**Expansion-assisted iterative fluorescence *in situ* hybridization (EASI-FISH) in *Drosophila* CNS.**

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**Project Technical Resources**

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A straightforward, robust, and reliable protocol (EASI-FISH) that utilizes expansion microscopy and the hybridization chain reaction for multiplexed *in situ* hybridization for thick slices of 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.

**Fly brain dissection**

(for more details see uploaded dissection protocol and <https://www.janelia.org/project-team/flylight/protocols>). (Note: dissected brains for EASI-FISH are stored in 70% EtOH, not PBS).

*Solutions:*

2% PFA (Electron Microscopy Services; 15710).

Schneider’s Drosophila medium (S2 medium, ThermoFisher; 21720024).

PBT (0.5% Triton).

70% Ethanol (in nuclease free water).

1. Dissect fly brains or CNS in S2 medium.
2. Fix up to 20 brains or 10 CNS in 2 ml of 2% PFA/S2 medium for 55 min in the dark on a nutator.
3. Rinse sample 1 x 2 ml PBT (0.5% Triton).
4. Wash sample with 1 ml PBT 4 x 15 min, on a nutator.
5. Rinse sample 2 x 2 ml with 70% EtOH.
6. Store brains in 2 ml 70% EtOH @ 4C for up to 6 months.

**Day 1: Labelling RNA**

*Solutions and materials:*

20mM MOPS Buffer (Fisher; BP308-100). Store @ RT.

Melphalan-X (Stock 2 mg/ml; working solution 1 mg/ml). Store @ -20C.

Acryloyl-X (ThermoFisher; A20770), Stock 10 mg/ml; working 0.1 mg/ml. Store @ -20C.

PBT (0.1% Triton).

Silicone Gaskets (Invitrogen; P24743) 9 mm diameter, 0.5 mm deep.

Poly-Lysine (Pelco; 18026, 1.6 ml + 3.2 ml Photoflo-200 (EMS, 74257). Store @ 4C.

0.2 ml PCR tubes (USA Scientific; 1402-4700).

RNase Away (Thermo Scientific; 7003).

1. Transfer brains to a 0.2 ml PCR tube (2-4 brains per tube).
2. Rehydrate with 2 x 5 min wash in 150 ml PBT (0.1%).
3. Incubate brains 1 x 30 min in 150 ml 20 mM MOPS Buffer.
4. Thaw Melphalan-X (MelphX) and Acryloyl-X (Ac-X) solutions.
5. Using a P20 pipette, take off as much MOPS buffer from the brains as possible.
6. Dilute Melphalan-X stock 1:1 with MOPS Buffer.
7. Add 1/100 Ac-X (10 mg/ml) to Melphalan-X working solution. Vortex, mix, and spin.
8. Add 30 ml of Melphalan-X/AcX solution to each PCR tube and gently mix.
9. Incubate overnight @ 37C.
10. Prepare gel chambers for gelation the next day: Wipe a non-charged slide with RNase away. Adhere gasket (4-6 wells max) and coat glass surface of chamber with 1 ml poly-lysine using a P20 pipette tip. Air dry and repeat.

**Day 2: Gelation and Proteinase K digestion**

*Solutions and materials:*

Stock-X (see materials) (Store @ -20C).

10% Ammonium persulphate (APS) (Sigma; A3678) (Store @ -20C).

10% N, N, N’, N’-Tetramethylethylenediamine (Temed) (Sigma; T22500) (Store @ -20C).

0.5% 4-Hydroxy-TEMPO (4HT) (Sigma; 176141) (Store @ -20C).

Proteinase K (NEB; P8107S, 800U/ml) (Store @ -20C).

50mM Proteinase K Buffer (Store @ RT).

Small paint brush.

