ExFISH - Cultured Cells

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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

Cell Culture Preparation

Step 1.

Cells can be grown as desired to suit experimental needs.

NOTES
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For ease of gelation and imaging, and handling, we have found 16-well Culturewell removable chambered coverglass from Grace Bio Labs (Sigma catalog: GBL112358-8EA) to be great for before imaging, as well as subsequent gelation and digestion. In cases where smFISH is performed before gelation and expansion, we found it convenient to plate cells in 8 well Nunc Lab-Tek Chambered Coverglasses (Thermo Fisher: 155411).

Fixation and Permeabilization of Cultured Cells

Step 2.

Wash cells once with PBS warmed to 37°C.

TEMPERATURE

37 °C Additional info: PBS

Fixation and Permeabilization of Cultured Cells

Step 3.

Fix with 10% formalin for 10 mins.

Fixation and Permeabilization of Cultured Cells

Step 4.

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

Fixation and Permeabilization of Cultured Cells

Step 5.

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

Fixation and Permeabilization of Cultured Cells

Step 6.

Replace buffer with 70% ethanol. For immediate use, cultured cells can be permeabilized with 70% ethanol for 1hr at room temperature.

NOTES

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Alternatively, cells can be stored in 70% ethanol at 4°C for up to 2 weeks.

LabelX Treatment of Cultured Cells

Step 7.

Rehydrate cells permeabilized with 70% ethanol by washing with 1x PBS for 5 minutes at room temperature. (1/2)

LabelX Treatment of Cultured Cells

Step 8.

Rehydrate cells permeabilized with 70% ethanol by washing with 1x PBS again for 5 minutes at room temperature. (2/2)

LabelX Treatment of Cultured Cells

Step 9.

Pre-incubate cultured cells with MOPS buffer for 5 minutes.

LabelX Treatment of Cultured Cells

Step 10.

Prepare LabelX by diluting in MOPS buffer at the desired concentration.

NOTES

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We have observed nearly complete RNA retention when using LabelX at a final Label-IT amine concentration of 0.006 - 0.02 mg/ml. For smFISH experiments, we recommend using a final Label-IT amine concentration of 0.006 mg/ml, since higher concentrations result in somewhat dimmer smFISH staining.

LabelX Treatment of Cultured Cells

Step 11.

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

- For cells grown in Grace Bio labs removable coverglasses, use **80µl**.
- For cells grown in Nunc Lab-Tek chambered coverglasses, use **120µl**.

LabelX Treatment of Cultured Cells

Step 12.

Incubate overnight at 37°C.

TEMPERATURE

37 °C Additional info: Incubation

LabelX Treatment of Cultured Cells

Step 13.

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

LabelX Treatment of Cultured Cells

Step 14.

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

Gelling and Digestion

Step 15.

Remove wells and upper structure from chambered coverslips.

NOTES

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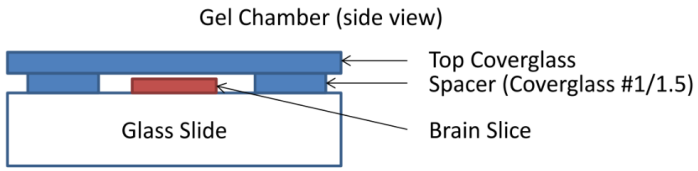
Expansion speed is generally limited by the diffusion time of salt and water out/into the gel, thus casting thin gels will generally speed up expansion time. The Grace Bio labs removable coverglass has a removable chamber upper structure which can be removed via a removal tool (Sigma catalog: GBL103259). After removal of the chamber, a 1 mm silicone spacer remains with the coverslip and can be used to cast the gel. If cells are grown on coverglass, then a chamber similar to the slice gelation chamber can be made using coverslip spacers. If cells are grown in Nunc Lab-Tek Chambered Coverglass, we have found that the bottom coverglass along with its 1 mm gasket can be removed from the rest of the plate.



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- For **Grace Bio labs removable coverglass,** use the removal tool to remove the top wells leaving behind the black silicone gasket. Leave 40µl of 1x PBS to keep cells hydrated.

- For the **Lab-Tek Chambered Coverglass**, first glue the bottom of the coverglass to a 1mm thick microscope glass slide for mechanical support using epoxy glue. After the glue has cured, use a razor blade to tease apart the coverglass from the top wells leaving behind the plastic gasket. Leave 100µl 1x PBS to keep cells hydrated.

