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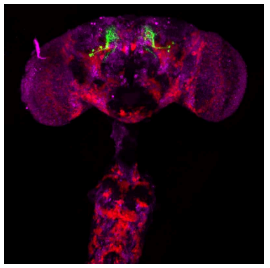
# Expansion-assisted iterative fluorescence in situ hybridization (EASI-FISH) in Drosophila CNS V.1



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

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## Abstract

A straightforward, robust, and reliable protocol (EASI-FISH) that utilizes expansion microscopy and the hybridization chain reaction for multiplexed *in situ* hybridization in thick slices of the mouse brain has recently been described (Wang et al., 2021). Below details a modified version of the EASI-FISH protocol for adult *Drosophila* CNS, which includes antibody detection of fluorescent reporters. The protocol also works well for larval CNS and is expected to be applicable to other tissue types.

## Attachments

DOCX
Fly_EASI_FISH_publis...
39KB

XLSX
Materials_for_EASI_F...
10KB

ZIP
LIGHTSHEETHOLDER_V18...
14.2MB

## Guidelines

**Reference:**

Wang et al., EASI-FISH for thick tissue defines lateral hypothalamus spatio-molecular organization. ***Cell***184 (2021) 6361-6377.e24.

https://pubmed.ncbi.nlm.nih.gov/34875226/

# Materials

### Solutions for Fly brain dissection

- 2% PARAFORMALDEHYDE 16% Aqueous SOL. EM GRADE **Electron Microscopy Sciences Catalog #**15710 in S2 medium.
- Schneider's Drosophila Medium **Thermo Fisher Catalog #**21720024
- PBT (0.5% Triton).
- 70% Ethanol (in nuclease-free water).

### Solutions for Day 1: Labelling RNA

- MOPS (Fine White Crystals/Molecular Biology) **Fisher Scientific Catalog #**bp308100 . Store Room temperature .
- Melphalan-X (Stock [M] 2 mg/mL ; working [M] 1 mg/mL ). Store -20 °C .
- Acryloyl-X, SE **Thermo Fisher Scientific Catalog #**A20770 , Stock [M] 10 mg/mL ; working [M] 0.1 mg/mL . Store -20 °C .
- Press-to-Seal™ Silicone Isolator with Adhesive, eight wells, 9 mm diameter, 0.5 mm deep **Thermo Fisher Catalog #**P24743
- Poly-L-Lysine solution 10ml **TEDPELLA Catalog #**18026 , 1.6 mL + 3.2 µL PHOTO-FLO 200 SOLUTION **Electron Microscopy Sciences Catalog #**74257 . Store 4 °C .
- PBS-Triton (0.1%)
- 0.2 ml PCR tubes (USA Scientific; 1402-4700).
- RNase Away (Thermo Scientific; 7003).

### Solutions for Day 2: Gelation and Proteinase K digestion

- Stock-X. Store -20 °C .
- 10% Ammonium Persulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A3678 . Store -20 °C .
- 10% NNN'N'-Tetramethylethylenediamine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T22500 . Store -20 °C .
- 0.5% 4-Hydroxy-TEMPO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**176141 ). Store -20 °C .
- Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #**P8107S , [M] 800 U/ml ). Store -20 °C .
- [M] 50 millimolar (mM) Proteinase K Buffer: Store Room temperature .
- Small paint brush.

**Solutions for Day 3: Hybridization**

- Hybridization Buffer (Molecular Instruments, Store -20 °C ).
- Probe Wash Buffer (Molecular Instruments, Store -20 °C ).
- DNA oligo probes (Designed and made by Molecular Instruments, Store -20 °C ).
- DAPI (Sigma D9534)/ PBS **Fisher Scientific Catalog #**BP24384 at [M] 500 ng/ml .

**Solutions for Day 5: Hybridization Chain Reaction (HCR)**

- Amplification Buffer (Molecular Instruments, Store 4 °C ).
- Fluorescent Hairpins (448 or 546 from Molecular Instruments or 669 conjugated in lab, Store -20 °C ).
- 5x SSCT (5x SSC, 0.1% Tween in Nuclease Free Water). Store Room temperature .
- 0.5x SSCT (0.5x SSC, 0.1% Tween in Nuclease Free Water). Store Room temperature .
- Invitrogen GFP Polyclonal Antibody Alexa Fluor™ 488 **Thermo Fisher Scientific Catalog #**A-21311 .
- UltraPure™ BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #** AM2616 .

