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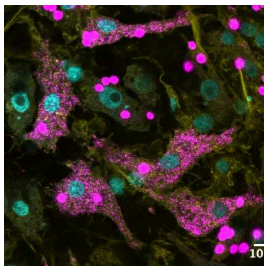
HCR-fluorescent in situ hybridization (HCR-FISH) of gemmule-hatched freshwater sponges



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We use this protocol and it's working

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Abstract

This HCR-FISH protocol using probes and amplifiers from Molecular Instruments is intended for gemmule-hatched freshwater sponges grown in 35 mm coverslip bottom cell-culture dishes with a 10 mm inner-well diameter.

Guidelines

We take reasonable measures to use RNAse-free reagents, but have successfully used this protocol with and without filter-tips.

Materials

Gemmule-hatched freshwater sponges

Sterile Filtered or autoclaved lakewater or store-bought spring water

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.

10x HF buffer (590mM NaCl; 6.7mM KCL; 7.6mM CaCl2; 2.4mM NaHCO) *note, make with RNAse-free molecular biology grade water

Cellbrite-Fix 640 (Biotium)

20% paraformaldehyde solution, methanol free (Electron Microscopy Solutions 15713-S)

SUPERase.in (AM2694 ThermoFisher)

100% Methanol

Reagent-grade (95%) Ethanol

1X PBS (RNAse-free)

1x PBS + 0.1% Tween (PTw) (RNAse-free)

Proteinase K, recombinant, PCR Grade,Solution (Sigma-Aldrich 03115828001)

2 mg/mL glycine (in PTw) (RNAse-free)

Probe Hybridization Buffer and Probe Wash Buffer (Molecular Instruments, HCR Buffer bundle #1 for whole-mount samples)

HCR probe sets with appropriate amplifiers and amplification buffer (Molecular Instruments)

Molecular Biology Grade water (RNAse/DNAse free)

20X SSC Buffer (RNAse-free) (e.g., Sigma-Aldrich S6639-1L)

Can use filter tips to minimize the risk of RNAse contamination

Thermalcycler

0.2 mL PCR tubes

Kim Wipes

100 mm petri dishes

Parafilm

Vectashield or other glycerol-based mounting medium with anti-fade properties

Hoechst (10 mg/mL stock solution)

Before start

Order probes and HCR-FISH kit from Molecular Instruments

Grow sponges in coverslip bottom dishes

1

Note

For detailed information refer to "**Growing sponges from Gemmules**."

Briefly, add 3-4 mL of culture medium to a 35 mm, coverslip bottom culture dish with an inner well diameter of 10mm. Place 1-2 gemmules in the center of the inner well.

2 Grow the sponges for 168:00:00 ~ 1 week in the dark (this reduces the growth of Chlorella-like algal symbionts that autofluoresce (particularly in the far-red channel). 1w

Note

Different gemmules develop at quite different 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.

Membrane counterstaining and fixation

3 Remove culture medium from the outer well and inner well areas by aspiration or pipetting.

Note

Throughout this protocol, try to only remove liquid from the outer well area whenever possible, as pipetting and (particularly) aspiration from the inner well area can damage the sponge tissue. Always pipette slowly when adding and removing solutions.

4 Add 90-100 µL of 1:1000 Cellbrite-Fix 640 (Biotius) in lake/spring water to the inner well area for 00:15:00 at Room temperature in the dark. 15m

Note

This step is optional, but useful because it allows you to see cell shape/boundaries to better understand to localization of your FISH signal. But, if you are interested in an easy-to-identify cell type such as choanocytes or archeocytes, a membrane counterstain may not be necessary.

Note

Depending upon your experimental design you may choose to use a different conjugate of Cellbrite-Fix other than 640 (e.g., 488). But, since Chlorella symbionts in cells autofluoresce strongly in the far-red channel it may make sense to counterstain in this channel to save "cleaner" channels for your FISH probes.

5 To wash, it is not necessary to remove the staining solution. Instead, add 3 mL lake/spring water directly to the outer well area.

6 Immediately remove the wash by pipetting or aspiration from the outer well area.

7 Add 3 mL of cold 4% paraformaldehyde in 1/4x HF Buffer to the outer well area. Place at 4 °C Overnight in the dark.

Safety information

You should not breathe the fumes of Formaldehyde. Work inside a chemical fume hood.

