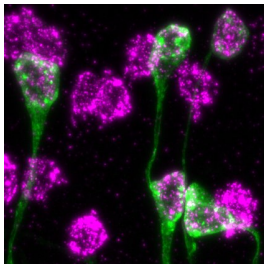
Aug 21, 2025 Version 2

Multiplex Detection of Gene Expression in the Intact Drosophila Brain using EASI-FISH V.2



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We use this protocol and it's working

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Abstract

Expansion-Assisted Iterative Fluorescence *In Situ* Hybridization (EASI-FISH) is a powerful technique that integrates expansion microscopy with multiplexed FISH to investigate gene expression in thick tissue samples. We describe an updated fly EASI-FISH protocol suitable for high throughput experimentation. Up to six brains or two CNS can be embedded in a single gel and several gels can be processed in parallel. We use a new gel recipe that improves gel robustness and permits at least 10 rounds of hybridization. Using the GAL4-UAS system for co-detection of green fluorescent protein (GFP), gene expression can be visualized in cell types of interest. Due to the high resolution and sensitivity of the method, single transcripts can be detected, and spot counting can be used to quantify expression levels.

Attachments

ZIP
LIGHTSHEETHOLDER_V18...
14.2MB

XLSX
EASI_FISH_Recipes.xl...
20KB

XLSX
Sequence of Drosophi...
8KB

XLSX
Table of materials.x...
11KB

Image Attribution

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Materials

Solutions for Fly brain dissection

- 4% PARAFORMALDEHYDE 16% Aqueous SOL. EM GRADE **Electron Microscopy Sciences Catalog #**15710 in PBS
- 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.

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
	Nucelase Free Water	183 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

	A	B
	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
	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
	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 Mass Percent 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

Fly brain dissection

15m

1 Dissect fly CNS in cold S2 medium. Use steps that reduce chance of contamination with RNases: wear gloves, use RNase free reagents when available, clean bench and dissecting forceps with RNase away prior to use.

2 Fix up to 20 brains or 10 CNS in 2 mL of 4 °C 4% PFA/PBS medium Overnight in the dark on a nutator @ 4 °C. Transfer brains into cold fixative after each dissection to prevent RNA degradation.

3 The next day rinse sample 1 x 2 mL 4 °C PBT (0.5% Triton).

4 Wash sample 4 x 00:15:00 2 mL 4 °C PBT (0.5%Triton) on a nutator. 15m

5 Rinse sample 1 x 2 mL 4 °C 70% EtOH.

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

Day 1: Mounting brains and adding RNA and protein anchors

20m

7 Prepare gel chambers by wiping non-charged slide with RNase away, coat slide twice with poly-lysine allowing to air dry between coating, preferably allowed to dry overnight after second coating.

8 The next day adhere silicone gasket with up to 4 chambers to slide.

9 Transfer brains from 70% EtOH to a 0.2ml PCR tube (2-4 brains per tube).

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

11 Rehydrate with 2 x 00:05:00 wash in 150 µL PBS.

12 Add 40ul of PBS per chamber and then mount brains by gently sticking them down in a row in the center of the chamber using fine tipped forceps. Note- Mounting up to 6 brains or 2 CNS per chamber is possible.

13 Remove PBS from chamber and add 50 µL [M] 20 millimolar (mM) MOPS Buffer, incubate 1 x 00:30:00

14 While brains are equilibrating in MOPS buffer, thaw Melphalan-X (MelphX) and Acryloyl-X (Ac-X) solutions.

15 Dilute Melphalan-X stock 1:1 with MOPS Buffer (to 1mg/ml). Add 1:100 Ac-X ([M] 10 mg/mL) to Melphalan-X working solution. Vortex, mix, and spin. Note- time the preparation such that the solution is ready at the end of the 30 minute MOPS incubation.

16 Remove MOPS buffer and add 50 µL of Melphalan-X/AcX solution to each chamber.

17 Cover the chamber by gently placing a 25mm x 25mm coverslip on top, do not use gasket adhesive to seal.

18 Place slide in humidified box protected from light and incubate Overnight @ Room temperature 5m

Day 2: Gelation and Proteinase K digestion 2h 8m

19 The next day carefully remove the coverslips from the chambers and remove Melphalan-X/AcX solution.

20 Wash mounted brains in chambers 2 x 00:02:00 100 µL PBT (0.1% Triton). 4m

21 Wash mounted brains in chambers 2 x 00:02:00 100 µL PBS. 4m

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

23 Mix together TREx-1000 and 4HT, Temed and APS at a ratio of 94:2:2:2. Vortex and keep on ice for the duration of the incubations @ 4 °C

