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Hybridization Chain Reaction (HCR) In Situ Protocol

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ABSTRACT

Hybridization Chain Reaction (HCR) In Situ Protocol

This protocol largely follows the HCR v3.0 protocol for whole-mount Drosophila embryos (Choi et al. 2018) with a few adaptations that simplify the procedure and improve signal to noise ratio in our hands. Our lab has used it with good success in *Drosophila melanogaster* (fruit fly) embryos and imaginal discs, *Junonia coenia* (butterfly) embryos and imaginal discs, *Antheraea polyphemus*, (moth) pupal wings, *Parhyale hawaiensis* (amphipod crustacean) embryos, *Tribolium castaneum* (beetle) embryos, *Artemia franciscana* (brine shrimp) nauplii and adults, *Acanthoscurria geniculata* (tarantula) embryos, *Daphnia magna* (water flea) embryos, and *Danio rerio* (zebrafish) embryos. For some examples, please refer to Bruce & Patel 2020 and images below.

Fixation:
Dissect tissue and fix for 10-40 minutes as you would for standard in situ or antibody staining. For fixation, we generally use 3.2% paraformaldehyde mixed in 1X artificial seawater (for *Artemia* and *Parhyale*) or 1X PBS (for *Junonia*, *Antheraea*, and *Anthoscurria*). For *Drosophila* and *Tribolium*, a standard heptane/PEM-FA fixation, with subsequent methanol devitillenization is followed (detailed protocol in Patel 1994). We routinely dehydrate the embryos/tissue into 100% methanol after fixation and store the samples at -20°C for several years without a problem. This dehydration in methanol is not strictly necessary, but often makes the samples less prone to floating in later steps of the protocol. We no longer carry out the xylene treatment or ProteinaseK digestion steps found in other protocols.

Probes:
We have had our probes synthesized by Molecular Instruments (Los Angeles, CA. USA). They recommend starting with a minimum of 1.4kb of sequence with 20 probe sets created from this. We have had success with shorter sequences – our shortest has been 777bp with just a 9 probe set, but obviously longer is better.

We have used five different hairpin amplifier colors (Alexa 488, 514, 546, 594, and 647) available from Molecular Instruments, and all have worked well. We routinely carry out three color in situs using the combination of Alexa 488, 546, and 647. Additionally we have had success with five color labeling experiments with up to nine probes. To create clear separation of five color channels, spectral imaging and linear unmixing have been necessary.

For some species, we have had issues with autofluorescence at shorter wavelengths. In these cases we have found that the Alexa 488 hairpins have led to a poor signal to noise ratio, and favor using the longer wavelength probes (546, 594, and 647).

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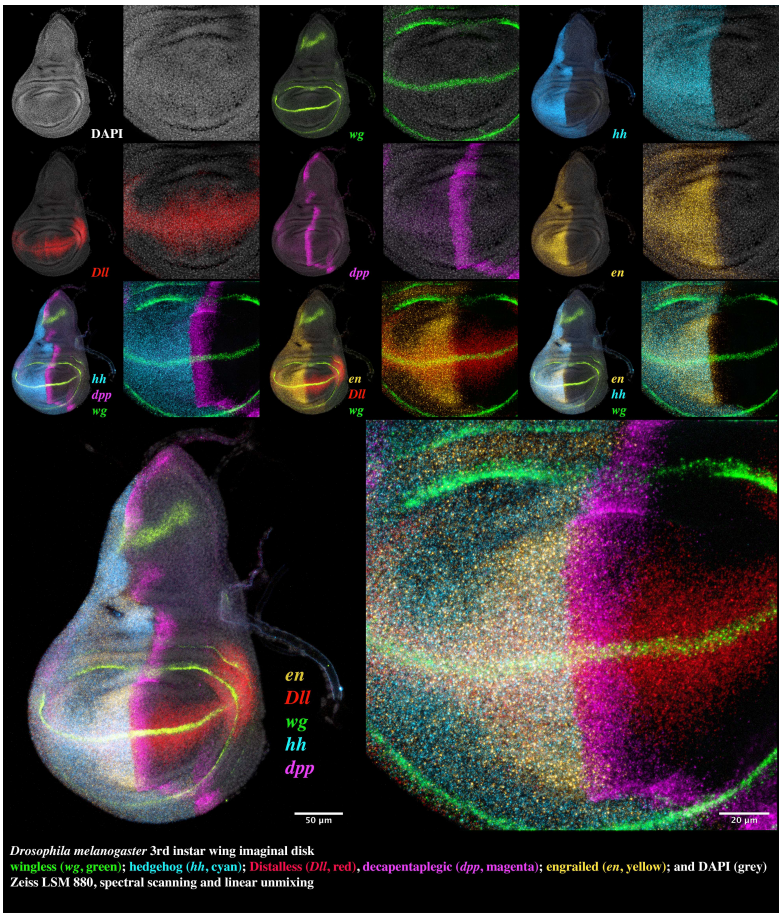

