HCR RNA-FISH protocol for the whole-mount brains of Drosophila melanogaster

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

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ABSTRACT

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).

CITATION

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.

LINK

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GUIDELINES

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.

MATERIALS

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**

Safety information

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

Note

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**

Safety information

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

HCR Amplification Buffer **Molecular Instruments**

Buffer to prepare:

Note

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

- 1% PBTx (1X PBS with 1**%** v/v Triton X-100 and 1mM glycine)
- 5X SSCT (5X SSC with 0.1% v/v Tween-20)
- 1X PBS

SAFETY WARNINGS

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 ste 2m
case below)
250 µL / sample (If preparing a new probe mixture)
100 µL / sample (If reusing probes)
37 °C

3 Dissect brains in cold Schneider's medium 10m

Note

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

4 Fix brains in 800 µL of 4% PFA / Schneider's medium 20m

00:20:00
Room temperature
24 rpm Nutator or 60 rpm Orbital shaker

Note

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.

5 Rinse 3x with 150 µL of 1% PBTx 3m
Room temperature

6 Wash 3x 15 min with 150 µL of 1% PBTx 45m
00:15:00
00:15:00
00:15:00
Room temperature
24 rpm Nutator or 60 rpm Orbital shaker

7 Pre-hybridize samples by incubating them with 100 µL of warm Probe Hybridization Buffer from step 2 10m
00:10:00 can be extended to 30 min
37 °C

Note

Hybridization buffer is viscous, and the brains might be stuck on the wall. Be careful not to remove them or let them dry out by accident.

8 In the meantime, prepare a 8nM probe solution by adding 0.4 pmol of each probe mixture (e.g. 0.4 µL of 1 µM stock) to the warm Probe Hybridization Buffer for the total volume of 100 µL.
0.4 µL 1µM probe per sample
100 µL Warm probe hybridization buffer per sample

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

Note

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

10 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

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

Room temperature

60 µL per amplifier per sample (if preparing new) + 110 µL per sample

Note

If using 3 different amplifiers for 2 samples, 60 * 3 * 2 + 110 * 2 = 580µL is to be equilibrated.

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

37 °C

450 µL per sample

13 Remove excess probes by washing 4 x 10 min with 100µL pre-heated Probe Wash Buffer from step 12 40m

00:10:00 Wash 100µL pre-heated probe wash buffer

00:10:00 Wash 100µL pre-heated probe wash buffer

00:10:00 Wash 100µL pre-heated probe wash buffer

00:10:00 Wash 100µL pre-heated probe wash buffer

37 °C
24 rpm Nutator or 60 rpm Orbital shaker

14 Wash samples 2 × 5min with 1mL of 5X SSCT at room temperature. 10m
00:05:00 5x SSCT
00:05:00 5x SSCT

15 Equlibrate each sample with 100 µL of Amplification Buffer 10m
00:10:00 can be extended to 30 min
Room temperature
24 rpm Nutator or 60 rpm Orbital shaker

16 During sample equilibration, warm up the thermo cycler to 95°C.

17 Prepare **separately** each hairpin (h1 and h2) of each amplifier. Each sample require 2 µL of **each** 3 µM sto 30m
of hairpin to be mixed in a final volume of 100µL/sample amplification buffer. Aliquot the required amount of hairpin to **individual** PCR tubes. 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

18 Briefly spin down the hairpin solutions, add 100 µL/sample amplification buffer, and use **the same** buffer to resuspend all hairpins used.
Room temperature

19 Remove 100 µL liquid from the samples and add the Amplification Buffer + hairpins from step 17 2m

Note

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

20 **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 Prepare 1.2 ml/sample 5X SSCT

23 Save hairpin mixture and keep at -20°C 2m

24 Wash 2 x 5 min with 150 µL of 5X SSCT 10m

00:05:00

00:05:00

Room temperature

24 rpm Nutator or 60 rpm Orbital shaker

25 Wash 2 x 15 min with 150 µL of 5X SSCT 30m

00:15:00

00:15:00

Room temperature

24 rpm Nutator or 60 rpm Orbital shaker

26 If not using DAPI, skip this step and the next and proceed to step 28. 2h

If using DAPI: incubate samples with 150 µL of SSCT + DAPI (1:1000 of the 5 mg/mL stock - final concentration 10 µg/mL) for 1 h

02:00:00

Room temperature

24 rpm Nutator or 60 rpm Orbital shaker

27 Wash 2 x 10 min with 150 µL of 5X SSCT 20m

00:10:00

00:10:00

Room temperature

24 rpm Nutator or 60 rpm Orbital shaker

28 Rinse with 2X Nuclease-Free PBS to remove the detergent 2m

29 Remove the PBS and add Slowfade (or another antifade mountant) 2m

30 If necessary, finish dissecting to remove extra tissue. Mount brains on slides. 10m

31 Proceed with imaging