

Protocol 1: Planarian maintenance

1.1. Planarian housing

Planarians are shipped by Carolina Biological Supply Company or by Ward Scientific inside a plastic container filled with water. For a short-term maintenance (< 4 weeks) the worms can be kept in this water without feedings and water changes (Table 1). For medium- and long-term storage (> 4 weeks) the worms can be maintained in 0.5 g/l Instant Ocean[®] Sea Salt (Spectrum Brand) in distilled water (King and Newmark, 2013) or in 1x Montjuïc water (1.6 mmol/l NaCl, 1.0 mmol/l CaCl₂, 1.0 mmol/l MgSO₄, 0.1 mmol/l MgCl₂, 0.1 mmol/l KCl and 1.2 mmol/l NaHCO₃ in Milli-Q water, pH 6.9-8.1) (Cebrià and Newmark, 2005) (Table 1).

All four planarian species can be maintained at approximately room temperature (RT) (18-22°C). The worms can be housed in plastic storage containers (Ziploc[®] Brand Containers) or in Petri dishes.

Because each 6-8 mm planarian requires 1 ml of water, we can place about:

- 5 worms in Small Petri dish (35 x 10 mm, 5 ml)
- 10 worms in Medium Petri dish (60 x 15 mm, 11 ml)
- 50 worms in Big Petri dish (100 x 15 mm, 50 ml)
- 100 worms in Extra Small Bowl Ziploc[®] (9 cm diameter x 5 cm depth, 150 ml)
- 500 worms in Small Square Ziploc[®] (12 cm x 12 cm x 6 cm, 600 ml)
- 1000 worms in Large Rectangular Ziploc[®] (15 cm x 26 cm x 8 cm, 1300 ml)

Planarians can be transferred from one container to another using plastic Pasteur pipettes with openings big enough to accommodate the worms without injuring them. In case the opening of the Pasteur pipette is not big enough, the pipette tip can be cut with

scissors. Once the worms are settled, the lids are placed on the storage containers or the dishes without being hermetically closed in order to reduce the evaporation and worms escaping. It is important, do not completely seal the Ziploc containers, as the lack of oxygen will kill your planarians.

If the planarians are not healthy, they show lesions on the dorsal side of their body (as showed in Fig. 3 of the paper “Hands-on, classroom studies of regeneration, and stem cell biology using freshwater planarians”) the speedy removal of the sick individuals from the culture reduces the risk of losing all the animals.

REQUIRED MATERIALS 1.1:

Instant Ocean[®] Sea Salt or 1x Montjuïc water (for medium- and long-term storage)

Plastic storage containers or Petri dishes

Plastic Pasteur pipettes

1.2. Water changing

Long-term planarian maintenance requires water changes twice a week, but the culture can be maintained for a few months, replacing the water only once a week (Table 1).

The water is usually changed two days after feeding in order to remove excrement.

The protocol for the water change is:

1. Fill a squeeze wash bottle or a standard bottle with fresh planarian water
2. Slowly pour off the dirty planarian water into a waste container
3. Using the squeeze wash bottle (or a transfer pipette), rinse the worms, pushing them to one corner of the container/dish

4. Use paper towels or lab wiper KimWipes™ (KimTech Science) to wipe mucus off of the exposed area inside the container/dish
5. Squirt the planarians to the opposite corner of the container and repeat step 4
6. Fill the container/dish with fresh planarian water
7. Examine the waste container for any animals that were accidentally poured off and move them back to the container/dish
8. Discard waste water into the sink

Note: If new Petri dishes are available, worms could also be placed into a new Petri dish instead of wiping the dirty one.

REQUIRED MATERIALS 1.2:

Squeeze bottle or standard bottle

Waste container

Plastic Pasteur pipettes

Paper towels or lab wiper KimWipes™

Instant Ocean® Sea Salt or 1x Montjuic water

Optional: new Petri dishes

1.3. Planarian feeding

Feeding is required for the medium- and long-term maintenance of planarians, but they are completely fine without food for 3-4 weeks (Table 1). The planarians can be fed with chopped calf liver, with egg yolk from hard-boiled eggs or with brine shrimp, usually sold as frozen fish food.

If the calf liver is used for the feeding, it is chopped into small pieces (4 x 4 x 4 mm) and stored at -20°C. On the day of feeding:

1. Remove liver from the freezer and thaw it.
2. Large containers receive five pieces of liver; Medium containers receive two pieces of liver; Small containers and dishes receive one piece of liver. The animals eat the food only if it sits on the bottom of the container/dish.
3. Monitor the feeding behavior of the animals.
4. Leave the worms undisturbed with the food until they are finished eating or no longer interested in it. The feeding lasts between 20 min and 2 h.
5. Remove any residual food with a pipette.
6. Change the water and wipe the bottom of the box or replace the Petri dish as described in the Section 1.2.
7. Two days after feeding, change the water and wipe the bottom of the box or replace the Petri dish as described in the Section 1.2, in order to remove excrement and keep the culture clean.

