

Chapter 11

RNA Fluorescence In Situ Hybridization in Cultured Mammalian Cells

Vidisha Tripathi, Jingyi Fei, Taekjip Ha,
and Kannanganattu V. Prasanth

Abstract

It is now clear that long noncoding RNAs (lncRNAs) regulate a number of aspects of nuclear organization and gene expression. An important tool for the study of the distribution and function of lncRNAs is RNA fluorescence in situ hybridization (RNA-FISH). The protocols presented in this chapter describe this method in detail and also mention a number of critical points that must be considered when performing this technique.

Key words Long noncoding RNA, lncRNA, In situ hybridization, RNA-FISH, Fluorescence

1 Introduction

In situ hybridization provides a unique link between cell biology and molecular genetics and is being extensively used by many laboratories for basic cell biology research to study structural and dynamic properties of cells and subcellular entities and also for diagnostic purposes. RNA-fluorescence in situ hybridization (RNA-FISH) is a technique utilized for the detection of RNA within cells allowing the visualization of transcripts that are localized either in the nucleus or in the cytoplasm. RNA-FISH has been used to monitor gene expression and analyze transcriptional activity of endogenous [1] and exogenous genes, for example, genes integrated to viral genomes [2, 3] and transgenes [4] at the individual cell level. This has been the standard method employed in studying different functional aspects of genome organization and nuclear architecture [5–14]. This technique has also been used to investigate allelic-specific expression of genes [15, 16] and gene expression profiling in single cells [17].

RNA-FISH involves the hybridization of fluorochrome- or enzyme-labeled nucleic acid probes to RNA target sequences in

cells, followed by microscopic visualization. The availability of advanced fluorescence microscopes, sensitivity of immunocytochemical detection systems, and development of various labeling procedures have been instrumental in the successful utilization of this technique at single cell level. With the advent of fluorescence-based detection methods, detection of different nucleic acid target sequences together with proteins or other cellular components simultaneously in the same cell is possible. Different cellular components can be marked using combinations of fluorochromes having different excitation and emission spectra. To provide information about the phenotypic analysis of the cells expressing the transcript of interest, an RNA-FISH procedure is often combined with immunofluorescent detection of proteins. This combined detection of RNAs and proteins is also used to study basic cell-biological processes viz. transcription, RNA processing and translation [18–20]. Several of the antibody sera may contain trace amounts of RNases that could degrade target RNA and will result in loss of RNA hybridization signal. Therefore, during the combined detection of an RNA and protein, the RNA-FISH is performed first followed by the immunocytochemical detection of the protein of interest. However, some of the antigens are sensitive to RNA-FISH procedures. In such instances, the immunofluorescence staining of protein should be performed first, followed by the RNA-FISH. In such instances, all of the solutions, including the antibody should contain RNase inhibitors (please see below for details).

Finally, single-molecule FISH (smFISH), first developed by Singer and coworkers [1] and then further improved by Raj and coworkers [21, 22] has become a very popular tool to study gene expression by direct visualization of individual RNA transcripts [23–27]. In this chapter, we will also describe the protocol for smFISH adapted from Raj and coworkers' method [21] in which large number (typically larger than 30) of fluorescently labeled short DNA oligonucleotides are hybridized to different regions of the target RNA, such that spatially separated RNA transcripts appear as individual diffraction-limited spots that are readily detectable using conventional wide-field fluorescence microscope.

Over the past decade, several lines of evidence have suggested that lncRNAs regulate various aspects of nuclear organization and gene expression. We have extensively used RNA-FISH and IF as tools to study the distribution and function of lncRNAs in mammalian cells [28].

RNA in situ hybridization involves a series of steps and each of them are critical to the final success of RNA detection. These steps can be categorized as:

Probe preparation.

Pretreatment of cells.

Hybridization of the probe.

Detection.

The protocols presented in this chapter describe these steps in detail and also mention the critical points to be taken care of.

2 Materials

2.1 Cell Culture on Coverslips

Culture cells for at least 24–48 h on acid-treated sterilized coverslips (18 × 18 mm). For smFISH, 2-well imaging chambers with #1 Borosilicate cover glass (Thermo Scientific, USA) are used for cell culturing and then directly for imaging.

