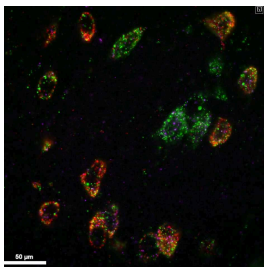
Feb 18, 2025 Version 1

Multiplexed RNA FISH on Expanded Mouse Brain Slices V.1



DOI
https://dx.doi.org/10.17504/protocols.io.dm6gpzj28lzp/v1

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.dm6gpzj28lzp/v1

Protocol Citation: Rachel Flannery, Molly Logsdon, Laura Roy, Kevin Cao, Jayaram Chandrashekar 2025. Multiplexed RNA FISH on Expanded Mouse Brain Slices. **protocols.io** https://dx.doi.org/10.17504/protocols.io.dm6gpzj28lzp/v1

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

Created: May 16, 2024

Last Modified: February 19, 2025

Protocol Integer ID: 99985

Keywords: In Situ Hybridization, FISH, Tissue Slice, Tissue Clearing, Hydrogel, RNase Free, Tissue Expansion, Spatial Transcriptomics, multiplexed rna fish, expanded mouse brain slices recent advance, high resolution imaging of cell morphology, single cell rna sequencing, thick slices of brain tissue, spatial transcriptomic, expansion microscopy, brain tissue, retrospective fish, multiscale anatomy, cell morphology, multiple rounds of multiplexed fluorescent, brain slice, multiplexed fluorescent, field of neuroscience, high resolution imaging, neuroscience, rna, tissue clearing, cell, robust trex gel chemistry, situ hybridization, tissue, mfish, microscopy, cell rna

Abstract

Recent advances in single cell RNA sequencing, taxonomic classifications of cell types, and high resolution imaging of cell morphology has primed the field of neuroscience for new discoveries. Spatial transcriptomics has the potential to bridge these data modalities to each other by linking the molecular identity of cells to their native biological context.

This protocol describes a method that utilizes expansion microscopy to perform multiple rounds of multiplexed fluorescent in situ hybridization (mFISH) on thick slices of brain tissue. It is adapted from **EASI-FISH**, where we have incorporated the more robust **TREx** gel chemistry to achieve better sample durability for ease of sample handling and extended rounds of probing. The protocol is optimized for retrospective FISH on fixed tissue using modalities such as optical physiology and tissue clearing/multiscale anatomy.

Materials

Note

All solutions and equipment must be kept RNase free to preserve RNA quality. Prepare solutions in a PCR hood that has been sprayed thoroughly with RNAse Away™. Wear a lab coat, mask, and gloves to prevent contamination. Spray gloves thoroughly with RNAse Away™ and dry with a Kimwipe® regularly. Milli-Q water can be substituted for nuclease free water.

- RNase AWAY™ Spray Bottle, RNase in spray bottle; 475mL **Thermo Fisher Catalog #**7002PK
- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #**AM9625
- N,N′-Methylenebisacrylamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M7279
- Acrylamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A9099
- Sodium chloride solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S6546
- UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #**15575020
- 10% SDS solution **Thermo Fisher Scientific Catalog #**15553027
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787-50ML
- Tris-HCl, 1M Solution, pH 8.0, Molecular Biology Grade, Ultrapure, Thermo Scientific Chemicals **Thermo Fisher Scientific Catalog #**AAJ22638K2
- 5M Sodium Chloride, 1000ml **Promega Catalog #**V4221
- 10% SDS solution **Thermo Fisher Scientific Catalog #**15553027
- Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #**P8107S
- HCR Probe Hybridization Buffer **Molecular Instruments**
- HCR Probe Wash Buffer **Molecular Instruments**
- HCR Amplification Buffer **Molecular Instruments**
- DNase I Reaction Buffer - 6.0 ml **New England Biolabs Catalog #**B0303S
- DNase I (RNase-free) - 5,000 units **New England Biolabs Catalog #**M0303L

Equipment

22×22 mm Square Cover Glass

NAME

Corning

BRAND

2855-22

SKU

https://ecatalog.corning.com/life-sciences/b2b/US/en/Glassware/Cover-Glass/Corning%C2%AE-Square-%232-Cover-Glass/p/2855-22