Drosophila melanogaster 3rd instar wing imaginal disk
wingless (*wg*, green); hedgehog (*hh*, cyan); Distalless (*Dll*, red), decapentaplegic (*dpp*, magenta); engrailed (*en*, yellow); and DAPI (grey)
Zeiss LSM 880, spectral scanning and linear unmixing

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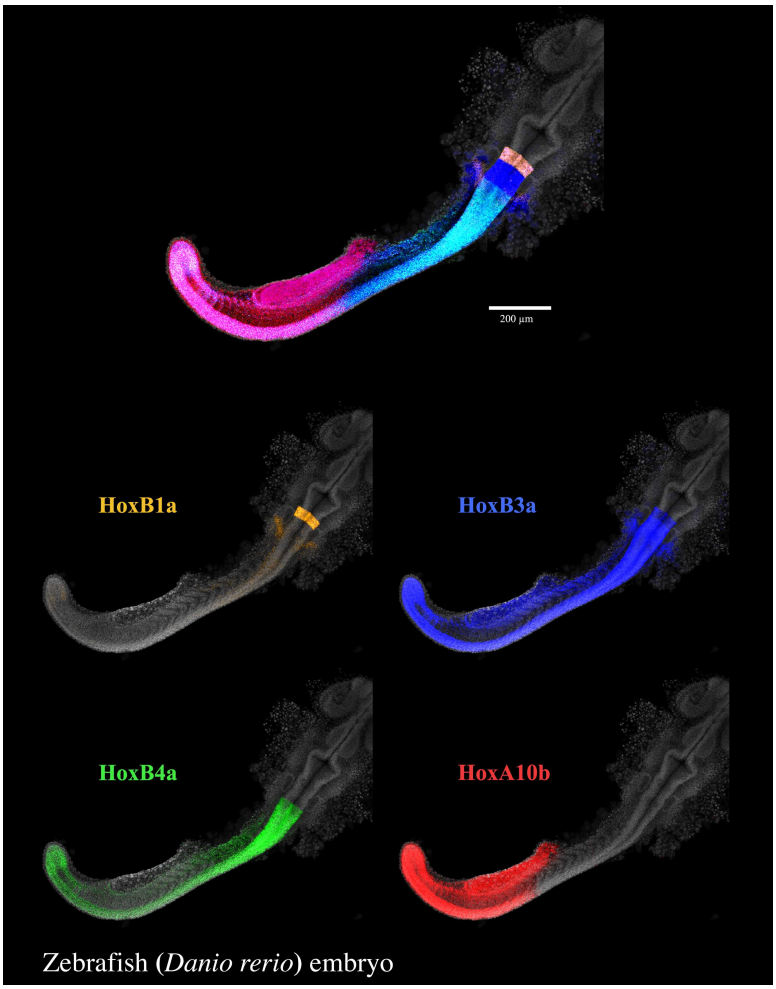

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MATERIALS TEXT

Recipes:

10X PBS	For 1 L of solution:
18.6 mM NaH_2PO_4	2.56 g of $NaH_2PO_4 \cdot H_2O$
84.1 mM $Na_2H_2PO_4$	11.94 g of $Na_2H_2PO_4$
1750.0 mM NaCl	102.2 g of NaCl
	Fill up to 50 mL with distilled H_2O

Prepare **1X PBS** by diluting 1:10 with distilled H2O. Adjust pH to 7.40 with NaOH or HCl after dilution to 1X. Both 1X and 10X PBS can be kept indefinitely at room temperature.