2.2 Fixation and Permeabilization

1. Fresh 1× Phosphate buffered saline (PBS), pH 7.4: We use 10× PBS (diluted to 1× in nuclease-free water for cell culture).
2. Fixation solution: Freshly prepared and filter-sterilized 4 % paraformaldehyde (PFA) in 1× PBS.
3. Fresh permeabilization solution: PBS or CSK buffer containing, 0.2–0.5 % v/v Triton X-100 (Sigma). Add an RNase inhibitor, 2 mM Vanadyl Ribonucleoside Complex (VRC) (New England Biolabs) and 2 mM EGTA.

CSK buffer: Cytoskeletal buffer (CSK): 10 mM PIPES, pH 7.0, 100 mM NaCl, 300 mM Sucrose, 3 mM MgCl₂. Filter, sterilize, and store in aliquots at –20 °C.

2.3 Immunofluorescence (IF)

1. Fresh blocking solution: 1–5 % w/v Bovine serum albumin (BSA) or 1–5 % Normal Goat Serum (Invitrogen) in PBS.
Use an RNase inhibitor (0.4 U/mL RNAGuard, Amersham; GE Healthcare) or VRC, if IF will be performed prior to RNA-FISH.
2. Secondary antibodies: Conjugated fluorescent secondary antibodies (Molecular Probes; Invitrogen or Jackson Research labs).

2.4 FISH Probes

1. We label DNA probes by nick translation using nick translation kit (cat number: 32-801-300) and fluorescent nucleotides (SpectrumGreen- and SpectrumRed-dUTP) from Abbott Molecular Inc.
2. Deionized Formamide (Ambion Inc., USA): Once opened, store sterile formamide in aliquots at –20 °C.
3. Hybridization buffer: 50 % Formamide, 2× SSC prepared from a stock solution of 20× SSC, 10 % w/v dextran sulfate (Sigma-Aldrich), 1 mg/mL yeast t-RNA (Sigma-Aldrich, USA).

2.5 RNA-FISH

1. Fresh 2× SSC diluted from 20× SSC (Sigma-Aldrich) in nuclease-free water.
2. Deionized Formamide for washes: once opened, store sterile formamide in 50-mL aliquots at −20 °C.

2.6 DNA Counterstaining and Mounting

1. Fresh DNA staining solution: 4',6-diamidino-2-phenylindole dihydrochlorid (DAPI) (Sigma-Aldrich), 0.2 mg/mL in PBS or 4× SSC.
2. Mounting medium: (a) 0.1 % w/v p-phenylenediamine (Sigma), pH 9. Store in small aliquots at −20 °C. (b) Vectashield (Vector Laboratories, USA).

2.7 For smFISH

1. *smFISH wash solution*: 10 % formamide in 2× SSC. This should be stored at 4 °C.
2. *smFISH hybridization solution*: 10 % dextran sulfate, 1 mg/mL *E. coli* tRNA, 0.2 mg/mL BSA, 2 mM VRC and 10 % formamide in 2× SSC.

Note: mix the dextran sulfate in water with gentle agitation at RT until dissolved, and then add other components (make 500 µL aliquot store in −20 °C).

3. *100× Gloxy*: 16 U/µL glucose oxidase, 74 U/µL catalase dissolved in 5 mM Tris-HCl (pH_{RT}=8.0), 25 mM NaCl and 50 % glycerol and stored in −20 °C.
4. *smFISH imaging solution*: 0.4 % glucose, 10 mM Tris-HCl (pH_{RT}=8.0) in 2× SSC.
Right before imaging, add 100× Gloxy to smFISH imaging solution to a final concentration of 1×.

3 Methods**3.1 Preparation of Probes for RNA-FISH****3.1.1 Part I: Preparation of Nick-Translated cDNA Probes**

Several types of probes synthesized by various methods can be used for the detection of transcripts by RNA in situ hybridization: dsDNA probes, ssRNA probes, or oligonucleotide (DNA/RNA) probes. Commonly, dsDNA probes that are generated from a cDNA (or genomic) clones are the preferred choice for RNA-FISH because they can be prepared easily and they cover a large portion of the target sequence increasing their accessibility to bind to their complementary target sequence. The only disadvantage is that the larger size may lead to penetration problems into cells under certain fixation conditions. Single-stranded antisense RNA probes have an advantage over dsDNA probes because they form stronger hybrids with the RNA target than the DNA probes. Additionally, sense-strand probes can be used as perfect negative controls when using ssRNA probes. The drawback is that RNA probes are extremely unstable and sometimes give higher background signal, especially

when used in cultured mammalian cells. Oligonucleotide probes due to their small size and specific sequence have an advantage over cDNA probes. However, they have a limited target size leading to lesser signal generation. Nick translation of cDNA probes using fluorescent nucleotides can be easily performed using the nick translation kits sold by several companies, including Abbott Molecular Inc. and Roche. The median probe size should be between 100 and 500 bp.

