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Optimizing Tissue Preservation for High-Resolution Confocal Imaging of Single-Molecule RNA-FISH

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Abstract

Over the past century, formalin-fixed, paraffin-embedded (FFPE) tissue samples have represented the standard for basic histology and immunostaining. However, FFPE has several limitations and less stringent tissue preservation methods are required for the visualization of nucleic acids at high resolution, particularly those that are expressed at low levels. Here, we describe the FFPE properties that negatively impact RNA integrity, an alternative tissue preservation technique that prevents RNA loss, and the steps necessary to optimize slide preparation for single-molecule RNA fluorescent in situ hybridization (smRNA-FISH) and imaging by confocal microscopy. This strategy retains RNA quality and eliminates formalin-induced artifacts, thereby producing high-resolution, diffraction-limited confocal images of even rare RNA transcripts in tissues. As non-coding RNAs and alternative splicing of gene isoforms continue to emerge as important regulators of human health and disease, a reliable, cost-effective approach is required to examine the expression and localization of RNA targets in patient samples.

Keywords

alcohol fixation; formalin fixation; RNA degradation; single-molecule RNA-FISH; tissue preservation

Basic Protocol 1: Preparing an RNase-free workstation

Support Protocol 1: Diethyl pyrocarbonate water treatment

Support Protocol 2: Removing RNase contamination from glassware

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Basic Protocol 2: BE70 tissue fixation and processing

Basic Protocol 3: Cutting slide sections from paraffin blocks

Basic Protocol 4: Specimen pre-treatment

Basic Protocol 5: RNA fluorescent in situ hybridization labeling

Basic Protocol 6: Slide mounting

Basic Protocol 7: Generating deconvolution-capable confocal micrographs

INTRODUCTION

The fixation of tissue specimens with aldehydes, especially formalin, followed by paraffin embedding is still one of the most commonly used techniques for processing and analyzing biopsies and intact tissues. While formalin-fixed, paraffin-embedded (FFPE) samples are generally suitable for immunohistochemistry and other protein-based assessments, there are several disadvantages of using this approach in evaluating RNA expression and localization. In this article, we give step-by-step instructions for evaluating RNA transcripts in tissue sections using an alternative fixation protocol that retains both subcellular structure and RNA integrity. BE70 is a phosphate-buffered fixation solution consisting of 70% ethanol, glycerol, and glacial acetic acid that was developed at the National Cancer Institute for use in clinical pathology, allowing both microscopy and biomolecular assays to be produced from a single biopsy. As a coagulative fixative, BE70 does not cause overfixation, which greatly reduces the risk of damaging precious and/or sensitive samples (Perry et al., 2016). We also describe important considerations for the processing of specimens following fixation such as low-temperature/pressure embedding, acetylation treatment, and confocal image deconvolution that maximize visualization of RNA targets at the single-cell level (see Fig. 1). As a proof-of-principle, we provide a side-by-side comparison of RNA fluorescent in situ hybridization (RNA-FISH) micrographs captured after either the standard FFPE protocol or the RNA-specific strategy we present here.

BASIC PROTOCOL 1

PREPARING AN RNase-FREE WORKSTATION

Before starting your first experiment, great care must be taken to ensure all RNA-degrading compounds are removed from your workstation and supplies. Exogenous RNases are released from shed skin cells and are ubiquitous in the modern laboratory environment. Always assume that where there is dust, there are also RNA-degrading enzymes. It is assumed that these precautions detailed in the section below will be closely followed throughout the protocol.

Materials—Aerosol-resistant, RNase-free pipet tips

RNase Away

70% ethanol (EtOH)

Milli-Q-filtered water (mQH₂O) or diethyl pyrocarbonate (DEPC)-treated water

1. Choose your workstation's location carefully to avoid areas with high foot traffic or work benches located below air vents.
2. Designate the chosen workspace and a set of pipets to be only used on RNA-FISH experiments.

Maintaining a sterile and RNase-free workstation requires much less effort than repeating experiments lost due to RNA degradation. Consider placing a sign near your workstation to remind colleagues of the necessary precautions or to avoid the area altogether.

3. Use aerosol-resistant, RNase-free pipet tips. Do not use them with other experiments.
4. Wear gloves and lab coat. Periodically rub RNase Away on gloved hands while working. Replace gloves whenever they contact bare skin.
5. Clean workstation surfaces and pipets with 70% EtOH, followed by RNase Away, before and after every experiment.
6. Prepare all buffers and solutions with either Milli-Q-filtered water (mQH₂O) or diethyl pyrocarbonate (DEPC)-treated water.

DEPC treatment must occur before making buffers and solutions (see Support Protocol 1).

7. Store all buffers and solutions in small aliquots and discard aliquot after each use. Never use stock solutions used for other purposes that do not implement the same necessary precautions.
8. Heat-sterilize all glassware you expect to use, including measuring vessels and storage bottles (see Support Protocol 2).
9. Whenever possible, purchase reagents that are certified RNase-free.

SUPPORT PROTOCOL 1

DIETHYL PYROCARBONATE WATER TREATMENT

Labs without access to Milli-Q or Nanopure-filtered water must take extra steps to ensure their solutions are RNase free. DEPC chemically inhibits RNase by altering the histidine residues of the enzyme. This protocol must be followed to completion before making any solutions. Do NOT treat finished solutions with DEPC. For best results, treat water in glassware already sterilized (see Support Protocol 2).

Materials—Diethyl pyrocarbonate (DEPC; MilliporeSigma, cat. no. 40718)

Deionized water

1-L storage bottle

Autoclave

1. Add 1 ml 0.1% DEPC to 1,000 ml distilled water in a 1-L storage bottle. Mix thoroughly.
Use a glass pipet, as DEPC will dissolve some plastics. DEPC does not immediately dissolve in water; consider placing bottle on a shaker, if available, after mixing.
2. With the cap on, invert bottle three times, then let solution sit overnight at room temperature.
This ensures that RNases under the cap are also treated. During this time, DEPC deactivates RNases but must be removed from the treated water before making solutions with it.
3. Loosen cap on the bottle and autoclave solution 30 min at 15 psi.
This relieves pressure from within the bottle and allows the hydrolyzed DEPC products to escape.
4. Remove bottle from the autoclave while wearing insulated gloves. Allow water to reach room temperature then close the cap tightly.
The water is now considered RNase free and ready for use in experiments. Practice sterile technique when opening the bottle to avoid recontamination. DEPC-treated water can be stored at room temperature for up to 12 months.

