Dec 22, 2025

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

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

DOI
https://dx.doi.org/10.17504/protocols.io.n92ld1b4ol5b/v1

Amanda A. G. Ferreira[1], Bogdan Sieriebriennikov[2], Hunter Whitbeck[1], Yen-Chung Chen[1]

[1]New York University; [2]NYU Grossman School of Medicine

Yanqiqi Zeng
NYU

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.n92ld1b4ol5b/v1

External link: https://doi.org/10.1371/journal.pgen.1010802

Protocol Citation: Amanda A. G. Ferreira, Bogdan Sieriebriennikov, Hunter Whitbeck, Yen-Chung Chen 2025. HCR/IF RNA-FISH combined protocol for the whole-mount brains of Drosophila melanogaster. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n92ld1b4ol5b/v1

Protocol status: Working
We use this protocol and it's working

Created: December 20, 2025

Last Modified: December 22, 2025

Protocol Integer ID: 235559

Keywords: brain, drosophila, ant, hcr, in situ, larva, rna fish, rna fluorescent in situ hybridization, rna fluorescent, hcr rna, drosophila melanogaster, fly drosophila melanogaster, hcr reagent, rna, hcr, other insect, mount samples of the brain, ant harpegnathos saltator, molecular instrument, hybridization chain reaction, jumping ant harpegnathos saltator, different probe set, using hybridization chain reaction, probe, larger concentrations of probe, fish protocol, situ hybridization, mount brains of drosophila melanogaster

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
(Invalid date). Non-canonical odor coding ensures unbreakable mosquito attraction to humans. bioRxiv.

https://doi.org/10.1101/2020.11.07.368720 LINK

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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**X100
- 10% Tween 20 **Bio-Rad Laboratories 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**
- HCR Antibody Buffer **Molecular Instruments**
- HCR 2 Antibody Probe **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

3h 40m

1 Pre-warm HCR Antibody Buffer to room temperature

300 µL per sample

2 Dissect brains in cold Schneider's medium

30m

Note

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

3 Fix brains in 500 µL of 4% PFA / Schneider's medium in a spot plate

20m

00:20:00

Room temperature

24 rpm Nutator

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.

4 Transfer the brains to a PCR tube and rinse 3x with 150 µL of 0.3% PBST

5m

Note

Make 0.3% PBST using 1X Nuclease-Free PBS.

Room temperature

5 Wash 3× 15 min with 150 µL of 0.3% PBST

45m

Room temperature

00:15:00

00:15:00

00:15:00

24 rpm Nutator

6 Block samples with 200 µL of HCR Antibody Buffer for 2-4h at 4°C

2h

Note

Blocking time might vary, suggestion for minimum is 1.5h, >2h for optimal signal-to-noise quality

7 Prepare working concentration of 1° antibodies in HCR Antibody Buffer. Prepare 100 µL per sample

8 Remove HCR Antibody Buffer and add 100 µL of 1° antibody solution to sample; incubation sample over two nights at 4°C with gentle rotation

4 °C

24 rpm Nutator

Overnight

Note

For fly optical lobe, primary antibody incubation for two nights is optimal; can be adjusted based on the tissue types

Day 3

2h

9 Pre-warm HCR Antibody Buffer to room temperature

100 µL per sample

10 Remove excess antibodies by washing 4×30 min with 150 µL of 0.3% PBST at room temperature

2h

Room temperature

00:30:00

00:30:00

00:30:00

00:30:00

24 rpm Nutator

Note

1° Antibody solution can be reused for up to 2-3 times.

11 Prepare working concentration of HCR Antibody Probe with specific amplifier in 100 µL of HCR Antibody Buffer; antibody probe e.g. anti-mouse B1

Note

Plan out the use of amplifiers for all antibodies and HCR RNA probes for the multiplex experiment; avoid overlap across all antibodies and RNA probes

12 Remove PBST and add HCR 2° Antibody Probe solution to samples; incubate samples over two nights at 4°C with gentle rotation

4 °C

24 rpm Nutator

Overnight

Day 5

1h 15m

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

250 µL / sample (If preparing a new probe mixture)

110 µL / sample (If reusing probes)

37 °C

14 Remove excess 2° Antibody solution by washing 5 × 5 min with 150 µL of 0.3% PBST 25m

00:05:00

00:05:00

00:05:00

00:05:00

00:05:00

Room temperature

24 rpm Nutator

15 Remove PBST and post-fix sample with 500 µL of 4% PFA, incubate for 10 min at room temperature 10m

Note

Use 500 µL of 4% PFA if transferring back to glass dish, use 200 µL instead if fixing in tube.

16 Remove fixative and rinse sample quickly 3x with 150 µL of 0.3% PBST 5m

17 Pre-hybridize samples by incubating them with 100 µL of warm Probe Hybridization Buffer from step 2. Pipet several times to ensure the brains are submerged in Probe Hybridization Buffer instead of residual PBTx. 30m

00:30:00

37 °C

Note

Hybridization buffer is viscous, and the brains might be stuck on the wall or float to the surface. Make sure you see where the brains are during this step. Be careful not to remove them or let them dry out by accident.

18 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

Note

If the signal is weak, consider increase the probe concentration by 2X - 4X.

19 Remove the pre-hybridization solution from the sample and add the probe solution from step 18. 3m

Note

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

20 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

Day 6

1h 20m

21 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.

22 Pre-heat an aliquot of Probe Wash Buffer

2m

37 °C

450 µL per sample

23 Remove probe solution and store it at -20°C.

Note

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

Probe solution can be reused for > 3 times without apparent decrease

24 Remove excess probes by washing 4 × 10 min with 100µL pre-heated Probe Wash Buffer from step 22

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

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

26 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

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

28 Prepare **separately** each hairpin (h1 and h2) of each amplifier. Each sample require 3 µL of **each** 3 µM stock 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. 30m

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

Note

For lowly-expressed targets, consider increase the amplifier concentration by 2X.

29 Briefly spin down the hairpin solutions, add 100 µL/sample amplification buffer, add 2 µL of each hairpin into the 100 µL amplification buffer to make the final mixture.
Room temperature

30 Remove 100 µL liquid from the samples and add the Amplification Buffer + hairpins from step 29 2m

Note

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

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

32 Incubate in **the dark.**

Overnight

Room temperature

24 rpm Nutator or 60 rpm Orbital shaker

Day 7

2h 12m

33 Save hairpin mixture and keep at -20°C

2m

Note

Hairpin mixture can be reused for > 3 times without apparent decrease of signal.

34 Wash 2 × 5 min and 2 × 15 min with 150 µL of 5X SSCT

40m

00:05:00

00:05:00

00:15:00

00:15:00

Room temperature

24 rpm Nutator

35 If not using DAPI or GFP/RFP booster, skip this step and the next and proceed to step 37.

1h

If using DAPI: incubate samples with 150 µL of SSCT + DAPI (1:500 from [M] 5 µg/µL stock) at room temperature for 1hr.

If using GFP/RFP booster, add 0.3µL to the butter above (1:500 dilution).

Room temperature

01:00:00

24 rpm Nutator or 60 rpm Orbital shaker

Note

DAPI and GFP/RFP booster incubation works well with 1h RT or 4 degree overnight.

36 Wash 3 × 10 min with 150 µL of 5X SSCT 30m

00:10:00

00:10:00

00:10:00

Room temperature

24 rpm Nutator

37 Rinse with 2X Nuclease-Free PBS to remove the detergent; store in PBS with foil to protect sample from light or proceed to mounting and imaging.