Xenopus Whole-Mount *In Situ* Hybridization Chain Reaction (HCR-FISH)

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1 Introduction

This document outlines a protocol for using third-generation *in situ* Hybridization Chain Reaction (HCR-FISH v3.0) in whole-mount *Xenopus* embryos. In addition to use in whole-mount embryos, this protocol has also been adapted for use in *Xenopus* animal caps and lamprey embryos. Unless otherwise noted, all steps are performed at room temperature.

2 Protocol

Day 0 - Embryo collection

Fixation

- Fix embryos[1] in MEMFA fixative[2] for 45 min.-1 hr.
- Wash 3 x 5 min. in PBS-T[3]

Dehydration and storage

- Wash 3 x 5 min. in MeOH
- Incubate overnight in MeOH at -20°C
- Proceed to Day 1 or store long-term at -20°C

Day 1 - Hybridization

Rehydration

- Wash 1 x 5 min. in 75% MeOH/25% PBS-T
- Wash 1 x 5 min. in 50% MeOH/50% PBS-T
- Wash 1 x 5 min. in 25% MeOH/75% PBS-T
- Wash 3 x 5 min. in PBS-T

Bleaching

- Mix fresh bleaching solution.[4]
- Bleach under bright fluorescent light for 2 hrs.[5]

Acetic anhydride treatment

- Wash 2 x 5 min. in 0.1M TEA
- Wash 1 x 5 min. in 25 μL acetic anhydride/10 mL 0.1 M TEA[6]
- Wash 1 x 5 min. in 50 μL acetic anhydride/10 mL 0.1 M TEA
- Wash 3 x 5 min. in PBS-T

Hybridization

- Wash 1 x 5 min. on ice in 50% PBS-T/50% 5x SSC-T[7]
- Wash 1 x 5 min. on ice in 5x SSC-T
- Wash 1 x 5 min. on ice in Hybridization Buffer
- Wash 1 x 30 min. at 37°C in Hybridization Buffer
- Dilute probes in Hybridization Buffer and incubate at 37°C overnight [8][9]

Day 2 - Amplification

To prepare, pre-heat Probe Wash Buffer at 37°C for 30 min. and bring Amplification Buffer to room temperature.

Probe washes

- Remove probe solution and store at -20°C for future re-use.[10]
- Wash 4 x 15 min. at 37°C in Probe Wash Buffer

Signal Amplification

- Wash 1 x 30 min. in Amplification Buffer
- Snap cool hairpins by heating to 95°C for 30 sec. and allowing to cool to room temperature for 30 min. in a dark place/drawer[11]
- Dilute snap-cooled hairpins in Amplification Buffer[12]
- Add hairpin solution and incubate overnight in the dark at room temperature.

Day 3 - Imaging Prep

Hairpin Removal

- Wash 2 x 5 min. in 5x SSC-T
- Wash 1 x 30 min. in 5x SSC-T w/ DAPI[13]
- Wash 1 x 30 min. in 5x SSC-T
- Wash 1 x 5 min. 5x SSC-T
- Wash 3 x 5 min. in PBS[14]
- Either image immediately in PBS for surface layer imaging, store at 4°C for up to 2 weeks, or proceed to optical clearing

Optical Clearing

- Wash 1 x 30 min. in RIMS[15]
- Incubate overnight in RIMS
- Mount on slide in RIMS and image on confocal microscope.

3 Buffer recipes

RIMS buffer recipe is adapted from (**?**), while all other buffer recipes are adapted from (**?**). All recipes are for 40 mL of solution, but can be scaled up or down as necessary.[16]

5x SSC-T

- 10 mL of 20x SSC
- 400 μL of 10% Tween-20
- Fill up to 40 mL with DEPC H_2O.

Hybridization Buffer

- 12 mL formamide
- 10 mL 20x SSC
- 360 μL 1 M citric acid, pH 6.0
- 400 μL 10% Tween-20
- 40 μL 50 mg/mL heparin
- 400 μL 100x Denhardt's solution
- 8 mL 50% dextran sulfate
- Fill up to 40 mL with DEPC H_2O.

