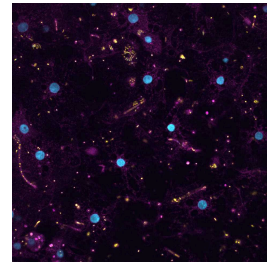


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Bacterial fluorescent in situ hybridization (FISH) in *Ephydatia muelleri*

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Protocol status: Working

We use this protocol and it's working

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Abstract

This protocol is used to visualize bacteria in and around gemmule-hatched freshwater sponges using a Eubacteria FISH probe.

Materials

Freshwater sponge gemmules.

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].

PBS-Tw [1x PBS (41620012 bioPLUS) containing 0.1% Tween-20 (P1379-500ML Sigma-Aldrich)].

Formamide, Deionized (0606-950mL Amresco).

SSC (S24022-1000.0 RPI).

Eub338 probe (Amann et al., 1995. *Microbiological reviews.*).

Hybridization Buffer (Found in Molecular Instruments HCR™ buffers pack in the HCR™ RNA-FISH bundle).

Hoechst 33342 stock solution [10mg/mL] (40046 Biotium).

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



Safety warnings

- ⚠ Work with formaldehyde and formamide (also present in Hybridization Buffer) in a chemical fume hood and dispose of waste appropriately.



Grow sponges

- 1 Add  3-4 mL mL of spring water to a 35mm coverslip bottom dish and place 1-3 gemmules into the center of the inner well.
- 2 Let grow in the dark (to reduce autofluorescence of algae) at  Room temperature for about  168:00:00 (1 week) or until tissue appears developed.

1w

Day One

6h

- 3 Make fixative of 4% formaldehyde in 95% EtOH
 150 µL per sample

Safety information

Do not breathe in fumes from formaldehyde, handle this chemical in a fume hood.

Day One

6h

- 4 Remove water from the outer well area, add  150 µL of fixative to the sponge, and let sit at room temperature for  04:00:00

4h

Note

When pipetting from the wells, do so slowly from the outer well to not disturb sponge tissue. Also pipette slowly when adding solutions to the inner well.

- 5 Closer to the end of the incubation, prepare a 0.1% solution of Tween-20 in 1X PBS (now referred to as PBS-Tw)
 12 mL per sample
Prepare 10% formamide in 2X SSC
 2 mL per sample

**Safety information**

Do not breathe in fumes from formamide, handle this chemical in a fume hood.

- 6 Remove the fixative from the outer well area and replace with  4 mL of PBS-Tw to each well
- 7 Remove the PBS-Tw and repeat the wash two more times
- 8 Add  2 mL of freshly prepared 10% formamide in 2X SSC to each sample and incubate at  37 °C for  02:00:00
- 9 Prepare 1:100 eub338 probe [1µM final concentration] in Hybridization Buffer (Molecular Instruments) and heat to 37°C

2h

Note

After this point, keep samples out of the light to protect the conjugated FITC probe

- 10 Remove the 10% formamide in 2X SSC from the samples and add  80-100 µL of probe mixture to the inner well, being careful to leave the samples flat
- 11 Place lid on sample and leave in humid chamber  Overnight at 37°C

Note

It may be helpful to place damp KimWipes in between samples in a Tupperware container to keep the samples from drying out

Day Two

50m

- 12 Prepare 10% formamide in 2X SSC
 3 mL per sample
Prepare 2X SSC
 3 mL per sample



Prepare 0.2X SSC

🧪 12 mL per sample

Warm all solutions to 🌡️ 37 °C in a water bath

Safety information

Do not breathe in fumes from formamide, handle this chemical in a fume hood.

- 13 Remove probe mixture from samples and add 🧪 3 mL of pre-warmed 10% formamide in 2X SSC. Incubate for ⌚ 00:10:00 at 🌡️ 37 °C 10m
- 14 Remove the 10% formamide in 2X SSC from the wells and replace with 🧪 3 mL of pre-warmed 2X SSC. Incubate for ⌚ 00:10:00 at 🌡️ 37 °C 10m
- 15 Remove the 2X SSC from the wells and replace with 🧪 3 mL of pre-warmed 0.2X SSC. Incubate for ⌚ 00:15:00 at 🌡️ 37 °C 15m
- 16 Repeat step 15 two more times, for a total of three washes 30m
- 17 Prepare a 1:200 dilution of Hoechst (stock concentration of 10mg/mL) in 0.2X SSC
🧪 100 µL per sample
- 18 Remove 0.2X SSC from wells and add about 🧪 100 µL of Hoechst solution to each inner well. Incubate for ⌚ 00:15:00 at 🌡️ Room temperature 15m
- 19 Wash samples with 3mL of 0.2X SSC
- 20 Remove wash and add mounting medium
- 21 Samples are ready to be imaged, or can be left in the dark at 🌡️ 4 °C for preferably up to a week