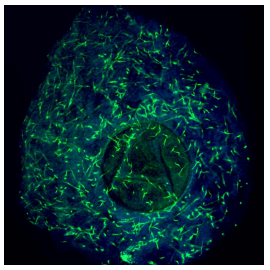
Feb 09, 2024

Fluorescent in situ hybridization in sponge (Ephydatia muelleri) tissues with tyramide signal amplification



DOI
dx.doi.org/10.17504/protocols.io.j8nlkonkxv5r/v1

Vanessa R Ho[1], Sally P Leys[1], April Hill[2]

[1]Department of Biological Sciences, University of Alberta, Canada; [2]Bates College

Vanessa R Ho
University of Alberta

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.j8nlkonkxv5r/v1

Protocol Citation: Vanessa R Ho, Sally P Leys, April Hill 2024. Fluorescent in situ hybridization in sponge (Ephydatia muelleri) tissues with tyramide signal amplification. **protocols.io https://dx.doi.org/10.17504/protocols.io.j8nlkonkxv5r/v1**

Protocol status: Working
We use this protocol and it's working

Created: October 26, 2023

Last Modified: February 09, 2024

Protocol Integer ID: 89973

Keywords: fluorescence, in situ hybridization, molecular techniques, developmental biology, sponge, porifera, ephydatia, invertebrate biology, cultured sponges such as ephydatia muelleri, fluorescent in situ hybridization, many other tissue samples such as embryo, many other tissue sample, tissues with tyramide signal amplification, cultured sponge, ephydatia muelleri, sponge, situ hybridization, embryo, fluorescent, reagent, tissue, tyramide signal amplification, delicate tissue, formamide

Funders Acknowledgements:
Gordon and Betty Moore Foundation "New Tools for Advancing Model Systems in Aquatic Symbiosis"
Grant ID: Hill #9332
NSERC Discovery Grant
Grant ID: Leys 2022-03414

Abstract

Cultured sponges such as *Ephydatia muelleri* (Demospongiae) have delicate tissues and are attached to coverslips, unlike many other tissue samples such as embryos that are typically free floating. This protocol also uses urea in place of formamide, which is not only safer, but is just as effective at reducing background signals and also improves signal detection.

It is strongly recommended to have all the reagents prepared at least a day before they are needed. Large quantities of some solutions like PBS can be made at one time but some solutions such as the proteinase K solution are to be prepared fresh.

Image Attribution

Vanessa Ho

Materials

Reagents are from Sigma Aldrich unless otherwise noted

STERILE + RNase-FREE **materials** - handle with gloves only and keep in a separate, dedicated space
Clean bench space with RNase decontamination solution such as RNaseZap before working

- 6-well cell culture plate
- 15 mL Falcon tubes
- 50 mL Falcon tubes
- 1.7 mL Eppendorf tubes
- Dedicated RNase-free pipette tips
- Airtight plastic container (RNase decontaminated) as a humidity chamber when incubating tissues
- Jeweler's forceps

Sterile, RNase-free **stocks**

- DEPC-treated water
- 10X phosphate buffered solution (PBS)
- 1X PBS
- 20% Tween20
- PbTween (PBS + 20% Tween20)
- 100% ethanol
- 20X saline sodium citrate (SSC) pH 7.0
- 20X SSC pH 4.5
- 10X blocking buffer
- 10X Maleic acid buffer (MAB)
- Hoechst 33342 nuclear stain*
- anti-DIG-POD* - Roche 11207733910
- 8M Urea*
- Post-fix solution (PF)**
- Hybridization buffer (HB)**
- Post-hybridization buffer (PHB)**
- HPLC-grade DMSO
- TSA Plus Stock Solution (Akoya Biosciences TSA Plus Fluorescence Kit)
- TNT buffer (Akoya Biosciences, included in TSA Plus kit)
- Mowiol + DABCO anti-fade agent (or other mounting medium of choice)

Reagents and antibodies, prepared FRESH

- Glycine in PbTw (20 uL/mL)
- Proteinase K in PbTw (3 uL of 20 mg/mL ProK per 12 mL PbTw) - thawed on ice
- 1% TEA in PBS (label **tube #1**)
- 1% TEA in PBS + acetic anhydride (3 uL/4 mL ratio) (**tube #2**)
- 1% TEA in PBS + acetic anhydridge (6 uL/4mL ratio) (**tube #3**)
- DIG-labeled riboprobes - THAW ON ICE

