

FEB 09, 2024

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## DOI:

[dx.doi.org/10.17504/protocols.io.j8nlkonkxv5r/v1](https://doi.org/10.17504/protocols.io.j8nlkonkxv5r/v1)

**Protocol Citation:** Vanessa R Ho, April Hill, Sally P Leys 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>

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

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

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### 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

Created: Oct 26, 2023

Last Modified: Feb 09, 2024

PROTOCOL integer ID: 89973

**Keywords:** fluorescence, in situ hybridization, molecular techniques, developmental biology, sponge, porifera, ephydatia, invertebrate biology

**Funders Acknowledgement:**

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

## 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 anhydride (6 uL/4mL ratio) (**tube #3**)
- DIG-labeled riboprobes - THAW ON ICE
- TSA Plus Working Solution

**\*store at 4°C**

**\*\*store at -20°C**

### Solution recipes

#### 1:4 Holtfreter's Solution (HS)

- 875 mg NaCl, 12.5 mg KCl, 25 mg CaCl<sub>2</sub>, 50 mg NaHCO<sub>3</sub> fill to 1.0 L with dH<sub>2</sub>O

#### 10X PBS

- 18.6 mM NaH<sub>2</sub>PO<sub>4</sub> (2.56 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O per liter dH<sub>2</sub>O)
- 84.1 mM Na<sub>2</sub>HPO<sub>4</sub> (11.94 Na<sub>2</sub>HPO<sub>4</sub> per liter dH<sub>2</sub>O)
- 1750 mM NaCl (102.2g NaCl per liter dH<sub>2</sub>O)

Mix phosphates in about 800ml of dH2O 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 O.

#### 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<sub>2</sub>O

- 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<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>·2H<sub>2</sub>O)
- 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<sub>2</sub>O to 50 mL

\*Store at -20°C

#### 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<sub>2</sub>O to 50 mL

\*Store at -20°C

#### Maleic acid buffer (MAB) - for 500 mL

- 50 mL 1M maleic acid
- 15 mL 5M NaCl
- DEPC H<sub>2</sub>O 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 -20°C

#### TNT Buffer - for 200 mL

- 20 mL 1 M TRIS-HCl, pH 7.5
- 6 mL 5 M NaCl
- DEPC H<sub>2</sub>O 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 4°C

## TSA Plus Working Solution

- Dilute TSA Plus Stock Solution 1:50 in Amplification Diluent
- Use 100-300 µL solution per sample; discard unused portion

## 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 -80°C 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<sub>2</sub>O<sub>2</sub> 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

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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 54°C
- 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<sub>2</sub> 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

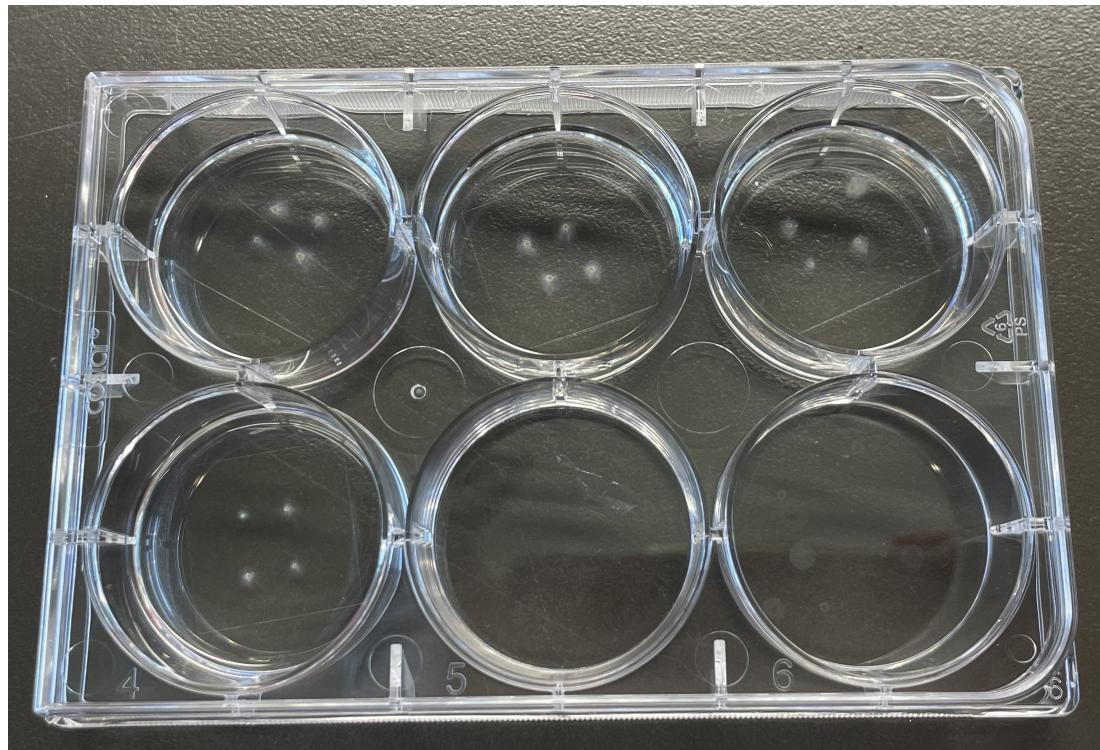
Quickly add probe (final well volume 3 mL)