SUPPORT PROTOCOL 2

REMOVING RNase CONTAMINATION FROM GLASSWARE

RNase-free reagents are only as reliable as the containers they are stored in. Make sure all glassware to be used is treated before adding any reagents.

Materials—Contrad 70 labware cleaner

Diethyl pyrocarbonate (DEPC; MilliporeSigma, cat. no. 40718)

Aluminum foil

Isotemp heating/drying oven (Thermo Fisher Scientific, Fisherbrand, cat. no. 15-103-0503)

1. Preheat isotemp oven to 250°C.
2. Clean glassware with Contrad 70 then thoroughly rinse.
3. Remove all plastic and tape from the glassware. Wrap glassware mouths with a small piece of aluminum foil.
Plastic melts at this temperature so we recommend removing RNases from bottle caps and other plasticware via DEPC treatment (see Support Protocol 1).

4. Place glassware in oven once temperature is reached. Incubate glassware overnight.
5. Remove glassware from oven with insulated gloves. Allow glassware to reach room temperature before use.

Cool glassware on a clean towel to avoid scorching or melting work surfaces.
Remove foil cover under sterile conditions only.

BASIC PROTOCOL 2

BE70 TISSUE FIXATION AND PROCESSING

Samples are fixed in a specific solution (BE70) to maximally retain mRNA integrity and cell morphology. Fixed samples are then infiltrated with, and embedded in, a block of paraffin wax that does not exceed mRNA denaturation point.

Materials—Tissue samples

BE70 tissue fixative (see recipe)

Ethanol

Milli-Q water (mQH₂O)

Xylenes

Paraffin block molds

Paraplast X-tra (Leica Biosystems, cat. no. 39603002)

Tissue processing cassettes (Tissuetek, cat. no. 4173)

Graphite pencil

Dissection scalpel (Thermo Fisher Scientific, Fisherbrand, cat. no. 12-000-161)

RNase-free Coplin jar

Tissue embedding cassettes

Vacuum infiltration tissue processor (e.g., Sakura Tissue-Tek VIP 6)

Tissue embedding module (e.g., Sakura Tissue-Tek TEC 5)

Collect tissue

1. Prior to scheduled biopsy or sample procurement, label enough processing cassettes to collect expected tissue volume.

In our experience, solvent- and heat-resistant ink pens have inconsistent performance. Use a pencil for more reliable labeling.

2. Using a dissection scalpel, begin dividing tissue into sub-samples immediately after it is excised; each sub-sample should fit completely in the tissue cassette without having to force the lid closed.

Samples must not exceed a 20-mm × 30-mm area, or 3-mm thickness. This facilitates predictable fixation times and avoids physical damage due to sample compression within the cassette.

3. Once tissue samples are placed into cassettes, add all cassettes to a sufficiently large container (e.g., an RNase-free Coplin jar), and add a volume of BE70 fixative greater than or equal to ten times the tissue volume.

Work quickly, minimizing the time between excision and fixation. During this transition, oxidative stress can impact cellular morphology and endogenous RNases remain active until fixation is complete.

Fix tissue

4. Leave small samples that comprise less than half of the tissue cassette in fixative ~6 hr at room temperature, while larger samples should be fixed 24 hr at room temperature.

Unlike aldehyde-based fixatives, BE70 does not produce over-fixation, so samples may remain in BE70 until tissue processing is convenient. RNA integrity and cell morphology were shown to be identical between 24 hr and 6 months of fixation (Chung et al., 2018). Thus, when there is doubt of sample size or cellular density, extend fixation time.

Process tissue

5. Turn on vacuum infiltration tissue processor. Fill each station's solvent reservoir and program time and temperature of each station accordingly (see Table 1).
6. Delay processor schedule such that the 14-hr program finishes the following morning. Load tissue cassettes into the processor retort and start program.
7. Turn on tissue embedding module and allow paraffin reserves to melt completely.
8. Remove tissue cassettes from the retort once processor's program completes. Transfer cassettes to warm holding chamber on the embedding module.

Prevent tissue sections from reaching room temperature before embedding. Tissue adheres to block better when same temperature as the embedding wax.

9. For each sample processed, place one paraffin block mold and one embedding cassette on the embedder's warming plate. Label each embedding cassette for future identification.
10. Remove one sample from its processing cassette and lay it flat in the center of one paraffin block mold. Ensure sample's region of interest, if any, is oriented toward the bottom of the mold.

11. Dispense enough wax from the embedder into the block mold to cover the bottom of the mold. Slide mold onto the embedder's chill plate to partially solidify the wax.

This prevents the sample from shifting during the next few steps.
12. Place an embedding cassette on top of the block mold and return them to the wax dispenser.
13. Slowly dispense more wax into the mold until the surface level rises through the cassette's holes. Ensure wax completely covers the cassette and no air gaps are present.

Wax blocks will break off from the cassette if not completely attached.
14. Transfer mold and cassette to chilling plate for 5 min or until wax is completely solid. Gently tap mold against the benchtop to break the block loose; the cassette should remain attached to the block.
15. Repeat steps 10 to 14 until all samples are in blocks.
16. Transfer all blocks to a zip-top plastic bag and store them at 4°C until ready to cut slide sections.

BASIC PROTOCOL 3

CUTTING SLIDE SECTIONS FROM PARAFFIN BLOCKS

Very thin slices, called sections, are cut from the sample blocks and deposited onto microscope slides. Extra steps are taken to minimize RNase, thermal, as well as water damage to the samples.