Note

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

24 Remove PBS from chamber with pipette tip and carefully wick away remaining PBS with a lint free wipe.

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

26 Take off gel solution and add 40 µL of gel solution, on top of the brains, to each chamber. Incubate slide in the fridge (4 °C) for another 00:10:00 . 10m

Note

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

27 Take off the gel solution and peel the clear plastic protective film off the gasket's surface adhesive. Add a final 45 µL of gel solution and gently place a coverslip over the chamber, avoiding trapping any air bubbles. Gently press to seal the coverslip and incubate @ 4 °C for another 00:10:00 (for a total of 30 minutes equilibration @ 4 °C 10m

Note

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

28 Remove slides from fridge and place in incubator to polymerize the gel @ 37 °C for 01:30:00 . 1h 30m

29 Once gels have set, allow to cool on the bench for a few minutes.

30 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.

31 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.

32 Incubate each gel with 500 µL ProK Buffer and 5 µL (1:100) ProK Enzyme @ 37 °C Overnight .

Day 3: DNA digestion and probe hybridization 4h 30m

33 The next day wash gels 3 × 10 min with 1 mL PBS @ Room temperature . Note- use a fine flexible tip pipette to avoid damaging the gels when changing solutions. 30m

Note

Omit DNAse treatment before the first round of hybridization if nuclear DAPI staining needs to be preserved (e.g., for segmentation). In this case, samples should be washed with PBS 4x 15 min before proceeding.

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

35 Add 450 µL of DNase Buffer to 50 µL DNase1. Gently mix but do not vortex

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

37 Wash 4 × 15 min with 1 mL PBS. Note- gels can be stored in 5x SSC @ 4 °C before or after Digest/DNase washes for several weeks. 1h

38 Thaw hybridization (hyb) buffer.

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

40 Dilute probes 1:100 in 300 µL hyb buffer per gel. Vortex.

41 Incubate gels with hybridization buffer containing probes overnight @ 37 °C , no shaking necessary.

42 Place probe wash buffer and PBS @ 37 °C for washes next day.

Day 4: Probe Washing 6h

43 The next day wash 3 × 30 min 750 µL Probe Wash Buffer @ 37 °C . 1h 30m

44 Wash 3 × 30 min 1 mL PBS @ 37 °C . 1h 30m

45 Wash 3 × 1 hr 1 mL PBS @ 37 °C . 3h

46 Keep gels in PBS @ Room temperature Overnight or @ 4 °C over the weekend.

Day 5: Hybridization Chain Reaction (HCR) 5h 46m 30s

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

48 In a PCR machine, heat the fluorescent hairpins to 95 °C for 00:01:30 and snap cool to 25 °C for 00:30:00. Spin briefly to collect hairpins. 31m 30s

49 For each probe dilute matching hairpins h1 and h2 at 1:50 in 300 µL Amplification Buffer per gel. Vortex.

50 Incubate gel with hairpins for 03:00:00 @ Room temperature protected from light. 3h

51 Wash gels 2 × 15 min in 750 µL 5x SSCT @ Room temperature protected from light. 30m

52 Wash gels 2 × 30 min in 1 mL 0.5x SSCT @ Room temperature protected from light. 1h

53 If brains express nls-GFP to mark/and or segment cells of interest, stain sample with 500 µL of directly labeled anti-GFP-488 Ab (1:500) in PBT (0.1% Triton) containing [M] 5 mg/mL Ultrapure BSA and incubate Overnight (or over the weekend) @ 4 °C. 15m

54 If brains do not require antibody staining, they can be kept in 1 mL PBS overnight or over the weekend @ 4 °C. Alternatively, they can be DAPI stained with 1 mL PBS/DAPI [M] 5 µg/µL @ 4 °C overnight or over the weekend.

Day 6: Mount and Image 2h 30m

55 Prepare light sheet holder or glass bottom dish by coating with poly-lysine 2x allowing to dry @ 37 °C between coats.

56 If antibody stained wash 1 × 30 min with 1 mL PBT (0.1% Triton). 30m
If no antibody stain skip to mounting steps.

57 If antibody stained wash 3 × 30 min with 1 mL PBS. 1h 30m

58 If antibody stained, DAPI stain gel for at least 00:30:00 with 1 mL PBS/DAPI ([M] 5 µg/µL). 30m

59 To mount gels remove most of PBS from tube and allow gel to slide out and place them gently onto a coverslip and carefully dry the edges with a lint free wipe.

60 Ensure the gel is oriented such that the tissue will be closest to the objective and using a paintbrush gently slide the gel onto the poly-lysine surface using one movement.

61 Return gel holder to 2ml tube and ensure the sample is submerged in PBS or alternatively bathe the glass bottom dish in PBS to prevent desiccation.