- TSA Plus Working Solution

***store at 4ºC**
****store at -20ºC**

Solution recipes

1:4 Holtfreter's Solution (HS)
- 875 mg NaCl, 12.5 mg KCl,, 25 mg $CaCl_2$, 50 mg $NaHCO_3$ fill to 1.0 L with dH_2O

10X PBS
- 18.6 mM NaH_2PO_4 (2.56 g NaH_2PO_4-H_2O per liter dH_2O)
- 84.1 mM Na_2HPO_4 (11.94 Na_2HPO_4 per liter dH_2O)
- 1750 mM NaCl (102.2g NaCl per liter dH_2O)

Mix phosphates in about 800ml of dH20 for a 1L volume. Check the pH, it should be 7.4 ±0.4. If it is more than 0.4 off then start over. Otherwise, adjust pH to 7.4 with NaOH or HCl. Add the NaCl and the rest of the H 2 0.

PbTween
- 1x PBS + 0.1% Tween 20 detergent

(100ml 10 X PBS + 895ml dH 2 0, DEPC treat/autoclave; when cool add 5 ml 20% Tween)

DEPC H_2O
- 0.5% DEPC (0.5ml in 1L); shake the bottles at 250rpm at 37°C O/N, autoclave

20X SSC - for 1L (0.3M Sodium citrate + 3M NaCl)
- 175.3 g NaCl
- 88.2 g Sodium Citrate, dihydrate ($CH_6H_5Na_3$-$2H_2O$)
- pH to 7.0 and sterilize by autoclaving

∗Dilute 1:10 for 2X

Post-fix solution
- 3.7% paraformaldehyde + 0.3% gluteraldehyde in PbTween

Hybridization Buffer (HB) - for 50 mL
- 25 mL 8M Urea
- 12.5 mL 20X SSC pH 4.5
- 0.125 mL 20 mg/mL heparin
- 0.25 mL 20% Tween20
- 0.5 mL 100X Denhardt's solution
- 0.25 mL 10 mg/mL yeast tRNA
- dH_2O to 50 mL

∗Store at -20ºC

Post Hyb Buffer (PHB) - for 50 mL

- 25 mL 8M Urea
- 12.5 mL 20X SSC pH 4.5
- 0.25 mL 20% Tween20
- DEPC H_2O to 50 mL

*Store at -20ºC

Maleic acid buffer (MAB) - for 500 mL

- 50 mL 1M maleic acid
- 15 mL 5M NaCl
- DEPC H_2O to 500 mL
- use NaOH pellets to raise pH to 7.5

*Just before use add 0.1% Tween20

10X Blocking Buffer

- Dissolve 10g blocking reagent (10% w/v; Roche) in ~80ml MAB (for final volume of 100ml), shaking and heated (can microwave)
- Autoclave

*Store aliquots at -20ºC

TNT Buffer - for 200 mL

- 20 mL 1 M TRIS-HCl, pH 7.5
- 6 mL 5 M NaCl
- DEPC H_2O to 200 mL

*Just before use add 0.05% Tween20 (e.g., 125 µL 20% Tw20 in 50 mL TNT buffer)

