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HCR RNA-FISH protocol for the whole-mount brains of *Drosophila* and other insects

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This is a protocol to perform RNA fluorescent in situ hybridization (RNA-FISH) using hybridization chain reaction (HCR) on whole-mount samples of the brains of the fly *Drosophila melanogaster* and other insects, e.g. the jumping ant *Harpegnathos saltator*. Probes and HCR reagents are purchased from Molecular Instruments. This protocol is loosely based on the "generic sample in solution" protocol published by Molecular Instruments. Our modifications include the description of fixation conditions, counterstaining by Hoechst, and altered washes. Additionally, we use larger concentrations of probes and hairpins following the protocol described by Younger, Herre et al. 2020. We have successfully employed this protocol to stain insect brains with up to 4 different probe sets simultaneously (hairpins conjugated with Alexa Fluor 488, 546, 496, and 647).

Meg A. Younger, Margaret Herre, Alison R. Ehrlich, Zhongyan Gong, Zachary N. Gilbert, Saher Rahiel, Benjamin J. Matthews, Leslie B. Vosshall (2020). Non-canonical odor coding ensures unbreakable mosquito attraction to humans. bioRxiv. https://doi.org/10.1101/2020.11.07.368720

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brain, drosophila, ant, hcr, in situ, larva, rna fish

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WORKING PRACTICES:

Prepare all buffers using nuclease-free water. Use filter tips and nuclease-free tubes. If using spot plates, pre-clean them first with household bleach diluted 1:10 in water and then with 70% ethanol. Wear gloves and adhere to other practices aimed at minimizing RNA degradation in the sample. Working in a clean bench is not required if other RNAse-free practice are followed.

PROBE DESIGN:

We select the target sequence or isoform of the gene of interest and let Molecular Instruments design the probes. For genes with multiple isoforms, either target the isoform that includes as many as possible constitutive exons and as few as possible alternatively spliced exons, or the isoform that has the highest RNA-seq coverage (assessed visually in IGV) if RNA-seq data are available. Aim for the highest number of probes in a set, ideally 40, although we have successfully performed experiments with probe sets containing <20 probes.

AMPLIFIER CHOICE:

We routinely perform multiplexed stainings with up to 4 different probe sets and a Hoechst counterstain. We use amplifiers conjugated with Alexa Fluor 488, 546, 594, and 647. We are able to detect clearly distinguishable signals with minimal bleed-through on our confocal microscope (Leica SP8). However, be aware that simultaneously using fluorophores with partially overlapping spectra (e.g. AF 546 and 594) requires setting narrower detection ranges, which reduces the amount of signal detected.

REAGENTS TO PURCHASE:

Nuclease-Free Water (not DEPC-Treated) **Thermo Fisher Scientific Catalog #AM9937**

20X PBS (Phosphate Buffered Saline) pH 7.4 **growcells.com Catalog #MRGF-6396**

Schneider's Drosophila Medium **Thermo Fisher Catalog #21720024**

Paraformaldehyde, 16% (wt/vol) **Electron Microscopy Sciences Catalog #15710**

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Paraformaldehyde is toxic, consult the SDS sheet for proper handling instructions

Avoid long-term storage of the paraformaldehyde solution after opening the ampoule

Triton X-100 **Sigma Aldrich Catalog #X100**

10% Tween 20 **BIO-RAD Catalog #1662404**

20X SSC **Quality Biological Catalog #351-003-131**

SlowFade™ Gold Antifade Mountant **Invitrogen - Thermo Fisher Catalog #S36936** -
or any other antifade mountant

Hoechst 33258, Pentahydrate (bis-Benzimide), 100 mg **Thermo Fisher Catalog #H1398**
- dissolve in DMSO to 5 mg / mL, aliquot and store at -20 ºC

Methanol **Fisher Scientific Catalog #A412-4**

HCR Probe Hybridization Buffer **Molecular Instruments**

HCR Probe Wash Buffer **Molecular Instruments**

Hybridization and Wash Buffers contain formamide, consult the SDS sheet for proper handling instructions

HCR Amplification Buffer **Molecular Instruments**

BUFFERS TO PREPARE ("Fly" version of the protocol, see Steps for details):

Prepare fresh using nuclease-free water, store at 4 ºC if required after the 1st day of the protocol.

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- 0.3% PBSTX (1X PBS with **0.3%** v/v Triton X-100)
- Fixation Buffer (4% paraformaldehyde prepared from the 16% solution by diluting it with 0.3% PBSTX, i.e. 1 volume 16% PFA + 3 volumes 0.3% PBSTX)
- 0.3% PBST (1X PBS with **0.3%** v/v Tween-20)
- 5X SSCT (5X SSC with 0.1% v/v Tween-20)
- 1X PBS

BUFFERS TO PREPARE ("Ant" version of the protocol, see Steps for details):

Prepare fresh using nuclease-free water, store at 4 ºC if required after the 1st day of the protocol.

- Fixation Buffer (1X PBS with **0.03%** v/v Triton X-100 and 4% paraformaldehyde)
- 0.1% PBST (1X PBS with **0.1%** v/v Tween-20)
- 5X SSCT (5X SSC with 0.1% v/v Tween-20)
- 1X PBS

This protocol uses solutions of paraformaldehyde and formamide, which are highly toxic chemicals. Consult the SDS sheets of the reagents used in this protocol for proper handling instructions.

