ExFISH - Tissue Slice

Fei Chen,Asmamaw T Wassie,Allison J Cote,Anubhav Sinha,Shahar Alon,Shoh Asano,Evan R Daugharthy,Jae-Byum Chang,Adam Marblestone,George M Church,Arjun Raj,Edward S Boyden

Abstract

The ability to image RNA identity and location with nanoscale precision in intact tissues is of great interest for defining cell types and states in normal and pathological biological settings. Here, we present a strategy for expansion microscopy of RNA. We developed a small-molecule linker that enables RNA to be covalently attached to a swellable polyelectrolyte gel synthesized throughout a biological specimen. Then, postexpansion, fluorescent *in situ* hybridization (FISH) imaging of RNA can be performed with high yield and specificity as well as single-molecule precision in both cultured cells and intact brain tissue. Expansion FISH (ExFISH) separates RNAs and supports amplification of single-molecule signals (i.e., via hybridization chain reaction) as well as multiplexed RNA FISH readout. ExFISH thus enables super-resolution imaging of RNA structure and location with diffraction-limited microscopes in thick specimens, such as intact brain tissue and other tissues of importance to biology and medicine.

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Guidelines

Preparation of LabelX:

- Label-IT amine nucleic acid labeling reagent purchased from Mirus Bio.: https://www.mirusbio.com/products/labeling/label-it-nucleic-acid-modifying-reagent
- Resuspend Label-IT amine at 1 mg/mL using the provided resuspension buffer. Add the resuspension buffer and vortex to mix.
- Resuspend acryloyl X SE (AcX) (https://www.lifetechnologies.com/order/catalog/product/A20770) in 500 µL anhydrous DMSO (10 mg/mL stock solution). Aliquot in 20 µL and store desiccated at -20°C.
- React the Label-IT to AcX at equal mass ratio. For example, add 1 µL of acryloyl X to 10 µL of Label-IT (both are resuspended in DMSO so the reaction is carried out in DMSO). React for >6 hrs at room temperature on benchtop shaker. Store the LabelX at -20°C with desiccant for

future use.

Before start

*Sodium Acrylate, purity note: check for yellow color upon resuspension: that indicates poor quality; solution should be clear (see http://expansionmicroscopy.org)

Stock solutions:

MOPS Buffer: 20 mM MOPS pH 7.7

- For conveniently adjusting the final volume, prepare the MOPS buffer at 10 times the final concentration (i.e. 200mM MOPS pH 7.7). When diluting LabelX in the MOPS buffer add nuclease-free water to bring the final concentration of the buffer to 20 mM MOPS pH 7.7.

Hybridization Buffer for smFISH: 10% (w/v) dextran sulfate, 10% (v/v) formamide, 2X SSC

- Prepare the hybridization buffer without formamide and store it at 4°C. Add formamide right before performing FISH.

Hybridization Buffer for HCR-FISH: 10% (w/v) dextran sulfate, 20% (v/v) formamide, 2X SSC

- Prepare the hybridization buffer without formamide and store it at 4°C. Add formamide right before FISH.

Wash Buffer for smFISH (WA-10): 10% (v/v) formamide, 2X SSC

- Can be stored at room temperature for up to a week.

Wash Buffer for HCR-FISH (WA-20): 20% (v/v) formamide, 2X SSC

- Can be stored at room temperature for up to a week.

5x SSCT: 5x SSC, 0.1% Tween-20

- Can be stored at room temperature.

0.05x SSCT: 0.05x SSC, 0.1% Tween-20

- Can be stored at room temperature.

Amplification buffer: 5x SSC, 10% (w/v) dextran sulfate, 0.1% Tween-20

- Store at 4°C.

Digestion Buffer: 50 mM Tris pH 8.0, 1 mM EDTA, 0.5% Triton X-100, 1M NaCl

- Can be stored at room temperature.
- Add Proteinase K (NEB, 1:100, final concentration 8 units/mL) to digestion buffer before use.

