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

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 100 mL)
36–38 % formaldehyde	3.7 mL	30.03	3.7 %
25 % gluteraldehyde	0.2 mL	100.12	0.05 %
10× PBS	10.0 mL	–	1×
Ultrapure water	–	–	–

1. Add 3.7 mL of 36–38 % formaldehyde to a graduated cylinder.
2. Add 0.2 mL of 25 % gluteraldehyde to a graduated cylinder.
3. Fill up to 100 mL with ultrapure water.
4. Store the fixative solution at 4 °C.

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

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