Gelling and Digestion

Step 16.

[Prepare gelation solution on ice and degas]

Prepare gelation solution by mixing monomer solution and VA-044 solutions on ice. Prepare VA-044 stocks fresh at 25% (w/v) in water. Dilute to a final concentration of 0.5% (w/v) in monomer solution. To prepare 1ml of gelation solution, add 20µl of VA-044 solution and 40µl of water to 940µl of monomer solution.

Gelling and Digestion

Step 17.

[Prepare gelation solution on ice and degas]

In a cooling rack, or on ice, distribute the gelling solutions into 200µl aliquots in Eppendorf tubes.

Degas for 10 mins in a vacuum desiccator.

Gelling and Digestion

Step 18.

[Prepare gelation solution on ice and degas]

Remove any remaining PBS from the wells and add the gelling solution.

- For Grace Bio labs removable coverglass, add 40µl of gelling solution.
- Add 200µl of gelling solution if using Lab-Tek Chambered Coverglass.

Place the coverglass in a vacuum desiccator and degas for another 10 mins.

In the meantime, prepare a glass slide covered in parafilm. Immediately, remove the coverglass from the dessicator and place the parafilm glass slide directly on top of the well. Any excess gelling solution might spill out to the sides. After this step, proceed to the following steps without delay.

Nitrogen perfusion and gelling

Step 19.

The following steps require the use of a humidified chamber. The chamber provides a nitrogen filled environment for gelling and also minimizes evaporation.

NOTES

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We use a tupperware as a humidified chamber. To make the chamber humidified, we add a small amount of water to the bottom of the tupperware. We position an empty 24-well plastic bottom plate at the very bottom to provide a raised platform onto which we place the coverglass with the cells and gelling solution. Furthermore, we have bored two holes onto the cover of the tupperware using syringe needles to perfuse the chamber with nitrogen. The holes can be sealed with tape.

Nitrogen perfusion and gelling

Step 20.

After placing the parafilm-covered glass slide on top of the coverglass, place the coverglass onto the platform in the tupperware. Seal the tupperware shut.

Nitrogen perfusion and gelling

Step 21.

Remove the tapes covering the holes.

Nitrogen perfusion and gelling

Step 22.

Insert the inlet of the nitrogen line through one hole.

Nitrogen perfusion and gelling

Step 23.

Slowly, turn on the regulator to start the flow of nitrogen into the chamber. Increase the flow until you can feel the airflow coming out of the other hole. Flush the chamber with nitrogen for 10 mins. When done, immediately remove the inlet and tape the holes shut with electric tape.

Nitrogen perfusion and gelling

Step 24.

Place the chamber in a 60°C oven for 2hrs to initiate gelation.

TEMPERATURE

60 °C Additional info: Oven

Proteinase K Digestion and Expansion

Step 25.

When gelation is finished, take out the cover glass from the humidified chamber and gently remove the parafilm-covered glass slide from the top of the wells.

NOTES

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For **Grace Biolabs removable coverglass**, gently remove the black silicone gasket by peeling it off using your hands or a pair of tweezers.

For the **Lab-Tek Chambered coverglass**, do not attempt to remove the plastic gasket.

Proteinase K Digestion and Expansion

Step 26.

For digestion, place the coverglass in a Nunc 4-well rectangular plate (Thermo Fisher: 267061).

Proteinase K Digestion and Expansion

Step 27.

Prepare digestion buffer with Proteinase K.

NOTES

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Proteinase K (NEB, 1:100, final concentration 8 units/mL)

Proteinase K Digestion and Expansion

Step 28.

Add 6ml of digestion buffer into the well containing the coverglass.

AMOUNT

6 ml Additional info: Digestion buffer

NOTES

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If using any other container, add enough digestion buffer to completely cover the coverglass and gels.

Proteinase K Digestion and Expansion

Step 29.

Leave to digest at room temperature overnight.

NOTES

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During the digestion, the gels formed in the wells on the coverglass will pop off.

Proteinase K Digestion and Expansion

Step 30.

After digestion, gels are expanded in a Petri dish, or any container large enough to hold the gels.

Proteinase K Digestion and Expansion

Step 31.