Materials—Tissue blocks (see Basic Protocol 2)

RNase Away surface decontaminant (Thermo Fisher Scientific, Invitrogen brand, cat. no. 10328011)

PARA/Gard paraffin repellent (Thermo Fisher Scientific, cat. no. 15-183-15)

Kimwipes (Thermo Fisher Scientific, cat. no. 06-666)

HM-325 rotary microtome (Thermo Fisher Scientific, cat. no. 902100)

Low profile microtome blades (Tissuetek, cat. no. 4980)

Generic fine-tipped artist's paintbrush

Positively charged glass slides, Fisherbrand Superfrost Plus (Thermo Fisher Scientific, cat. no. 12-550-15)

Slide box

Tissue floating bath (Premiere, model no. XH-1001)

Incubator, set at 37°C

4°C refrigerator

1. Remove tissue blocks from 4°C and allow them to reach room temperature.
Cold blocks tend to produce rolled up sections instead of continuous ribbons.
2. Add RNase Away to the empty tissue floating bath, spreading it evenly across the entire surface with Kimwipes. Allow surface to dry.
3. Place tissue floating bath within reach of the microtome and fill it with RNase-free water. Heat bath to 42°C. Preheat incubator to 37°C.
4. Load a single, low-profile microtome blade into the blade holder. Ensure blade holder is oriented to zero degrees. Set microtome to the desired section thickness.
Ideal section thickness depends on the cellular density, intracellular material concentration, and nuclear diameter of the tissue sample; 5 to 10 µm is typical. Avoid sections thicker than 20 µm until more experienced.
5. Apply RNase Away to all surfaces below the blade that tissue sections are likely to touch. Allow surfaces to dry. Apply PARA/Gard to the same surfaces by spreading a minimal volume into a thin layer. Remove excess with a clean Kimwipe.
Paraffin sections tend to cling to PARA/Gard when in liquid form.
6. When tissue blocks and floating bath have reached room temperature and 42°C, respectively, install block into the specimen clamp.
Release clamp slowly to avoid fracturing the tissue block.
7. Slide blade holder forward and bring it close to the tissue block without touching it. Lock blade holder into place, ensuring the blade is parallel with the tissue block surface.
8. Begin sectioning by gently grasping the handwheel and rotating it in a clockwise direction.
Maintain constant speed throughout each rotation. Moving the handwheel too fast or too slow will gouge pieces from the block or produce wrinkled sections, respectively.
9. Continue slicing away excess wax until you observe sections containing tissue. Once you have a section with a relevant sample, gently lift section off the microtome and, with the shiny side down, drape it onto the surface of the water bath.
The sections will be very fragile and tend to cling to most objects, making it difficult to cleanly release the tissue onto the water surface. A simple, fine-tipped paintbrush works much better than forceps. Avoid touching sections with hands (gloved or otherwise) at all costs.

10. Grasp a positively charged glass microscope slide by the label and slowly dip the other end into the water bath near the tissue section. Make sure the top face of the slide is facing the section. Allow one edge of the tissue section to make contact with the slide; it should adhere immediately. Slowly lift slide out of the water, allowing the tissue section to adhere in a single, evenly spaced layer.

Tissue sections should spend <1 min in the bath to preserve morphology and RNA integrity.

11. Repeat steps 9 and 10 until you have enough slides for your labeling experiment. Place all slides in the pre-warmed incubator overnight to dry.
12. Remove dry slides, place them in a slide box, seal box in a zip-top bag, and store them at 4°C until ready to proceed.

While tissue blocks remain stable for months in cold temperatures, tissue sections do not respond well to a damp atmosphere. Prepare just enough slides that you can process within a week to maximize RNA retention. For particularly sensitive and/or valuable samples, include a small silica gel packet in the slide box. This helps mitigate residual moisture exposure.

BASIC PROTOCOL 4

SPECIMEN PRE-TREATMENT

Some amino groups in the tissue sample carry a positive charge that may cause the RNA probe to bind non-specifically. The following process uses acetic anhydride (AA) to acetylate these groups, leaving only RNA with sufficient charge to attract the probe. See the Sample Acetylation video in Supplementary Material for a visual demonstration of this process.

CAUTION: AA is toxic, and this process should only be carried out in a fume hood with proper personal protective equipment. Dispose waste properly according to your research institution's guidelines.

Materials—Tissue samples (see Basic Protocol 3)

0.1 M triethanolamine (TEA) buffer, freshly prepared (see recipe)

Dewaxing/rehydration series (consisting of two staining dishes of xylenes, two staining dishes of 100% ethanol, and one staining dish each of 95%, 70%, and 50% ethanol)

Acetic anhydride (AA; Thermo Fisher Scientific, cat. no. A10-500)

RNase-free PBS

Magnetic mixer

Stir bar

Glass slide staining dish with removable rack (Thermo Fisher Scientific, cat. no. 08-812)

Tube clamp

Prepare acetylation apparatus

1. Move magnetic mixer into the fume hood. Plug mixer into AC outlet and confirm it powers on.
2. Place RNase-free glass staining dish in the center of the magnetic mixer. Place a 3-cm stir bar in the dish. Confirm stir bar rotates freely and evenly.
3. Add a tube clamp to the support rod and hang glass slide staining rack from the tube clamp. Orient clamp such that the staining rack lowers straight into the dish when the clamp is slid down along the support stand.
4. Test apparatus by lowering the staining rack into the dish while the stir bar is spinning. Ensure rack completely fits within the dish while neither disturbing the stir bar nor contacting the dish floor. Lock tube clamp at this position and remove staining rack.

Dewax and rehydrate specimens

5. Remove slides from 4°C and allow them to reach room temperature. Prepare dewaxing/rehydration series during this time.
6. Dewax slides by submerging them in two changes of xylenes, 5 min each.
7. Rehydrate slides through the following sequence:
100% EtOH: Twice, 2 min each;
95% EtOH: 2 min;
70% EtOH: 2 min;
50% EtOH: 2 min.

Sample acetylation

8. During the last rehydration wash of step 7, pour 0.1 M TEA solution into the glass staining dish. Adjust stir bar to 60 to 120 rpm.
9. When rehydration is complete, load slides into the glass staining rack and submerge in TEA buffer by hanging rack from tube clamp. Allow slides to equilibrate 2 min.
10. Add 1.25 ml AA to the glass staining dish.

