Multiplexed mRNA *in-situ* Hybridization Chain Reaction (HCR) in *Drosophila melanogaster* adult brain

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– PROTOCOL –

Day 1 – Sample preparation

> *For fixation and the following steps, samples can be placed in Eppendorf tubes or in wells of a spot plate.*
>
> *In each step, samples can be rocked on a nutator or on an orbital (horizontal) shaker.*

1. Dissect adult brains in Schneider's *Drosophila* medium or in nuclease-free 1× PBS.
2. Fix samples for 20 min with 800 µL of **4% paraformaldehyde in PBST** at RT.

> *Pre-heat probe-hybridization buffer to 37 °C (you will need 500 µLper tube [250 µL for the pre-hybridization, and 250 µL for the probe solution]).*

3. Rinse samples for 3 times with 500 µL of **PBST** at RT (no incubation).
4. Wash samples for 3 × 15 min with 500 µL of **PBST** at RT.

Day 1 – HCR detection stage

1. Pre-hybridize samples for 30 min with 250 µL of **pre-warmed probe hybridization buffer** at 37 °C.

> *If reusing probes, pre-warm probe solution to 37 °C, then skip to Step 3.*
>
> *At this step, samples can be stored in probe hybridization buffer at –20 °C for several months.*

2. Prepare probe solution by adding 1 µL (1 pmol) of each **probe set 1 µM stock solution** to 250 µL of **probe hybridization buffer** (final concentration of 4 nM) at 37 °C.
   Note: if using 500 µL of probe solution, add 2 µL (2 pmol) of each probe set.
3. Incubate samples for >12 h (overnight) with 200 µL of **pre-warmed probe solution** at 37 °C.

> *Pre-heat probe wash buffer at 37 °C (you will need 500 µLper tube per 4 washes).*

Day 2 – HCR amplification stage

> *Pre-equilibrate amplification buffer to room temperature (you will need 450 µL per tube [250 µL for pre-amplification, and 200 µL for the hairpin solution]).*

1. Wash samples for 4 × 20 min with 500 µL of **probe wash buffer** at 37 °C.

> *Probe solution can be saved and reused for 2–3 times; store at –20 °C.*

2. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **5× SSCT** at RT.
3. Pre-amplify samples for 30 min with 250 µL of **amplification buffer** at RT.

4. Snap cool in separate tubes 4 µL of **hairpin H1** (30 pmol) and 4 µL of **hairpin H2** (30 pmol) **3 µM stock solutions**: heat at 95 °C for 90 s and cool to RT in the dark for 30 min.

> *If reusing hairpin solutions, heat at 95 °C and cool at RT, then skip at Step 6.*

5. Prepare hairpin solution by adding **snap-cooled H1 hairpins** and **snap-cooled H2 hairpins** to 200 µL of **amplification buffer** (final concentration of each hairpin of 60 nM) at RT.
   Note: if using 100 µL of amplification buffer, add 2 µL (15 pmol) of each hairpin.
   Note: if using 500 µL of amplification buffer, add 10 µL (75 pmol) of each hairpin.
6. Incubate samples for >12 h (overnight) with 200 µL of **hairpin solution** at RT.

Day 3 – HCR conclusion

1. Wash samples for 2 × 5 min, 2 × 30 min, and 1 × 5 min with 500 µL (or 1 mL) of **5× SSCT** at RT.

> *Hairpin solution can be saved and reused for 2–3 times; store at –20 °C.*
>
> *At this step, samples can be stored in the dark in 5× SSCTfor several days at room temperature.*

Day 3 – Sample mounting

1. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **1× PBS** at RT.

> *If using VECTASHIELD® PLUS Antifade Mounting Medium with DAPI (H-2000), skip directly to Step 4.*

2. Incubate samples for 20 min with 500 µL (or 1 mL) of **DAPI staining solution** at RT.
3. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **1× PBS** at RT.
4. Incubate samples for 30–60 min with 1 mL of **50 % glycerol in 1× PBS** at RT.
5. Incubate samples for 30–60 min with 1 mL of **75 % glycerol in 1× PBS** at RT.

> *Maintaining samples at pH 7.40 is critical for the stability of fluorophores and long-term storage of mRNA in-situ HCR samples.*
>
> *At this step, samples can be stored in the dark in 75 % glycerol for several month at 4 °C.*

6. Collect samples from the 75 % glycerol solution and place them on a cleaned (bridged, if necessary) slide.
7. Add ~15–20 µL of mounting medium (or VECTASHIELD® PLUS Antifade Mounting Medium with DAPI [H-2000]) directly on samples.

> *Adjust the amount of embryo suspension and mounting medium according to the cover glass dimensions, the quantity of samples, and the thickness of the bridge.*

8. Seal the slide with nail polish and store in the dark at 4 °C.

– RECIPES –

Fixative solution

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 100 mL)
36–38 % formaldehyde	3.7 mL	30.03	3.7 %
25 % gluteraldehyde	0.2 mL	100.12	0.05 %
10× PBS	10.0 mL	–	1×
Ultrapure water	–	–	–

1. Add 3.7 mL of 36–38 % formaldehyde to a graduated cylinder.
2. Add 0.2 mL of 25 % gluteraldehyde to a graduated cylinder.
3. Fill up to 100 mL with ultrapure water.
4. Store the fixative solution at 4 °C.

1× PBS with 0.1 % Tween 20 (PBST or PTw)

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 50 mL)
Tween 20	50.0 µL	1,227.54	0.01 %
20× PBS	5.0 mL	–	1×
Ultrapure water	–	–	–

1. Add 5 mL of 20× PBS to a graduated cylinder.
2. Add 50 µL of Tween 20.
3. Fill up to 50 mL with ultrapure water.

5× SSC 0.1 % Tween 20 (SSCT)

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 50 mL)
Tween 20	50.0 µL	1,227.54	0.01 %
20× SSC	12.5 mL	–	5×
Ultrapure water	–	–	–

1. Add 12.5 mL of 20× SSC to a graduated cylinder.
2. Add 50 µL of Tween 20.
3. Fill up to 50 mL with ultrapure water.

1:500 DAPI staining solution

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 500 µL)
DAPI	1.0 µL	–	1:500
1× PBS	499.0 µL	–	–

– RESOURCES –

1. **mRNA fluorescent *in-situ* HCR in *Drosophila* brains.**
    - Ferreira, A. A., Sieriebriennikov, B., & Whitbeck, H. (2021). HCR RNA-FISH protocol for the whole-mount brains of *Drosophila* and other insects. https://slack.protocols.io:8443/view/hcr-rna-fish-protocol-for-the-whole-mount-brains-o-bzh5p386.html (accessed on Feb 11th, 2024)