Materials

Sodium Acrylate (purity note:*) 408220 by Sigma
Acrylamide A9099 by Sigma
N,N′-Methylenebisacrylamide M7279 by Sigma
Ammonium Persulfate A3678 by Sigma
N,N,N′,N′-Tetramethylethylenediamine T7024 by Sigma
VA-044 27776-21-2 by Wako
4-Hydroxy-TEMPO 176141 by Sigma
Dextran Sulfate D8906-50g by Sigma
SSC AM9765 by Thermo Fisher Scientific
Formamide AM9342 by Thermo Fisher Scientific
Paraformaldehyde 15710 by Electron Microscopy Sciences
Tissue-prep Buffered 10% Formalin 15742-10 by Electron Microscopy Sciences
Triton X-100 93426 by Sigma
Ethyl Alcohol E7023 by Sigma
Glycine 50046 by Sigma
10x PBS AM9624 by Thermo Fisher Scientific
Proteinase K P8107S by New England Biolabs
Ethylenediaminetetraacetic acid EDS by Sigma
Sodium Chloride S9888 by Sigma
Tris-HCl AM9855 by Life Technologies
Amplification Buffer N/A by Molecular Instruments
Tween 20 P1379 by Sigma
Label-IT ® Amine MIR 3900 by Mirus Bio
Acryloyl-X, SE A20770 by Thermo Fisher Scientific
MOPS M9381-25G by Sigma
DNAse I 4716728001 by Sigma
Bind-Silane GE17-1330-01 by Sigma

Protocol

Perfusion and Slicing

Step 1.

Transcardially perfuse with 1xPBS (5-10 mL) followed by 4% paraformaldehyde in 1xPBS (30 mL).

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Perfusion and Slicing are essentially the same as conventional histology.

Perfusion and Slicing

Step 2.

Post-fix the brain in 4% paraformaldehyde overnight.

Perfusion and Slicing

Step 3.

Wash with 1x PBS before cutting slices.

Perfusion and Slicing

Step 4.

Cut brain slices on a vibratome (can cut 50 - 300 micron slices).

Perfusion and Slicing

Step 5.

Store slices in 1x PBS if they are used immediately (1-2 days), or store in 70% ethanol for longer times for improved RNA stability at 4 °C.

LabelX Treatment of Slices

Step 6.

If slices were stored in ethanol, rehydrate slices permeabilized with 70% ethanol by washing twice with 1x PBS for 15 minutes per wash at room temperature.

LabelX Treatment of Slices

Step 7.

Pre-incubate slices with MOPS buffer for 30 minutes.

LabelX Treatment of Slices

Step 8.

Prepare LabelX by diluting in MOPS buffer at 0.1 mg/mL.

LabelX Treatment of Slices

Step 9.

Remove the preincubation MOPS buffer and add to the cultured cells LabelX in MOPS buffer.

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We like to process our tissue in 24 well or 48 well plates. (Use enough volume to cover the slices,

for example in a 24 well plate, 4 sections can be incubated in 250 µL solution.)

LabelX Treatment of Slices

Step 10.

Incubate slices with LabelX in MOPS overnight at 37°C.

LabelX Treatment of Slices

Step 11.

Wash slices with 1x PBS for 5 minutes. (1/2)

LabelX Treatment of Slices

Step 12.

Wash slices with 1x PBS for 5 minutes. (2/2)

LabelX Treatment of Slices

Step 13.

Proceed to gelation and digestion as described in the "ExM Slice Protocol."

Gelling

Step 14.

Make sure to remove excess PBS from brain slices before incubation with gelling solution.

Gelling

Step 15.

Incubate slices in gelling solution in an Eppendorf tube for 5 mins at 4°C.

TEMPERATURE

4 °C Additional info: Incubation

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Use freshly prepared gelling solution, immediately after adding APS at 4°C. (Make sure at least 100-fold excess volume of monomer solution is used. E.g., ~200µl of gelling solution for each brain slice. Need ~100µl for each of two incubations.)

Gelling

Step 16.

Replace the gelling solution with new gelling solution and incubate for another 25 mins at 4°C.

TEMPERATURE

4 °C Additional info: Incubation

Gelling

Step 17.

Transfer slices from Eppendorf tube into a gel chamber and then incubate at 37°C for 2 hours.

TEMPERATURE
37 °C Additional info: Incubation

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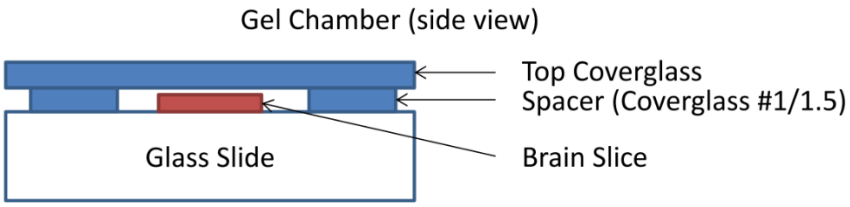

Gel chambers are constructed by sandwiching the slice between a slide and a coverglass, with spacers on either side of the tissue section to prevent compression of tissue slice (see schematic below). For 30 and 100 µm sections, pieces of #1 coverglass were used for spacers and for 200 µm sections, pieces of # 1 coverglasses can be stacked two coverglasses thick. (Spacers are easy to make from full coverglasses with a diamond scribe.) Make sure the slices are flat, and avoid air bubbles trapped inside the chamber.