Note

At this point, and in all subsequent steps, it is important to keep your samples shielded from light as much as possible to protect your Cellbrite signal

Treat with RNAse inhibitor

8 Remove 3mLs of fixative from the outer well area. It is not necessary to first remove the residual liquid in the inner well area around the sponge. (Dispose of formaldehyde-containing waste properly)

9 Add 4 mLs of 1/4x HF Buffer to the outer well area of the dish. Incubate 00:05:00 5m

10 Remove the wash from the outer well area, and repeat step 9.

11 Remove the wash from the outer and inner well areas (be very careful pipetting near the tissue).

12 Add 100µl of ¼ x HF buffer containing an RNAse inhibitor (1 U/µl final concentration) to the inner well. Incubate at Room temperature for 00:10:00 . 10m

13 Wash by adding 3 mL ¼ x HF Buffer to the outer well area for 00:03:00 at Room temperature . 3m

14 Remove wash by pipetting from the outer well area only, then repeat wash (step 13) once. 3m

Dehydrate

15 Remove the wash buffer from the outer well area of the dish and add 2 mL of 30% Methanol diluted in ¼ x HF Buffer. Incubate 00:05:00 at Room temperature 5m

Safety information

Work in a fume hood to avoid breathing methanol fumes, and dispose of waste properly.

16 Remove 30% Methanol solution from the outer well area and add 2 mL of 70% Methanol diluted in ¼ x HF Buffer. Incubate 00:05:00 at Room temperature 5m

17 Remove 70% Methanol solution from the outer well area and add 3 mL of 100% Methanol. Incubate 00:05:00 at Room temperature 5m

> **Note**
>
> This could be a stopping point if you want to parafilm the sample and store at -20 C overnight.

18 Remove the Methanol from the outer well area and replace with 3 mL 100% Ethanol. Incubate 00:05:00 at Room temperature 5m

Rehydrate and Permeabilize

19 Remove the 100% Ethanol from the outer well area and replace with 2 mL of 70% Ethanol diluted in PTw (1x PBS + 0.1% Tween-20). Incubate 00:05:00 at Room temperature 5m

20 Remove the 70% Ethanol from the outer well area and replace with 2 mL of 30% Ethanol diluted in PTw. Incubate 00:05:00 at Room temperature 5m

21 Remove the 30% Ethanol from the outer well area and replace with 2 mL of 30% Ethanol diluted in PTw. Incubate 00:05:00 at Room temperature 5m

22 Remove 30% Ethanol from the outer well area and replace with 2 mL of PTw. Incubate 00:05:00 at Room temperature 5m

23 Repeat PTw wash, 1 time.

Proteinase K treatment

24 Remove the PTw from the outer well area and replace with 1 mL of 5 µg/mL Proteinase K (in PTw) for 00:01:30 at Room temperature — 1m 30s

25 Remove the Proteinase K treatment from the inner and outer well area and replace with 2 mL of 2 mg/mL glycine (in PTw) for 00:05:00 at Room temperature — 5m

26 Remove the glycine solution from the outer well area and add an additional 2 mL of 2 mg/mL glycine (in PTw) for 00:05:00 at Room temperature — 5m

27 Remove the glycine solution from the outer well area and replace with 2 mL of PTw for 00:05:00 at Room temperature — 5m

28 Remove the PTw from the outer well area and add an additional 2 mL of PTw for 00:05:00 at Room temperature — 5m

Post-fixation and Wash

29 Remove PTw from outer well area and replace with 3 mL of 4% paraformaldhyde in PTw. Incubate for 01:00:00 at Room temperature . — 1h

Safety information

Formaldehyde fumes are toxin. Work in a chemical fume hood, and dispose of waste appropriately.

30 Remove fixative from the outer well area, and replace with 3 mL of PTw. Incubate for 00:10:00 at Room temperature . — 10m

31 Remove PTw from outer well area and add an addition 3 mL PTw to the outer well area. Incubate for 00:10:00 at Room temperature . — 10m

32 Remove PTw from outer well area and replace with 3 mL of 2x SSC. Incubate for 00:10:00 at Room temperature 10m

33 Remove 2x SSC from the outer well area and replace with 3 mL of 2x SSC. Incubate for 00:10:00 at Room temperature 10m

Prehybridization

34 Bring 30% hybridization buffer (HB) to Room temperature

Safety information

Contains formamide. Use inside a chemical fume hood and dispose of waste properly.