Complete this step within 30 s to avoid dehydrating the tissue sections. TEA buffer should be circulating at the highest possible rate without creating a visible vortex in the staining dish. Do not add AA before lowering the staining rack, as the half-life of AA in TEA is very short.
11. Once AA is completely dissolved, reduce stir bar rpm and incubate slides in the solution 10 min.

The last remaining AA droplets may be difficult to see. Do not reduce rpm until complete dissolution is observed.

12. Remove slides from the glass stain rack and wash them two times, 3 min each time in RNase-free PBS. Only remove slides from the fume hood after washes are complete.

Slides are now prepared for your RNA-FISH labeling system of choice. While some details vary between labeling systems, certain best practices should be followed to improve the outcome (see Critical Parameters).

BASIC PROTOCOL 5

RNA FLUORESCENT IN SITU HYBRIDIZATION LABELING

There are several choices that exist for fluorescent RNA labeling of samples. While certain experimental details vary between systems, their basic chemistry is functionally the same and this article should enhance resolution and signal intensity in all RNA-FISH protocols. Hybridization chain reaction (HCR) RNA-FISH was chosen to validate this protocol based on its high specificity, short RNA sequences, and the ability to bind a single RNA transcript with multiple fluorescent probes (Choi, Beck, & Pierce, 2014).

The HCR RNA-FISH system used here has sustained multiple modifications over three different versions (Choi et al., 2010, 2014, 2018) and was previously commercialized by Molecular Instruments (<https://www.molecularinstruments.com>). Briefly, HCR is an enzyme-free, two-stage protocol comprised of a detection phase, followed by signal amplification. It is composed of: (1) a synthetic RNA probe containing both a sequence complementary to the RNA target and an initiator sequence that interacts with (2) a pair of fluorescently labeled, metastable DNA hairpins (HPs). Each HCR amplifier set operates in parallel and is labeled with one of the Alexa Fluor dyes (Alexa 488, 514, 546, 594, and 647) or can be custom made with other fluorescent labels. The initiator sequence on the HCR probe first binds to Hairpin 1, causing it to unfold. Hairpin 2 uses this release of kinetic energy to then bind to Hairpin 1, producing a chain reaction that expands the length of the hybridized fluorescent molecules. This results in a single RNA transcript bound to many fluorophores, which maximizes signal visualization for RNA quantification and allows the detection of small or lowly expressed RNA transcripts. In addition, multiple probes, and HP pairs specific to them, can be applied to the same sample to visualize different RNAs simultaneously. Lastly, the third-generation HCR RNA-FISH system also incorporates automatic background suppression using probes and amplifiers that do not amplify background signal, even if they bind non-specifically, to reduce noise in samples (Choi et al., 2018).

Below, we provide the HCR protocol for RNA-FISH that was used to validate this article.

Materials—Tissue sample slides for labeling (see Basic Protocol 4)

Probe hybridization buffer (see recipe)

5× saline-sodium citrate Tween 20 (SSCT) buffer (see recipe)

Hybridization chain reaction (HCR) probes (designed by and ordered from Molecular Instruments)

Probe wash buffer (see recipe)

Amplification buffer (see recipe)

Fluorescent hairpin pairs (HPs; Molecular Instruments)

RNase-free glass coverslips

Drybath

Thermobrite or incubator with humidified chamber

Thermocycler

Day 1: Detection stage

1. Pre-warm 1 ml probe hybridization buffer to 37°C in drybath.
2. Bring Thermobrite or incubator to 37°C.
3. Thaw HCR probes slowly over ice.
4. Saturate Thermobrite humidity pads with mQH₂O.
If using an incubator, add a humidified tip box according to Critical Parameters.
5. Dry slides by blotting edges with a Kimwipe.
6. Add enough hybridization buffer to completely cover the sample. Incubate 10 min at 37°C.
7. Prepare probe solution by adding 1 µl of each probe to a 500-µl volume of pre-warmed hybridization buffer. Mix gently by slowly pipetting up and down.
8. Remove pre-hybridization buffer from experimental slides. Drain excess by blotting.
Leave pre-hybridization buffer on negative control slide. This prevents it from drying out and will be used to assess both successful labeling and baseline confocal parameters.
9. Add 50 to 100 µl probe solution to the section, depending on coverslip size.
10. Gently lower coverslip onto surface of section. Incubate overnight (~16 hr) at 37°C.
Coverslip reduces evaporation and prevents contaminants from falling into probe solution.

Day 2: Amplification stage

11. Allow 250 μ l amplification buffer to reach room temperature.
12. Pre-heat thermocycler to 95°C.
13. Prepare the following probe washes and pre-warm them to 37°C in a drybath:
750 μ l wash buffer/250 μ l 5 $\times$ SSCT;
500 μ l wash buffer/500 μ l 5 $\times$ SSCT;
250 μ l wash buffer/750 μ l 5 $\times$ SSCT;
1000 μ l 5 $\times$ SSCT.
14. Remove excess probes from sections by incubating slides in all washes 15 min each at 37°C.
15. Immerse slides in 5 $\times$ SSCT 5 min at room temperature.
16. Blot slide edges until dry. Add enough amplification buffer to completely cover the section. Place slide in room temperature humidified chamber and incubate in dark drawer 30 min at room temperature.
17. Prepare fluorescent HP solutions:
 - a. Add 4 μ l of each 3 μ M HP stock to individual PCR tubes. Heat all HP stocks 90 s at 95°C in thermocycler.
 - b. Cool HPs to room temperature in dark drawer for 30 min.
 - c. Combine all HPs in 100 μ l of aliquoted, room temperature amplification buffer. Mix well.
18. Remove pre-amplification buffer from slides. Drain excess by blotting slide edges.
19. Add 50 to 100 μ l HP solution to tissue, depending on coverslip size.
20. Gently lower coverslip onto surface of section. Incubate overnight (~16 hr) in dark drawer at room temperature.

Day 3

21. Immerse slide in 5 $\times$ SSCT at room temperature to allow coverslip to float off.
22. Remove excess HPs by incubating sections in 5 $\times$ SSCT 30 min two times, then once for 5 min.