TSA Plus Stock Solution

- Add 150 µL DMSO per tube of provided Amplification Reagent

Store at 4ºC

TSA Plus Working Solution

- Dilute TSA Plus Stock Solution 1:50 in Amplification Diluent

Use 100-300 µL solution per sample; discard unused portion

Troubleshooting

Tissue fixation and preparation

1 Fix sponges in 4% paraformaldehyde in 1:4 Holtfreter's solution (HS; diluted 1 in 4) overnight

2 Wash once in 1:4 HS

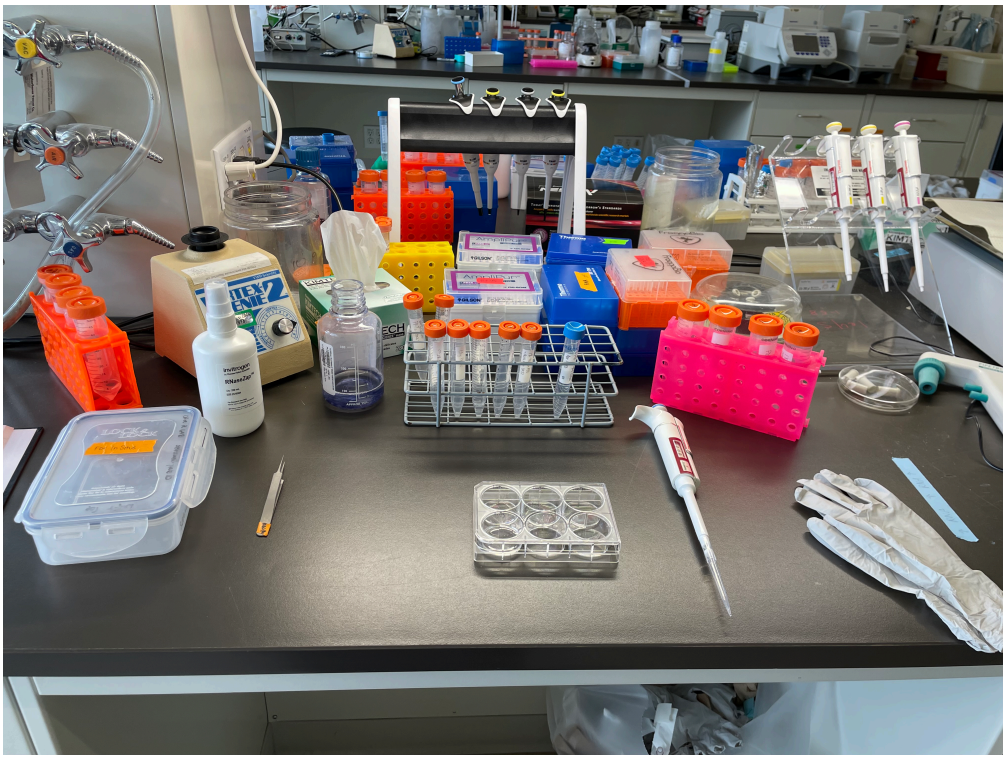
> Note
>
> All washes are 5 min unless otherwise indicated

3 Dehydrate the tissues
1. 25% ethanol (EtOH) and 75% 1:4 HS
2. 50% EtOH and 50% 1:4 HS
3. 75% EtOH and 25% 1:4 HS
4. 100% EtOH

3.1 Dehydrated tissues can be stored at -80ºC until ready to use.

ISH Day 1a - tissue pre-treatment

4



Setup for ISH day 1 in a dedicated RNA-clean space with RNA-clean supplies

Rehydrate sponges - carefully and quickly transfer coverslips into the wells of the cell culture plates

1. 100% EtOH
2. 75% EtOH:25% phosphate buffered saline (PBS)
3. 50% EtOH:50% PBS
4. 25% EtOH: 75% PBS
5. 100% PBS

5 Quench endogenous peroxidases 15 min with 2% H_2O_2 in EtOH

6 Rinse tissues

1. PBS **x2**
2. 2 min PBTw **x3**

7 1 min proteinase K digestion

8 Wash with Gly:PBTw solution **x2** to stop digestion

9 Acetylation

1. 1% TEA:PBS **x2 (Tube #1)**

2. 1% TEA:PBS + acetic anhydride **(Tube #2)** - 3 µL acetic anhydride per 4 mL 1%TEA:PBS
3. 1% TEA:PBS + acetic anhydride **(Tube #3)** - 6 µL acetic anhydride per 4 mL 1% TEA:PBS

10 Rinse with PBTw **x2**

11 Post-fix in 3.7% paraformaldehyde (PFH) + 0.3% gluteraldehyde, at least 1 h at room temperature (RT)

Note

- Can be left overnight in post-fix if needed
- Use this time to thaw hybridization buffer (HB) to RT and pre-heat a separate tube of HB if continuing past post-fix in the same day

ISH Day 1b - pre-hybridization

12 Wash with PBTw **x5**

13 Wash with HB 10 min at RT

14 Replace HB with fresh, pre-heated (55°C) HB, pre-hybridize at 55°C 2-3 h

Note

Use this time to prepare probes (closer to the end of pre-hyb period)

ISH Day 1b - probe preparation and hybridization

15 Dilute probe (we use 1:1000; 3 µL 50 ng/µL probe in a final volume of 3 mL HB)
1. in **labeled** 1.5 mL Eppendorf tubes, add 3 µL probe to 0.97 mL **pre-warmed (55°C)** HB
2. Make sure the lid is shut tight

Note

Probe template synthesized using gBlocks Gene Fragments (Integrated DNA Technologies)
- Reverse primer design includes T7 tail for RNA polymerase binding
- PCR amplification of gBlocks using Phusion HF polymerase
- PCR thermocycler melting temp is 54ºC
- PCR clean up using MinElute column (Qiagen) according to manufacturer's instructions