**Solutions for Stripping Probes and Hairpins for Multiplexing**

DNAse I, RNAse-free **Qiagen Catalog #**79254 .

Note

Do **not** use RDD buffer supplied.

**DNAse1 buffer**

| | A | B |
|---|---|---|
| | Tris-HCL pH 8.0 | 10 mM |
| | MgCl2 | 2.5 mM |
| | CaCl2 | 0.5 mM |

## Recipes and Reagents

**200mM MOPS Buffer (10X Stock)**:

1046.5 mg in 25 mL in NFW, pH to 7.7 with [M] 10 Normality (N) NaOH. Store -20 °C .

## Melphalan stock (2.5mg/ml)

Melphalan **Cayman Chemical Company** **Catalog #**16665

DMSO, Anhydrous **Thermo Fisher** **Catalog #**D12345

- Dissolve 2.5 mg per ml in anhydrous DMSO (Invitrogen, D12345).
- Dissolve, heat to 37 °C and vortex vigorously and place on a shaker.
- May take an hour to dissolve.
- Aliquot in 800 µL batches.
- Store in a desiccated environment at -20 °C .

## Acryloyl-X (AcX) stock (10 mg/ml)

Acryloyl-X, SE **Thermo Fisher Scientific** **Catalog #**A20770

- Dissolve in anhydrous DMSO.
- Aliquot in 200 µL batches.
- Any extra aliquot in 5 µL batches (for extra AcX).
- Store in a desiccated environment at -20 °C.
- Don't re-use AcX after thawing.

**Melphalan-X (2mg/ml):**

- Combine an equal concentration of Acryloyl-X ( [M] 10 mg/mL ) and Melphalan ( [M] 2.5 mg/mL ) (1-part AcX to 4-parts Melphalan (ie. 200 µL : 800 µL ).
- Incubate Overnight at Room temperature with shaking.
- Store in 50 µL aliquots in a desiccated environment at -20 °C.
- Use at [M] 1 mg/mL by 1:1 dilution with [M] 20 millimolar (mM) MOPS.

**Stock X:**

[M] 4.04 Molarity (M) Sodium Acrylate* – made from Acrylic Acid as it is made at variable purity
40% Acrylamide Solution **Bio-Rad Laboratories Catalog #**1610140
2% Bis Solution **Bio-Rad Laboratories Catalog #**1610142
NaCl (5 M) RNase-free **Thermo Fisher Scientific Catalog #**AM9760G
Nuclease-free water **Ambion Catalog #**AM9932
10X PBS (ThermoFisher; AM9625)

**4.04M Sodium Acrylate stock solution**

- In a fume hood, place 5.5 mL of acrylic acid into a 50mL tube. Place tube in a Room temperature water bath (e.g. a beaker).
- Add 4.5 mL water.
- Add 7.2 mL [M] 10 Molarity (M) NaOH gradually to prevent excessive heating and evaporation/boiling.
- Remove tube from hood (at this point most of the acrylic acid has been converted to non-volatile sodium acrylate).
- Add [M] 1 Molarity (M) NaOH (nominally 1 mL ) gradually until the pH is between 7.5 and 8 using a pH meter, at Room temperature. Do not use pH test strips.

- Add water up to a final volume of 20 mL .

Note

Acrylic acid has a pKa of 4.76 -- at pH 7.75 this solution has about [M] 4 millimolar (mM) remaining buffering capacity.