35 Remove 2x SSC from outer and inner well area.

36 Add 1 mL HB to the dish. Incubate for 00:10:00 at Room temperature 10m

37 Remove HB from outer well area and replace with 1 mL HB. Incubate for 00:30:00 at 37 °C 30m

Probe hybridization

38 During Prehybridization, prepare probe solutions.

Note

Molecular Instruments provides probes at 1 µM, with the recommendation to use 0.5 µl in a 100 µl final volume. We find that this concentration works well, but for characterizing a new probe we often also test a higher concentration, such as 5 µl probe in a 100 µl final volume.

If using more than one probe, combine both in the same 100 µl volume per sample. (e.g., 1 µl probe 1 + 1 µl probe 2 + 98 µl HB)

39 After 30 min incubation in HB (minimum), remove HB from the outer and inner wells, then add 100 µL probe solution to the inner well area only.

40 Incubate on a very gently rocking platform at 37 °C Overnight .

> **Note**
>
> It is important that the rocking platform VERY slow and that the angle of incline is not so great that the probe solution drains out of the inner well. It is probably fine to leave the sample stationary, but we haven't tested this.

Washes

41 Heat 30% Probe Wash Buffer (PWB) to 37 °C (4 mL per sample).

42 Add 1 mL preheated 30% PWB to the outer well area (it is not necessary to first remove the probe solution from the inner well area). Incubate at 37 °C for 00:05:00 5m

43 Remove PWB from outer well area and replace with 1 mL 30% PWB to outer well area. Incubate at 37 °C for 00:05:00 5m

44 Remove PWB from outer well area and replace with 1 mL 30% PWB to outer well area. Incubate at 37 °C for 00:05:00 5m

45 Remove PWB from outer well area and replace with 1 mL 30% PWB to outer well area. Incubate at 37 °C for 00:05:00 5m

46 Remove PWB from outer well area and replace with 1 mL 2x SSC. Incubate at Room temperature for 00:05:00 5m

47 Remove 2x SSC from outer well area and replace with 1 mL 2x SSC. Incubate at Room temperature for 00:05:00 5m

Prepare Amplifiers

48 Bring Amplification Buffer (AB) to Room temperature

49

Note

If you are preparing more than one sample, calculate for the # of samples + 1 so that you have plenty of amplifier solution after accounting for pipetting error.

Add 6 pmol (2 µL of a 3 µM stock) of the appropriate amplifier hairpin 1 to 0.2 mL PCR tube.

50 Add 6 pmol (2 µL of a 3 µM stock) of the appropriate amplifier hairpin 2 to a 0.2 mL PCR tube.

51 Add amplifier samples to preheated 95 °C thermal cycler for exactly 00:01:30 . 1m 30s

52 Remove samples from thermal cycler and place in the dark at Room temperature for 00:30:00 30m

53 Combine 2 µL of hairpin 1 and 2 µL of hairpin 2 (and additional hairpins if multiplexing) into 100 µL final volume of AB at Room temperature .

Note

This assumes 1 sample. If doing more samples, just scale the volume of hairpins and AB needed. Also, you will combine amplifiers with different fluorophores into the same tube of AB at this point if you are multiplexing.

Amplification

54 Remove 2x SSC from the outer and inner well area, then add 100 µL of AB to the inner well. Incubate for 00:05:00 at Room temperature 5m

55 Remove AB from the inner well area and replace with 100 µL of AB. Incubate for 00:05:00 at Room temperature 5m

56 Remove AB from the inner well area and add 100 µL of the hairpin solution from step 53.

57 Place up to 3 samples into a 100 mm Petri dish. Add water-soaked kimwipes to the space between two samples and close the lid. Seal the Petri dish with a strip of parafilm, being very careful not to spill the amplifier solution out of the inner well.

58 Incubate in the dark at Room temperature Overnight .

Counterstaining, Mounting, and Imaging 30m

59 Add 2 mL 2x SSC to the outer well area (it is not necessary to first remove the amplifier hairpin solution). Incubate 00:05:00 at Room temperature 5m

60 Remove 2x SSC from the outer well area and replace with 2 mL 2x SSC. Incubate 00:05:00 at Room temperature . 5m

61 Remove 2x SSC and add 1 mL PBS containing 1:100 Hoechst solution. Incubate 00:20:00 at Room temperature . 20m

62 Remove PBS/Hoechst solution and replace with 2 mL PBS.

63 Remove PBS from outer and inner well areas.

64 Add 100 µL Vectashield (or other antifade mounting medium) to the inner well area. Store at 4 °C until ready to image.