Digestion and Expansion

Step 18.

Take off the cover of the gel chamber, and submerge it in digesting buffer, overnight at room temperature.

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(Make sure at least 10-fold excess volume of digestion buffer is used, and make sure to completely submerge the slice; make sure it does not dry out.) A small slide box, or a plastic well can be used to incubate the gel and digestion buffer.

Digestion and Expansion

Step 19.

Wash slices with excess volume of ddH_2O (we usually use at least 10x the final gel volume), 3-5 times, for 15mins each time.

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Slice expansion reaches plateau after about the 3 rd or 4th wash. The expansion chamber needs to be of adequate size for the sample, we found that for hemislices of mouse brains, the expanded gel fits nicely in a glass bottom 6 well plate (In Vitro Scientific) when the excess gel around the

brain is trimmed away (a Glass Slide Gel Chamber (side view) Top Coverglass Spacer (Coverglass #1/1.5) Brain Slice razor blade works well for this). Gels can be immobilized with 2% low melt agarose in water to prevent drift during imaging.

Staining gelled slices with HCR-FISH
Step 20.

After digestion, wash gels with 1x PBS for 15 minutes.

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Gels can be stored at 4°C after this step.

Staining gelled slices with HCR-FISH
Step 21.

Pre-hybridize gels by incubating with wash buffer (WA-20) for 30 minutes at room temperature.

Staining gelled slices with HCR-FISH
Step 22.

Prepare probes by diluting in HCR-FISH hybridization buffer at the desired concentration (1 nM per probe). Vortex to mix.

Staining gelled slices with HCR-FISH
Step 23.

Remove the wash buffer from the gels.

Staining gelled slices with HCR-FISH
Step 24.

Add the hybridization buffer with probes onto the gels.

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Add enough volume to completely cover the gel to be stained (for 24-well plates, 300µl).

Staining gelled slices with HCR-FISH
Step 25.

Incubate overnight (or for > 6hrs) at 37°C.

TEMPERATURE
37 °C Additional info: Incubation

Staining gelled slices with HCR-FISH
Step 26.

Wash gels with excess volume (eg. 500µl for 24-well plates) of WA-20 at 37°C for 30 mins. (1/2)

TEMPERATURE
37 °C Additional info: WA-20 wash (1/2)

Staining gelled slices with HCR-FISH

Step 27.

Wash gels with excess volume of WA-20 at 37°C for 30 mins. (2/2)

TEMPERATURE
37 °C Additional info: WA-20 wash (2/2)

Staining gelled slices with HCR-FISH

Step 28.

Wash once with excess volume 1x PBS at 37°C for 2 hours.

TEMPERATURE
37 °C Additional info: PBS wash

Staining gelled slices with HCR-FISH

Step 29.

Wash once with excess volume of 1x PBS at RT for 2 hours.

HCR amplification

Step 30.

Pre-amplify slices with amplification buffer for 30 minutes at room temperature.

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HCR amplification is adapted from: **Choi et al.**, ACS Nano, 2014, 8 (5), pp 4284–4294, "Next generation in situ hybridization chain reaction;" see http://molecularinstruments.org

HCR amplification

Step 31.

Prepare fluorescently labeled hairpin by snap cooling each hairpin (heat at 95°C for 90 seconds and cool to room temperature on the benchtop for 30 min).

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For each 500 µL of amplification reaction, 10 µL of each hairpin is needed; we use 150 µL reactions per 48 well or 250 µL per 24 well.

HCR amplification

Step 32.

Prepare hairpin solution by adding all snap-cooled hairpins to amplification buffer at room temperature.

HCR amplification
Step 33.

Remove the pre-amplification solution and add the hairpin solution to the gels.

HCR amplification
Step 34.

Incubate for 2-4 hours at room temperature.

HCR amplification
Step 35.

Stop amplification with 30-minute washes with 5x SSCT. (1/4)

HCR amplification
Step 36.

Stop amplification with 30-minute washes with 5x SSCT. (2/4)

HCR amplification
Step 37.

Stop amplification with 30-minute washes with 5x SSCT. (3/4)

HCR amplification
Step 38.

Stop amplification with 30-minute washes with 5x SSCT. (4/4)

HCR amplification
Step 39.

Slices can now be imaged. To expand slices, wash 3x 10 minutes with .05X SSCT (1:100 dilution of 5x SSCT).

NOTES
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Expansion factor can be tuned by salt level; imaging can also be performed in 1x PBS for ~2x expansion.

Warnings

Please refer to the SDS (Saftey Data Sheet) for safety warnings and hazard information.