PTw	For 50 mL of solution:
1X PBS	5 mL of 10X PBS
0.1% Tween 20	500 µL of 10% Tween 20
	Fill up to 50 mL with distilled H_2O

20x SSC	For 50 mL of solution
3 M NaCl	8.77 g of NaCl
0.3 M sodium citrate	4.41 g of sodium citrate
	Fill up to 50 mL with distilled H_2O
	Adjust pH with 14 N HCl to 7.0

5X SSCT	For 40 mL of solution
5X sodium chloride sodium citrate (SSC)	10 mL of 20X SSC

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0.1% Tween 20	400 µL of 10% Tween 20
	Fill up to 40 mL with distilled H_2O

Probe hybridization buffer (Store at -20°C)	For 40 mL of solution:
30% formamide	12 mL formamide
5x sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 µL 1 M citric acid, pH 6.0
0.1% Tween 20	400 µL of 10% Tween 20
50 µg/mL heparin	200 µL of 10 mg/mL heparin
1X Denhardt's solution	800 µL of 50X Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate (high molecular weight)
	Fill up to 40 mL with distilled H_2O

Probe wash buffer (Store at -20°C)	For 40 mL of solution:
30% formamide	12 mL formamide
5X sodium chloride sodium citrate (SSC)	10 mL of 20X SSC
9 mM citric acid (pH 6.0)	360 µL 1 M citric acid, pH 6.0
0.1% Tween 20	400 µL of 10% Tween 20
50 µg/mL heparin	200 µL of 10 mg/mL heparin
	Fill up to 40 mL with distilled H_2O

Amplification Buffer (Store at 4°C)	For 40mL of solution:
5X sodium chloride sodium citrate (SSC)	10 mL of 20X SSC
0.1% Tween 20	400 µL of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with distilled H_2O

50% dextran sulfate	For 40 mL of solution:
50% dextran sulfate	20 g of dextran sulfate powder (high molecular weight)
	Fill up to 40 mL with distilled H_2O

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Detergent Solution	For 500 mL of Solution
1.0% SDS	50.0 mL 10% SDS (filtered)
0.5% Tween	12.5 mL 20% Tween
50.0 mM Tris-HCl (pH 7.5)	25.0 mL 1M Tris-HCl, pH 7.5
1.0 mM EDTA (pH 8.0)	1.0 mL 0.5 M EDTA, pH 8.0
150.0 mM NaCl	15.0 mL 5 M NaCl
	Fill up to 500 mL with distilled H_2O

Glycerol Solutions
Prepare 50% and 70% glycerol solutions by mixing the appropriate volumes of ultrapure glycerol with 1X PBS (pH 7.40). Acidic glycerol will cause rapid loss of HCR probe signal.

Day 1 2m

1 20m
Perform PTw Washes:

Note: If your embryos/tissue are stored in methanol, first rehydrate into PTw. For larger embryos, we recommend rehydrating step-wise 75/50/25% methanol in PTw).

1 × 10min PTw wash **00:10:00**
1 × 5min PTw wash (Sonicate here if necessary due to cuticle, see Step 18) **00:05:00**
1 × 5min PTw wash **00:05:00**

2 30m
Permeabilize in 300-500µL Detergent Solution for 30min at **room temperature**. **00:30:00**
During this step, pre-warm probe hybridization buffer to **37°C** (200µL/tube).

3 30m

Pre-hybridize embryos in pre-warmed 200µl of probe hybridization buffer for 30min at **37°C**.
1 X 30min Probe Hybridization Buffer **00:30:00**

*Note for **reusing probe**: Probe is presumably already in correct concentration, pre-warm to* **37°C**, then *skip to Step 5. Number of reuses will depend on sample type and concentration used.*

4

Prepare probe solution by adding 0.8pmol (0.8µl of probe from 1uM stock solution) of each probe mixture to 200µL of probe hybridization buffer at **37°C**.

Troubleshooting tip: If signals are weak, try increasing probe concentration up to 2-3x. We have found this increased

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probe concentration to be useful when dealing with shorter than recommended starting sequences (fewer probe sets).