LINK

Product Number: 2855-22; Qty./Pk: 100 / Pk; Qty./Cs: 1000 / Cs; Size: 22×22 mm (approx); Sterile: No; Thickness: 0.17 - 0.25 mm

SPECIFICATIONS

Equipment

Synthetic Sable Brushes, #3

NAME

Ted Pella

BRAND

11816

SKU

https://www.tedpella.com/brush_html/brush.aspx#_11806

LINK

Synthetic Sable Brushes, #3, 2.0mm W x 13.0mm L

SPECIFICATIONS

Equipment

Electron Microscopy Sciences SuperFrost™ End Slide End Slide 1 mm thick

NAME

Electron Microscopy Sciences 7186701

BRAND

50-949-343

SKU

https://www.fishersci.com/shop/products/end-slide-1-mm-thick-1gs-1/50949343?searchHijack=true&searchTerm=50-949-343&searchType=RAPID&matchedCatNo=50-949-343

LINK

Equipment

Sterile Microcentrifuge Tubes with Screw Caps

NAME

Fisherbrand

BRAND

02-681-374

SKU

https://www.fishersci.com/shop/products/fisherbrand-sterile-microcentrifuge-tubes-screw-caps-6/02681374

LINK

Max. RCF 18,000 x g Autoclavable Autoclavable Color Natural Includes O-ring Closure Type Screw Cap Closure Material Polypropylene Capacity (Metric) 2 mL Material Polypropylene Sterility Sterile Bottom Shape Skirted

SPECIFICATIONS

Equipment

AirClean Systems AC600 Series UPUV Class 1 Biological Safety Workstation	NAME
PCR hood	TYPE
Midwest Production Supply	BRAND
AC632UPUV	SKU
https://aircleansystems.com/products/combination-pcr-workstation	LINK
No ductwork required; No installation required – plugs directly into a standard 110V or 220V electrical outlet; Clear polycarbonate shell for 360° visibility; UVTect® microprocessor controller UV and fluorescent lights; Seamless plastic design -no joints or gaps in construction; Lab event timer; Digital UV light timer :01-59 minutes; Access port for electrical cords; Weight: 163 lbs; External Size: 32"Wx24"Dx30"H or 48"Wx24"Dx32"H	SPECIFICATIONS

Equipment

SimpliAmp™ Thermal Cycler

NAME

Thermal Cycler

TYPE

Applied Biosystems™

BRAND

A24811

SKU

https://www.thermofisher.com/order/catalog/product/A24811

LINK

Thermal Accuracy ±0.25°C (35°C to 99.9°C); High-throughput Compatibility Not High-throughput Compatible (Manual); For Use With (Equipment) GeneAmp 2700, GeneAmp 9700, Veriti Thermal Cycler, 2720 Thermal Cycler; Thermal Uniformity <0.5°C (20 sec. after reaching 95°C); Dimensions 18.1 × 9.5 × 8.3 in. (46 × 24 × 21 cm); Capacity 96-well, 3 Zones VeriFlex; Voltage 100/240 V; Display Type 8 in. Color TFT LCD Touchscreen; Product Type Thermal Cycler; Warranty Limited Warranty; Thermal Range 0°C to 100°C; User Interface USB, Wi-Fi, Ethernet; Wattage 600 W; Max. Ramp Rate 4°C/sec. (Block), 3°C/sec. (Sample); No. of Programs >1000; Weight 18.3 lb. (8.3 kg); Block Configurations 96-well, 0.2 mL VeriFlex Block

SPECIFICATIONS

RECIPES

4.04M Sodium Acrylate:

Safety information

- Acrylic acid is toxic if swallowed, inhaled, or absorbed through the skin. It is flammable, corrosive, and an environmental hazard. Handle only in a fume hood while wearing a mask, chemically resistant gloves, protective clothing, and eye protection. Keep away from heat, sparks, open flames, and hot surfaces. Dispose of acrylic acid and any contaminated consumables in a hazardous waste stream.

	A	B
	14.6M Acrylic Acid	13.75 mL
	Nuclease Free Water	11.25 mL + extra to reach 50 mL
	10N NaOH	18 mL

	A	B
	1N NaOH	2.5 mL to 5 mL

50% Acrylamide:

Safety information

- Acrylamide powders and solutions are toxic if swallowed, inhaled, or absorbed through the skin. It is a mutagen, teratogen and a carcinogen. Handle only in a fume hood while wearing a mask, chemically resistant gloves, protective clothing, and eye protection. Dispose of acrylamide and any contaminated consumables in a hazardous waste stream.

