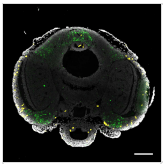

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Hybridization Chain Reaction on paraffin sections

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ABSTRACT

This protocol has been optimized for HCR-IHC on paraffin sections of *Octopus vulgaris* embryos. More detailed information about our experiments can be found in the following publication: https://elifesciences.org/articles/69161

This protocol also led to successful results on killifish and mouse brain paraffin sections. The protocol is largely based on the recommended protocol by Molecular Instruments (https://www.molecularinstruments.com/hcr-rnafish-protocols) and on the protocol currently being used by the laboratory of Cris Niell (https://nielllab.uoregon.edu/).

For designing our HCR probes, we make use of the probe generator (https://github.com/rwnull/insitu_probe_generator; https://www.biorxiv.org/content/10.1101/2021.04.22.439825v1) and we order our probe sets from IDT (Integrated DNA Technologies).

EXTERNAL LINK

https://elifesciences.org/articles/69161

PROTOCOL CITATION

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https://protocols.io/view/hybridization-chain-reaction-on-paraffin-sections-bxd7pi9n

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Deryckere, A. et al. (2021) 'Identification of neural progenitor cells and their progeny reveals long distance migration in the developing octopus brain', eLife. Available at: https://doi.org/10.1101/2021.03.29.437526.

KEYWORDS

HCR, Hybrization Chain Reaction, Paraffin, FISH

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IMAGE ATTRIBUTION

In situ hybridization (HCR v3.0) for neuroD combined with immunostaining against PH3 on a transversal paraffin section of an Octopus vulgaris embryo (Stage XV.2). Overview image showing expression of neuroD (green) and presence of mitotic cells (yellow) together with a DAPI (grey). Scale bar represents 100 µm. B2 amplifier - 647 nm.

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GUIDELINES

All glassware used was baked overnight at 180°C. Plastic lids, the bench, etc. were cleaned thoroughly with EtOH and RNase away. Work as RNase-free as possible during the duration of the experiment.

MATERIALS TEXT

BUFFER RECIPES FOR IN SITU HCR v3.0

Glassware should be baked at 180°C overnight.

Probes, amplifiers, probe hybridization buffer and probe wash buffer should be stored at -20°C.

Amplification buffer should be stored at 4°C.

Keep these reagents on ice at all times during probe and amplifier preparation. Make sure all solutions are well mixed before use.

Probe hybridization buffer (aliquot and store at -20°C) => Prepare in 50 ml falcon

Final concentration	*For 40 ml*
30% formamide	12 ml formamide
5x SSC	10 ml of 20 x SSC
9 mM citric acid pH 6	360 ul 1 M citric acid pH 6
0,1% Tween 20	400 ul of 10% Tween 20
50 ug/ml heparin	200 ul of 10 mg/ml heparin
1 x Denhardts solution	800 ul of 50x Denhardt's solution
10% dextran solution	8 ml of 50% dextran sulfate
	Fill up to 40 ml with ultrapure H20

Probe wash buffer (100 ml per experiment/ freeze in 50 ml aliquots) Store at -20°C => Prepare in 0.5l bottle

Final concentration	*For 400 ml*
30% formamide	120 ml formamide
5x SSC	100 ml of 20 x SSC
9 mM citric acid pH 6	3,6 ml of 1 M citric acid pH 6
0.1% Tween 20	4 ml of 10% Tween 20
50 ug/ml heparin	2 ml of 10 mg/ml heparin (4°C)
	Fill up to 400 ml with ultrapure H20 (170,4 mls)

Amplification buffer (4°C) - prepare in 50 ml falcon - make 10 ml aliquots

Final concentration	*For 40 ml*
5 x SSC	10 ml of 20 x SSC
0,1% Tween 20	400 ul of 10% Tween 20
10% Dextran sulfate	8 ml of 50% Dextran sulfate
	Fill up to 40 ml with ultrapure H20

5x SSCT (RT) - prepare 1 L

Final concentration	*For 1 L*
5x SSC	250 ml of 20 x SSC
0,1% Tween 20	10 ml of 10% Tween 20
	Fill up to 1L with ultrapure H20 (740 ml)

50% Dextran sulfate => best to dissolve overnight (add water gradually) - prepare in falcon

20 g of dextran sulfate powder
Fill up to 40 ml with ultrapure H20.