Riboprobe synthesis
- MEGAscript T7 transcription kit (Thermo Fisher)
- DIG-labeling mix (Roche)
- $LiCl_2$ precipitation and EtOH extraction

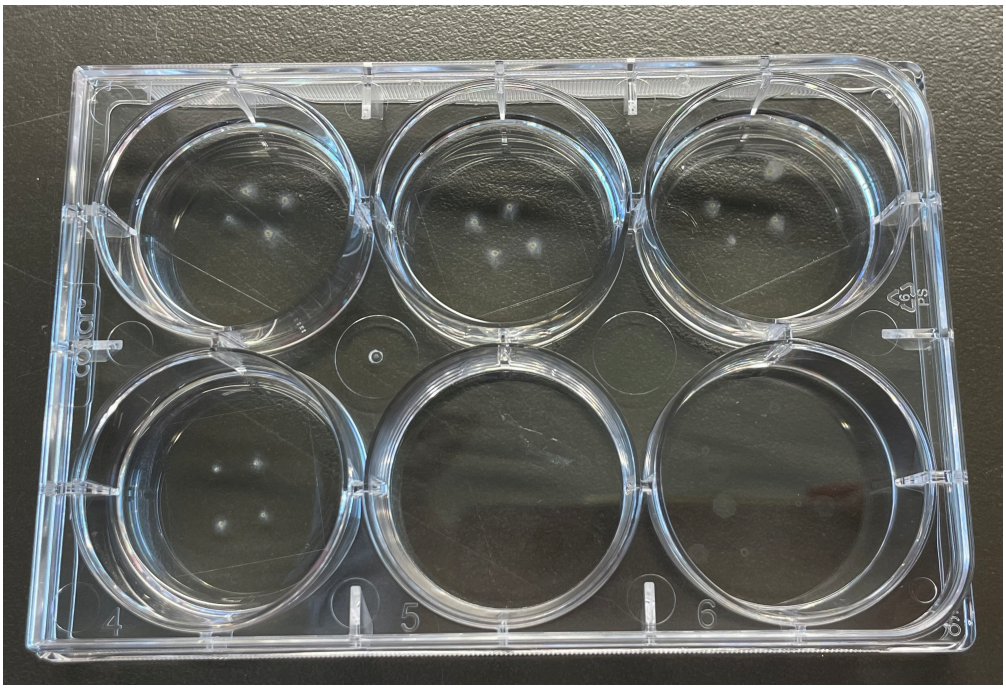
15.1 Immediately denature probe after adding to Eppendorf tube by boiling at 80°C for 10 min
- Replace the pre-hyb HB with 2 mL fresh, **pre-warmed** HB in the wells in the meantime

15.2 Quickly add probe (final well volume 3 mL)

Note

- Dry off the tube with a kimwipe before opening
- Remember to label the wells to the corresponding probe

16 Hybridize 16-72 h at 55°C



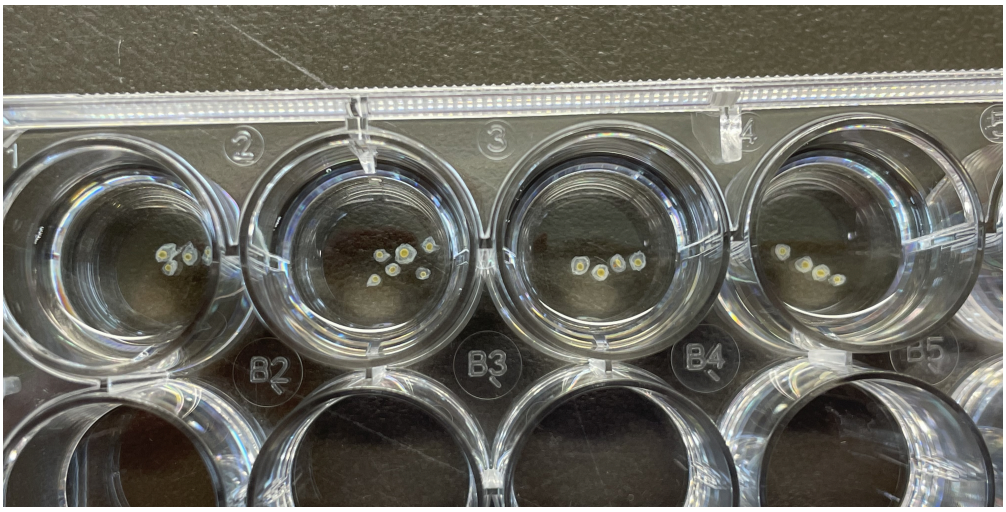
Sponges on coverslips in a 6-well dish prior to hybridization