**For 9.4 mls Stock X (not including APS +TEMED + 4HT):**

| | A | B |
|---|---|---|
| | 4.04M Sodium Acrylate | 2275 ul |
| | 40% Acrylamide | 625 ul |
| | 2% N,N MethylBisacrylamide | 750 ul |
| | 5M NaCl | 4000 ul |
| | 10x PBS | 1000 ul |
| | Nuclease Free Water | 750 ul |

**10% APS**:

| | A | B |
|---|---|---|
| | APS | 100 mg |
| | H20 | 900 ul |

**10% Temed**:

| | A | B |
|---|---|---|
| | TEMED | 100 ul |
| | H20 | 900 ul |

**0.5% 4HT**:

| | A | B |
|---|---|---|
| | 4HT | 5 mg |
| | H20 | 995 ul |

Note

Store APS, TEMED and 4HT in 150 µL aliquots in PCR tubes @ -20 °C .

**50mM ProK/SDS Buffer (50ml)**

EDTA (0.5 M, pH 8.0, nuclease-free) **Thermo Fisher Scientific Catalog #**AM9260G

10% SDS solution **Thermo Fisher Scientific Catalog #**15553027

| | A | B |
|---|---|---|
| | Tris-HCL-pH8 | 50 mM |
| | EDTA | 1 mM |
| | TritonX | 0.5 % |
| | NaCl | 50 mM |
| | SDS | 0.3 % |

| | A | B |
|---|---|---|
| | 1 M Tris> 50 mM | 2.5 ml |
| | 10 % Triton> 0.5 % | 2.5 ml |
| | 5 M NaCl> 50 mM | 0.5 ml |
| | 0.5 M EDTA | 100 ul |
| | 10% SDS > 0.3% SDS | 1.5 ml |

| | A | B |
|---|---|---|
| | Nuclease Free Water | 42.9 ml |

Note

If brains have TdTomato as a reporter, in the buffer solution increase the NaCl to 500mM and remove SDS to preserve endogenous fluorescence. Depending on expression level, Myr-GFP fluorescence tends to withstand ProK digestion, but we also detect it with a directly conjugated GFP antibody to ensure a good signal.

**RNase-Free DNase1**

- Add 550 µL DNAase1 Buffer to DNase1 powder. Mix.
- Aliquot 50 µL per PCR tube, store @ -20 °C .

**DNase1 buffer (50ml)**

| | A | B |
|---|---|---|
| | 1 M Tris-HCL > 10 mM | 500 ul |
| | 1 M MgCl2 > 2.5 mM | 125 ul |
| | 1 M CaCl2 > 0.5 mM | 25 ul |
| | Nuclease Free Water | 49.350 ml |

**HCR Wash Buffers (50 ml)**

**5x SSCT**:

SSC, RNase-free, 20× **Ambion Catalog #**AM9763

| | A | B |
|---|---|---|
| | 20x RNase free-SSC (ThermoFisher, AM 9763) | 12.5 ml |

| | A | B |
|---|---|---|
| | 10% Tween | 500 ul |
| | Nuclease Free Water | 37 ml |

**0.5x SSCT**:

| | A | B |
|---|---|---|
| | 20x RNase free-SSC | 1.25 ml |
| | 10% Tween | 500 ul |
| | Nuclease Free Water | 48.25 ml |

**Reagents for JF-669 conjugation to unlabelled hairpins:**

- Janelia Fluor® 669 **Tocris Catalog #**6420 Store -20 °C .
- [M] 1 nanomolar (nM) unlabelled amine-modified hairpins (~70mer, Molecular Instruments, Store -20 °C ).
- Acetonitrile anhydrous **Thermo Fisher Scientific Catalog #**042311-K7
- [M] 0.1 Molarity (M) Sodium Bicarbonate pH 8-9.
- DMSO, Anhydrous **Thermo Fisher Catalog #**D12345
- QIAquick Nucleotide Removal Kit (250) **Qiagen Catalog #**28306
- SCREW CAP MICROCENTRIFUGE TUBES SELF-STANDING AMBER (0.5 ml) **USA Scientific Catalog #**1405-9707

**Equipment**

Equipment

| | |
|---|---|
| Savant™ SpeedVac™ SPD120 Vacuum Concentrator and Kits | NAME |
| Vacuum Concentrator | TYPE |
| Savant | BRAND |
| SPD120-230 | SKU |
| https://www.thermofisher.com/order/catalog/product/SPD120-230 | LINK |