1. Wash brains 2 x 2 min 150 ml PBT and 1 x 2 min 150 ml PBS.
2. Thaw Stock-X and 4HT, Temed and APS. Vortex well and keep on ice.
3. Gently stick down brains in centre of the chamber, once stuck down, carefully add a drop of PBS to prevent dehydration. It is possible to mount 4 brains per chamber.
4. Mix Stock-X and 4HT, Temed and APS at a ratio of 94:2:2:2. Vortex. Each chamber needs ~120 ml of Stock-X, make excess. (i.e., for two chambers make 300 ml gel solution)
5. Remove PBS from chamber with pipette tip and carefully wick away remaining PBS with a tissue.
6. Pipette 38 ml gel solution, on top of brain, to each chamber. Incubate in a fridge for 10 min.
7. Take off gel solution and repeat step 6. Note: Collect gel waste solution in an Eppendorf and let polymerise before discarding.
8. Take off gel solution and gasket surface adhesive. Add a final 38 ml of gel solution and gently place a cover slip over chamber (adding 5 ml of gel solution to the underside of the coverslip can help prevent air bubbles when sealing). Gently press coverslip to seal and incubate in fridge for a final 10 min.
9. Polymerize the gel @ 37C for 1.5-2 hr.
10. Cool gels on the bench for a few minutes.
11. Take off chamber lid and gasket with a razor blade. Trim the gels into a rectangle and nick the top right-hand corner to track orientation.
12. Take off gel from slide with a paintbrush that has been wetted with a small amount of ProK Buffer and transfer to a 2 ml Eppendorf.
13. Incubate each gel with 1 ml ProK Buffer and 10 ml (1/100) ProK Enzyme @ 37C overnight.

**Day 3: Hybridization**

*Solutions:*

Hybridization Buffer, Probe Wash Buffer and HCR probe sets (from Molecular Instruments, see <https://www.molecularinstruments.com/hcr-rnafish-products>. Store @ -20C)

DAPI (Sigma; D9534)/PBS (Fisher; BP24384) at 500 ng/ml.

1. Wash gels 4 x 15 min with 1 ml PBS @ RT.
2. DAPI stain gels for 10 min with 1ml DAPI/PBS (500 ng/ml).
3. Rinse with PBS.
4. Use a dissection scope with a UV bulb to neatly trim gel edges with razor blade.
5. Thaw and mix hybridization (hyb) and probe wash buffer. Make sure hyb buffer is clear.
6. Incubate gel in 500 ml hyb buffer for 30 min @ 37C.
7. Dilute probes 1/100 (10 nM), in 300 ml hyb buffer per gel. Vortex. Incubate @ 37C.
8. Incubate gel with probes overnight @ 37C, no shaking necessary.
9. Put probe wash buffer and PBS @ 37C.

**Day 4: Probe Washing**

*Solutions:*

Probe Wash Buffer @ 37C.

PBS@ 37C.

1. Wash 3 x 30 min 750 ml Probe Wash Buffer @ 37C
2. Wash 3 x 30 min 1 ml PBS @ 37C.
3. Wash 3 x 1 hr 1 ml PBS @ 37C.
4. Keep gels in PBS @ RT overnight.

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

(for principals see <https://www.molecularinstruments.com/hcr-rnafish>)

*Solutions:*

Amplification Buffer (Molecular Instruments; Store @ 4C).

Fluorescent Hairpins (448 and 546 from Molecular Instruments, or conjugated to 669, see protocol below. Store @ -20C).

5x SSCT (5x SSC, 0.1% Tween in Nuclease Free Water). Store @ RT.

0.5x SSCT (0.5x SSC, 0.1% Tween in Nuclease Free Water). Store @ RT.

GFP Polyclonal Antibody, Alexa Fluor-488 (ThermoFisher; A-21311).

Ultrapure BSA (50 mg/ml) (ThermoFisher; AM2616).

1. Incubate gels in 500 ml Amplification buffer for at least 30 min @ RT.
2. Snap cool hairpins with PCR machine @ 95C for 90 sec and cool @ RT for 30 min.
3. For each fluor mix hairpins h1 and h2 @ 1/100 in 300 ml Amp Buffer per gel. Vortex.
4. Incubate gel with hairpins for 3 hr @ RT in the dark.
5. Wash gels 2 x 20 min in 750 ml 5X SSCT @ RT.
6. Wash gels 2 x 40 min in 1 ml 0.5X SSCT @ RT.
7. Stain sample with 500 ml of anti-GFP-488 Ab (1/500) in PBS-Triton (0.1%) containing 5 mg/ml Ultrapure BSA and incubate overnight (or weekend) @ 4C.

