Multiplexed mRNA *in-situ* Hybridization Chain Reaction (HCR) in *Sycon ciliatum*

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– PROTOCOL –

Day 1 – Sample preparation (version with xylene clearing)

> *From Kojima & Funayama, 2022.*
>
> *This protocol refers to samples that have been already fixed and stored in pure methanol at –20 °C.*

1. Wash samples for 15 min with 500 µL (or 1 mL) of **100 % ethanol** at RT.
2. Clear samples for 30 min with 500 µL (or 1 mL) of **50 % xylene in ethanol** at RT.

> *Always use xylene in glass containers (as it dissolves plastic) and under a chemical safety hood.*

3. Wash samples for 15 min with 500 µL (or 1 mL) of **ice-cold 100 % ethanol** at 4 °C.
4. Wash samples for 15 min each with a graded series of 500 µL (or 1 mL) **ice-cold ethanol (75, 50 and 25 %) in 0.25× HS** at 4 °C.
5. Wash samples for 10 min with 500 µL (or 1 mL) of **ice-cold 0.25× HS** at 4 °C.
6. Wash samples for 10 min with 500 µL (or 1 mL) of **ice-cold 1× PBS** at 4 °C.
7. Post-fix samples for 30 min with 500 µL (or 1 mL) of **ice-cold fixative solution** at 4 °C.

> *Pre-heat probe-hybridization buffer to 37 °C (you will need 700 µL per tube [500 µLfor the pre-hybridization, and 200 µL for the probe solution]).*

8. Wash samples for 4 × 10 min with 500 µL (or 1 mL) of **PBST** at 4 °C.

Day 1 – Sample preparation (version with proteinase-K permeabilisation and acetic anhydride acetylation)

> *From Fortunato et al., 2012; Voigt et al., 2017.*
>
> *This protocol refers to samples that have been already fixed and stored in pure methanol at –20 °C.*

1. Wash samples for 15 min with 500 µL (or 1 mL) of **ice-cold 100 % ethanol** at 4 °C.
2. Wash samples for 15 min each with a graded series of 500 µL (or 1 mL) **ice-cold ethanol (75, 50 and 25 %) in PBST** at 4 °C.
3. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.
4. Rock samples for 15 min with 500 µL (or 1 mL) of **7.5 µg/mL proteinase K in PBST** at 37 °C.
5. Wash samples for two times without incubation with 500 µL (or 1 mL) of **PBST** at RT.
6. Quench samples for 10 min with 500 µL (or 1 mL) of **2 mg/mL glycine in PBST** at RT.

7. Wash samples for 3 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.
8. Acetylate samples for 15 min each with a graded series of 500 µL (or 1 mL) **0.1 M triethanolamine containing 0, 1.5 and 3 µL/mL acetic anhydride** ar RT.
9. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.
10. Post-fix samples for 30 min with 500 µL (or 1 mL) of **fixative solution** at RT.

> *Pre-heat probe-hybridization buffer to 37 °C (you will need 700 µL per tube [500 µLfor the pre-hybridization, and 200 µL for the probe solution]).*

11. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.

Day 1 – Sample preparation (version with proteinase-K permeabilisation and sodium borohydride clearing)

> *This protocol refers to samples that have been already fixed and stored in pure methanol at –20 °C.*

1. Wash samples for 15 min with 500 µL (or 1 mL) of **ice-cold 100 % ethanol** at 4 °C.
2. Wash samples for 15 min each with a graded series of 500 µL (or 1 mL) **ice-cold ethanol (75, 50 and 25 %) in PBST** at 4 °C.
3. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.
4. Rock samples for 15 min with 500 µL (or 1 mL) of **7.5 µg/mL proteinase K in PBST** at 37 °C.
5. Wash samples for two times without incubation with 500 µL (or 1 mL) of **PBST** at RT.
6. Wash samples for 10 min with 500 µL (or 1 mL) of **PBST** at RT.
7. Post-fix samples for 30 min with 500 µL (or 1 mL) of **fixative solution** at RT.

> *Pre-heat probe-hybridization buffer to 37 °C (you will need 700 µL per tube [500 µLfor the pre-hybridization, and 200 µL for the probe solution]).*

8. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.
9. Treat samples for 20 min with 500 µL (or 1 mL) of **fresh 0.1 % SBI** at RT.

> *SBI solutions should be prepared fresh, and used always under a fume hood, as it release molecular hydrogen, which is a highly flammable gas.*

10. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.

Day 1 – HCR detection stage

1. Pre-hybridize samples for 30 min with 500 µL of **pre-warmed probe hybridization buffer** at 37 °C.

> *If reusing probes, pre-warm probe solution to 37 °C, then skip to Step 3.*

> *At this step, samples can be stored in probe hybridization buffer at –20 °C for several months.*

2. Prepare probe solution by adding 0.8 µL (0.8 pmol) of each **probe set 1 µM stock solution** to 200 µL of **probe hybridization buffer** (final concentration of 4 nM) at 37 °C.