- Combine the following reagents and vortex to combine at Room temperature until fully dissolved. Store at -20 °C .

	Reagents	Amount
	Acrylamide	5.0 g
	Nuclease Free Water	10 mL
	Total	**10 mL**

2% N,N-Methylenebisacrylamide (N,N-Mba):

Safety information

- N,N-Methylenebisacrylamide (N,N-Mba) is toxic if swallowed, inhaled, or absorbed through the skin. It is a mutagen, carcinogen, and teratogen. Handle only in a fume hood while wearing a mask, chemically resistant gloves, protective clothing, and eye protection. Dispose of N, N - Mba and any contaminated consumables in a hazardous waste stream.

- Combine the following reagents and vortex to combine at Room temperature until fully dissolved. Store at -20 °C .

	Reagents	Amount
	N,N-Methylenebisacrylamide	0.2g
	Nuclease Free Water	10 mL
	Total	**10 mL**

10% VA-044:*

- Add reagents and mix to combine until solution is clear. Aliquot into 500 µL aliquots and store at -20 °C .

	Reagents	Amount
	VA-044	0.1 g
	Nuclease Free Water	10 mL

TREx Gel Solution: 1.1 M Sodium Acrylate, 2.0M Acrylamide, 0.32 mM N,N-Mba, 1X PBS in water (9.4 mL)

- Combine the following reagents and invert the tube several times to combine. 10% VA-044 will activate the TREx gel solution and allow it to polymerize. Do not add 10% VA-044 until right before applying to sample. Store at -20 °C .

Safety information

- Acrylamide powders and solutions are toxic if swallowed, inhaled, or absorbed through the skin. It is a mutagen, teratogen and a carcinogen. Handle only in a biosafety fume hood while wearing a mask, chemically resistant gloves, protective clothing, and eye protection. Dispose of acrylamide and any contaminated consumables in a hazardous waste stream.
- Sodium acrylate is an environmental toxin. Handle only while wearing a mask, gloves, and protective clothing. Dispose of sodium acrylate and any contaminated consumables in a hazardous waste stream.

	Reagents	Amount
	Sodium Acrylate	2.7 mL
	50% Acrylamide	2.5 mL
	2% N-N Methylenebisacrylamide	24.6 µL
	10x PBS	1 mL
	Nuclease Free Water	3.4 mL
	10% VA-044*	110 µL
	Total	**9.4 mL**

* Do not add until right before applying to sample

50 mM ProK Buffer - 0.5% TritonX, 50 mM Tris-HCl-pH8, 50 mM NaCl, 1mM EDTA, 0.3% SDS in water (100 mL):

- Add ingredients to a glass bottle on a stir plate, starting with some of the Nuclease Free Water to aid in dissolving. Stir until the solution is clear or Overnight .

- Store at Room temperature .

	Reagents	Amount
	10% Triton X-100*	5 mL
	1M Tris Base pH 8	5 mL
	5M Sodium Chloride	1 mL
	0.5M EDTA	200 µL
	10% SDS	2 mL
	Water	85.8
	Total	**100 mL**

**Note:* *Triton X-100 is highly viscous*

Proteinase K Solution (ProK Solution): 0.01% ProK in ProK Buffer

- Make the solution in each tube containing a gelled sample. Begin by adding 50 mM ProK Buffer to the tube, then add Proteinase K (ProK). Invert tube several times to gently mix.
- Keep ProK On ice .

	Reagents	Amount
	50 mM ProK Buffer	742.5 µL
	ProK (800 U/mL)	7.5 µL
	Total	**750 µL**

FISH Probe Solution: 50 µM Rn28s, 10 nM Probes in Hybridization Buffer (300 µL):

- Add 1 µM Rn28s Solution and Probes to Hybridization Buffer. Invert tube several times to combine. Use 300 µL probe solution per sample. If using fewer probes, replace lost volume with hybridization buffer.

