Fluorescent Labelling of *Ephydatia muelleri*

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Equipment:

Fine forceps (e.g. Dumont #5)
5 cm Petri dishes or 6 well culture plates
Pasteur pipettes or transfer pipettes for ~1-2 ml volumes
Pipetters: P-1000, P-200, P20 (and possibly P-2)

nail polish
slides
Plasticine
Locloc ® or Ziploc ® box
Parafilm ®
Scissors
tin foil
Kimwipes®
orbital shaker
fridge

Reagents:

20% Paraformaldehyde
8% glutaraldehyde
10% BSA (bovine serum albumin)
100mM PBS (phosphate buffered saline)
Triton X-100 detergent
MOWIOL (see recipe below)

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1. **Fix sponges**

i. About 2 hours before fixing the sponges, move the sponges in culture dishes to the place they will be fixed - they contract when moved, so they need this time to relax before you fix them.

ii. Prepare 4 % Paraformaldehyde fixative 'spiked' with glutaraldehyde.

For 50 ml of fix:

a) 39 ml of PBS
b) 9.2 ml of 20% paraformaldehyde
c) 1.8 ml of 8% glutaraldehyde

iii. Fill each well with 3-5 ml of fixative and quickly but gently using forceps transfer 1 coverslip with live sponges from culture medium into each well of a six-well plate, gently.

iv. Let sponges fix for a minimum of 5 hours in a 6°C refrigerator (can be overnight). We use a Locloc ® or Ziploc ® container to keep the dishes in so that fixative vapour is not released into the fridge.

v. After fixation, using remove fixative leaving enough to cover the sponge (never expose it to air) and gently add PBS to fill the dish. Repeat to rinse 3 times 15 minutes each using PBS.

Note: *Fixed sponges are able to sit in PBS in the refrigerator for weeks and will be still usable.*

2. **Block sponge tissue (to prevent non-specific binding).**

i. Place a piece of parafilm on a flat surface, such as a 6-well plate lid, and put the coverslips with sponges facing upright onto the parafilm. This holds the coverslips in place and keeps the solutions on the coverslips.

ii. Prepare block by mixing together in a tube:

a) 30 μl BSA (per coverslip)
b) 270 μl of PBTx100(= 300 μl) (per coverslip)

iii. Gently place the 300 µl of block onto each coverslip (not directly onto the sponge, but adjacent to it on the coverslip) using a P-1000 micropipette. Place the dish with coverslips gently into a Locloc ® box together with a moistened Kimwipe (prevents coverslips from drying out). (*Important to do this slip by slip so the sponges are not exposed to the air for a long period of time.)*

iv. Let the sponges block for 30 minutes on an orbital shaker at a slow gentle speed.

3. Apply primary antibody.

i. Prepare primary antibody label at desired concentration directly after blocking. Prepare antibody mixture at a final volume of 300 µl (per coverslip).

ii. Centrifuge the primary antibody for 20 minutes at 300 rpm before using, this will assist in avoiding background labeling.

iii. For 1:100 concentration a primary antibody solution was prepared by combining in an Eppendorf:

a) 3 µl of primary antibody
b) 30 µl of 30 % BSA
c) 276 µl of PBTx100

iv. Mix solution by gently pipetting mixture up and down.

v. Remove the block from the coverslips by tipping the flat dish with the coverslip sideways and drawing off the liquid with a pipette.

vi. Add the antibody as previously (2 iii) by gently pipetting it at the edge of the sponges on the coverslips.

vii. Put the dish with coverslips back in the LocLoc ® box with a moistened Kimwipe and leave for a minimum of 5 hours or overnight (at 4 °C) gently rotating on an orbital shaker.

4. Apply secondary antibody.

i. Rinse sponges: Draw off the primary antibody by tipping the dish as above (3v) and gently add 3-500ul of PBS to the coverslips; leave for 10minutes. Repeat 3 times for 10 minutes each leaving the coverslips rotating gently on an orbital shaker each time.

ii. Prepare secondary antibody label: For a final concentration of 1:300 use:

1:300 antibody	1 µl
10% BSA (block)	30 µl
PBS-TX-100 buffer to 300 µl	269 µl

iii. Add secondary antibody label. Now keep the samples covered using tin foil and in the dark for the rest of the labeling process to avoid photo bleaching.

iv. Label for minimum of 5 hours on a gentle speed on the orbital shaker.