Aliquot into 8-10 mL volumes and store at -20°C.

Probe Wash Buffer

- 12 mL formamide
- 10 mL 20x SSC
- 360 μL 1 M citric acid, pH 6.0
- 400 μL 10% Tween-20

- 40 μL 50 mg/mL heparin
- Fill up to 40 mL with DEPC H_2O.

Aliquot into 8-10 mL volumes and store at -20°C.

Amplification Buffer

- 10 mL 20x SSC
- 400 μL 10% Tween-20
- 8 mL 50% dextran sulfate
- Fill up to 40 mL with DEPC H_2O.

Aliquot into 8-10 mL volumes and store at 4°C.

50% Dextran Sulfate

- 20 g dextran sulfate powder
- 24 mL DEPC H_2O
- Heat to dissolve dextran sulfate into solution.
- Fill up to 40 mL with DEPC H_2O.

Aliquot into 8 mL volumes and store at -20°C.

RIMS - Refractive Index Matching Solution

- 40 g histodenz powder
- 24 mL 20 mM Phosphate Buffer
- Heat to dissolve histodenz powder into solution.
- Fill up to 40 mL with DEPC H_2O.

Notes

[1] This protocol is optimized for 1.8 mL borosilicate glass vials (VWR #82028-426), with 5 embryos/vial.
[2] While this protocol used MEMFA, it is possible that other fixatives such as PFA will also work.
[3] We used 0.1% Tween-20 as a detergent, but it is possible that Triton may also work, especially when combining HCR with immunofluorescence.
[4] Combine 6.5 mL DEPC H_2O, 2 mL 2x SSC, 1 mL H_2O_2 and 0.5 mL formamide.
[5] We used a 100W LED flood light.
[6] Prepare the acetic anhydride mixtures fresh immediately before treatment
[7] We pre-cool the solutions on ice for 10-15 minutes, and place vials on ice during the wash. We saw good results without the need to rock the vials while on ice.
[8] Probes ordered from Molecular Instruments are typically shipped as a 1 μM stock solution. We dilute this stock solution 1:250 by adding 1 μL probe stock to 249 μL Hybridization Buffer, for a final probe concentration of 4 nM.
[9] We saw good results both with and without rocking vials at 37°C.
[10] Signal degraded after several uses, but we typically saw signals after re-using probe dilutions 3-5 times. For quantitative imaging purposes, use fresh probes for each experiment.
[11] For use in 1.8 mL vials, snap cool 2 μL each of H1 and H2 hairpins. Keep H1 and H2 hairpins separate at this stage, but you can mix multiple fluorophores to reduce the number of tubes. E.g. for multiplexed fluorophores using B1-AlexaFluor647 and B2-AlexaFluor546 hairpins, we would snap cool B1-H1 and B2-H1 hairpins in one vial, and B2-H1 with B2-H2 hairpins in a second vial.
[12] We diluted hairpins to a final concentration of 60 nM. We diluted 3 μL stock solutions from Molecular Instruments into 100 μL volumes by diluting 1:50, adding 2 μL of each hairpin into 100 μL of Amplification Buffer. E.g. for multiplexed fluorophores using B1-AlexaFluor647 and B2-AlexaFluor546 hairpins, each 100 μL volume will contain 2 μL B1-H1 hairpins, 2 μL B1-H2, 2 μL B2-H1 and 2 μL B2-H2.
[13] Dilute DAPI stock 1:1000 in relevant volume of SSC-T
[14] For combining the HCR protocol with immunofluorescence, wash in PBS-T and then proceed with blocking and then primary antibody incubation.
[15] We used 88% histodenz dissolved in 20 mM phosphate buffer. Lower concentrations of histodenz can be used to adjust the refractive index for different microscope objectives, but are less efficient at increasing imaging depth.
[16] Dextran sulfate and 5x SSC-T can be stored indefinitely, but all other buffers should be re-made approximately every month for the best signal. When the lab

is only performing sporadic HCR-FISH experiments, we will typically cut volumes down to 10 mL.

References

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