Day 1 1h 45m

1 Prepare all solutions 15m

2 Pre-heat an aliquot of Probe Hybridization Buffer if proceeding with hybridization on the same day (see step case below) 2m

37 °C

3 Dissect brains in cold Schneider's Medium 10m

Dissection can also be done in 1X Nuclease-Free PBS.

4 Choose the version of the protocol you would like to follow (Fly is default)

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Step 4 includes a Step case.
Fly
Ant

step case

Fly

We follow this path when working with *Drosophila melanogaster* brains.
This path is 2 days shorter than the other one.
Fixation is shorter, there is no dehydration-rehydration, and final washes are in both 5X SSCT and Probe Wash Buffer.

5 Fix brains in 800 µL of Fixation Buffer 20m

00:20:00

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

For fixation and all subsequent steps, samples can be placed either in Eppendorf tubes or in wells of a spot plate (e.g. Pyrex spot plate with 9 depressions, Catalog #CLS722085). Tubes are incubated on a nutator and plates are incubated on an orbital (horizontal) shaker.

6 Rinse 3x with 500 µL of PBST 3m

Room temperature

7 Wash 3x 15 min with 500 µL of PBST 45m

00:15:00

00:15:00

00:15:00

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

8 Pre-hybridize samples by incubating them with 250 µL of warm Probe Hybridization Buffer from step 2 10m

00:10:00 can be extended to 30 min

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37 °C

9 In the meantime, prepare a 16 nM probe solution by adding 8 pmol of each probe mixture (e.g. 8 µL of 1 µM stock) to the warm Probe Hybridization Buffer for the total volume of 500 µL

4 µL of each probe in 250 µL total volume should work as well.

10 Remove the pre-hybridization solution from the sample and add the probe solution from step 9 3m

Take extra care while removing the liquid. Hybridization Buffer is viscous and brains may float.

11

Incubate samples with the probes

Overnight We usually do ~24 h, but incubation can be extended to ~48 h. Minimum recommended is 12 h

37 °C

24 rpm Nutator or **60 rpm Orbital shaker**

Day 2 1h 20m

12 Pre-heat an aliquot of Probe Wash Buffer 2m

37 °C

13 Equilibrate an aliquot of Amplification Buffer to room temperature 2m

Room temperature

14 Remove excess probes by washing 5x 10 min with pre-heated Probe Wash Buffer from step 12 50m

00:10:00

00:10:00

00:10:00

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00:10:00

00:10:00

37 °C

24 rpm Nutator or **60 rpm Orbital shaker**

15 ⚠ 30m

In the meantime, prepare **separately** each hairpin (h1 and h2) of each amplifier. Add 7 µL / sample of the 3 µM stock of each hairpin to a **separate** PCR tube. For example, if amplifiers B1 and B2 are being used, prepare 4 PCR tubes that contain B1-h1, B1-h2, B2-h1, and B2-h2, respectively. Incubate the tubes in a thermocycler at 95 ºC for 90 sec. Immediately take them out of the machine (while it is still at 95 ºC), place them in a rack and incubate them at room temperature **in the dark** for at least 30 min.

00:30:00 or longer

Room temperature

16 Wash samples 2x 5 min with 500 µL of 5X SSCT 10m

00:05:00

00:05:00

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

17 Pre-amplify each sample with 250 µL of Amplification Buffer 10m

00:10:00 can be extended to 30 min

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

18 In the meantime, add 6 µL of each hairpin from step 15 to 100 µL of Amplification Buffer (keep the volume of Amplification Buffer at 100 µL even if multiple hairpin sets are being used)

Room temperature

19 Remove 250 µL liquid from the samples and add the Amplification Buffer + hairpins from step 18 2m

Take extra care while removing the liquid. Amplification Buffer is viscous and the brain may float.

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All the next steps are light sensitive and must be done in the dark!

21

Incubate in **the dark**

Overnight

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

Day 3 3h 15m

22 Equilibrate an aliquot of Probe Wash Buffer to room temperature 20m

Room temperature

23 Add 300 µL of 5X SSCT to samples, which at this point still contain Amplification Buffer + hairpins. This will make the solution inside the tubes less viscous. Remove as much liquid as possible. 2m

24 Wash 1x 5 min with 500 µL of 5X SSCT 5m

00:05:00

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

25 Wash 1x 15 min with 500 µL of Probe Wash Buffer 15m

00:15:00

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

26 2h

If not using Hoechst, skip this step and the next and proceed to step 28.

If using Hoechst: incubate samples with 500 µL of Probe Wash Buffer + Hoechst (1 µL of the 5 mg/mL stock - final concentration 10 µg/mL) for 2 h

02:00:00

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Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

27 15m

Wash 1x 15 min with 500 µL of Probe Wash Buffer

00:15:00

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

28 10m

Wash 1x 10 min with 500 µL of 5X SSCT

00:10:00

Room temperature

24 rpm Nutator or **60 rpm Orbital shaker**

29 2m

Rinse with 1X Nuclease-Free PBS to remove the detergent

30 2m

Remove the PBS and add Slowfade (or another antifade mountant)

31 10m

If necessary, finish dissecting to remove extra tissue. Mount brains on slides.

32

Proceed with imaging

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