Safety information

- Hybridization buffer contains 10-30% formamide. Formamide is toxic, carcinogenic, and teratogenic. Handle probe wash buffer in a well-ventilated area. Wear chemical resistant gloves, a mask, and protective clothing when handling. Dispose of hybridization buffer and any contaminated consumables in a hazardous waste stream.

	Reagents	Amount
	1 µM Rn28s Solution	15 µL
	Probe 1	3 µL
	Probe 2	3 µL
	Probe 3	3 µL
	Hybridization Buffer	282 µL
	Total	**300 µL**

Hairpin Solution: 30 nM Hairpins

- Add hairpins to amplification buffer, taking care not to cross contaminate pipette tips. If less hairpin is used, replace lost volume with amplification buffer. Similarly, if more hairpins are being used, remove amplification buffer to ensure total volume per sample remains 200 µL

Note

- The hairpin initiator sequence should match the corresponding probe initiator sequence. However, only one hairpin initiator sequence should match the probe.
- For example: Gad1 - B1 probe needs to be paired with the B1 - 488 hairpin to be viewed in the 488 channel. No other B1 hairpins should be applied.

	Reagents	Amount
	Hairpin 1 H1	2 µL
	Hairpin 1 H2	2 µL
	Hairpin 2 H1	2 µL
	Hairpin 2 H2	2 µL
	Hairpin 3 H1	2 µL
	Hairpin 3 H2	2 µL
	Amplification Buffer	188 µL
	Total	200 µL

5x SSCT - 5x SSC, 0.2% Tween 20 (50 mL):

- Add 10% Tween 20 to 20x SSC and nuclease-free water. Vortex until combined. Store at Room temperature .

	Reagents	Amount
	20x SSC	12.5 mL
	10% Tween 20	1 mL
	Nuclease Free Water	37 mL
	Total	**50 mL**

0.5x SSCT - 0.5x SSC, 0.2% Tween 20 (50 mL):

- Add 10% Tween 20 to 20x SSC and nuclease-free water. Vortex until combined. Store at Room temperature .

	Reagents	Amount
	20x SSC	1.25 mL
	10% Tween 20	1 mL
	Nuclease Free Water	47.75 mL
	Total	**50 mL**

DNase Buffer Solution (750 µL):

- Thaw DNase Buffer at room temp. Vortex until combined.

	A	B
	10x DNase buffer	75 µL
	Water	675 µL
	Total Volume	**750 µL**

DNase Solution - 1X DNase Buffer, (500 µL):

- Thaw DNase Buffer at room temperature. Keep DNAse I on ice. Combine water and DNase Buffer and add to sample tubes. Add DNase I directly to sample tubes. Rock gently to combine.

	A	B
	DNase I*	20 µL

	A	B
	10x DNase buffer	50 µL
	Water	430 µL
	Total Volume	**500 µL**

Note: Keep DNase I on ice

Safety warnings

Formamide:
Hybridization buffer and probe wash buffer contains 10-30% formamide by weight. Formamide is toxic, carcinogenic, and teratogenic. Wear gloves, a mask, and protective clothing when handling. Dispose of formamide and any contaminated consumables in a hazardous waste stream. Store probe wash buffer and hybridization buffer at -80 °C .

Acrylamide:
Acrylamide powders and solutions are toxic if swallowed, inhaled, or absorbed through the skin. It is a mutagen, teratogen, and a carcinogen. Handle solid acrylamide in a chemical fume hood while wearing a mask, chemically resistant gloves (glove specs), protective clothing, and eye protection. Dispose of acrylamide and any contaminated consumables in a hazardous waste stream. Store aqueous solutions at -20 °C ; solid acrylamide can be stored at Room temperature .

Sodium Acrylate:
Sodium acrylate is an environmental toxin. Handle only while wearing a mask, gloves, and protective clothing.
Dispose of sodium acrylate and any contaminated consumables in a hazardous waste stream. Store sodium acrylate at -20 °C .

N,N-Methylenebisacrylamide:
N,N-Methylenebisacrylamide (N,N-Mba) is toxic if swallowed, inhaled, or absorbed through the skin. It is a mutagen, carcinogen, and teratogen. Handle only in a biosafety fume hood while wearing a mask, chemically resistant gloves, protective clothing, and eye protection. Dispose of N,N-Mba and any contaminated consumables in a hazardous waste stream. Store at -20 °C .