**Day 6:** **Mount and Image**

*Solutions and materials:*

PBT (O.1% Triton)

DAPI/PBS (500 ng/ml)

Sample holders for Z.1 Microscope (files attached).

8mm circular coverslips (CS-8R, Warner Instruments, 64-0701).

1. Wash 2 x 30 min with 1 ml PBT (0.1%).
2. Wash 2 x 30 min and 1 x 1 hr with 1 ml PBS.
3. DAPI stain gels for 15 min with 1 ml DAPI/PBS.
4. Mount gels on an 8mm poly-lysine coated coverslip superglued to Z1 sample holder.
5. Image on a Zeiss Z1 Lightsheet Microscope.
6. For gel removal from holder incubate gels in 750ul of 10% Dextran Sulphate
7. For longer term storage, keep gels in 10% Dextran Sulphate @ 4C.

**Stripping Probes and Hairpins for Multiplexing**

*Solutions:*

RNase-Free DNase1: (Qiagen, 79254)\* Note: Do **not** use RDD buffer supplied.

DNAse1 buffer:(10 mM Tris-HCL pH8.0; 2.5 mM MgCl2; 0.5 mM CaCl2)

1. Incubate gel for 30 min in 1ml of DNAse1 Buffer @ 37C
2. Add 450ml of DNase Buffer to 50 ml DNase1. Mix.
3. Incubate gel in DNase1 for 2 hr @ 37C.
4. Wash 4 x 15 min with 1 ml PBS.
5. Hybridize (Day 3, step 5).

**Recipes and Reagents**

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

1046.5 mg in 25 ml in NFW, pH to 7.7 with 10N NaOH. Store @ -20C.

**Melphalan stock** (2.5 mg/ml)(Cayman Chemicals; 16665).

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

**Acryloyl-X** (**AcX**) **stock** (10 mg/ml) (Thermo Fisher; A20770).

Dissolve in anhydrous DMSO. Aliquot in 200 ml batches. Any extra aliquot in 5 ml batches (for extra-AcX step). Store in a desiccated environment @ -20C. Don’t re-use AcX after thawing.

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

Combine an equal concentration of Acryloyl-X (10 mg/ml) and Melphalan (2.5 mg/ml) (1-part AcX to 4-parts Melphalan (i.e., 200 ml:800 ml). Incubate overnight @ RT with shaking. Store in 50 ml aliquots in desiccated environment @ -20C. Use at 1 mg/ml by 1:1 dilution in 20 mM MOPS.

**Stock X**:

4.04 M Sodium Acrylate\* – made from Acrylic Acid as it is made at variable purity

40% Acrylamide (Bio-Rad; 1610140)

2% N,N Methylene-Bis-acrylamide (Bio-Rad; 1610142)

5M NaCl RNase-free (Thermofisher; AM97060G)

10x PBS (ThermoFisher; AM9625)

Nuclease Free Water (NFW) (ThermoFisher; AM9932)

**4.04 M Sodium Acrylate stock solution**

1. In a fume hood, place 5.5 ml of acrylic acid into a 50 ml tube. Place tube in a room temperature water bath (e.g. a beaker).
2. Add 4.5 ml water.
3. Add 7.2 ml 10M NaOH gradually to prevent excessive heating and evaporation/boiling.
4. Remove tube from hood (at this point most of the acrylic acid has been converted to non-volatile sodium acrylate).
5. Add 1M NaOH (nominally 1 ml) gradually until the pH is between 7.5-8 using a pH meter, at RT. Do not use pH test strips.
6. 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 4 mM remaining buffering capacity

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

4.04M Sodium Acrylate 2275 ml

40% Acrylamide 625 ml

2% N,N MethylBisacrylamide 750 ml

5M NaCl 4000 ml

10x PBS 1000 ml

Nuclease Free Water 750 ml

**10% APS**: 100 mg APS + 900 ml H20

**10% Temed**: 100 ml TEMED + 900 ml H20

**0.5% 4HT**: 5 mg 4HT + 995 ml H20

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

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

50mM Tris-HCL-pH8, 1mM EDTA, 0.5% TritonX, 50mM NaCl, 0.3% SDS.