To fully expand, wash gels with excess volume nuclease free water for at one hour. (1/3)

Proteinase K Digestion and Expansion

Step 32.

To fully expand, wash gels with excess volume nuclease free water for at one hour. (2/3)

Proteinase K Digestion and Expansion

Step 33.

To fully expand, wash gels with excess volume nuclease free water for at one hour. (3/3)

smFISH Staining of Cultured Cells after ExFISH

Step 34.

[Reducing thickness of expanded gels]

To shave down fully expanded gels to a thickness of 1mm, we use 1mm thick microscope glass slides as spacers as follows.

Prepare a large plastic dish to use as a cutting board. We often use the plastic covers of Nunc 4-well rectangular plates (Thermo Fisher: 267061), although any similarly sized flat plastic surface will work.

NOTES
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In the following steps the thickness of expanded gels is reduced: since the gels formed in either of the above chambered coverglass have a thickness of ~1 mm before expansion, we want to facilitate efficient diffusion of probes through the gel by shaving the fully expanded gel to a thickness of 1mm.

smFISH Staining of Cultured Cells after ExFISH

Step 35.

[Reducing thickness of expanded gels]

Use epoxy to glue two 1mm thick microscope glass slides on their flat slide onto the plastic surface. Position the two glass slides such that they are aligned along their longest dimension with 2.5-3cm space in between. Allow the epoxy to dry.

smFISH Staining of Cultured Cells after ExFISH

Step 36.

[Reducing thickness of expanded gels]

Position a fully expanded gel in between the two glass slides on the plastic cover.

NOTES
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The expanded gel should fit in between the two glass slides. If not, cut the gel such that it fits.

Position the gel such that the side of the gel with cultured cells is facing the bottom.

smFISH Staining of Cultured Cells after ExFISH

Step 37.

[Reducing thickness of expanded gels]

Place a razor blade across the glass slides such that it bridges the slides. Slide the razor blade along the glass slides and through the gel to shave off everything except the bottom 1 mm of the gel containing the cells.

smFISH Staining of Cultured Cells after ExFISH

Step 38.

[Reducing thickness of expanded gels]

Carefully collect the shaved gel and move to 1x PBS. Proceed to smFISH staining (next step).

smFISH Staining of Cultured Cells after ExFISH

Step 39.

[smFISH staining] Pre-hybridize gels by incubating with wash buffer (WA-10) for 30 minutes at room temperature.

NOTES

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Note to smFISH staining after gelation and expansion: staining can be performed in any container, though we often use glass bottom 24-well plates for the convenience of imaging right after staining.

smFISH Staining of Cultured Cells after ExFISH

Step 40.

[smFISH staining] Prepare probes by diluting in smFISH hybridization buffer at the desired concentration.

Vortex to mix.

smFISH Staining of Cultured Cells after ExFISH

Step 41.

[smFISH staining] Remove the wash buffer from the gels.

smFISH Staining of Cultured Cells after ExFISH

Step 42.

[smFISH staining] Add the hybridization buffer with probes onto the gels.

NOTES

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

smFISH Staining of Cultured Cells after ExFISH

Step 43.

[smFISH staining] Incubate overnight (or for > 6hrs) at 37°C.

TEMPERATURE

37 °C Additional info: Incubation

smFISH Staining of Cultured Cells after ExFISH

Step 44.

[smFISH staining] Wash gels with excess volume (eg. 500µl for 24-well plates) of WA-10 at 37°C for 30 mins. (1/2)

TEMPERATURE

37 °C Additional info: Wash

smFISH Staining of Cultured Cells after ExFISH

Step 45.

[smFISH staining] Wash gels with excess volume of WA-10 at 37°C for 30 mins. (2/2)

TEMPERATURE

37 °C Additional info: Wash

smFISH Staining of Cultured Cells after ExFISH

Step 46.

[smFISH staining] Wash once with excess volume 1x PBS at 37°C for 30 mins.

TEMPERATURE

37 °C Additional info: PBS wash

smFISH Staining of Cultured Cells after ExFISH

Step 47.

[smFISH staining] Perform imaging in 1x PBS or any other buffer of choice.

NOTES

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Determine expansion factor by salt concentration; 1x PBS results in ~2x expansion; dilute PBS in water to 0.02x PBS for ~ 3x expansion while still preserving hybridization.

Warnings

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