Note

- 24-48 h is optimal, 36 h is usually most convenient
- use a clean airtight container such as a plastic snap-lock container (wiped with RNase Zap or equivalent) and a damp kimwipe (wetted with leftover HB works great) to maintain humidity and prevent drying out/urea crystallization
- incubate the day 2 wash solutions at the same time so they are ready to go

ISH Day 2 - post-hybridization stringency washes

17



Sponges after hybridization often detach from the coverslips. These were transferred to fresh HB in a 24-well dish using a sterile, trimmed pipette tip to prevent damaging the tissues. Make sure to note which probes and sponges are which in the new culture plate.

Stringency washes - **at 55°C**

1. 30 min fresh HB
2. 20 min PHB1
3. 20 min PHB2
4. 20 min PHB3
5. 20 min 2X SSC

Note

Preheat all solution
PHB1 - 75% PHB: 25% 2X SSC pH 7.0
PHB2 - 50% PHB: 50% 2X SSC pH 7.0
PHB3 - 25% PHB: 75% 2X SSC pH 7.0

17.1 Stringency washes **at RT**

1. 50% 2X SSC: 50% MAB 10 min
2. 100% MAB **x3** (5 min)

18 Block 1 hr blocking buffer (bb) on rocker at RT

19 Incubate overnight in antibody (anti-DIG-POD) at 4°C on rocker

1. Make the stock at 1:500 dilution in bb
2. Replace half the bb in the well from the previous step with the anti-DIG-POD for final antibody dilution of 1:1000

ISH Day 3

20 Wash 20 min MAB **x5**

21 Wash 5 min TNT buffer **x3**

ISH Day 3 - signal development - photosensitive steps; **keep dark**

22 Incubate in 250 µL 1:50 TSA Plus working solution 20 min at RT

Note

This was calculated for use in 24-well plates, follow manufacturer's instructions for determining amounts needed

23 Stop reaction

1. Wash 5 min TNT buffer **x3** on rocker
2. Rinse with PBS

24 Incubate in Hoechst 33342 (1:1000) at 15 min RT for nuclear counterstaining

Safety information

Handle with gloves. As a cell-permeant DNA dye, Hoechst 33342 can be potentially carcinogenic/mutagenic