The worms can be fed once per week for 6-7 months, but a twice a week feeding schedule is suggested if planarians are maintained for a longer period of time.

Note: The worms have to be starved for 7-10 days before any experiment, because the residual food in the gut dirties the water when the animals are cut, interferes with the chemical treatment in the *in situ* hybridization (WISH) protocol, and reduces the amount of food eaten during the RNA interference feeding.

REQUIRED MATERIALS 1.3:

Calf liver, egg yolk from a hard boiled egg or brine shrimp

Waste container

Plastic Pasteur pipettes

Paper towels or lab wiper KimWipes™

Instant Ocean® Sea Salt or 1x Montjuïc water

Optional: new Petri dishes

1.4. Planarian amplification

All the planarian species reported in this paper can be cut into several fragments, and each fragment will regenerate an entirely new organism. This technique is used for both increasing the number of worms in your culture and obtaining smaller worms for the WISH protocol. The worms are ready to be cut 48 h after feeding and the following steps have to be followed:

1. Wipe the blade with ethanol.
2. Move the animals to a Petri dish filled with fresh planarian water.
3. Cut the animals into the desired number of fragments, wiping the blade frequently with a fresh paper towel or a KimWipe™ (KimTech Science) to remove the mucous layer.

Note: *Girardia sp.* and *D. dorocephala* can be cut into several tiny fragments and all of them regenerate; the regenerative ability of *P. morgani* and *P. gracilis* is inferior and, in medium size worms we suggest generating 6-7 fragments from *P. morgani* and 10-20 fragments from *P. gracilis*.

4. Transfer the fragments into the new container/dish by gently squirting them from the Petri dish with a squeeze bottle or plastic Pasteur pipette.

5. The first, second, third, fifth and seventh days post amputation (dpa) the water needs to be changed as described in Section 1.2.

Note: *Girardia sp.*, *D. dorotocephala* and *P. morgani* fully regenerate in 2 weeks, while *P. gracilis* requires 4-6 weeks.

6. Check regeneration regularly.
7. Do not feed the planarians before the gut and the pharynx are regenerated. After 2 weeks all the species can be regularly fed again.

Regeneration is complete when the animal is properly proportioned and the body pigmentation is homogeneous.

REQUIRED MATERIALS 1.4:

Ethanol

Blade

Paper towels or lab wiper KimWipes™

Plastic Pasteur pipettes

Petri dishes

Squeeze bottle or standard bottle and plastic Pasteur pipettes

Instant Ocean® Sea Salt or 1x Montjuïc water

Waste container

1.5. Image acquisition of live worms

Planarians are clearly visible to the human eye when they are longer than 1 mm and a microscope is not required during water changes and feedings. When overall health, pigmentation, behavior, and regeneration are analyzed, a higher resolution is recommended. A lens magnification or a dissecting microscope together with a camera

allows the observation and the documentation of the results. A smartphone works as well if it is used properly as showed in the video “Turn your smartphone in a digital microscope!” (https://www.youtube.com/watch?v=KpMTkr_aiYU).

If you are using a dissecting microscope, the light should come from above the samples; the background can be white (for looking at dark worms like *P. gracilis* or at the eye regeneration) or black (for looking at white worms like *P. morgani* or at the blastema formation and regeneration of new tissues). The working magnification is usually about 10x.

When the sample consists of living planarians, a movie can be recorded to show the movement and behavior of the worms. For acquiring single images of living worms, the replacement of the planarian water with cold planarian water immediately before the picture acquisition is necessary. The cold water reduces animal movement allowing for the acquisition of clearer images.

REQUIRED MATERIALS 1.5:

Lens magnification or dissecting microscope or smartphone

Optional: Camera

Plastic Pasteur pipettes

Petri dishes

Squeeze bottle or standard bottle and plastic Pasteur pipettes

Cold Instant Ocean[®] Sea Salt or 1x Montjuïc water

Waste container

Table 1: Planarian maintenance

	Long-term (> 28 weeks)	Medium-term (4-28 weeks)	Short-term (<4 weeks)
Planarian water	0.5 g/ml Instant Ocean® Sea Salt in water or 1x Montjuïc water	0.5 g/ml Instant Ocean® Sea Salt in water or 1x Montjuïc water	Carolina Supply Biological Company water or Ward Scientific water
Water changes	Twice a week (two days after the feeding)	Once a week (two days after the feeding)	Any water change is required
Food schedule	Twice a week with calf liver, boiled egg yolk or brine shrimp	Once a week with calf liver, boiled egg yolk or brine shrimp	Any feeding is required

References

- King, R.S. & Newmark, P.A. (2013). *In situ* hybridization protocol for enhanced detection of gene expression in the planarian *Schmidtea mediterranea*. *BMC Developmental Biology*, 13, 8.
- Cebrià, F. & Newmark, P.A. (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, 3691-3703.