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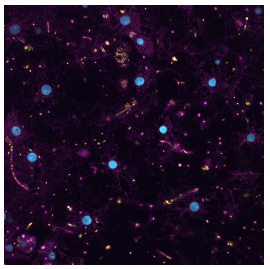
Bacterial fluorescent in situ hybridization (FISH) in Ephydatia muelleri



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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.

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 2h

9 Prepare 1:100 eub338 probe [1µM final concentration] in Hybridization Buffer (Molecular Instruments) and heat to 37°C

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