   Note: if using 500 µL of probe solution, add 2 µL (2 pmol) of each probe set.

3. Incubate samples for >12 h (overnight) with 200 µL of **pre-warmed probe solution** at 37 °C.

> *Pre-heat probe wash buffer at 37 °C (you will need 500 µLper tube per 4 washes).*

Day 2 – HCR amplification stage

> *Pre-equilibrate amplification buffer to room temperature (you will need 700 µL per tube [500 µL for pre-amplification, and 200 µL for the hairpin solution]).*

1. Wash samples for 4 × 20 min with 500 µL of **probe wash buffer** at 37 °C.

> *Probe solution can be saved and reused for 2–3 times; store at –20 °C.*

2. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **5**× **SSCT** at RT.
3. Pre-amplify samples for 30 min with 500 µL of **amplification buffer** at RT.
4. Snap cool in separate tubes 4 µL of **hairpin H1** (30 pmol) and 4 µL of **hairpin H2** (30 pmol) **3 µM stock solutions**: heat at 95 °C for 90 s and cool to RT in the dark for 30 min.

> *If reusing hairpin solutions, heat at 95 °C and cool at RT, then skip at Step 6.*

5. Prepare hairpin solution by adding **snap-cooled H1 hairpins** and **snap-cooled H2 hairpins** to 200 µL of **amplification buffer** (final concentration of each hairpin of 60 nM) at RT.

   Note: if using 100 µL of amplification buffer, add 2 µL (15 pmol) of each hairpin.

   Note: if using 500 µL of amplification buffer, add 10 µL (75 pmol) of each hairpin.

6. Incubate samples for >12 h (overnight) with 200 µL of **hairpin solution** at RT.

Day 3 – HCR conclusion

1. Wash samples for 2 × 5 min, 2 × 30 min, and 1 × 5 min with 500 µL (or 1 mL) of **5**× **SSCT** at RT.

> *Hairpin solution can be saved and reused for 2–3 times; store at –20 °C.*
>
> *At this step, samples can be stored in the dark in 5× SSCTfor several days at room temperature.*

Day 3 – Sample mounting

1. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **1**× **PBS** at RT.

If using VECTASHIELD® PLUS Antifade Mounting Medium with DAPI (H-2000), skip directly to Step 4.

2. Incubate samples for 20 min with 500 µL (or 1 mL) of **DAPI staining solution** at RT.
3. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **1× PBS** at RT.
4. Incubate samples for 30–60 min with 1 mL of **50 % glycerol in 1× PBS** at RT.
5. Incubate samples for 30–60 min with 1 mL of **75 % glycerol in 1× PBS** at RT.

Maintaining samples at pH 7.40 is critical for the stability of fluorophores and long-term storage of mRNA in-situ HCR samples.

At this step, samples can be stored in the dark in 75 % glycerol for several month at 4 °C.

6. Collect precipitated samples from the 75 % glycerol solution and place them on a cleaned (bridged, if necessary) slide.
7. Add ∼15–20 µL of mounting medium (or VECTASHIELD® PLUS Antifade Mounting Medium with DAPI [H-2000]) directly on samples.
8. Seal the slide with nail polish and store in the dark at 4 °C.

– RECIPES –

Fixative solution (as per Voigt et al., unpublished)

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 100 mL)
MOPS buffer	2.0930 g	209.30	100 mM
NaCl	2.9220 g	58.44	0.5 M
$MgSO_4 \cdot 7\,H_2O$	0.0493 g	246.50	2 mM
Paraformaldehyde	4.0000 g	–	4 %
25 % gluteraldehyde	200.0000 µL	–	0.05 %
Ultrapure water	–	–	–

1. Preparation and handling of the fixative solution should be done under a fume hood. Everything that come into contact with aldehydes should be discarded into the appropriate waste bins.
2. Add 2.093 g of MOPS buffer, 2.922 g of NaCl, and 0.0493 g of $MgSO_4 \cdot 7\,H_2O$ to a becker.
3. Add 4.0 g of paraformaldehyde to the becker.
4. Add 200 µL of 25 % gluteraldehyde to the becker.
5. Add 80 mL of ultrapure water to the becker and start stirring with a magnetic bar.
6. Heat the solution to approximately 35–40 °C to allow the parafolmadehyde to dissolve.
7. Add 1 M NaOH dropwise, to increase the pH of the solution and aid parafolmadehyde full solubilization.
8. Keep stirring until the solution clears.
9. Fill up to 100 mL with ultrapure water, using a graduated cylinder.
10. Adjust the pH to 7.0 by adding dropwise 1 M HCl.
11. *optional* Filter the solution.
12. Store at 4 °C (or −20 °C for long term).

