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HCR - Embryo/Larvae fixation and permeabilisation

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ABSTRACT

In situ hybridization chain reaction (HCR) is a method to visualize specific mRNAs in diverse organisms by applying a HCR that is an isothermal enzyme-free nucleotide polymerization method using hairpin DNAs. This specific protocol was developed by Chintan Trivedi based on previous work by Tsuneoka, Y. [1] and Choi [2]. It has many strengths over traditional (F)ISH: probe design is easier and doesn't require optimisation or purification (contamination is not an issue), it is more specific (no off-target labelling), you can label up to six different targets in the same fish (multiplexing).

1. Tsuneoka, Y. and H. Funato, Modified in situ Hybridization Chain Reaction Using Short Hairpin DNAs. Frontiers in Molecular Neuroscience, 2020. **13**.
2. Choi, H.M.T., et al., Third-generationin situhybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. Development, 2018. **145**(12): p. dev165753.

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Notes

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- Aggressive permeabilization leads to an increase in background autofluorescence
- HCR Probes and hairpins do not require excessive permeabilization in dissected brains or whole-mount embryos/larvae

- Transgenic/genetically driven fluorescence (GFP/RFP) persists after the HCR protocol.

Buffer Recipes

2 **20X SSC**

Dissolve in 300 mL ddH2O

- 70.12g NaCl
- 35.3g Sodium citrate
- Adjust pH to 7.0 with 1M HCl

Adjust volume to 400 mL

3 **5X SSCTw**

- 12.5 mL 20X SSC
- 100 µL 50% Tween Up
- 50 mL ddH2O

4 **1X PBSTw**

- 50 mL 1X PBS*
- 100 µL 50% Tween

*no $CaCl_2$ or $MgCl_2$ as it leads to increased autofluorescence

Prepare Stock Probe stock solution

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- *Plate takes a long time to defrost properly, leave at room temperature for a few hours*
- *Each well in 96 well plate is normalised to 100 µM*

1. Add 10 uL from each well to 1.5 mL Eppendorf
2. Make up to 1 mL with nuclease-free water after all the individual probes have been added

(i.e. 20 probe pairs = 40 individual probes = 40 wells = 400 µL probes + 600 µL water)

Day 0

6 Cull embryos using an overdose of Tricaine (4-5 mL 25X in a 50 mL Petri dish)

- *Note. Zebrafish larval brain anatomy is standardised at day 6 or 7 post fertilisation.*

Fix embryos/larvae in 2 mL of 4% paraformaldehyde (PFA) **overnight** at 4° C on a rocker (12 – 18 hours)

- *Note: Use sweet PFA (4% (w/v) sucrose, 1.6b sucrose in 40 mL) for fixing larvae that are to be dissected later*
- *Note: Use fresh PFA (no more than three days old) and cool to 4° C before use to avoid increased autofluorescence*

Day 1

7 **Wash** embryos/larvae 3 x 5 min with 1 mL PBS to stop the fixation
- *Note: Ensure PFA is disposed of* safely

8 (Optional) **Dissect** the brains in PBS
- *Note: If left undissected, skip to step 8*

9 (Optional - The HCR probes are very small, so HCR will work with no permeabilisation at all, even in undissected brains. Only perform this step if you plan immunostaining or DAPI labelling alongside HCR. Artur does permeabilise for DAPI staining in dissected brains)

Permeabilise, treat with 1 mL of proteinase K (30 µg/mL) for 15 minutes at room temperature.
- *Note: Optimised for 6 dpf larvae*

Wash with PBST (1 mL each) x2 without incubation (rinse)

10 (Optional - postfix important for dissected brains to harden and increase structural stability)

Postfix with 1 mL of 4% PFA for 20 minutes at room temperature

11 **Wash** x3 for 5 minutes in 1 mL PBST

Day 1 - HCR Detection

12 For each sample, transfer 8 - 10 embryos/larvae to a 1.5 mL Eppendorf tube
- *Note: Probe concentration optimised for 8 - 10 fish. Less fish is ok, as excess will be washed out*

13 **Pre-hybridise** with 500 µL of probe hybridization buffer for 30 min at 37° C.

14 Simultaneously, **prepare probe solution** by adding 2-4 pmol of each probe set (e.g., 2 µL of 1 µM stock) to 500 µL of probe hybridisation buffer at 37° C
- *Note: For fewer probe pairs (<10), use a higher concentration of probe to improve probe*

hybridization efficiency

15 Remove the pre-hybridisation solution and **add the probe solution.**

16 Incubate embryos/larvae **overnight** (12 – 16 hours) at 37° C

- *Note: Hybridisation timing is flexible – can be left longer if needed*

Day 2

17 Remove excess probes by washing larvae 4 x 15 min with 500 µL of probe wash buffer at 37°C.

- *Note: preheat probe wash buffer to 37° C before use.*

18 **Wash** embryos/larvae 2 x 5 min with 5x SSCT at room temperature.

Day 2 - HCR Amplification

19 Pre-amplify embryos/larvae with 500 µL of amplification buffer for 30 min at room temperature.

- *Note: equilibrate amplification buffer to room temperature before use.*

20 Prepare 30 pmol** of each hairpin, h1 and h2, in separate tubes
Snap cool by heating to 95° C for 90 seconds and cool to room temperature for ~30 minutes (there is a protocol in the Themocyclers machines)

- ***we routinely use 4 µL of each haprin, later diluted in 190 µL amplification buffer, for 6-8 larvae*
- *HCR hairpins h1 and h2 are provided in hairpin storage buffer ready for snap cooling.*
- *h1 and h2 should be snap cooled in separate tubes. DO NOT mix them before snap cooling*

21 Prepare the hairpin solution by adding snap-cooled h1 and snap-cooled h2 to 190 µL amplification buffer (for 6-8 larvae)

22 Remove the pre-amplification buffer solution and add the hairpin solution

23 Incubate the embryos/larvae overnight (12-16 hours) in the dark at room temperature.

- *Note: amplification timing is flexible, can be left longer if needed*

Day 3

24 Remove excess hairpins by washing with 500 µL SSCT at room temperature:

1. 2 x 5 min
2. 2 x 30 min
3. 1 x 5 min

25 Wash embryos/larvae with PBS (1 mL each) 2 x without incubation

26 **Samples can be stored in PBS at 4° C protected from light before microscopy for 2-3 days.**

(Optional) DAPI Staining

27 *This protocol is for dissected brains only*

1. Dilute 1 µL DAPI stock (5 mg/mL) in 500 µL 5 x SSCT at room temperature (Final conc 10 µg/mL)
2. Remove PBS and add DAPI solution
3. Incubate for 2 hours at room temperature or 4°C overnight
4. Wash embryos/larvae with PBS (1 mL each) 2 x without incubation

Samples can be stored at 4° C protected from light before microscopy for 2-3 days