2.5 ml 1M Tris > 50 mM

2.5 ml 10% Triton > 0.5%

0.5 ml 5M NaCl > 50 mM

100 ml 0.5M EDTA > 1 mM

1.5 ml 10% SDS > 0.3% SDS

42.9 ml Nuclease Free Water

Note: If brains have TdTomato as a reporter, in the buffer solution increase the NaCl to 500mM and remove SDS to preserve endogenous TdTomato 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 ml DNAase1 Buffer to DNase1 powder. Mix. Aliquot 50 ml per PCR tube, store @ -20C.

**DNase1 buffer (50 ml)**

500 ml 1M Tris-HCL > 10 mM

125ml 1M MgCl2 > 2.5 mM

25 ml 1M CaCl2 > 0.5 mM

49.350 ml Nuclease Free Water

**HCR Wash Buffers (50 ml)**

**5xSSCT**:

12.5 ml 20x RNase free-SSC (ThermoFisher; AM 9763)

500 ml 10% Tween

37 ml Nuclease Free Water

**0.5xSSCT**:

1.25 ml 20x RNase free-SSC

500 ml 10% Tween

48.25 ml Nuclease Free Water

**JF-669 conjugation to unlabelled hairpins**

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

**Reagents**

JF-669, SE (Tocris; 6420, Store @ -20C)

1 nmol unlabelled amine-modified hairpins (~70mer, Molecular Instruments; Store @ -20C).

Acetonitrile, anhydrous (Thermofisher; 042311-K7).

0.1M Sodium Bicarbonate pH 8-9.

Anhydrous DMSO (Invitrogen; D12345).

QIAquick Nucleotide Removal Kit (250) (Qiagen; 28306).

0.5ml screw-cap microcentrifuge tubes, Amber (USA Scientific; #1405-9707).

1. Turn on speed vacuum (eg. Thermofisher; SPD120) and defrost dye @ RT for 30 min.
2. Resuspend 2 mgs of JF-669, SE in 630 ml Acetonitrile, mix and vortex, and aliquot 30 ml into labelled skirted 0.5 ml screw-cap centrifuge tubes > each will contain 0.1 mg of dye.
3. Evaporate acetonitrile in speed vacuum (in organic solvent mode) for 45 min. Store @ -20C.
4. In two 1.5 ml Eppendorf tubes, evaporate 5 ml (500 pmol/10 mg) of unlabelled hairpins h1 and h2 using a speed vac, in aqueous mode, for 30 min. If you use 10 ml (1 nmol) use two tubes per hairpin. Check they have been fully evaporated.
5. Add 3 ml of 0.1M Sodium Bicarbonate pH 8-9 to each evaporated hairpin. Mix.
6. Add 2 ml of anhydrous DMSO to 0.1 mg of dye. Mix.
7. Add 2 ml dye mix (100 mg) to each 3 ml of hairpin (10 mg). Mix. It will change colour.
8. Leave hairpin-dye mixture to react overnight @ RT in the dark.
9. The next morning, add 5 ml nuclease-free water to bring to 10 ml.
10. Remove excess dye with a QIAquick Nucleotide removal kit (add 100 ml PN1).
11. Elute dye-oligo conjugate in 50 ml nuclease-free water.
12. Check the hairpin concentration on a spectrophotometer (eg. NanoDrop One, ThermoScientific) and dilute to 60 ng/ml.
13. Separately store hairpins h1-669 and h2-669 in 25 ml aliquots in a PCR tube @ -20C.
14. Test conjugation by HCR.

**Reference**

Wang et al., EASI-FISH for thick tissue defines lateral hypothalamus spatio-molecular organization. ***Cell*** 184 (2021) 6361-6377.e24. DOI: [10.1016/j.cell.2021.11.024](https://doi.org/10.1016/j.cell.2021.11.024)