1× Holtfreter's solution (HS)

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 1 L)
NaCl	3.46 g	58.44	0.05900 M
KCl	0.05 g	74.55	0.00067 M
$CaCl_2$	0.10 g	110.98	0.00076 M
or $CaCl_2 \cdot 2\,H_2O$	0.13 g	120.04	0.00090 M
$NaHCO_3$	0.20 g	84.00	0.00240 M
Ultrapure water	–	–	–

1. Dissolve solutes in 800 mL of ultrapure water, by continuous stirring.
2. Fill up to 1 L with ultrapure water.
3. Store the 0.25× HSindefinitely at RT.

1× PBS with 0.1% Tween 20 (PBST or PTw)

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 50 mL)
Tween 20	50.0 µL	1,227.54	0.1%
20× PBS	5.0 mL	–	1×
Ultrapure water	–	–	–

1. Add 5 mL of 20× PBS to a graduated cylinder.
2. Add 50 µL of Tween 20.
3. Fill up to 50 mL with ultrapure water.

5× SSC 0.1% Tween 20 (SSCT)

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 50 mL)
Tween 20	50.0 µL	1,227.54	0.1%
20× SSC	12.5 mL	–	5×
Ultrapure water	–	–	–

1. Add 12.5 mL of 20× SSC to a graduated cylinder.
2. Add 50 µL of Tween 20.
3. Fill up to 50 mL with ultrapure water.

Proteinase K working solution

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 10 mL)
20 mg/mL proteinase K stock solution	3.75 µL	–	7.5 µg/mL
PBST	–	–	–

1. Add 3.75 µL of 20 mg/mL proteinase K stock solution to 8 mL of PBST.
2. Fill up to 10 mL with PBST.

1:500 DAPI staining solution

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 500 µL)
DAPI	1.0 µL	–	1:500
1× PBS	499.0 µL	–	–

– RESOURCES –

1. **mRNA fluorescent *in-situ* HCR in *Sycon* spp.**

   - *****Fortunato, S., Adamski, M., Bergum, B., Guder, C., Jordal, S., Leininger, S., ... & Adamska, M. (2012). Genome-wide analysis of the sox family in the calcareous sponge *Sycon ciliatum*: multiple genes with unique expression patterns. *EvoDevo*, *3*, 1-11. 10.1186/2041-9139-3-14
   - Voigt, O., Adamski, M., Sluzek, K., & Adamska, M. (2014). Calcareous sponge genomes reveal complex evolution of α-carbonic anhydrases and two key biomineralization enzymes. *BMC Evolutionary Biology*, *14*, 1-19. 10.1186/s12862-014-0230-z
   - Voigt, O., Adamska, M., Adamski, M., Kittelmann, A., Wencker, L., & Wörheide, G. (2017). Spicule formation in calcareous sponges: coordinated expression of biomineralization genes and spicule-type specific genes. *Scientific Reports*, *7*(1), 45658. 10.1038/srep45658
   - Voigt, O., Wilde, M. V., Fröhlich, T., Fradusco, B., Vargas, S., & Wörheide, G. (2025). Genetic parallels in biomineralization of the calcareous sponge *Sycon ciliatum* and stony corals. *bioRxiv*. 10.1101/2025.02.06.636789

2. **mRNA *in-situ* HCR in other sponges.**

   - Kojima, C., & Funayama, N. (2022). *In situ* hybridization to identify stem cells in the freshwater sponge *Ephydatia fluviatilis*. In: Blanchoud, S., & Galliot, B. (eds.), *Whole-Body Regeneration*, 335-346. 10.1007/978-1-0716-2172-1
   - Larroux, C., Fahey, B., Adamska, M., Richards, G. S., Gauthier, M., Green, K., & Degnan, B. M. (2008). Whole-mount *in situ* hybridization in *Amphimedon*. *CSH protocols*, pdb-prot5096. 10.1101/pdb.prot5096
   - Musser, J. M., Schippers, K. J., Nickel, M., Mizzon, G., Kohn, A. B., Pape, C., & Arendt, D. (2021). Profiling cellular diversity in sponges informs animal cell type and nervous system evolution. *Science*, *374*(6568), 717-723. 10.1126/science.abj2949
   - Funayama, N., Nakatsukasa, M., Hayashi, T., & Agata, K. (2005). Isolation of the choanocyte in the fresh water sponge, *Ephydatia fluviatilis* and its lineage marker, *Ef annexin*. *Development, growth & differentiation*, *47*(4), 243-253. 10.1111/j.1440-169X.2005.00800.x