BASIC PROTOCOL 6**SLIDE MOUNTING**

Most of the “blurriness” found in poor quality micrographs is caused by diffraction. When light from a fluorescent probe travels through a given medium, its trajectory bends, or diffracts, at a specific angle according to that medium’s refractive index. The presence of

multiple media with mismatched indices causes unpredictable diffraction angles, resulting in fewer of the probes' photons reaching the objective and a weaker, undefined signal. This chaotic diffraction can be mitigated by using a mounting medium with the same refractive index as glass ($N_d = 1.518$), so the light emitted from a fluorescent probe bends at only one angle as it travels to the microscope objective. We recommend CFM-3, one of several high-index media available, for a few reasons. CFM-3 does not require the sample to be dehydrated before mounting, as incomplete dehydration with other mounting media can result in the formation of bubbles and residual water movement on the slide. It also contains an anti-bleaching agent that strongly preserves both Hoechst and Alexa probes from extended laser exposure. Follow these steps immediately after completing RNA-FISH.

Materials—Tissue sample slides (see Basic Protocol 5)

Double-stranded DNA (dsDNA) counterstain (e.g., DAPI or Hoechst 34580)

CFM-3 mounting medium (EMS, cat. no. 17979)

Clear nail polish

Slide coverslips, thickness #1.5

Optical lens cleaner

1. Counterstain slides with an appropriate dsDNA counterstain.

Counterstain selection is dependent on the wavelengths of your RNA probe(s) and the lasers installed on your microscope. Make sure their emission spectra do not overlap to avoid crosstalk between channels. Online tools, such as Thermo Fisher Scientific's Fluorescence SpectraViewer, are useful in this selection process (see Online Resources). dsDNA-specific stains are recommended to enhance the contrast between nuclear DNA and mRNA transcripts. DAPI or Hoechst 34580 perform well on confocal microscopes equipped with UV lasers.

2. Add the minimum volume of CFM-3 required to completely cover the sample. Look carefully for the formation of a meniscus layer within the mounting medium. Place slides in a dark drawer 30 min.

The observed liquid layer is residual water in the sample displaced by the mounting medium.

3. Gently tap slide against a Kimwipe to remove excess CFM-3. Add a fresh volume of CFM-3 to each sample and check for further water displacement. Return slides to drawer for 10 min.

4. Repeat step 3 until CFM-3 appears completely homogenous.

The number of repeated steps is dependent on section volume and tissue type. Expect this number to vary some between samples.

5. Place one edge of a #1.5 glass coverslip to one side of the section, making contact with the mounting medium. Slowly lower coverslip onto the section to avoid bubbles.

Confocal microscope objectives are calibrated for #1.5 coverslips. Other thicknesses impact image quality.

6. Place a Kimwipe on the slide to leech excess medium from under the coverslip. Repeat until Kimwipe remains dry.
7. Paint a thin stripe of clear nail polish around the coverslip perimeter to seal the sample. Allow polish to dry completely.
8. Pour a minimal volume of optical lens cleaner onto a clean Kimwipe. Gently clean the underside of the slide directly beneath the sample. Use another Kimwipe to wipe away any excess.

BASIC PROTOCOL 7

GENERATING DECONVOLUTION-CAPABLE CONFOCAL MICROGRAPHS

Now that you have high quality, optically clear slides, you need to configure some of the confocal microscope's parameters to image them accordingly. Specifically, each pixel in the image must be smaller than the resolvable distance between two adjacent probes. This will generate enough visual data for post-processing software (e.g., ImageJ) to calculate the exact position of each probe and remove the blurry "halo" that surrounds it. The minimum pixel size required by this process, known as oversampling, can be calculated with two equations. One determines the pixel's width (lateral sampling distance) and the other determines the pixel's depth (axial sampling distance).

Materials

Optically clear sample slides (see Basic Protocol 6)

Type F immersion oil (Leica, cat. no. 11513859)

Laser-scanning confocal microscope

High capacity portable USB drive

1. Calculate the lateral sampling distance using the following formula:

$$\Delta_{x,y} = \frac{\lambda_{ex}}{8n \sin(\alpha)}$$

Where λ_{ex} is the probe's excitation wavelength, n is the lens refractive index, and α is the half-aperture angle of the objective. The value of α can be found via:

$$\alpha = \sin^{-1}(NA / n)$$

Where NA is the numeric aperture of the microscope's objective. This value can be found in the microscope software or printed on the objective itself.

For example, consider a sample with the following parameters:

$$\begin{aligned}\lambda_{ex} &= 488 \text{ nm} \\ n &= 1.515 \\ \text{NA} &= 1.3\end{aligned}$$

Using the equations above:

$$\begin{aligned}\alpha &= \sin^{-1}_{\circ}(1.3 / 1.515) \\ &= 59.1\end{aligned}$$

And:

$$\begin{aligned}\Delta_{x,y} &= 488 \text{ nm} / [8 \times 1.515 \times \sin(59.1)] \\ &= 488 \text{ nm} / 10.4 \\ &= 46.9 \text{ nm}\end{aligned}$$

Therefore, the lateral sampling distance (the width of each pixel) must be 47 nm. Any higher values will result in an under sampled image and digital deconvolution will not be possible.

2. Calculate the axial sampling distance using the following formula:

$$\Delta_z = \frac{\lambda_{ex}}{4n[1 - \cos(\alpha)]}$$

For example, consider the same parameters used in step 1:

$$\begin{aligned}\Delta_z &= 488 \text{ nm} / (4 \times 1.515)[1 - \cos(59.1^{\circ})] \\ &= 165.5 \text{ nm}\end{aligned}$$

Therefore, each single image or z-stack slice must be 165 nm thick. Any higher values will result in an under-sampled image and digital deconvolution will not be possible.

3. In the confocal microscope software, adjust the digital zoom and image resolution until the lateral sampling distance is below your calculated value.

Increasing the zoom reduces the required resolution and vice versa. However, a very high zoom may cut important regions from your field of view and high resolutions have long scan times, increasing probe photobleaching. Therefore, a balance must be struck between these two parameters to produce an amply sampled micrograph of sufficient size.

4. Set up your z-stack so each optical slice is below your calculated axial sampling distance.

5. Rotate an oil immersion objective into the active position. Dispense a small volume of immersion oil such that only the lens opening is covered.