Ethics statement

The protocols.io team notes that research involving animals and humans must be conducted according to internationally-accepted standards and should always have prior approval from an Institutional Ethics Committee or Board.

Before start

- This protocol enables the visualization of individual mRNA transcripts in slices of fixed brain tissue. We anchor our brain slices via the [Gel and RNA Anchor Perfusion] protocol, but anchoring methods such as EASI-FISH or uniExM can also be used. See references for more information.

- All enzymes are kept on ice.

Protocol Overview

5d

1 This protocol describes Multiplexed Fluorescence In Situ Hybridization (mFISH) on brain slices for retrospective transcriptomic analyses of neurophysiology and neuromorphology, with single transcript resolution. It is adapted from the Expansion Assisted Iterative FISH (EASI-FISH) protocol , where tissue is integrated into an expandable gel and transcript detection is amplified using the Hybridization Chain Reaction (HCR).

5d

Citation

Wang Y, Eddison M, Fleishman G, Weigert M, Xu S, Wang T, Rokicki K, Goina C, Henry FE, Lemire AL, Schmidt U, Yang H, Svoboda K, Myers EW, Saalfeld S, Korff W, Sternson SM, Tillberg PW
(2021)
. EASI-FISH for thick tissue defines lateral hypothalamus spatio-molecular organization.
Cell.

10.1016/j.cell.2021.11.024 LINK

The first round of HCR RNA mFISH can be completed in one work week and each subsequent round can be completed in as few as two days.

Day 1
Gelation (~ 03:30:00) and Proteinase K (ProK) Digestion (Overnight)

Day 2
Hybridization with DNA probes(~ 02:30:00 + Overnight)

Day 3
Hybridizing hairpin amplifiers (~ 06:00:00 + Overnight) or (~ 09:00:00)

Day 4
Expansion and imaging (~ 02:30:00)

Day 5
Probe removal and hybridization of next round's probes

Note

- All aspects of this protocol should be performed RNase free. Whenever possible, handle samples and reagents in a PCR hood that has been sprayed thoroughly with RNase Away™. All tools and equipment should be sprayed thoroughly with RNase Away™ and all solutions should be kept RNase free. Where applicable, nuclease free water or Milli-Q water should be used. Wear a lab coat, mask, and gloves to prevent contamination. Spray gloves thoroughly with RNase Away™ and dry with a Kimwipe® regularly.

Note

The total volumes of the solutions are not specific. What is important is that the sample is completely submerged for these incubations.

Preparation of gelled tissue slice

1d 7h

2 First, the brain tissue slice is embedded in a robust expandable hydrogel to anchor biomolecules and allow easy handling. Following gel polymerization, protein digestion via a ProK Solution allows isotropic expansion to occur.

Citation

Damstra H, Mohar B, Eddison M, Akhmanova A, Kapitein LK, Tillberg PW (2022) . Visualizing cellular and tissue ultrastructure using Ten-fold Robust Expansion Microscopy (TREx). eLife.

10.7554/eLife.73775 LINK

2.1 **Prepare solutions:**
TREx monomer solution (9.4 mL):

	Reagents	Amount
	Sodium Acrylate	2.7 mL
	50% Acrylamide	2.5 mL
	2% N-N Methylenebisacrylamide	24.6 µL
	10x PBS	1 mL
	Nuclease Free Water	3.4 mL
	10% VA-044*	110 µL
	Total	**9.4 mL**

* **Note:** Add VA-044 immediately before use; once it is added, it is referred to as "activated TREx".

The TREx solution recipe can also be found in the Materials section.

Safety information

TREx gels contain acrylamide, sodium acrylate, and N,N-methylenebisacrylamide. See Guidelines & Warnings for proper chemical safety. While handling TREx solutions, wear a mask, gloves, and protective clothing and dispose of TREx solutions in a dedicated hazardous waste stream.

50 mM ProK Buffer (100 mL):

	Reagents	Amount
	10% Triton X-100	5 mL
	1M Tris Base pH 8	5 mL
	5M Sodium Chloride	1 mL
	0.5M EDTA	200 µL
	10% SDS	3 mL
	Water	85.8 mL
	Total	**100 mL**

Note: Store 50 mM ProK Buffer at room temperature.

The ProK Buffer Solution recipe can also be found in the Materials section.

