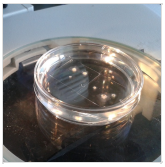

Nov 12, 2019

Hatching and freezing gemmules from the freshwater sponge *Ephydatia muelleri*

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1 *Works for me* dx.doi.org/10.17504/protocols.io.863hzgn

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ABSTRACT

Freshwater sponges are found in lakes and rivers worldwide. Many species produce an asexual cyst called a gemmule that contains stem cells which allow the sponge to survive harsh periods in a dormant state. For over a century gemmules from freshwater sponges have been collected to grow sponges in controlled laboratory conditions for research and teaching. The practicality of this method of culturing sponges has led to a rich literature on the physiology, cell and developmental biology of freshwater sponges. A few species have gemmules that withstand extreme cold. The freshwater sponge *Ephydatia muelleri* can be frozen at -80°C and hatched with good success even several years later. A high quality assembly of the genome of this species makes this an especially attractive tool for a wide range of studies. While there are many publications reporting hatching of freshwater sponges, there is no universally available protocol, especially for freezing and thawing this species.

Here we outline a protocol for cleaning and hatching gemmules from freshwater sponges and for freezing gemmules for storage at -80C and subsequently thawing frozen gemmules to grow sponges. Sponge gemmules can be collected during late fall and winter months and kept in water at ~3 degrees Celcius for many months prior to cleaning and hatching, but it is recommended that the water is aerated and exchanged for new ice-cold water every few weeks. The best hatching rate is found with gemmules cleaned within the first 6 months of collection. Hatching success varies with population of sponges. Once cleaned, gemmules can be kept in the fridge (below 6 degrees Celcius) and hatched as needed for several weeks. Cleaned gemmules have a greater tendency to hatch earlier than gemmules left in the native sponge skeleton, but even gemmules left below 6 degrees Celcius in the native sponge skeleton can often hatch after washing months later. For best hatching success, gemmules frozen at -80C should be no older than 4-6 weeks. Gemmules frozen at -80C tend to take slightly longer to hatch, but all aspects of sponge development are identical to those in sponges hatched from freshly collected gemmules.

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KEYWORDS

Ephydatia muelleri, Porifera, Hatching, Freshwater sponge, Cryo-preservation

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MATERIALS TEXT

Ice cold distilled water
Vacuum flask and hose (or pipettes, but that is slower)
Wide bore Pasteur pipettes and bulbs
Ice and small cooler
Glass bowl (~15mm diameter)
Corduroy-lined Petri dish and corduroy-covered 50ml orange cap Falcon tube.
Petri dishes
250ml squirt bottle with ice cold distilled water (keep chilled on ice)
Kim wipes or equivalent
95% ethanol
Alcohol lamp
22x22 coverslips
Forceps (kept for this work only)
Hydrogen peroxide (H_2O_2)
Cryovials 2mL (e.g. Corning 430488)
Dimethylsulfoxide (DMSO) sterile, filtered

Strekal's medium:
Strekal TA, McDiffett W. 1974. Factors affecting germination, growth, and distribution of the freshwater sponge, *Spongilla fragilis* Leidy (Porifera). Biological Bulletin 146:267-278.

'RR Medium' – concentrations of cations given and concentration of whole compounds calculated.

Mg/L		MG/L of whole compound
Mg 2.0	$MgSO_4 \cdot 7H_2o$	20.3
Ca 2.0	$CaCO_3$	5.0
Na 0.5	$Na_2SiO \cdot 9H_2O$	3.1
K 0.5	KCl	0.96

For M-medium see Rasmont R. 1961. Une technique de culture des éponges d'eau douce en milieu contrôlé. Annales de la Société Royale Zoologique de Belgique 91:147-156.
A commonly used version of this medium is 1 mM $CaCl_2 \cdot H_2O$, 0.5 mM $MgSO_4 \cdot 7H2O$, 0.5 mM $NaHCO_3$, 0.05 mM KCl, 0.25 mM $Na_2SiO_3 \cdot 9H_2O$.

We generally make up the Strekal's or M-medium as a 10x stock stirred with a stir bar used only for this. When diluted to 1X the pH should be around 7.2 but will depend on the quality of the water in which it is made.

SAFETY WARNINGS

Sterile technique is used at plating, which involves flame sterilizing alcohol-washed coverslips.

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Here we outline a protocol for cleaning and hatching gemmules from freshwater sponges and for freezing gemmules for storage at -80C and subsequently thawing frozen gemmules to grow sponges. Sponge gemmules can be collected during late fall and winter months and kept in water at ~3 degrees Celcius for many months prior

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BEFORE STARTING

It is assumed gemmules are in the adult skeleton framework and kept in bags of water in an incubator at 3°C.

Throughout the whole cleaning process gemmules should be kept on ice to avoid warming to room temperature and causing premature hatching.