62 Gels will be expanded 2 x in PBS. For 3 x expansion wash gels in 0.05x SSC instead of PBS.

63 For gel removal gently slide paintbrush between surface of gel and gel holder or glass bottom dish, gels will easily dislodge. Transfer to 2ml tube for storage.

64 For long-term storage, keep gels in 1 mL 5x SSC @ 4 °C

For Cell Segmentation and Spot Quantification 1h

65 Omit DNAse treatment before the first round of hybridization. After step 32, samples should be washed with PBS 4 × 10 min with 1 mL PBS before proceeding to hybridization with a 28S ribosomal RNA probe ([M] 1 micromolar (µM)) directly conjugated to a fluorophore (e.g., CF633) diluted 1:50.

66 After probe washing (Steps 43-45) stain with 1 mL PBS/DAPI ([M] 500 ng/ml) for 01:00:00 1h

67 After imaging, cellular masks are created using segmentation software, utilizing the information from both the cytoplasmic stain (ribosomal RNA probe) and the nuclear DAPI stain to identify and outline individual cells. Fluorescent spots reporting single transcripts can be counted within these masks.

Note

We are currently developing a segmentation and spot counting protocol using CellposeSAM and fishspot, respectively; see additional information in the "References" section. Upon completion, this protocol will be made available at 10.17504/protocols.io.6qpvrwenzlmk/v1.

Stripping Probes and Hairpins for Multiplexing 4h 30m

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

69 Add 450 µL of DNase Buffer to 50 µL DNase1. Gently mix but do not vortex.

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

71 Wash 4 × 15 min with 1 mL PBS. 1h

72 Hybridize with next round of probes (Day 3, step 38).

CF633 Succinimidyl Ester conjugation to unlabeled hairpins 1h 45m

73 30m

Note

647 dyes quickly photobleach in the gel. For a far red alternative, conjugate unlabelled hairpins to CF633 SE or Janelia Fluor 669 SE. Hairpins are amine modified, CF633/JF669 SE has an NHS ester group for conjugation.

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

74 Resuspend [M] 1 micromolar (µM) of CF633, SE in 330 µL Acetonitrile, mix and vortex, and aliquot 30 µL into labelled skirted 0.5ml screw-cap centrifuge tubes. Each will contain ~ 0.08 mg of dye.

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

76 In two 1.5ml Eppendorf tubes, evaporate 5 µL ([M] 500 picomolar (pM) / 10 µg) of unlabeled 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. 30m

77 Add 3 µL of [M] 0.1 molar Sodium Bicarbonate pH 8-9 to each evaporated hairpin. Mix.

78 Add 2 µL of anhydrous DMSO to 0.08 mg of dye. Mix well.

79 Add 2 µL of dye mix to each 3 µL of hairpin. Mix well.

80 Leave hairpin-dye mixture to react Overnight @ Room temperature in the dark.

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

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

83 Elute dye-oligo conjugate in 75 µL nuclease-free water.

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

85 Separately store hairpins h1-633 and h2-633 in 25 µL aliquot's in a PCR tube @ -20 °C .

86 Test conjugation by HCR.

Protocol references

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https://www.janelia.org/support-team/project-technical-resources/fly-easi-fish (The information on this page includes a video of the sample handling from step 7 to 60 in the protocol described here, as published in J. Vis. Exp. 219, e67656, doi: **10.3791/67656**.)

Yuhan Wang, Mark Eddison, Greg Fleishman, Martin Weigert, Shengjin Xu, Tim Wang,Konrad Rokicki, Cristian Goina, Fredrick E. Henry, Andrew L. Lemire, Uwe Schmidt, Hui Yang, Karel Svoboda, Eugene W. Myers, Stephan Saalfeld, Wyatt Korff, Scott M. Sternson, and Paul W. Tillberg (2021). **EASI-FISH for thick tissue defines lateral hypothalamus spatio-molecular organization.** Cell 184:6361, doi: **10.1016/j.cell.2021.11.024**

Claire Managan and Gudrun Ihrke (2025). **EASI-FISH Spot Counting in Drosophila Brain Using RS-FISH. dx.doi.org/10.17504/protocols.io.ewov1opz7lr2/v1**

Marius Pachitariu, Michael Rariden, Carsen Stringer (2025). **Cellpose-SAM: superhuman generalization for cellular segmentation.** bioRxiv preprint https://www.biorxiv.org/content/10.1101/2025.04.28.651001v1

Greg Fleishman (2023). **Fishspot** https://github.com/GFleishman/fishspot/blob/main/fishspot/detect.py

Sanna Koskela, Claire Managan, Carsen Springer, Mark Eddison, and Gudrun Ihrke. **Quantification of gene expression in Drosophila brain by EASI-FISH spot counting.** https://dx.doi.org/10.17504/protocols.io.6qpvrwenzlmk/v1 in progress

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