5. Counterstain: Actin (Optional, but effective for localizing structures labeled above).

i. Rinse off secondary antibody with PBS as above (4i) 3 times for 10 minutes each.

ii. Prepare the actin label:

a. Combine 1µL Bodipy Fl Phallacidin (green) or 594 Alexa phalloidin (red) in 270µL PBSTx-100 plus 30 µl 10% BSA (300µl per coverslip).
b. Mix the solution by gently pipetting up and down.
c. Add the actin label to each coverslip, 300uL per coverslip.
d. Leave covered in the dark for 5 hours on the orbital shaker.

6. **Counterstain: Nuclei.**

i. Rinse off the actin label with PBS as above (4i), repeat 3 times for 10 minutes each.
ii. Prepare the nuclei label at a concentration of 1:1000.
iii. Centrifuge the Hoechst for 20 minutes at 300 rpm before using.
iv. Combine in a 1.5 ml Eppendorf tube:
    1. 0.3 µl Hoechst 33342,
    2. 30 µl 30% BSA,
    3. 269.7 µl PBTx100
v. Mix solution by gently pipetting mixture up and down.
vi. Add the nuclei label to each coverslip, 300 µl per coverslip. Keep in the dark.
vii. Label for a maximum of 10 minutes only.
viii. Rinse with PBS as above (4i). Rinse 3 times for 30 minutes each.

7. **Mount coverslips onto slides.**

Coverslips are mounted onto slides using MOWIOL mounting medium, nail polish, and plasticine/dental wax. Do not expose the sponges to air for more than a moment during transfer.

i. Using a Pasteur pipette, put MOWIOL onto a slide. Be sure not to have any bubbles in the MOWIOL.
ii. Lift the coverslip from the parafilm using fine forceps and allow the PBS rinse to drain off. Tap the coverslip edge against a Kimwipe to absorb all the liquid (for a brief moment). Then, carefully holding the coverslip edges with the sponge facing down, lightly dab the underside of the coverslip with the Kimwipe to remove all traces of the PBS buffer. Be very gentle so as not to break the coverslip.
iii. Add plasticine legs to each corner of the coverslip, by taking the corner on the coverslip and scraping it against the plasticine. The legs should be on the side of the coverslip that the sponges are on.
iv. Using fine forceps place the coverslip sponge side down onto the MOWIOL, first lowering one side and slowly lowering it until the side you are holding contacts the MOWIOL to avoid air bubbles. The coverslips should be placed so the Plasticine legs and specimens are going directly onto the MOWIOL. Let the Mowiol spread throughout the coverslip.
v. Using the forceps tap gently on each of the corners of the coverslip to anchor it in place.

Note: It is advisable to practice this using an empty coverslip before using the labelled specimens to ensure that you have the correct amount of MOWIOL, not too much so that squeezes out from the coverslip nor any bubbles due to too little MOWIOL

vi. Seal the edges of the coverslips using nail polish. It is typically useful to apply a second seal after several hours.
vii. After the coverslips are mounted onto slides, allow them to dry in the dark at room temperature overnight. *If the coverslips are dried in the refrigerator condensation can form and create air bubbles.*
viii.

Once dry the slides can be stored in a slide box in the refrigerator and will be fluorescent for months or sometimes for years.

Mowiol Mounting medium:

Mowiol 4-88 is a high-quality mounting medium with good anti-fade characteristics. It hardens and matches the refractive index of immersion oil, and thus is particularly suited for this form of microscopy. Additional anti-fade (DABCO) is added to further retard photobleaching.

Mowiol 4-88 (Calbiochem; 475904)
Glycerol (Fisher Chemicals; G/0650/08)
Tris (Sigma; T-8524)
DABCO (Sigma; D-2522)

1. Add 2.4g Mowiol to 6g glycerol and stir briefly with a pipette.
2. Add 12ml dH_2O and stir at room temp for several hours.
3. Add 12ml 0.2M Tris (pH 8.5) and heat to 50^{o}C for 1-2 hrs while stirring.
4. When the Mowiol has dissolved, clarify by centrifugation @ 500 x g for 15mins