2.2 **Incubate anchored brain slices in TREx monomer solution:** 1h

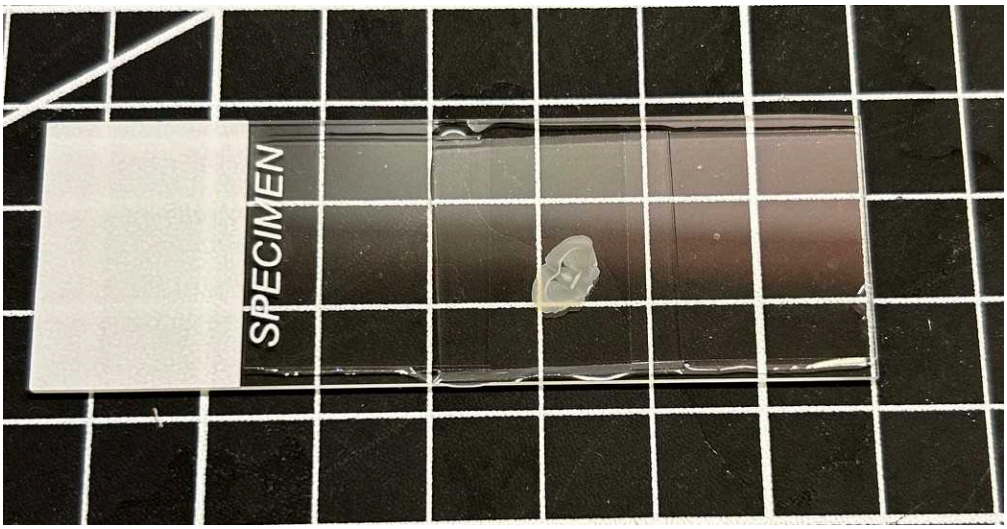
- Place the anchored brain slices in a sterile container.
- Incubate 3X 00:20:00 in activated TREx gel solution at Room temperature.

2.3 **Prepare the gelling mold:**

- Place a drop of activated TREx monomer solution on each end of a glass slide. Place a square coverslip (~22 mm x 22 mm) on each drop, leaving ~1 cm of space between the two coverslips. Place a drop of activated TREx monomer solution between the coverslips.
- Coverslips can be layered to accommodate tissue slices of different thicknesses (see https://microscopy.duke.edu/guides/coverslip-thickness for average glass thickness). We use #1.5 coverslips for 150 µm tissue slices.

2.4 **Prepare the tissue for gel formation:**

- Use a clean paintbrush to transfer the brain slice to the prepared glass slide, placing it between the coverslips on the drop of TREx monomer solution. Ensure it lays flat and no bubbles are trapped beneath the tissue slice.
- Place another coverslip on top of the brain slice. If bubbles are present, use a clean razor blade to slightly lift the edge of the top coverslip and allow bubbles to escape.



An assembled gel mold with a coronal mouse brain slice in TREx gel monomer solution. No bubbles are present.

2.5 **Polymerize the TREx gel in a humid chamber under inert atmosphere:** 1h 30m

- Create a humidified chamber by double bagging two Ziplock bags and then spraying the inside with RNase Away™ or nuclease-free water.
- Place the gel molds on a large petri dish and tuck a Kimwipe saturated with RNase Away™ on the edge.

- Place the petri dish and gel molds in the double bagged Ziplock bag and insert a tube connected to a nitrogen or argon line.
- Purge both bags by pressing on each Ziplock bag at least three times and allow it to refill with nitrogen to ensure no oxygen is present.
- Place this chamber in an oven and bake the prepared slice at 45 °C for 01:30:00 or until the gels have solidified.

Note

- The presence of bubbles can be an indication that the gel has solidified but isn't a consistent metric. We recommend lightly pressing on the surface of the top coverslip. If the gel solution beads or oozes out the sides of the mold, the gels should be returned to a humid chamber under inert atmosphere and continue to bake at 45 °C .