## Troubleshooting

## Fly brain dissection

1 Dissect fly CNS in S2 medium.
(for more details see attached protocol)

2 Fix up to 20 brains or 10 CNS in 2 mL of 2% PFA/S2 medium for 00:55:00 in the dark on a nutator. 55m

3 Rinse sample 1 x 2 mL PBST (0.5% Triton).

4 Wash sample 4 x 00:15:00 1 mL PBST(0.5%Triton) on a nutator. 15m

5 Rinse sample 1 x 2 mL 70% EtOH.

6 Store brains in 2 mL 70% EtOH @ 4 °C for up to 6 months.

## Day 1: Labelling RNA

7 Transfer brains to a 0.2ml PCR tube (2-4 brains per tube).

8 Rehydrate with 2 × 5 min wash in 150 µL PBT (0.1%).

8.1 Rehydrate with 2 x 00:05:00 wash in 150 µL PBT (0.1%) (1/2). 5m

8.2 Rehydrate with 2 x 00:05:00 wash in 150 µL PBT (0.1%) (2/2). 5m

9 Incubate brains 1 x 00:30:00 in 150 µL [M] 20 millimolar (mM) MOPS Buffer. 30m

10 Thaw Melphalan-X (MelphX) and Acryloyl-X (Ac-X) solutions.

11 Using a P20 pipette, take off as much MOPS buffer from the brains as possible.

12 Dilute Melphalan-X stock 1:1 with MOPS Buffer (to 1mg/ml).

13 Add 1/100 Ac-X ( [M] 10 mg/mL ) to Melphalan-X working solution. Vortex, mix, and spin.

14 Add 30 µL of Melphalan-X/AcX solution to each PCR tube and gently mix.

15 Incubate Overnight @ 37 °C . 2m

16 Prepare gel chambers for gelation the next day. Wipe a non-charged slide with RNase away. Adhere a gasket (4-6 wells max) and coat the glass surface of the chamber with 1 µL poly-Lysine using a P20 pipette tip. Air dry and repeat.

Chambers.jpg

## Day 2: Gelation and Proteinase K digestion

17 Wash brains 2 × 2 min 150 µL PBT and 1 × 2 mins 150 µL PBS.

17.1 Wash brains 2 x 00:02:00 150 µL PBT and 1 x 00:02:00 150 µL PBS. 4m

17.2 Wash brains 2 x 00:02:00 150 µL PBT and 1 x 00:02:00 150 µL PBS. 4m

18 Thaw Stock-X and 4HT, Temed and APS. Vortex well and keep On ice .

19 Gently stick down brains in the center of the chamber, once stuck down, carefully add a drop of PBS to prevent dehydration.

Note

It is possible to mount 4-5 brains per chamber.

20 Mix together Stock-X and 4HT, Temed and APS at a ratio of 94:2:2:2. Vortex.

Note

Each chamber needs ~ 120 µL of Stock-X, make excess. (ie. for two chambers make 300 µL gel solution).

21 Remove PBS from chamber with pipette tip and carefully wick away remaining PBS with a tissue.

22 Pipette 40 µL of gel solution, on top of the brain, to each chamber. Incubate slide in the fridge ( 4 °C ) for 00:10:00 . 10m

23 Take off gel solution and repeat step 22.

Note

Place waste gel solution in an Eppendorf. Before discarding, polymerize the gel waste @ 37 °C

24 Take off the gel solution and gasket surface adhesive. Add a final 40 µL of gel solution and gently place a cover slip over the chamber. Gently press to seal the coverslip and incubate @ 4 °C for 00:10:00 . 10m

Note

Adding 5 µL of gel solution to the center of the underside of the coverslip can help prevent air bubbles when sealing.

25 Polymerize the gel @ 37 °C for 01:30:00 to 02:00:00 . 3h 30m

26 Cool gels on the bench for a few minutes.

27 Take off the chamber lid and gasket with a razor blade. Trim the gels into a rectangle and nick the top right-hand corner to track the orientation of the sample.

28 Take off the gel from the slide with a paintbrush that has been wetted with a small amount of ProK Buffer and transfer it to a 2 mL Eppendorf.