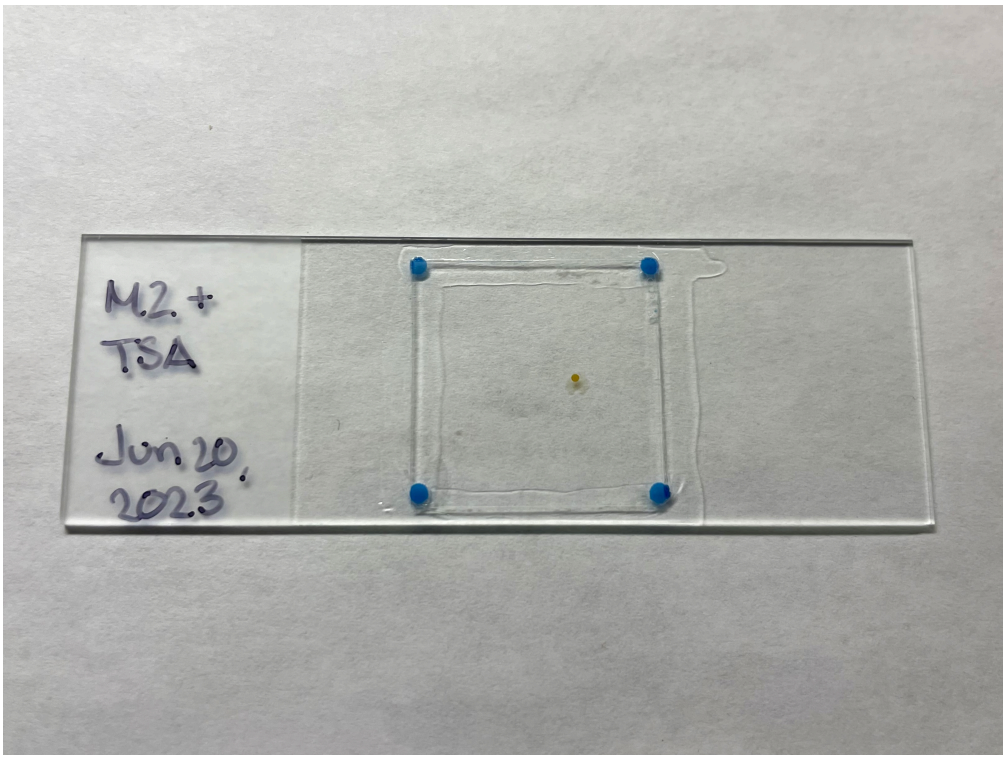
25 Rinse 5 min PBS **X4**

Note

May be stored at 4°C in the dark for a few days (eg. over the weekend) before mounting

26 Mount and store tissue samples

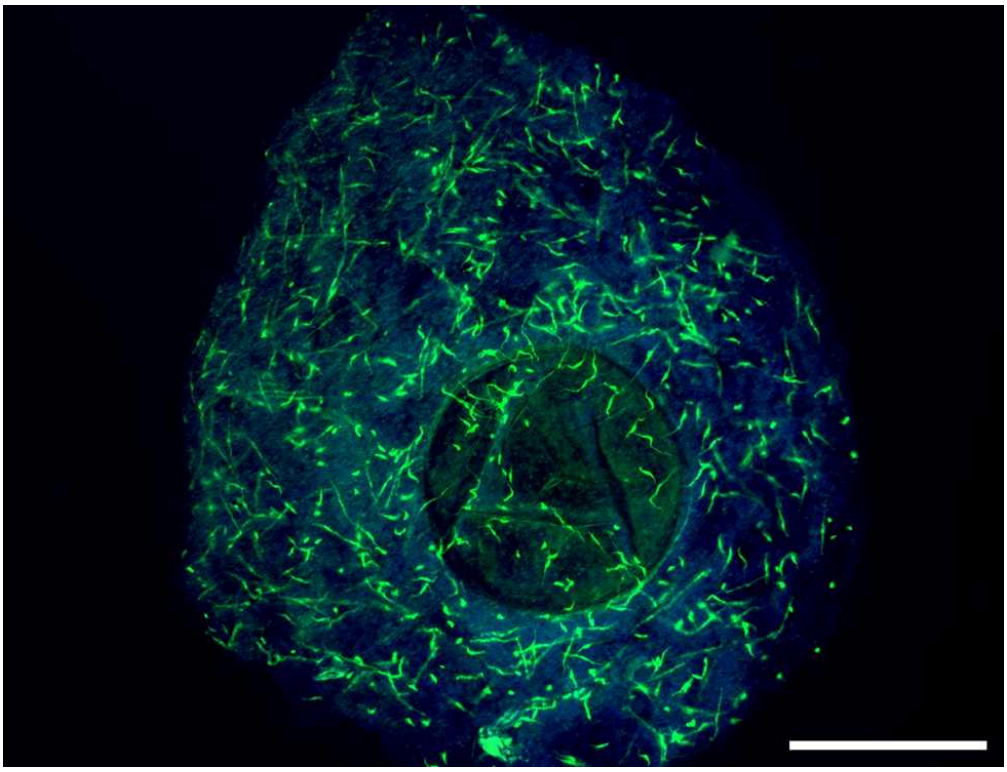
1. Clear tissues in Mowiol + DABCO anti-fade agent
2. Mount on microscope slide
3. Seal with nail polish and let dry
4. Store at 4°C in the dark



Single flattened sponge mounted on a microscope slide. To secure the glass coverslip scrape a small fragment of plasticine with each corner of the coverslip and gently press onto the sponge, avoiding air bubbles and shifting the sponge around.

Note

- cut the tip off a P1000 pipette tip to transfer sponges without damaging the tissues
- fine tip forceps and a 0.5 cm stab pen blade are helpful for flattening the sponge as it often curls up on itself



M2 Silicatein mRNA expression (green) in a 4-days post-hatch (stage 3 juvenile) *Ephydatia muelleri* specimen viewed with a standard FITC filter and merged with the image of Hoechst 33342-stained nuclei (blue) viewed with a DAPI filter. Scale bar = 0.5 mm

Protocol references

Sinigaglia, C., Thiel, D., Hejnol, A., Houliston, E., & Leclère, L. (2018). A safer, urea-based in situ hybridization method improves detection of gene expression in diverse animal species. *Developmental biology*, *434*(1), 15–23. https://doi.org/10.1016/j.ydbio.2017.11.015