### Note

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

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Hybridize 16-72 h at 55°C



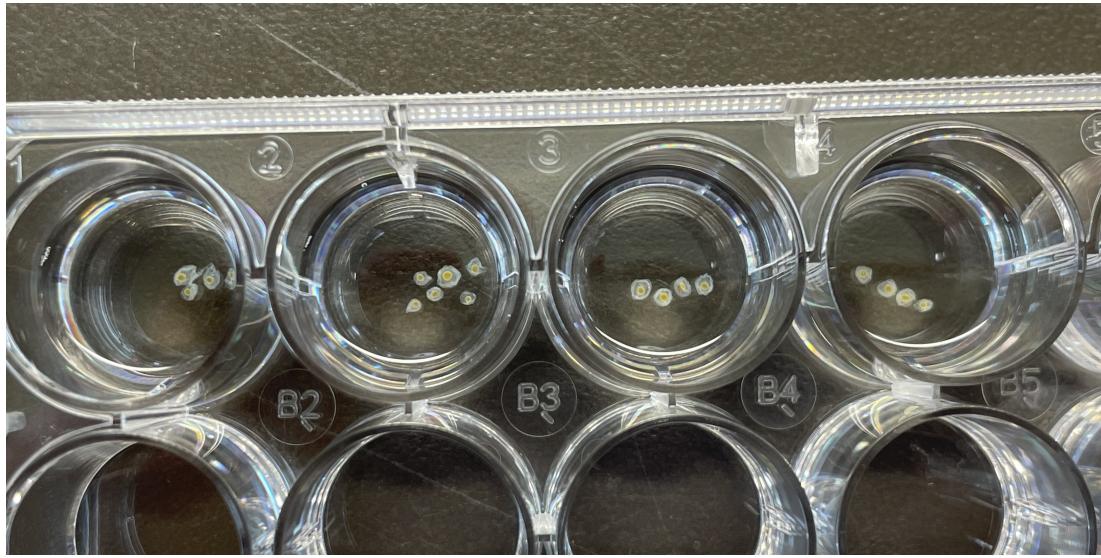
Sponges on coverslips in a 6-well dish prior to hybridization

#### 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

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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**