Dispensing directly onto the lens opening may trap an air bubble under the oil and the objective will not perform correctly. To avoid this, dispense just to the side of the opening and drag the oil across the opening to the opposite side.
6. Load negative control slide (see Critical Parameters) onto the stage with the coverslip oriented toward the objective. Slowly raise objective until the oil contacts the lens.

Immersion oil has the same refractive index as glass and CFM-3 mounting medium, further reducing index mismatch induced by dry objectives. CFM-3 is also approved as an immersion oil; however, consult your microscopy manager before use.
7. Activate counterstain laser and filter channel. Slowly increase the signal gain until minimal signal is visible. Bring image into focus. Reduce gain if focused image is too bright. Record signal gain value. Deactivate counterstain laser.
8. Activate each probe's specific laser and/or filter one at a time. Slowly increase the signal gain until signal is just beneath visibility. Record signal gain value. Deactivate probe laser.

This establishes the baseline excitation for the experiment; all fluorescence above this value is a reliable, true signal.
9. Repeat steps 5 and 6 with experiment slide. Use recorded signal gain values for each laser used.
10. Save all images as microscope's native file format.

Images are now ready for application of deconvolution software (see Current Protocols article: Hartig, 2013 and Internet Resources).

REAGENTS AND SOLUTIONS

Amplification buffer

10 ml 20× SSC

400 µl 10% Tween 20

8 ml 50% dextran sulfate (see recipe)

400 µl 10 mg/ml salmon sperm DNA

Fill to 40 ml with mQH₂O. Store at 4°C for 12 months.

Internet Resources

<https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html>

A useful tool for evaluating the excitation and emission spectra of various fluorophores, their intensity relative to excitation wavelength, and where they overlap, inducing crosstalk.

<https://imagej.net/Deconvolution> An introduction to deconvolution using ImageJ.

BE70 RNA-specific fixative

Combine 50 ml 10× PBS (pH 7.4), 12.5 ml 80% glycerol, and 5 ml glacial acetic acid in an RNase-free 1-L glass storage bottle. Adjust pH to 4.3 with NaOH. Adjust volume to 300 ml with mQH₂O. Add 700 ml 100% EtOH. Store up to 6 months at room temperature.

Dextran sulfate, 50%

20 g dextran sulfate powder

Fill to 40 ml with mQH₂O. Store at 4°C for 12 months.

Probe hybridization buffer

20 ml formamide

10 ml 20× SSC

360 µl 1 M citric acid, pH 6.0

400 µl 10% Tween 20

200 µl 10 mg/ml heparin

600 µl 50× Denhardt's solution

8 ml 50% dextran sulfate (see recipe)

Store at 4°C for 12 months.

Probe wash buffer

20 ml formamide

10 ml 20× SSC

360 µl 1 M citric acid, pH 6.0

400 µl 10% Tween 20

200 µl 10 mg/ml heparin

Fill to 40 ml with mQH₂O. Store at −20°C for 12 months.

Saline-sodium citrate Tween 20 (SSCT), 5×

10 ml 20× SSC

400 µl 10% Tween 20

Fill to 40 ml with mQH₂O. Store at 25°C for 12 months.

Triethanolamine (TEA) buffer, 0.1 M

Combine 3.33 ml 7.5 M TEA (MilliporeSigma, cat. no. 102-71-6) with 246.67 ml mQH₂O in a 250-ml storage bottle. Vortex until combined. Titrate solution to pH 8.0 with HCl. Keep sealed with lid until finished dewaxing and rehydrating specimens.

Store at 25 °C for 1 month.

COMMENTARY**Background Information**

RNA fluorescence in situ hybridization (RNA-FISH) has been a useful tool for analyzing gene expression and localization for decades. In that time, much progress has been made in optimizing the emission strength, binding efficiency, and signal-to-noise of RNA probes. However, little effort has been made to optimize the fixation and processing of tissue samples prior to RNA-FISH. As a result, many contemporary researchers are left struggling to improve RNA detection without realizing their sample was compromised from the beginning.

This lack of understanding extends to nearly every RNA analysis technique that relies on FFPE samples and has proven to be a significant challenge in both research and diagnostic medicine. Many alternative alcohol- or acetone-based fixatives with stabilizing agents have been proposed over the last decade to avoid the negative impact of formalin. However, none of these alternatives have been widely adopted for a variety of reasons, including economic issues, safety considerations, tissue specificity, and compromised histomorphology. While these fixatives excel at preserving nucleic acids, they do not adequately preserve cell morphology, leading to tissue shrinkage and hardening. Thus, these fixatives are sufficient for basic RNA work but lack the native histological context for accurate RNA quantification and localization.

Until relatively recently, the trade off between histomorphology and biomolecule retention was long considered a seemingly insurmountable obstacle. In 2016, BE70, a coagulative fixative composed of phosphate- buffered 70% ethanol, glycerol, and glacial acetic acid was developed as a potential solution to this apparent dichotomy (Perry et al., 2016). Unlike formalin, which induces protein cross-linking and the nicking of nuclei acids, BE70 fixation improved RNA recovery from tissues as determined by the extraction of longer RNA strands in comparison to FFPE samples, yet retained excellent morphology for basic microscopy. The application of PCR to extracted nucleic acids also resulted in the production of larger amplicons that were comparable in Ct value to those amplified from matched, flash frozen specimens. Furthermore, immunohistochemistry of BE70-fixed tissue proved superior to FFPE due to the lack of cross-linking of antigens, which remained open for antibody binding (Chung et al., 2018).

Taken together, we determined that these results could be extrapolated to an RNA-FISH protocol, where RNA integrity and cell morphology are critical for success. With the deleterious nature of formalin sufficiently mitigated, we demonstrate that it is now possible to further improve confocal resolution at higher magnifications than previously considered.

By eliminating the diffraction and background noise caused by formalin fixation, we also show that RNA transcripts can be visualized at the single-molecule level. As modern clinical pathology laboratories are often required to perform a wide range of tests on samples taken from the same biopsy, usually without the benefit of duplicates, a single fixative that can simultaneously preserve tissue morphology and RNA integrity for downstream analyses can finally be achieved.