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=> make aliquots of 8 ml and store at -20°C.

10 mg/ml Heparin (50 ml) => aliquot per 10 ml store at 4°C
500 mg heparin
Fill up to 50 ml with ultrapure H20.
Filter 0.2 uM and store at 4°C.
! store powder at room T, store solution at 4°C.

1M citric acid pH 6 (50 ml)
10.5 g in 30 ml
adjust pH to 6.0 with NaOH : make 20 ml of conc NaOH in Rnase-Dnase free water, let dissolve and add slowly.
Fill up to 50 ml

Autoclaved MQ

Hairpins

Ordered from MI:
B1 546 600 pmol
B2 647 600 pmol
B3 488 600 pmol
300 pmol (100 ul of 3 pmol/ul = 3 uM)
Aliquot per 15 ul and store at -20°C.

Probes
Ordered DNA oPools from IDT (without 5' mod): 50 pmol
Dissolved in 100 ul ultrapure water (or Tris pH 7.5). Final concentration is 0.5 pmol/ul. Aliquot per 10 ul and store at -20°C. We use 0.3 pmol = 0.6 ul per slide.

Reagents and supplies
Heparin (Sigma cat H3393): powder stored at room T, aliquots are stored at 4°C.
20x Sodium Chloride Sodium Citrate (SSC: Invitrogen) 1 L, stored at room T.
10 % Tween 20 (Bio-Rad). Protect from light. Stored at Room T.
50x Denhardt's solution (Aliquot and store at -20°C)
Dextran sulfate (Sigma cat D8906): powder stored at 4°C, aliquots are stored at -20°C.
HybriSlip™ Hybridization Covers (Grace Bio-labs)

SAFETY WARNINGS

Both formamide and xylol are toxic substances so make sure to take the appropriate measures.

Tissue preparation

1 Fix tissue overnight in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS-DEPC) at 4°C.

2 Wash and store in PBS-DEPC at 4°C.

3 Transfer tissues to embedding cassettes (Tissue-Tek Biopsy - Chamber Cassette, Sakura). Immerse the cassettes in 0.9% NaCl in DEPC-water overnight, and then in 50% EtOH for a couple of hours (depending on tissue size) before progressive dehydration and paraffin-embedding using an ExcelsiorTM AS Tissue Processor and HistoStarTM Embedding Workstation (Thermo Scientific).

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Make 6 µm-thick paraffin sections on superfrost plus slides and let dry overnight at 37°C. Slides can then be stored at room temperature.

Day 1 (2.5 hours)

5 Deparaffinization, Rehydration and permeabilization

5.1 Bake slides (in a glass holder) at 65°C for 30 min in an oven.

5.2

Rehydrate the tissues by immersing the slides in a series of glass coplin jars under a chemical fume hood.

4 minutes in Xylene 1 100%
4 minutes in Xylene 2 100%
4 minutes in EtOH 100%
4 minutes in EtOH 100%
4 minutes in EtOH 100%

5.3 Move slides back to the oven and bake for 5 min at 65°C.
Change the oven temperature to 37°C .
Prepare the humidified chamber with kimwipes and autoclaved MQ.

5.4 Draw a line with a PAP pen at the white border of the slides.

5.5 Air dry for 5 minutes.

5.6 Prepare the proteinase K solution: 1 µl of proteinase K in 3 ml of PBS-DEPC (Proteinase K rec PCR grade, art. 3115887001, Roche).

5.7 Add 800 µl of proteinase K solution per slide and make sure all sections are covered. Incubate slides in a humidified chamber for 5 min at 37°C. Move the hybridization buffer to ice.

5.8 Wash 2 x 2 min in autoclaved MQ in a glass coplin jar.

6 Hybridization

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6.1 Pre-warm oven and hybridization buffer to 37°C.