29 Incubate each gel with 1 mL ProK Buffer and 10 µL (1/100) ProK Enzyme @ 37 °C Overnight . 2h

## Day 3: Hybridization

30 Wash gels 4 × 15 min with 1 mL PBS at Room temperature .

30.1 Wash gels 4 x 00:15:00 in 1 mL PBS at Room temperature (1/4). 15m

30.2 Wash gels 4 x 00:15:00 in 1 mL PBS at Room temperature (2/4). 15m

30.3 Wash gels 4 x 00:15:00 in 1 mL PBS at Room temperature (3/4). 15m

30.4 Wash gels 4 x 00:15:00 in 1 mL PBS at Room temperature (4/4). 15m

31 DAPI stain gels for 00:10:00 with 1 mL DAPI/PBS (500 ng/ml). 10m

32 Rinse with PBS.

33 Use a dissection scope with a UV bulb to neatly trim gel edges with a razor blade.

34 Thaw and mix hybridization (hyb) and probe wash buffer. Make sure hyb buffer is clear.

35 Incubate gel in 500 µL hyb buffer for 00:30:00 @ 37 °C . 30m

36 Dilute probes 1/100 ( [M] 10 Nanomolar (nM) ), in 300 µL hyb buffer per gel. Vortex. Incubate @ 37 °C .

37 Incubate gels with probes overnight @ 37 °C , no shaking necessary.

38 Put probe wash buffer and PBS @ 37 °C .

## Day 4: Probe Washing

39 Wash 3 × 30 min 750 µL Probe Wash Buffer @ 37 °C .

39.1 Wash 3 x 00:30:00 750 µL Probe Wash Buffer @ 37 °C (1/3). 30m

39.2 Wash 3 x 00:30:00 750 µL Probe Wash Buffer @ 37 °C (2/3). 30m

39.3 Wash 3 x 00:30:00 750 µL Probe Wash Buffer @ 37 °C (3/3). 30m

40 Wash 3 × 30 min 1 mL PBS @ 37 °C .

40.1 Wash 3 x 00:30:00 1 mL PBS @ 37 °C (1/3). 30m

40.2 Wash 3 x 00:30:00 1 mL PBS @ 37 °C (2/3). 30m

40.3 Wash 3 x 00:30:00 1 mL PBS @ 37 °C (3/3). 30m

41 Wash 3 × 1hr 1 mL PBS @ 37 °C .

41.1 Wash 3 x 01:00:00 1 mL PBS @ 37 °C (1/3). 1h

41.2 Wash 3 x 01:00:00 1 mL PBS @ 37 °C (2/3). 1h

41.3 Wash 3 x 01:00:00 1 mL PBS @ 37 °C (3/3). 1h

42 Keep gels in PBS at Room temperature Overnight . 1h

## Day 5: Hybridization Chain Reaction (HCR)

43 Incubate gels in 500 µL Amplification buffer for at least 00:30:00 @ Room temperature 30m

44 Snap cool hairpins with PCR machine @ 95 °C for 00:01:30 and cool @ Room temperature for 00:30:00 . 31m 30s

45 Mix hairpins h1 and h2 @ 1/100 in 300 µL Amp Buffer per gel. Vortex.

46 Incubate gel with hairpins for 03:00:00 @ Room temperature in the dark. 3h

47 Wash gels 2 × 20 min in 750 µL 5X SSCT @ Room temperature .

47.1 Wash gels 2 x 00:20:00 in 750 µL 5X SSCT @ Room temperature (1/2). 20m

47.2 Wash gels 2 x 00:20:00 in 750 µL 5X SSCT @ Room temperature (2/2). 20m

48 Wash gels 2 × 40 min in 1 mL 0.5X SSCT @ Room temperature .

48.1 Wash gels 2 x 00:40:00 in 1 mL 0.5X SSCT @ Room temperature (1/2). 40m

48.2 Wash gels 2 x 00:40:00 in 1 mL 0.5X SSCT @ Room temperature (2/2). 40m

49 Stain sample with 500 µL of anti-GFP-488 Ab @ (1/500) in PBT (0.1%) containing [M] 5 mg/mL Ultrapure BSA and incubate Overnight (or the weekend) @ 4 °C . 15m