Critical Parameters

RNA integrity—Unlike DNA and proteins, mRNA is an inherently unstable macromolecule. This allows the cell to rapidly break down mRNA into reusable nucleotides once the mRNA is no longer needed for translation. However, the transient nature of mRNA presents several unique technical challenges to researchers and specific efforts must be taken to mitigate its degradation. It is therefore important to understand the processes that disrupt this fragile molecule.

In general, mRNA is primarily sensitive to three types of damage: Enzymatic, chemical, and thermal. RNases, a family of enzymes responsible for breaking down transcribed RNA, are abundant in both the tissue sample (endogenous) and the environment outside it (exogenous). It is therefore required that this endogenous enzymatic activity is disrupted by fixing the tissue as fast as possible to prevent any exogenous contamination while manipulating the sample (Green & Sambrook, 2019).

As discussed above, mRNA is highly incompatible with formalin, the most common chemical used for tissue fixation. Northern blot analysis of mRNA extracted from formalin-fixed tissue demonstrates that extensive RNA fragmentation increases significantly with increased fixation and storage times (Cronin et al., 2004). Further, formalin fixes tissue by forming covalent crosslinks with most macromolecules, reducing RNA yield (Evers, Fowler, Cunningham, Mason, & O'Leary, 2011). Formalin also permanently modifies the imino groups of nucleic acids, or those that have both an amide and carboxyl group, preventing the base pairing needed for hybridization techniques (Hoffman, Frey, Smith, & Auble, 2015).

Formalin crosslinks are often removed from FFPE samples for immunohistochemical staining by boiling the sample in antigen retrieval buffer. However, this temperature is far higher than the denaturation point of mRNA, causing further degradation. In fact, the temperature of molten paraffin wax typically used to infiltrate samples also exceeds mRNA's denaturation point.

RNA-FISH best practices—There are many RNA-FISH labeling systems that exist, with varying stringencies and proprietary buffers and probes, yet all are based on the same basic chemistry. While your labeling system is bound to have its own experimental challenges, follow these best practices to maximize hybridization efficiency.

1. Use a PAP pen to draw a hydrophobic ring around your sample. This will keep solutions centered on the sample, reducing necessary solution volume, and will prevent the sample from drying out during long incubation times.

2. Only work with your slides in a laminar flow hood (AirClean Systems, cat. no. AC624LFUV). RNA is susceptible to degradation once the sample is dewaxed. The hood allows you to handle the slides without the risk of introducing RNases or microbes. A biosafety cabinet may work as a substitute but remember that unrelated experiments may have introduced unknown contaminants (see step 2 of Basic Protocol 1).
3. Perform RNA-FISH steps with a Thermobrite slide hybridization system (Leica Microsystems, cat. no. 3800004852001) stored in the laminar flow hood. Most FISH protocols recommend using a repurposed, humidified pipet tip box in an incubator for hybridization steps. However, we found that the temperature in the tip box varies significantly with water volume and has a consistently lower temperature than the incubator around it, which limits probe binding efficiency. If you are unable to obtain a Thermobrite, you must experimentally determine the incubator temperature required to bring the tip box to the correct hybridization temperature. Always use the same volume of water in the tip box; 5 ml is usually sufficient.
4. Always include a negative control slide with your FISH experiments. Include slide with all steps, using probe-free hybridization buffer during the primary incubation. Using this slide to set the confocal microscope's baseline parameters greatly improves signal acquisition from other slides.
5. HCR probes are designed and synthesized by Molecular Instruments according to FASTA sequences provided by the researcher. Dozens of probe sequences are theoretically compatible with the gene target and offer a wide range of binding affinity dictated primarily by the percentage of GC content. In our experience, a subset of the most promising candidate probes will be presented to the researcher for selection. Whenever possible, obtain multiple probes for the same gene target to improve signal detection, especially of lowly expressed RNA transcripts.
6. Unless custom made, fluorescent DNA HPs are available in a subset of Alexa fluorophore wavelengths as mentioned above. Before ordering, make sure your confocal microscope is equipped with the appropriate lasers necessary to excite your chosen fluorophore. When planning to label multiple targets within a single sample, ensure the fluorophores' emission spectra do not overlap. Each target should only be excited by a single laser; fluorophores with similar wavelengths will produce signal in multiple filters, leading to confounding results known as crosstalk. Always consult a tool such as Thermo Fisher Scientific's SpectraViewer to confirm that only one fluorophore should be excited by a given laser.
7. When targeting lowly expressed or putative genes, consider including a probe set for a gene known to be highly expressed in the tissue type. This can be used to determine whether there was successful hybridization and assist in deciding if one should continue searching for positive signal.

Troubleshooting

Pieces of tissue break off during sectioning—Sample was under-fixed. Extend fixation time for next sample.

Section ribbons compress or crumple

1. Handwheel rotation is too slow. Maintain constant speed as the blade cuts through the block.
2. Dull blade; replace with new blade.

Sections fall off slides after dewaxing

1. Non-charged slides were used.
2. Sections were placed on the backside of the slide.

Acetic anhydride does not dissolve in TEA buffer—Increase stir bar rpm.

Signal intensity fades quickly when laser is on

1. Fluorescent molecules are incompatible with CFM-3.
2. Laser is focused on too small an area of the section.

Only some regions of the section are blurry—Not all residual water was removed during mounting.

No fluorescence is detected in the sample

1. Increase concentration of the probe, HPs, or both.
2. Probe for a known highly expressed gene to confirm optimal hybridization conditions.

Understanding Results

Only positively labeled structures should be visible at their expected wavelength. Any other signal detected is non-specifically bound probe, autofluorescence, or diffraction-induced noise (see Fig. 2).

Deconvolved micrographs should eliminate all noise and blurring from the original image. Labeled mRNA transcripts should resolve to a single pixel and intracellular space should be completely dark (see Fig. 3).

Even at very high magnifications, diffraction-limited oversampling should still produce deconvolvable images of subcellular structures. Colocalization of differentially labeled mRNA isoforms within the same cellular region should still be resolvable (see Fig. 4).

Time Considerations

Support Protocols 1 and 2 can be performed the afternoon before your first FISH experiment. Samples can be left in BE70 for up to 6 months without loss of RNA quality.