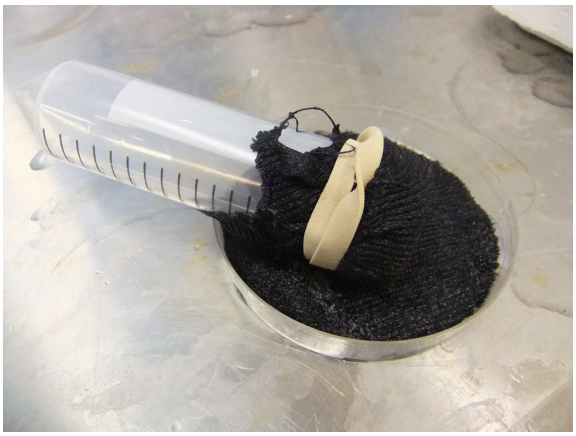
Cleaning and hatching sponge gemmules

1 Separate gemmules from the skeleton

Remove a portion of the skeleton with gemmules from the bag. Remove gemmules from the bag using sterile wide tipped forceps (wider is ideal because thinner forceps will rupture the gemmules). Sterile forceps are used in order to prevent contaminating the rest of the gemmules. Place the skeleton with gemmules into cold distilled water on ice.

1.1 i.Use two home-made tools for separating the gemmules from the skeleton: 50ml falcon tube with corduroy tied to the cap end, and a 15mm Petri dish with corduroy glued into the inner surface.
ii.Wet the corduroy on the tube and in the Petri dish with ice cold distilled water before the gemmules are placed onto the Petri dish.
iii.Transfer the skeleton and gemmules onto the corduroy and very gently rub the skeleton with a back and forth motion between the corduroy portion of the 50ml tube and the Petri dish. This action rolls the gemmules and skeleton between the corduroy and frees the gemmules from the skeleton.
It is important to not rub the gemmules to harshly, too much pressure will cause the gemmules to rupture.

iv. Alternatively, for gemmules that are more embedded in the host skeleton, sterile teasing needles or fine forceps can be used to carefully dissect the gemmules away from the skeleton under a compound microscope with the Petri dish sitting on ice. There may be gemmules that do not contain cells (non viable) and those should be discarded. Novices might try bursting a few gemmules with fine forceps to develop an ability to differentiate between viable and non viable gemmules.



Falcon tube and Petri dish with corduroy

1.2 Transfer freed gemmules from the Petri dish to a deeper glass bowl filled with ice cold distilled water. This is done my squirting ice cold water repeatedly onto the corduroy bottomed Petri dish until the gemmules fall out into the bowl of water.

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The glass bowl must keep kept on ice for the rest of process to ensure the gemmules to not warm.

Set up for keeping gemmules and water ice-cold throughout the cleaning procedure

2 Sort the gemmules from the skeleton

i.A pipette attached to a suction hose is used to draw off loose spicules and debris in the dish. By swirling the bowl, and by adding fresh ice cold water by squirt bottle to one side of the bowl, the healthy gemmules sink to the bottom and accumulate in the middle of the bowl, while the dead gemmule husks and spicule debris are drawn to the surface of the water making their removal easier. Continue this process repeatedly until all excess debris and skeleton is removed and only healthy gemmules are left in the bowl.
ii.If the suction is too aggressive a wide bore pipette can be used to capture individual gemmules and remove them to a new Petri dish with ice-cold water.

3 Sterilize healthy gemmules

i.Remove excess distilled water from the glass dish and replace with 1% H_2O_2 and shake gently for 5 minutes at 4°C.

By adding the H_2O_2*, dead or damaged gemmules are caused to 'pop', making dead gemmules float.*

ii.Dispose of floating gemmules by pouring off the sterilizing medium and quickly add ice-cold distilled water to rinse the healthy gemmules. Exchange water 4-5 times to completely remove the H_2O_2. Many, gentle rinses are essential to remove all the H_2O_2 .

Gemmules can be placed in a new sterile Petri dish with ice-cold water, parafilmed, and kept in the incubator at 3°C for several weeks until needed for hatching.

4 Plate gemmules: "growing sponges"

i.For a lawn of sponge tissue, place gemmules directly into a Petri dish with M-medium or Strekal's medium. Cover with tin foil to keep dark and leave to hatch for 2 days.
ii.For sponges that can be fixed for fluorescence microscopy or moved to other dishes for experiments, first soak the coverslips in 95% ethanol and then flame sterilize them by moving them quickly through the flame of an alcohol lamp to dry. Place coverslips into a new sterile Petri dish and, once they are cool, cover them with M-medium or Strekal's medium. Coverslips should be pushed down to prevent air bubbles from lifting them and rolling the gemmules off.
iii.Using a sterile, wide-bore pipette, place 3-4 gemmules onto each glass coverslip.
iv.Leave dishes undisturbed at 18-24°C in the dark, for 1-2 days until they have hatched. Gemmules should not be disturbed until they have hatched. Once hatched, medium should be changed every 48 hours. When changing the medium, take care to leave the gemmules submerged - never remove all the medium and never expose the sponges to air during changes.