## Day 6: Mount and Image

50 Wash 2 × 30 min with 1 mL PBS-Triton (0.1%).

50.1 Wash 2 x 00:30:00 with 1 mL PBS-Triton (0.1%) (1/2). 30m

50.2 Wash 2 x 00:30:00 with 1 mL PBS-Triton (0.1%) (2/2). 30m

51 Wash 2 × 30 min and 1 × 1hr with 1 mL PBS.

51.1 Wash 2 x 00:30:00 with 1 mL PBS (1/2). 30m

51.2 Wash 1 x 01:00:00 with 1 mL PBS (2/2). 1h

52 DAPI stain gels for 00:15:00 with 1 mL PBS/DAPI ( [M] 500 ng/ml ). 15m

53 Mount gels (sample up) on an 8mm poly-lysine coated coverslip superglued to a Z.1 light-sheet sample holder and image.

54 For gel removal from the holder, incubate gels in 750 µL of 10% Dextran Sulphate for 20 mins. Gels will shrink and fall off the coverslip.

55 For long-term storage, keep gels in 750 µL 10% Dextran Sulphate @ 4 °C

## Stripping Probes and Hairpins for Multiplexing 2h 45m

56 Incubate gel for 00:30:00 in 1 mL of DNAse1 Buffer @ 37 °C . 30m

57 Add 450 µL of DNase Buffer to 50 µL DNase1. Mix.

58 Incubate gel in DNase1 for 02:00:00 @ 37 °C . 2h

59 Wash 4 × 15 min with 1 mL PBS.

59.1 Wash 4 x 00:15:00 with 1 mL PBS (1/4). 15m

59.2 Wash 4 x 00:15:00 with 1 mL PBS (2/4). 15m

59.3 Wash 4 x 00:15:00 with 1 mL PBS (3/4). 15m

59.4 Wash 4 x 00:15:00 with 1 mL PBS (4/4). 15m

60 Hybridize with next round of probes (Day 3, step 34).

## JF-669 conjugation to unlabelled hairpins 1h 45m

61 30m

Note

Hairpins are amine modified, JF-669 has an NHS ester group.

Turn on speed vacuum (eg. Thermofisher, SPD120) and defrost dye at Room temperature for 00:30:00 .

62

Resuspend 2 mg of JF-669, SE in 630 µL Acetonitrile, mix and vortex, and aliquot in 30 µL into labelled skirted 0.5ml screw-cap centrifuge tubes > each will contain 0.1 mg of dye.

63 Evaporate acetonitrile in speed vacuum (in organic solvent mode) for 00:45:00 . 45m
Can store @ -20 °C .

64 In two 1.5ml Eppendorf tubes, evaporate 5 µL ( [M] 500 picomolar (pM) / 10 µg 30m
) of unlabelled hairpins h1 and h2 using a speed vac, in aqueous mode, for
00:30:00 . If you have 10 µL ( [M] 1 nanomolar (nM) ) use two tubes per
hairpin. Check they have been fully evaporated.

65 Add 3 µL of [M] 0.1 Molarity (M) Sodium Bicarbonate pH 8-9 to each evaporated
hairpin. Mix.

66 Add 2 µL of anhydrous DMSO to 0.1 mg of dye. Mix.

67 Add 2 µL dye mix ( 100 µg ) to each 3 µL of hairpin ( 10 µg ). Mix. It will
change colour.

68 Leave hairpin-dye mixture to react Overnight at Room temperature in the dark.

69 The next morning, add 5 µL nuclease-free water to bring to 10 µL .

70 Remove excess dye with a QIAquick Nucleotide removal kit (add 100 µL PN1).

71 Elute dye-oligo conjugate in 50 µL nuclease-free water.

72 Check the hairpin concentration on a spectrophotometer (eg. a NanoDrop One) and
dilute to [M] 60 ng/µl .

73 Separately store hairpins h1-669 and h2-669 in 25 µL aliquot's in a PCR tube@
-20 °C .

74 Test conjugation by HCR.