2.6 **Digest the gelled tissue with ProK Solution:** 1d 3h

- Fill a Microcentrifuge Tube with 742.5 µL 742.5 µL 50 mM ProK Buffer.
- Using a clean razor blade, disassemble the polymerization chamber and cut the gel to the edge of the tissue.
- Use a wetted clean paintbrush to gently dampen and lift the gelled tissue slice from the glass slide. Place the gelled tissue slice into the tube with ProK Buffer.
- Add 7.5 µL 7.5 µL ProK (800 U/mL) to the tube.
- Incubate the gelled tissue slice in ProK solution at 37 °C for Overnight .
- Place the gelled tissue slice into fresh ProK solution at 37 °C Overnight .

The ProK Solution recipe can be found in the Materials section.

2.7 **Wash out ProK Solution:** 1h 30m

- 3X 00:30:00 in 1X PBS at Room temperature .
- **Optional Pause Point:** Store samples in 1X PBS at 4 °C for up to 6 months.

FISH probe hybridization 1d 1h

3 Following gelation and digestion, DNA FISH probes are hybridized to RNA transcripts for genes of interest.

3.1 1h

Equilibrate gelled tissue in HCR Probe Hybridization Buffer:

- Thaw HCR Probe Hybridization Buffer to 37 °C
- Incubate samples for 01:00:00 in the HCR Probe Hybridization Buffer at 37 °C .

Safety information

- HCR Probe Hybridization Buffer contains 10-30% formamide. Formamide is toxic, carcinogenic, and teratogenic. Handle HCR Probe Wash Buffer in a well-ventilated area. Wear chemically resistant gloves, a mask, and protective clothing when handling.
- Dispose of HCR Probe Hybridization Buffer and any contaminated consumables appropriately as hazardous waste.

FISH probe hybridization

1d 1h

3.2 **Prepare FISH probes for hybridization:**

- During HCR Probe Hybridization Buffer equilibration, thaw the appropriate FISH probes to Room temperature and prepare the FISH Probe Solution.

FISH Probe Solution 300 µL :

- 10 nM of each FISH probe
- 50 nM Rn28s
- Remaining volume HCR Hybridization Buffer

Note

- When designing HCR RNA mFISH, it is important to select orthogonal initiator sequences for each probe within the same round of mFISH. For example: If a probe with a B1 initiator sequence is used, no other B1 probes should be included in that round of mFISH.
- We use a FISH probe against the Rn28s transcript for cell segmentation, but this is optional.

The FISH Probe Solution recipe can also be found in the Materials section.

FISH probe hybridization

1d 1h

3.3 **Replace HCR Hybridization Buffer with FISH Probe Solution:**

1d

- Incubate the sample in FISH Probe Solution Overnight at 37 °C

Note: Ensure the FISH Probe Solution volume completely covers the sample. Thicker samples will need a larger volume.

HCR amplification

1d 6h 15m 30s

4 Next, HCR amplification is performed to amplify detected transcripts. Before hybridizing fluorescent amplifiers to the FISH probes, the sample must first be washed thoroughly to remove any remaining FISH probe.

4.1 **Wash FISH probes from gelled tissue:** 5h

- Thaw HCR Probe Wash Buffer at 37 °C .
- Wash samples 6X 00:20:00 in HCR Probe Wash Buffer at 37 °C .
- Wash samples 6X 00:30:00 in 1X PBS at 37 °C .
- **Optional Pause Point:** Store samples overnight in 1X PBS at 4 °C for up to 6 months.

Safety information

- HCR Probe Wash Buffer contains 10-30% formamide. Formamide is toxic, carcinogenic, and teratogenic. Handle probe wash buffer in a well-ventilated area. Wear gloves, a mask, and protective clothing when handling.
- Dispose of HCR Probe Wash Buffer and any contaminated consumables appropriately as hazardous waste.

4.2 **Equilibrate gelled tissue in HCR Amplification Buffer:** 1h

- Thaw HCR Amplification Buffer to Room temperature .
- Remove 1x PBS and incubate samples in HCR Amplification Buffer for 01:00:00 at Room temperature .