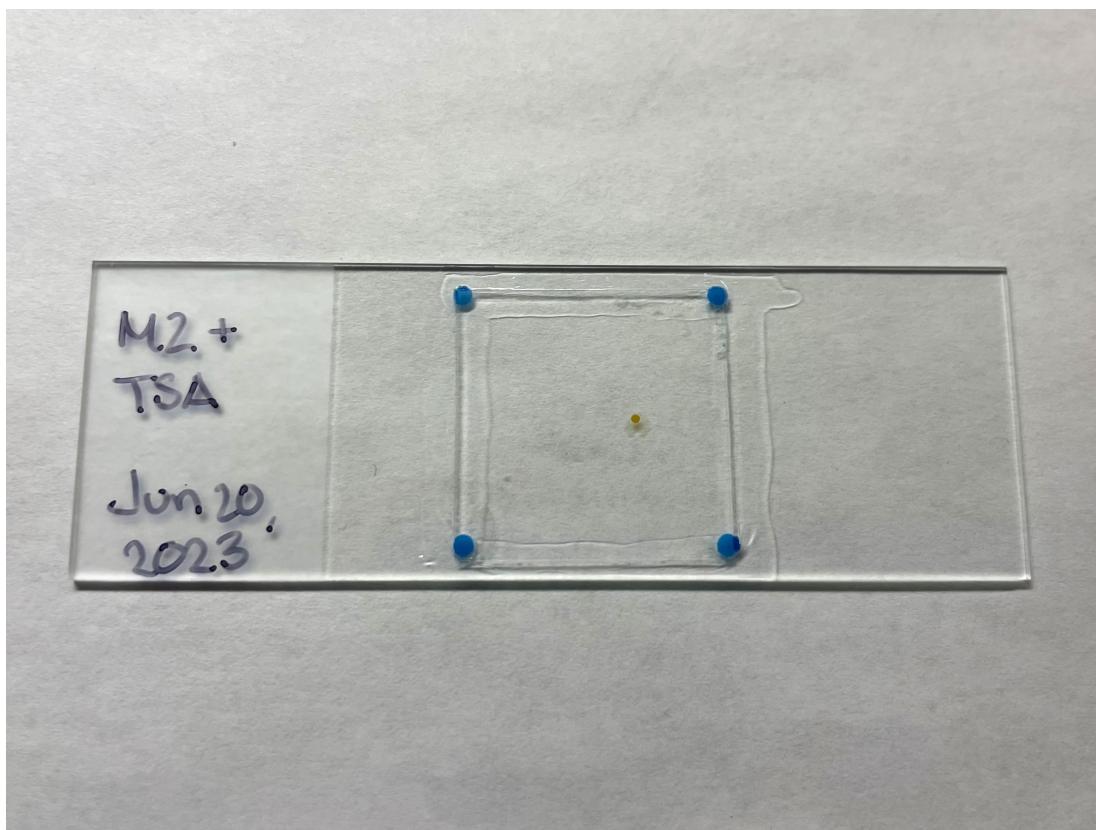
## Note

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

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Mount and store tissue samples

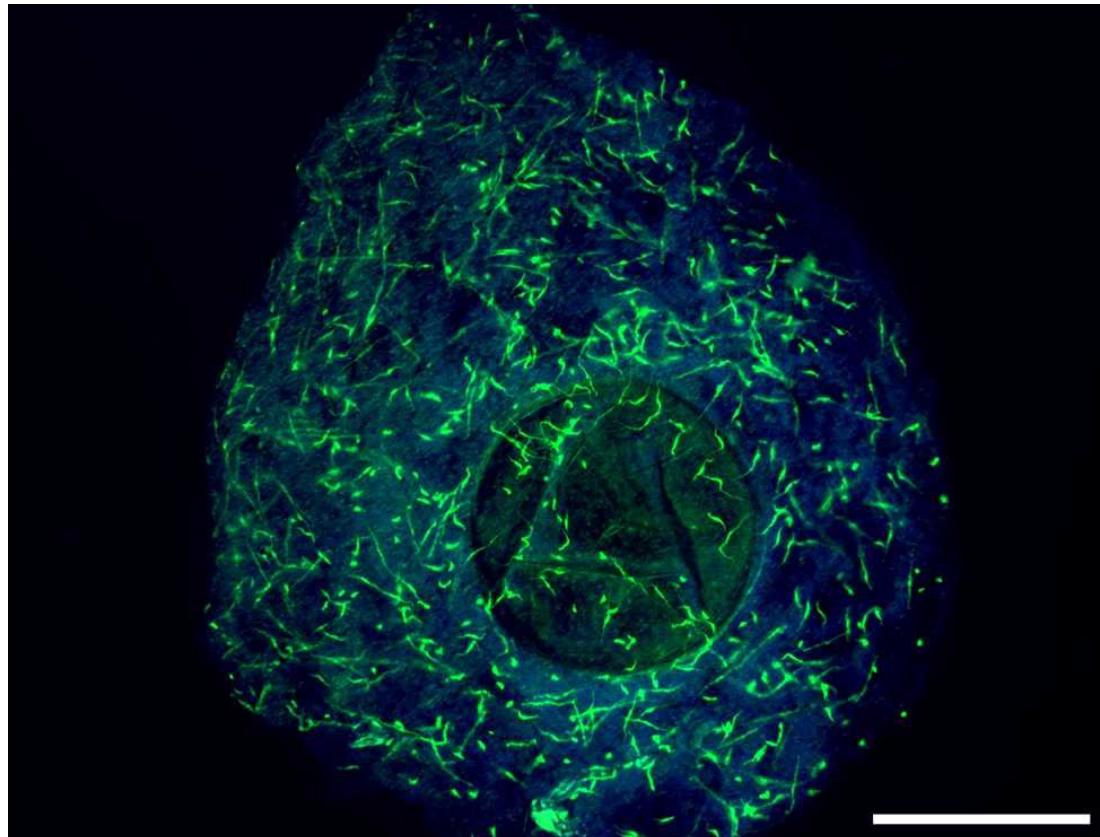
1. Clear tissues in Mowiol + DABCO anti-fade agent
2. Mount on microscope slide
3. Seal with nail polish and let dry
4. Store at 4°C in the dark



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