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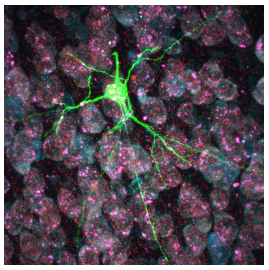
Expansion-assisted iterative fluorescence *in situ* hybridization (EASI-FISH) for morphoFISH



Forked from Expansion-assisted iterative fluorescence in situ hybridization (EASI-FISH) in Drosophila CNS

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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 this protocol for cleared mouse slices with GFP-labeled neurons fixed under morphoFISH prefusion (Ferreira et al., 2024). This uses a modified gel recipe (TREx-1000) for greater gel stability. We also include an updated design for the Z1 sample holder.

Attachments

XLSX
Table of materials_M...
10KB

ZIP
LIGHTSHEETHOLDER_V18...
14.2MB

Image Attribution

Mark Eddison

Materials

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

- Trex-1000. 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

	A	B
	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.

Trex-1000:

[M] 4.04 Mass Percent 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
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 Mass Percent 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 Mass Percent 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 Trex-1000 (not including APS +TEMED + 4HT):

	A	B
	4.04M Sodium Acrylate	2500 ul
	40% Acrylamide	3571 ul
	2% N,N MethylBisacrylamide	500 ul
	10x PBS	1000 ul
	Nuclease Free Water	1829 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 %

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
	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

Preparation of slices for EASI-FISH

1 To visualize the GFP neurons, rehydrate the slice in PBS for 10 mins.

2 Under a dissection scope and using a sharp knife, cut ROI with GFP cells from brain slice. The area should be about 2mm x 4mm and have a nick in the right hand corner to keep track of orientation. Take a photo of before and after to keep track of slice and cells.

3 Store ROI slice in 70% EtOH until ready to use

Day 1: Labelling RNA

4 Transfer each ROI slice to a 0.2ml PCR tube

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

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

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

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

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

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

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

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

11 Add 50 µL of Melphalan-X/AcX solution to each PCR tube and gently mix.

12 Incubate Overnight @ 37 °C . 2m

13 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

14 Wash slice 2 × 2 min 150 µL PBT and 1 × 2 mins 150 µL PBS.

14.1 Wash slice 2 x 00:02:00 150 µL PBT and 1 x 00:02:00 150 µL PBS. 4m

14.2 Wash slice 2 x 00:02:00 150 µL PBT and 1 x 00:02:00 150 µL PBS. 4m

15 Thaw TREx-1000 and 4HT, Temed and APS. Vortex well and keep On ice .

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

17 Mix together Trex-1000 and 4HT, Temed and APS at a ratio of 94:2:2:2. Vortex.

Note

Each chamber needs ~ 120 µL of Trex-1000, make excess. (ie. for two chambers make 300 µL gel solution).

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

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

20 Take off gel solution and repeat step 22.

Note

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

21 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.

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

23 Cool gels on the bench for a few minutes.

24 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.

25 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.

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

Day 3: Hybridization

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

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

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

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

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

28 DNAse treat sample before hybridization (see Step 48) for Cyto-DAPI staining/segmentation

29 Thaw and mix hybridization (hyb) and probe wash buffer.

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

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

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

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

Day 4: Probe Washing

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

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

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

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

35 Wash 3 × 30 min 1 mL PBS @ 37 °C .

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

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

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

36 Wash 3 × 1hr 1 mL PBS @ 37 °C .

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

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

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

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

Day 5: Hybridization Chain Reaction (HCR)

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

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

Note

Choice of far-red dyes: We found that JF669 tends to bleach faster in morphoFISH tissue. For this reason, we prefer to use AF647-conjugated hairpins.

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

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

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

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

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

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

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

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

Day 6: Mount and Image

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

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

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

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

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

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

46 DAPI stain gels for 01:00:00 with 1 mL PBS/DAPI ([M] 500 ng/ml). 1h

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

48 For long-term storage, keep gels in 750 µL 5X SSC @ 4 °C

Stripping Probes and Hairpins for Multiplexing 2h 45m

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

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

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

52 Wash 4 × 15 min with 1 mL PBS.

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

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

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

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

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

Protocol references

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