4.3 **Snapcool HCR hairpins for signal amplification during HCR Amplification Buffer wash:** 15m 30s

- Our team uses a thermocycler (SimpliAmp™ Thermal Cycler) with the following program:

Phase 1:

95 °C	Forever (skip this section)
95 °C	00:01:30

Phase 2 (14 cycles):

90 °C	00:01:00
85 °C	00:01:00

80 °C	00:01:00
75 °C	00:01:00
70 °C	00:01:00
65 °C	00:01:00
60 °C	00:01:00
55 °C	00:01:00
50 °C	00:01:00
45 °C	00:01:00
40 °C	00:01:00
35 °C	00:01:00
30 °C	00:01:00
25 °C	00:01:00

Phase 3:

20 °C	Forever/hold

4.4 **Prepare the Hairpin Solution:**

- Prepare the Hairpin Solution immediately before applying it to the samples. The hairpin initiator sequences must match the initiator sequences used in the FISH Probe Solution.
- Wait to remove the hairpins from the SimpliAmp™ Thermal Cycler until immediately before adding them to the Hairpin Solution.

Hairpin solution 200 µL :

- 30 nM H1 Hairpin
- 30 nM H2 Hairpin
- Remaining volume HCR Amplification Buffer

4.5 **Incubate gelled tissue in the Hairpin Solution:** 3h

- Incubate samples in the hairpin solution for 03:00:00 or Overnight at Room temperature .

Tissue expansion 3h

5 Following HCR amplification, samples are washed to remove excess hairpin, then expanded to enable high resolution imaging of individual transcripts.

5.1 **Wash off hairpins:** 2h

- 2X 00:30:00 in 5X SSCT at Room temperature .
- 2X 00:30:00 in 0.5X SSCT at Room temperature .

5.2 **Expand gelled tissue:** 1h

- 2X 00:30:00 in 1X PBS at Room temperature .
- At this point, the gel will be approximately 3x-expanded. For lower expansion factors, use PBS concentrations greater than 1X.

Imaging

6 At this point the sample is ready to be imaged. Our team uses an Olympus FV3000 confocal microscope or a Zeiss Lightsheet Z.1, but other fluorescence microscopy imaging systems can be used.

6.1 Mount the sample. The mounting protocol will vary depending on your imaging system.

6.2 Image the sample.

Probe Stripping with DNase 1d 2h

7 For carrying out additional rounds of mFISH, FISH probes and HCR amplifiers are removed using a DNase Solution and the process repeated for a new set of gene targets.

- **Optional Pause Point:** Store samples in 1X PBS at 4 °C up to 6 months.

7.1 **Prepare DNase solutions for probe removal:**
DNase Buffer Solution (750 µL):

	A	B
	10x DNase Buffer	75 µL
	Water	675 µL
	Total Volume	**750 µL**

The DNase Buffer Solution recipe can also be found in the Materials section.

DNase Solution (500 µL):

	A	B
	DNase I	20 µL

	A	B
	10X DNase Buffer	50 µL
	Water	430 µL
	Total Volume	**500 µL**

Note: We recommend making DNase Solution directly in each tube containing a gelled sample instead of making a stock. First add the water and 10X DNase Buffer, then spike the DNase I into each tube. Rock gently to combine. Keep DNase I On ice until addition to DNase Buffer.

7.2 **Equilibrate gelled tissue in DNase Buffer:** 1h

- Incubate the samples for 01:00:00 in DNase Buffer Solution at 37 °C .

7.3 **Incubate gelled tissue in DNase I:** 1d 1h

- Incubate the samples for 01:00:00 in DNase Solution at 37 °C .
- Refresh the DNase Solution and keep at 37 °C Overnight .

7.4 After this step, the probes and hairpins have been removed. Subsequent rounds of mFISH can be performed with different probes. To begin a subsequent round, go to step #3 .

Protocol references

Damstra H, Mohar B, Eddison M, Akhmanova A, Kapitein LK, Tillberg PW
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EASI-FISH for thick tissue defines lateral hypothalamus spatio-molecular organization. Cell. 2021 doi: 10.1016/j.cell.2021.11.024

Citations

Step 1

Wang Y, Eddison M, Fleishman G, Weigert M, Xu S, Wang T, Rokicki K, Goina C, Henry FE, Lemire AL, Schmidt U, Yang H, Svoboda K, Myers EW, Saalfeld S, Korff W, Sternson SM, Tillberg PW. EASI-FISH for thick tissue defines lateral hypothalamus spatio-molecular organization
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Step 2

Damstra H, Mohar B, Eddison M, Akhmanova A, Kapitein LK, Tillberg PW. Visualizing cellular and tissue ultrastructure using Ten-fold Robust Expansion Microscopy (TREx)
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