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in situ hybridization performed on the peri-rhopalial tissue of a scyphozoan jellyfish

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- 1 Fix tissue 2 h, RT, in freshly prepared 4% PF in 0.1 M PB (pH 9.5) + 0.42M NaCl + 2 mM MgSO₄
 - 1.1 Phosphate Buffer pH 9-9.5: made from phosphate dibasic (No pH adjustment required)
 - 1.2 PBS: 100 mM pH 7.5, 150 mM NaCl
- 2 Wash 3X 5' in PBST, pH 7.5
 - 2.1 PBST: PBS +0.1% Tween
- 3 Dehydration in PBS/methanol 3:1 → 1:1 → 1:3 10 min each then 100% methanol 5 min to ON in -20C.
- 4 Rehydration in PBS/methanol 1:3 → 1:1 → 3:1 10 min each then PBST, 10 min.
- 5 Heat treatment in 1 ml PBST, 5 min. Bain-marie at 95C with stopper. First 2 min, tissue should be

resuspended vigorously every 20 sec, every 60 sec during the rest of incubation in order to prevent glueing of specimens. Snap-cool tubes on ice.

6 Wash 2X in PBST

7 Incubation 5 min in TEAA

7.1 TEA: 0.1 M triethanolamine. Dissolve powder in water. Adjust pH at 7.8 with 5 N NaOH.
TEAA: 5 ml TEA + 12, 5 ul glacial acetic acid. Acetic acid must be added to TEA just before the incubation with tissues.

8 Wash 2X 10 min in PBST

9 Post-fixation in 4% PF in PBST pH 7.5 for 20 min.

10 Wash 3X in PBST, 10 min each

11 Saturation of unspecific binding with 75 ul PBST/HS-DNA (0,5 mg/ml) for 10 min

11.1 PBST/fish sperm: 0, 5 mg/ml fish sperm DNA in PBST. This DNA was made from herring and cod sperm and has a molecular weight distribution of 150-3000 bases.

- 12** Addition of the same amount of HybMix, incubate 5 min
- 12.1** HybMix:
50% formamide
5 x SSC
1mg/ml HS-DNA
0, 1% Tween 20
- 13** Prehybridization in 150 ul HybMix for 10 min RT
- 14** Prehybridization in 150 ul HybMix for 2h at 45C
- 15** Hybridization in 1 ml HybMix + DIG-probes sense or antisense for 1 day and 2 nights at 40C.
Before use, denature probe in HybMix at 70C for 10 min and snap-cool on ice.
- 16** 2 wash in 1 ml of Wash I 60 min at 45C
- 16.1** Wash I:
50% formamide
2 x SSC
0, 1 % Tween 20
- 17** 4 wash in 1 ml of Wash I, 15 min at 45C

- 18** Wash in PBST 20 min.
- 19** Saturation in PBST/10% NGS, 2h in fridge with shaking
- 19.1** Blocking solution: 10 % normal goat serum in PBST
- 20** Incubation in anti-DIG-AP-conjugated antibody diluted 1:2000 in PBST containing 1 % NGS/ 4C over the weekend with shaking. (preadsorption was not necessary).
- 21** Wash 4X in PBST for 20 min.
- 22** Incubation 3X 10min in AP-Buffer (containing 10mM MgCl₂)
- 22.1**
- 22.2**
- 23** Substrate reaction with NBT/BCIP solution

24 Stop reaction with PBST/50 mM EDTA. 3X 5min

24.1

24.2

24.3

24.4

24.5

25

26

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27.1