Fixation and staining of gemmule-hatched Ephydatia muelleri for fluorescence microscopy

Scott Nichols[1]

[1]University of Denver

Scott Nichols

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

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ABSTRACT

This protocol is intended for the preparation of gemmule-hatched freshwater sponges for imaging with an inverted scanning confocal microscope.

MATERIALS

Gemmule-hatched freshwater sponges

35 mm coverslip-bottom dishes with a 10 mm inner well diameter (Mattek #P35G-0-10-C). Note, you can use a different coverslip thickness, but the diameter of the inner well works with the volumes suggested in this protocol.

Fixative [4% formaldehyde (F8775-25ML Millepore] in 95% reagent alcohol).

PTw (1x PBS containing %0.1 Tween-20)

Block Solution (3% Bovine Serum Albumin in PTw).

Primary and secondary antibodies of choice (if immunostaining). We use Alexa Fluor secondary antibodies from ThermoFisher Scientific

Stock solution of appropriate phalloidin conjugate (we use Alexa Fluor Phalloidin conjugates from ThermoFisher Scientific, with the stock solution resuspended in 1 mL methanol)

Hoechst stock solution (10mg/mL)

Mounting medium [either Vectashield (H-1000 Vector Laboratories) or equivalent]

SAFETY WARNINGS

Work with formaldehyde in a chemical fume hood and dispose of waste appropriately.

Plate gemmules in coverslip-bottom culture dishes

1

Note

Details of cleaning and plating sponge gemmules can be found at "Growing Sponges from Gemmules".

Add 3-4 mL volume of culture medium to each dish, and place 1-2 gemmules in the center of the inner well.

2 Grow the sponges for 00:00:00 ~1 week, in the dark (this reduces the growth of Chlroella-like algal symbionts that autofluoresce (particularly in the far-red channel).

Note

Different gemmules develop at quite variable rates. If you are interested in fully developed tissues, you should wait to fix tissues until you see well developed oscula, choanocyte chambers, and water canals.

Fixation and washes

3 Remove the culture medium from the outer well by pipetting or aspiration. Then, carefully remove the residual medium from the inner well using a p200 pipette to avoid damaging the tissue.

4 Gently add 2 mL of fixative (4% formaldehyde in 95% alcohol) to the outer edge of the dish to avoid disrupting the sponge tissues.

Safety information

formaldehyde should be used in a chemical fume hood to avoid breathing toxic fumes

5 Replace the lid to the dish and incubate at Room temperature for 00:45:00 45m

6 Remove the fixative by carefully pipetting from the outer edge of the dish only. (It is better to leave the fixative in the inner well undisturbed to avoid damaging the tissue).

7 Add 3 mL of PTw to the outer edge of the dish, and incubate for 00:03:00 at Room temperature . Remove, and repeat. 3m

Permeabilization and Blocking

8 Add 3 mL of Block Solution to the outer edge of the dish, and incubate for 00:45:00 at Room temperature . 45m

Incubation with primary antibodies (if immunostaining)

9

> **Note**
>
> If you are only staining with dyes like phalloidin and DAPI/Hoechst, you can skip this step and proceed directly to the next section.

Dilute your primary antibody at an appropriate concentration in Block Solution. You will need 80-100 µL of diluted antibody per sample.

> **Note**
>
> If working with a new antibody, you may consider testing a range of concentrations such as 1:50, 1:200, and 1:400 to start.

10 Gently remove all Block Solution from the outer and inner well of your samples. It is important to

remove all residual block solution so that you don't further dilute your primary antibody to an unknown extent.

11 Add 80-100 µL of the diluted primary antibody solution to the inner well of the dish, being careful not to pipette directly onto the sponge tissue.

12 Incubate for 01:00:00 (or 2h if needed) at Room temperature . Alternatively, you can place the sample at 4 °C Overnight . 1h

13 At the end of incubation, it is not necessary to remove the primary antibody by pipetting. Instead, simply add 3 mL of PTw to the outer edge of the dish. Incubate 00:05:00 at Room temperature . Repeat 1x. 5m

Counterstaining for DNA, F-actin

14 Dilute Hoechst and Phalloidin stock solutions to 1:100 [final concentration], and (if antibody staining) the secondary antibody conjugate to 1:500-1:1000 [final concentration] in Block Solution. You will need to prepare at least 80-100 µL of this mixture for each sample. Protect this solution from light.

> **Note**
>
> Take into account the species your primary antibody was produced in. (e.g., if produced in rabbit, make sure to use a goat-anti-rabbit secondary). Also take into account the dye conjugates of the phalloidin you use, and the secondary antibody. (e.g., if using 568-phalloidin, make sure to use a 488 or 657 secondary antibody).

15 Carefully remove the final primary antibody wash from the outer and inner wells of the dish by pipetting.

16 Add 80-100 µL of the Hoechst/Phalloidin/secondary mixture (Staining Solution) to the inner well of the dish.

17 Incubate in the dark, for 00:45:00 at Room temperature . 45m

18 It is not necessary to remove the Staining Solution from the inner well. Instead, add 3 mL of PTw to the outer well area, and incubate in the dark for 00:03:00 . Repeat 1x. 3m

Mounting, storage, imaging

19 Remove the PTw wash from the outer and inner well area of the dish by carefully pipetting.

20 Add 80-100 µL of mounting medium to the inner well of the dish.

> **Note**
>
> Mounting medium is viscous so you should cut the tip off of a 200 µl pipette for this step.

21 Store the sample at 4 °C in the dark until imaging.

> **Note**
>
> Sponges prepared this way are best viewed on an inverted confocal microscope with the 60-100x objectives for seeing cellular-level detail.