6.2 Remove as much of the solution as possible from the slide (pipet/kimwipe).

6.3 

Add 200 ul of probe hybridization buffer on top of the tissue sample (contains formamide, dispose appropriately). Place a piece of parafilm on top to spread the volume across the slide.
Wear a mask suitable for organic solvents or work under a chemical fume hood.

6.4 Pre-hybridize slides for 30 min inside the humidified chamber at 37°C.

6.5 Thaw probes on ice, spin down before using.

6.6 Prepare probe solution by adding 0.3 pmol of each probe mixture to 75 ul of probe hybridization buffer at 37°C (0.6 ul per probe).

6.7 Remove as much of the pre-hybridization solution as possible (with a pipet/kimwipe). Do this slide per slide. Make sure the paper within the humidified chamber is wet enough.

6.8 Add 75 ul of probe solution on top of the tissue sample.

6.9 

Place a hybrislip (Grace Bio-labs) on the slide (this will cause the probe solution to distribute equally across all sections) and incubate overnight (12-16h) in the 37°C humidified chamber.

Day 2 (3 hours)

7 **Preparation**
Prepare wash solutions in coplin jars/falcons and pre-warm in the oven to 37°C before use.
Cover with parafilm to avoid evaporation.

8 **Wash steps**

8.1 

Remove the hybrislip without disturbing the tissue by immersing the slides in probe wash buffer. Wear

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a mask when dealing with the probe wash buffer, contains formamide!

8.2 Replace paper in the humidified chamber. Add autoclaved MQ to the chamber if necessary.

8.3 Removal of excess probes by incubating slides at 37°C in a glass coplin jar.

25% 5X SSCT (10 ml)/75% probe wash buffer (30 ml) for 15 min

50% 5X SSCT (20 ml)/50% probe wash buffer (20 ml) for 15 min

75% 5X SSCT (30 ml)/25% probe wash buffer (10 ml) for 15 min

(Thaw hairpins on ice in the dark and move the amplification buffer to room T)

100% 5x SSCT for 15 min (40 ml)

100% 5x SSCT for 5 min at room T

9 Amplification stage

9.1 Take off SSCT solution as much as possible and replace PAP pen if needed.

9.2 Add 800 µl of amplification buffer on top of the tissue sample and pre-amplify in a humidified chamber for at least 30 min at room T.

9.3 Separately prepare 9 pmol (per slide) of H1 and H2 in separate PCR tubes. Specifically, pipet 1.5 µl of each hairpin [3 µM stock (4.5 pmol for H1 and 4.5 pmol for H2) in hairpin storage buffer] in a separate PCR tube.

9.4 In a PCR thermocycler: heat hairpins at 95 °C for 90s. Immediately put hairpins on ice for 5 minutes, and then leave hairpins at room temperature for 30 minutes IN THE DARK.

9.5 Prepare the hairpin solution by adding all snap-cooled hairpins to 75 ul of amplification buffer at room T. (Add equal amounts of amplification buffer to both hairpin 1 and 2 and then add hairpin 1 to hairpin 2)

9.6 Remove pre-amplification buffer as much as possible (one by one).

9.7 Add 75 ul of the hairpin solution on top of the tissue sample.

9.8

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Place hybrislip on top and incubate overnight (12-16h) in a dark humidified chamber at room temperature.

Day 3 (2 hours)

10 Immerse slide in 5x SSCT at room T to release hybrislip.

11 Remove excess hairpins by incubating the slides in coplin jars with 5x SSCT: 3 x 10 min

12 Continue with IHC as normal if desired. To avoid loss of signal: try and minimize the incubation times and pay attention to the pH (PBS should have a pH of exactly 7.4).

13 Move slides to a coplin jar with SSCT-DAPI 1:1000. And incubate for 20 min in the dark.
(MQ decreases the signal intensity).

14 Incubate 5 min in SSCT.

15 Add 100 ul of Mowiol to the slide and add a cover slip. Let the slides dry and store at 4°C protected from the light.

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