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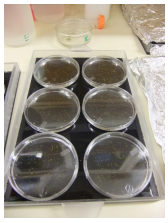
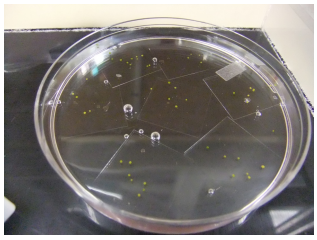


For a lawn of sponge



For individual sponges on coverslips

4.1 Staging sponges

Sponges hatch through stages defined by their development: Stage 1 = just pre-hatching or just hatching; Stage 2 = hatched with tissue surrounding the gemmule husk and attaching to the substrate; Stage 3 = chambers and 'pockets' formed but not united into an aquiferous system; Stage 4 = aquiferous system formed with osculum, but still being organized; Stage 5 = final form of the juvenile sponge with osculum and branching canals in their final positions. Sponges take roughly 1 week to reach stage 5 and will last for 10 days without feeding. Food can be provided as either heat-killed E coli, or as diluted Liquifry.

4.2 Feeding sponges

To feed the sponges, add two drops of Liquifry No. 1 to 200 ml of Strekal's Medium or filtered pond water. Add approximately 2 ml of this to the culture dishes every day.
Liquifry No. 1 contains dextrin, pea flower, egg whites, oil, yeast, and preservative (sulphur based).

Freezing gemmules at -80C for long-term storage

5 Prepare gemmules and cryoprotectant solution

*Note: Gemmules should be **fresh**, no older than 1-2 months, for successful cryopreservation. Gemmules require a period of ~6 weeks for 'vernalization' during which the cells become ready to hatch. If the gemmules are frozen prematurely, they may not hatch well when thawed. Similarly if gemmules are frozen many months after they have been stored in the fridge, hatching success is substantially diminished. Therefore it is important to know when during the autumn/winter months the gemmules first formed, and after collecting them, it is important to let them rest at 3°C until they have completed the vernalization period.*

This protocol uses DMSO as a cryoprotectant. Other cryoprotectants have been used (e.g. glycerol) with varying success. Other work has shown gemmules can tolerate freezing at -70°C or -80°C without any cryoprotectant, but best hatching success appears to be associated with use of a cryoprotectant. For references on this see:
Barbeau MA, Reiswig HM, Rath LC. 1989. Hatching of freshwater sponge gemmules after low temperature exposure: *Ephydatia mülleri* (Porifera: Spongillidae). Journal of Thermal Biology 14(4):225-231.
Ungemach LF, Souza K, Fell PE, Loomis SH. 1997. Possession and Loss of Cold Tolerance by Sponge Gemmules: A Comparative Study. Invertebrate Biology 116(1):1-5.

5.1 Clean gemmules as outlined above "Cleaning sponge gemmules". Immediately after the gemmules are rinsed to remove all hydrogen peroxide (Step 3 above), keep them in distilled water on ice and prepare the following solution.

5.2 Mix together 40 ml of cold 1x M-medium or 1x Strekal's medium and 10 ml of sterile DMSO, to have a final solution of 20% DMSO. Put the 20% DMSO solution into the refrigerator and leave for long enough for the solution to be at 3°C before using.
Ensure solution is cold before using, if used warm the gemmules could hatch prematurely.

5.3 Pre-label 20-50 cryovials and pre-label a box for the cryovials. Pre-chill the box in the -80 Freezer.

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6 **Mixing gemmules with cryoprotectant**
Use a wide bore pipette to place 15-20 gemmules into a 2mL cyrovial.
Add 750 mL of the cold 1x M-medium (or 1x Strekal's medium) and then immediately add 750 mL of the pre-chilled 20% DMSO to create a final solution of 10% DMSO, and gently invert the cryovial 4-5 times to mix the solution with the gemmules.

It is important to keep the gemmule/DMSO solution ice cold.

6.1 After mixing the gemmule/DMSO solution, keep the vials on ice for 30 minutes.

7 **Freeze the gemmules**
After 30 minutes on ice, place the cryovials into a box and put them into the -80°C freezer. Leave the box undisturbed to freeze the gemmule/DMSO mixture.

Frozen gemmules can be stored in the -80°C for months before use and still be viable.

8 **Thaw frozen gemmules for hatching**
Remove cryovials from the -80°C freezer and place them directly onto ice. Let the gemmules thaw completely on ice; this will take about one hour or longer.

8.1 Pre-chill high quality distilled water to 3 degrees Celcius.

9 As soon as the vials have thawed, use a sterile Pasteur pipette to remove the 10% DMSO solution from the cyrovial and immediately replace with cold distilled water. Gently invert the cyrovial to mix the distilled water and gemmules.

9.1 Place cryovials back onto ice and put on a shaker, set on a gentle speed, for 5 minutes.

9.2 Repeat step 9 five times to thoroughly remove the DMSO from the gemmules.
DMSO residue can interfere with hatching success and with development of the sponges.

10 **Plate gemmules**
After thoroughly rinsing the gemmules they can be either kept at 3°C for several days until needed, or plated immediately to grow sponges. To hatch follow the steps under plate gemmules as outlined above under Step 4 of "Cleaning and Hatching sponge gemmules".

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