This allows the scheduling of more convenient tissue processing times. Sample acetylation will add ~30 min to your RNA-FISH protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Literature Cited

- Choi HM, Beck JS, & Pierce NA (2014). Next-generation in situ hybridization chain reaction: Higher gain, lower cost, greater durability. *ACS Nano*, 27, 4284–4294. doi: 10.1021/nn405717p.
- Choi HM, Chang JY, le Trinh A, Padilla JE, Fraser SE, & Pierce NA (2010). Programmable in situ amplification for multiplexed imaging of mRNA expression. *Nature Biotechnology*, 28, 1208–1212. doi: 10.1038/nbt.1692.
- Choi HMT, Schwarzkopf M, Fornace ME, Acharya A, Artavanis G, Stegmaier J, ... Pierce NA (2018). Third-generation in situ hybridization chain reaction: Multiplexed, quantitative, sensitive, versatile, robust. *Development*, 145, dev165753. doi: 10.1242/dev.165753. [PubMed: 29945988]
- Chung JY, Song JS, Ylaya K, Sears JD, Choi L, Cho H, ... Hewitt SM (2018). Histomorphological and molecular assessments of the fixation times comparing formalin and ethanol-based fixatives. *Journal of Histochemistry and Cytochemistry*, 66, 121–135. doi: 10.1369/0022155417741467. [PubMed: 29125916]
- Cronin M, Pho M, Dutta D, Stephans JC, Shak S, Kiefer MC, ... Baker JB (2004). Measurement of gene expression in archival paraffin-embedded tissues. *The American Journal of Pathology*, 164, 35–42. doi: 10.1016/S0002-9440(10)63093-3. [PubMed: 14695316]
- Evers DL, Fowler CB, Cunningham BR, Mason JT, & O'Leary TJ (2011). The effect of formaldehyde fixation on RNA: Optimization of formaldehyde adduct removal. *The Journal of Molecular Diagnostics*, 13, 282–288. doi: 10.1016/j.jmoldx.2011.01.010. [PubMed: 21497290]
- Green MR, & Sambrook J (2019). How to win the battle with RNase. *Cold Spring Harbor Protocols*, 2019(2), pdb.top101857. doi: 10.1101/pdb.top101857.
- Hartig SM (2013). Basic image analysis and manipulation in ImageJ. *Current Protocols in Molecular Biology*, 102(1), 14–15. doi: 10.1002/0471142727.mb1415s102.
- Hoffman EA, Frey BL, Smith LM, & Auble DT (2015). Formaldehyde crosslinking: A tool for the study of chromatin complexes. *Journal of Biological Chemistry*, 290, 26404–26411. doi: 10.1074/jbc.R115.651679. [PubMed: 26354429]
- Perry C, Chung JY, Ylaya K, Choi CH, Simpson A, Matsumoto KT, ... Hewitt SM (2016). A buffered alcohol-based fixative for histomorphologic and molecular applications. *Journal of Histochemistry and Cytochemistry*, 64, 425–440. doi: 10.1369/0022155416649579. [PubMed: 27221702]

Figure 1. Comparison of FFPE and RNA-specific tissue fixation protocols.

A schematic that compares the drawbacks of FFPE fixation versus the benefits of RNA-specific tissue fixation, processing, and conditioning as well as the confocal microscopy modifications that are required to maximize fluorescent in situ hybridization imaging of single RNA molecules. FFPE, formalin-fixed, paraffin-embedded.

Figure 2. Micrographs of DNA counterstained samples produced from each fixation protocol. (A) Confocal image of rhesus macaque seminiferous tubules stained with Hoechst 34580 (blue) after formalin fixation. Note that significant formalin-induced AF and diffraction noise is present in the FFPE sample at nearly every visible wavelength. Individual cells, let alone subcellular structures, are not easily identified. (B) A similar confocal image taken following fixation with BE70. Formalin-related artifacts are absent and all cell types, each with a unique chromatin structure, are clearly recognizable. FFPE, formalin-fixed, paraffin-embedded; AF, autofluorescence.

Figure 3. RNA-FISH images comparing formalin versus RNA-specific fixation.

(A) Maximum-intensity projection (MIP) confocal image of rhesus macaque seminiferous tubules that underwent RNA-FISH of protamine 1 (PRM1; pink) and counterstained with Hoechst 34580 (blue). Chromatin resolution is only marginally improved by MIP. AF and noise is observed in intercellular spaces and the discrete location of fluorescently labeled mRNA transcripts cannot be adequately determined. (B) Another MIP confocal micrograph of a rhesus seminiferous tubule at the same magnification that was also deconvolved during confocal imaging. Note the single-molecule resolution of PRM1 mRNA transcripts shed by mature sperm cells (bright ovals) achieved by eliminating diffraction and background noise. RNA-FISH, RNA fluorescent in situ hybridization; AF, autofluorescence.

Figure 4. High magnification RNA-FISH micrographs from both fixation protocols. (A) Confocal image of a rhesus seminiferous tubule sub-section following PRM1 RNA-FISH (pink) and Hoechst 34580 counterstaining (blue) at higher magnification showing that diffraction-limited oversampling does not overcome formalin-induced artifacts. Colocalization of mRNA isoforms within individual cells is not resolvable. (B) A similar confocal micrograph following RNA-specific tissue fixation and processing reveals that high resolution is retained even at extreme magnification. PRM1 mRNA transcripts, adhered to mature sperm tails, sharply contrast against the DNA-rich sperm heads. RNA-FISH, RNA fluorescent in situ hybridization.

Table 1

Programmed Schedule and Contents for Vacuum Infiltration Tissue Processor

Station number	Treatment	Duration (min)	Temperature (°C)
1	70% EtOH	60	–
2	80% EtOH	60	–
3	95% EtOH	45	–
4	100% EtOH	60	–
5	100% EtOH	45	–
6	100% EtOH	60	–
7	100% xylenes	60	–
8	100% xylenes	60	–
9	100% xylenes	60	–
10	100% xylenes	60	–
11	100% Paraplast X-tra	60	55
12	100% Paraplast X-tra	60	55
13	100% Paraplast X-tra	60	55
14	100% Paraplast X-tra	60	55