5

Remove the pre-hybridization solution and add the probe solution.

6 16h

Incubate embryos overnight (12-16h) at **37°C**.

Troubleshooting tip: Extending incubation period up to 20 hours and/or increasing probe concentration up to 5-fold may be useful, especially with probes that have less than 20 probe pairs.

Day 2

7 Pre-heat probe wash buffer to **37°C** before use, equilibrate amplification buffer to **room temperature** before use, and pre-set heat block to **95°C**.

8

Remove and SAVE PROBE SOLUTION, which can be reused at least 3 times. Save used probe solutions at **-20°C**.

9 1h

Wash samples 4 × 15min with 1mL of pre-warmed probe wash buffer at **37°C**.

1 × 15min Probe Wash Buffer **00:15:00**

1 × 15min Probe Wash Buffer **00:15:00**

1 × 15min Probe Wash Buffer **00:15:00**

1 × 15min Probe Wash Buffer **00:15:00**

10 10m

Wash samples 2 × 5min with 1mL of 5X SSCT at **room temperature**.

1 × 5min 5x SSCT **00:05:00**

1 × 5min 5x SSCT **00:05:00**

11 30m

Pre-amplify embryos with 1mL of pre-equilibrated amplification buffer for 30min at room temperature.
Make sure to pre-equilibrate amplification buffer to **room temperature** before use.

1 × 30min Amplification Buffer **00:30:00**

12

During the pre-amplification step, prepare hairpins. Mix 2µL (3µM stock) of each hairpin h1 and 2µL of each hairpin h2 in 100µL of amplification buffer at **95°C** for 90sec, then cool to room temperature in a dark drawer for 30min.

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Troubleshooting tip: Doubling hairpin concentration can help to boost signal.
Note: If reusing hairpins from prior experiment, heat and cool, then skip to Step 13.

13 Remove the pre-amplification solution and add the hairpin solution.

14 16h

Incubate the embryos overnight (2–16h) in the **dark** at **room temperature**.

Day 3 1h 15m

15 1h 15m

SAVE HAIRPIN MIXTURE. Can be reused multiple times. Save used hairpin mixtures at **-20°C**.Remove excess hairpins by washing with 1mL of 5X SSCT at room temperature:

1 × 5min 5X SSCT **00:05:00**

1 × 5min 5X SSCT **00:05:00**

1 × 30min 5X SSCT **00:30:00**

1 × 30min 5X SSCT **00:30:00**

1 × 5min 5X SSCT **00:05:00**

16 Incubate embryos in 50% glycerol solution (in 1X PBS) with DAPI (skip if DAPI is not needed):
30min-1h if using 1.0µg/mL DAPI
2 h or overnight at **4°C** if using 0.1µg/mL DAPI

17 Replace DAPI glycerol with 50%-70% glycerol (in 1X PBS) and store at **4°C**.

Troubleshooting tip: 1X PBS must be at pH 7.40, deviation from this may result in rapid loss of signal.

Notes

18 If the sample has substantial cuticle that would normally block penetration of probes (such as Artemia), we follow the sonication procedure described in Patel 1994, as indicated in Step 1.

19 If you have problems with changing the solutions because the embryos do not settle, fold then twist a piece of Nitex mesh over a glass Pasteur pipette to make a filter, secure twisted Nitex with a ring of cut plastic transfer pipette, and use this to remove buffer without sucking up embryos.

20 You will note that there is no ProteinaseK treatment step in this protocol. Many years ago, when working with grasshoppers (and more standard DIG-RNA probes), we found that this ProteinaseK treatment time had to be optimized for each stage of development, and led to some degradation in morphology. We modified the DIG in situ protocol to instead use a Detergent Treatment step. In adapting the HCR protocol, we have continued with the Detergent step (Step2), and left out ProteinaseK treatment. Since there is no ProteinaseK treatment, a post-fixation step is unnecessary. Indeed, in our tests, we found that a post-fixation step (after Detergent treatment) results in a lower signal to noise ratio. Finally, addition of 0.1% SDS to the hybridization buffer was tested and deemed unnecessary.

21 If you would like to combine in situ and antibody staining, we have had good success with starting a standard immunofluorescence staining protocol following step 15 of this protocol.

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