in situ HCR v3.0 with *N. furzeri* embryos

Materials

- RNase free 8% Paraformaldehyde (PFA) (Thermo Fisher Scientific, 47347)
- 10× Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) (Research Products International, P32200)
- Tween 20 (Fisher Chemical, BP337)
- Proteinase K, RNA grade, 20 mg/mL (Invitrogen, 2553004)
- 20× Sodium chloride sodium citrate (SSC) (Sigma-Aldrich, S6639)
- Methanol (Fisher Chemical, A411)
- Glycerol (Fisher Scientific, BP229)
- Hoechst (Thermo Fisher Scientific, H3570)

Recipes

PBS (50 mL)	**Reagent**	**Amount**	**Final**
	10× PBS	5 mL	1× PBS
	Fill up to 50 mL with ultrapure H_2O		

PBST (50 mL)	**Reagent**	**Amount**	**Final**
	10× PBS	5 mL	1× PBS
	10% Tween 20	500 µL	0.1% Tween 20
	Fill up to 50 mL with ultrapure H_2O		

4% PFA (20 mL)	**Reagent**	**Amount**	**Final**
	8% PFA	10 mL	4% PFA
	10× PBS	2 mL	1× PBS
	ddH_2O	8 mL	

Proteinase K solution (1 mL)	**Reagent**	**Amount**	**Final**
	20 mg/mL proteinase K	1.5 µL	30 µg/mL
	Fill up to 1 mL with PBST		

5× SSCT (40 mL)	**Reagent**	**Amount**	**Final**
	20× SSC	10 mL	5× SSCT
	10% Tween 20	400 µL	0.1% Tween 20
	Fill up to 40 mL with ultrapure H_2O		

The storage conditions for HCR kits

- Store HCR probe sets, HCR amplifiers, HCR probe hybridization buffer, and HCR probe wash buffer at -20 °C.
- Store HCR amplification buffer at 4 °C. Please mix well and make sure there are no precipitates in the buffer when you bring it to room temperature before use.
- On the bench top, keep stock solutions on ice.
- Make sure all solutions are well mixed before use.

Methods

<u>This protocol is carried out at room temperature (RT) unless otherwise indicated.</u>

Fixation [Day 1]

1. Collect embryos and incubate at 27 °C in a petri dish with Ringer's solution.

2. Transfer the embryos into a clean glass dish with PBS. Use clean fine tip forceps to dechorionize the embryos and remove the enveloping layer membrane.

3. Transfer 5-10 embryos to a 1.5 mL Eppendorf tube and remove excess PBS using the 200 µL pipette.
   - ***Note****: Be aware that embryos might stick and stay in the pipette during transfer. Be careful not to accidentally discard them with the pipette tip. Early-stage embryos in the tube can be briefly spun down in a microcentrifuge for 3 s before any solution changes.*

4. Fix embryos in 250 µL 4% PFA made in PBS. Incubate for 2-3 h at RT or overnight at 4 °C.
   - ***Caution****: Use PFA with extreme care, as it is a hazardous material.*
   - ***Note****: Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*

5. Wash embryos in 250 µL PBS 3 times for 5 min each to stop the fixation.
   - ***Note****: Avoid using calcium chloride and magnesium chloride in PBS, as this leads to increased autofluorescence in the embryos.*

6. Dehydrate and permeabilize embryos in a series of graded 250 µL MeOH/PBS washes sequentially for 5 min each:
   (a) 25% MeOH / 75% PBS (e.g. 10 mL MeOH and 30 mL PBS for 40 mL solution)
   (b) 50% MeOH / 50% PBS (e.g. 20 mL MeOH and 20 mL PBS for 40 mL solution)
   (c) 75% MeOH / 25% PBS (e.g. 30 mL MeOH and 10 mL PBS for 40 mL solution)
   (d) 100% MeOH.

7. Store at -20°C overnight before use.

***Pause Point**: Samples in 100% MeOH can be stored for six months at -20 °C.*

Prepare to stain [Day 2]

8. Rehydrate embryos in a series of graded 250 µL MeOH/PBST washes sequentially for 5 min each:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 100% PBST.

9. Wash embryos in 250 µL PBST 2 times for 5 min each.

10. Treat embryos in 250 µL proteinase K. Wash embryos in 250 µL PBST 2 times without incubation. Postfix embryos with 250 µL 4% PFA for 20 min. Wash embryos in 250 µL PBST 5 times for 5 min each.
    - ***Note**: Skip the proteinase K treatment and the following postfixation step for embryos before pharyngula stage, including embryos in diapause.*
    - ***Note**: Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K or for samples at a different developmental stage.*

***Pause Point**: It is reported that samples in PBST can be stored for up to 4 days at 4 °C. However, the hybridization step is recommended to be started immediately after staining preparation.*

Detection Stage [Day 2]

