light, which may be useful in some circumstances.

Food Preparation and Allocation

Preparation of Beef Liver Paste—This method of preparation ensures that the liver will sink to the bottom of the dish, where the worms can consume it.

1. Place fresh organic beef liver on a chilled cutting surface.
2. Use a scalpel to cut away all visible veins and connective tissue (including the connective tissue of the capsule) as well as any fatty inclusions.
3. Cut the liver into small pieces (1-in. cubes). If you are cutting a significant amount of liver, make sure to collect the processed liver in a chilled and covered container that is protected from the air while you work.
4. Use a blender to purée the beef liver.
5. Strain the puréed liver to remove any remaining connective tissue.

6. A standard kitchen strainer can be used; only gentle pushing through the strainer should be used to avoid introducing connective tissue into the filtrate.
7. Centrifuge the liver paste at 4000 rpm to remove all air bubbles.
8. Five minutes at 4°C in a Sorvall RC-5B Refrigerated Superspeed Centrifuge is sufficient.
9. Aliquot the liver paste into 35-mm Petri dishes (filling each dish), being careful to avoid introducing new air bubbles.
10. Freeze individual aliquots at −80°C.

Feeding—Feed planarians once a week. Before any experiment, starve them for 7–15 d. This fasting period provides a more uniform metabolic status, thereby minimizing variability in data.

11. Thaw an aliquot of prepared liver paste (from Step 8).
12. Drop into the colony box as much liver as the colony can consume in 2 h (~2 mL per 2000-mL container with ~400–700 worms). Make sure that liver paste sinks to the bottom; floating food will not be eaten.
13. After 1 or 2 h, carefully remove any uneaten food with a pipette. Proceed immediately to Steps 12–17.

Cleaning—Planarian containers must be cleaned immediately after the feeding period. In addition, all species must have a second cleaning 2 d after feeding to remove metabolic debris and to prevent the water quality from degrading.

14. Gently agitate the water with a pipette to force any planarians from the surface to the bottom of the container.
15. Pour off all of the old water. If necessary, use the pipette to wash the worms back down to the bottom of the box as you pour off the water.
16. Add a small amount of water to the container (do not add any detergents or chemicals to the water). Use this water to rinse the sides and the bottom of the container and to wash the planarians down to one corner.
17. Pour off the rinse water.
18. Using paper towels, wipe out any mucus and debris attached to the walls and the bottom of the container.
19. Refill the container with fresh medium (prepared as described above in “Media,” using H_2O for planarian culture or bottled H_2O).

Reproduction

A choice may be made to keep either clonal colonies, where all worms derive from one original worm, or more genetically varied colonies. If clonal colonies are desired, expect one large *G. tigrina* to yield ~ 40 worms after 6 mo of weekly feedings. Both *S. mediterranea*

and *D. japonica* reproduce faster, doubling every ~2–3 wk. Although happy (and well-fed) worms will spontaneously reproduce by fissioning, cutting may also be used to accelerate the process of populating the colony with genetically similar worms.

TROUBLESHOOTING

Problem:

The planarians are lying limply on the bottom of the box, or a gentle tap on the box sends a large number of worms cascading off the sides of the box to land limply at the bottom. They may be scrunched up, looking ruffled around the edges rather than smooth. In advanced cases, they may lie on their sides curled into a “C” shape.

Solution:

Under normal conditions, ~50% of our worms will be on the sides of the box at any given time. If the worms appear stressed, consider the following:

1. Check the water quality. If the ammonia level of the water is too high, the dissolved oxygen content is too low, or the pH is too high or too low, change the culture medium immediately. Worms should recover from most water-quality problems by the next day or so.
2. The container may be overcrowded. Split the colony.

Problem:

Animals have white or black lesions on the dorsal side, or they are losing tissue at the anterior end. The lesions may be visible under a dissecting scope. At higher magnifications (~200X), protozoans may be seen clustered around the open wounds of the planarians. There may be a bad odor coming from the water.

Solution:

An infestation by protozoa is not uncommon, but it can lead to a secondary bacterial infection that can devastate a colony. The symptoms may vary from worm to worm; some succumb sooner. Worms that are feeling unwell for any reason will not eat until they are recovered from the incident. Because uneaten food can provide sustenance for bacteria, we do not feed worms suffering from either water-quality problems or microorganism attacks until the issue has been resolved. Remove any sick animals with abnormal behavior, and then treat the culture for microorganism attacks as described below. Keeping containers with sick worms and materials (e.g., pipettes) away from healthy animals is safe practice.

1. The antibiotics metronidazole (3 mg/L) and gentamicin (50 μg/mL) have been used to treat *G. tigrina* and are well tolerated, but are only marginally effective because they target the secondary bacterial infections and not the original problem. If antibiotic treatment is used, remember to bring the pH of the culture water back up to 7.5 after mixing in the antibiotics. Otherwise, these drugs can drop the pH low enough to harm the planarians.

2. The most effective treatment for infections in *G. tigrina* colonies is not chemical: Simply chill the colonies of worms overnight down to 10°C. Then, after slowly warming them back up to 18°C the next day, clean the containers thoroughly. Daily water changes during the next week are helpful in speeding the recovery of this species. Lost tissues should be regenerated shortly.

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MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with **<!>**, and recipes for reagents marked with **<R>**.

Reagents

Aquarium buffers (acid and alkaline; e.g., Seachem)

Beef liver (organic, very fresh)

<R>H_2O for planarian culture or bottled H_2O (Poland Spring)

Poland Spring H_2O gives better results than other types of bottled H_2O. Alternatively, H_2O for planarian culture can be prepared.

<R>Montjuïch salts (1X) (Cebrià and Newmark 2005) Planarians

Equipment

Blender

Centrifuge (e.g., Sorvall RC-5B Refrigerated Superspeed Centrifuge; DuPont)

Container (covered and chilled, for collecting processed liver) (optional; see Step 3)

Cutting surface (chilled)

Dissolved oxygen test kit (e.g., LaMotte)

Incubator or temperature-controlled light cabinet (preset to 17°C-20°C)

Paper towels (unbleached)

Petri dishes (35 mm)

Plastic containers (food grade; e.g., Rubbermaid 1.8-L rectangular boxes)

Scalpel

Strainer

Transfer pipettes (disposable)

Table 1.

Sources of planarians

Laboratories conducting research with planarians:	
Kiyokazu Agata	http://mdb.biophys.kyoto-u.ac.jp/index_E.html
Takashi Gojobori	http://www.cib.nig.ac.jp/dda/en/index.html
Brenton Graveley	http://genetics.uchc.edu/Graveley/Welcome.html
Michael Levin	http://www.drmichaellevin.org
Nico Michiels	http://www.uni-tuebingen.de/evoeco
Phillip Newmark	http://www.life.uiuc.edu/newmark
Robert Raffa	http://www.temple.edu/pharmacy/faculty_Raffa_Research.htm
Peter Reddien	http://inside.wi.mit.edu/reddien/pub/PWR_website/Rddn_home.html
Leonardo Rossi	http://www.unipi.it/english/university/index.htm
Emili Saló	http://planarian.bio.ub.es
Alejandro Sánchez Alvarado	http://planaria.neuro.utah.edu
Eva-Maria Schoetz	http://genomics.princeton.edu/schoetzlab/index.html
Ronald Vale	http://valelab.ucsf.edu
Ricardo Zayas	http://www.bio.sdsu.edu/cmb/research.html
Commercial sources of planarians:	
Carolina Biological Supply Company	http://www.carolina.com
WARD'S Natural Science	http://www.wardsci.com