11. For each sample, transfer 10 embryos to a 1.5 mL tube.

12. Pre-hybridize embryos in 250 µL probe hybridization buffer at 37 °C metal bath for 30 min.

- ***Caution****: Probe hybridization buffer contains formamide, a hazardous material.*
- ***Note****: Pre-heat enough probe hybridization buffer (250 µL for pre-hybridization + 250 µL for hybridization per tube) to 37°C before use.*
- ***Note****: Early-stage embryos tend to be suspended in the probe hybridization buffer. Briefly spinning embryos down will help the solution change.*

13. Prepare probe solution by adding 1 µL of 1 µM probe mixture to 250 µL of probe hybridization buffer at 37 °C. For multiplexing, add 1 µL of each 1 µM probe mixture to the same 250 µL of probe hybridization buffer at 37 °C.
    - ***Note****: For a 1 µM concentration stock, DNA oPools (50 pmol/oligo) are dissolved in 50 µL TE buffer and stored at -20 °C.*
    - ***Note****: The standard reaction concentration of each HCR probe is 4 nM. The HCR probe set scale depends on the reaction volume and the number of experiments. For example, for a 250 µL reaction volume, each experiment will require 1 pmol of each probe, so a 50 pmol probe set scale corresponds to 50 experiments.*

14. Remove the pre-hybridization solution and add 250 µL probe solution to each tube.

15. Incubate embryos overnight (12–16 h) at 37 °C.

Detection Stage [Day 3]

16. Remove excess probes by washing embryos in 250 µL probe wash buffer at 37 °C 4 times for 15 min each.
    - ***Caution****: Probe wash buffer contains formamide, a hazardous material.*
    - ***Note****: Pre-heat enough probe wash buffer (1000 µL per tube) to 37°C before use.*
    - ***Note****: Early-stage embryos tend to suspend in the probe wash buffer. Brief centrifugation can make solution change easier.*

17. Wash embryos in 250 µL 5× SSCT 3 times for 5 min each.

Pause Point*: Samples can be stored in SSCT for hours. However, the amplification step is recommended to be started immediately after the hybridization step.*

Amplification Stage [Day 3]

18. Pre-amplify embryos in 250 µL amplification buffer for 30 min.
    - ***Note****: Equilibrate enough amplification buffer (250 µL for pre-amplification + 250 µL for hairpin solution per tube) to RT before use.*

19. Add 5 µL hairpin h1 and 5 µL hairpin h2 from the 3 µM stock in separate 0.2 mL PCR tubes. For multiplexing, add 5 µL individual hairpin h1 and 5 µL individual hairpin h2 from the 3 µM stock in separate 0.2 mL PCR tubes. Snap cooling the hairpin tubes in a PCR machine as follows:
    (a) 95°C 1min 30s
    (b) 0°C 5min
    (c) 26°C 30min
    - ***Note****: Hairpins should be protected from light as much as possible.*
    - ***Note****: HCR hairpins h1 and h2 are provided in hairpin storage buffer ready for snap cooling. All hairpins should be snap cooled in separate tubes.*
    - ***Note****: The standard reaction concentration of each HCR amplifier hairpin is 60 nM. The HCR amplifier scale depends on the reaction volume and the number of experiments. For example, for a 250 µL reaction volume, each experiment will require 15 pmol of each hairpin, so 1 nmol amplifier scale corresponds to 66 experiments.*

20. Prepare hairpin solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 250 µL amplification buffer.

21. Remove the pre-amplification solution and add 250 µL the hairpin solution to each sample.
    - ***Note****: Keep in dark for the rest of the protocol.*

22. Incubate the embryos overnight (12–16 h) in a box in a dark drawer.

Amplification Stage [Day 4]

23. Remove excess hairpins by washing embryos in 250 µL 5× SSCT 3 times for 5 min each.

Pause Point*: Samples in SSCT can be stored at 4 °C for a week or longer protected from light before microscopy.*

Microscopy [Day 4]

24. Remove 5× SSCT and add 250 µL of 5 µg/mL Hoechst staining solution for around 3 h.

25. Clear embryos by submerging them in a series of 500 µL glycerol/SSCT solutions sequentially for 5 min each:
    (a) 30% glycerol / 70% SSCT

(b) 50% glycerol / 50% SSCT
(c) 70% glycerol / 30% SSCT

***Pause Point**: Samples in 70% glycerol / 30% SSCTPBST can be stored at 4 °C for 3 months or longer protected from light before microscopy.*

26. Place embryos on a clean glass slide without excess glycerol solution. Gently stage the embryos for imaging. Apply a small amount of vacuum grease to each corner of a clean coverslip (not over the edges) and place the coverslip on top of the embryos. Gently tap the corners to seal.
    - ***Note**: Embryos might deform when over-pressing the coverslip. Make sure the coverslip is horizontal or else the embryo will drift after mounting.*

27. Acquire images using a confocal microscope.

Adapted from the working protocol of

Benjamin Martin's Lab, Stony Brook University, 2021/03/02

v1.0: Lu Jia, 2022/08/05