3.1.2 Part II: Preparation of smFISH Probe Set

Design of smFISH Probe Set

Chemically synthesized smFISH probes are ordered from Biosearch Technologies (<http://www.biosearchtech.com>), from which smFISH probe set can be selected using Stellaris Probe Designer by simply inputting the sense-strand target RNA sequence. Probes are typically 20 nucleotides in length, with GC content around 45 %. Either 5' or 3' end of the oligonucleotide probe is conjugated with primary amine for labeling with the fluorophores. The minimum separation between two adjacent probes should be two bases. 10 nmol of each probe is synthesized and dissolved individually in water to a final concentration of 100 μ M in a 96-well plate.

Fluorescent Labeling of smFISH Probes

1. 5–10 μ L of each probe is pulled together, to which certain volume of 1 M sodium bicarbonate (pH 8.5) is added such that the final concentration of sodium bicarbonate is 0.1 M.
2. CyDye mono-reactive NHS ester (GE healthcare) or Alexa Fluor Succinimidyl Ester (Life Technologies) is used for labeling the primary amine conjugated oligonucleotide probes. Dissolve 0.1–0.3 mg of dye in 2–5 μ L of DMSO. The amount of dye is used such that in the final reaction, the dye is about 20–25 in excess to probes in molar concentration.
3. Mix the dissolved dye and the probe solution. Wrap the tube with aluminum foil, and incubate at room temperature for overnight. Gentle vortexing is recommended.
4. Add 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ethanol to the reaction, and incubate at -20°C for at least 2 h (up to overnight).
5. Spin the tube in microcentrifuge at 4°C at maximum speed for 20–30 min. A colored pellet containing both labeled and unlabeled probes will be precipitated.
6. To better purify the probes from unincorporated dyes, ethanol precipitation (**steps 4 and 5**) can be repeated once.
7. P-6 column (Bio-Rad) is used to further clean up the unincorporated dye. Typically, 2–3 such column purification steps are needed until no detectable color appears in the resin of the column.
8. The concentrations of the oligonucleotides and conjugated dye in the flow-through are measured by absorption at 260 nm

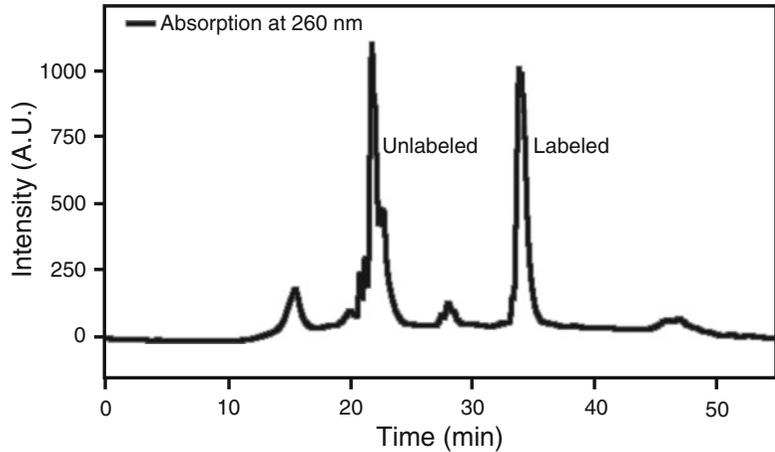


Fig. 1 HPLC purification of fluorescently labeled smFISH probes. The gradient for purification of labeled smFISH probes from unlabeled is described in text. Absorption at 260 nm is used to monitor the elution of each fraction. The absorption of the used fluorophore is typically monitored at the same time to verify the fraction corresponding to the labeled component (chromatograph not shown)

(for nucleic acid) and at the corresponding absorption wavelength of the specific dye to determine the labeling efficiency of the probes. Generally >80 % labeling efficiency can be achieved, which is normally sufficient for smFISH detection. If the labeling efficiency is too low, high-performance liquid chromatography (HPLC) can be used to purify the labeled probes.

9. In HPLC purification, labeled probes from **step 8** are directly diluted into 1–2 mL of 50 mM triethylammonium acetate (TEAA, pH 7.5) and injected onto Clarity 3 μm Oligo-RP C18 column (Phenomenex), and then eluted using methanol. Unlabeled and labeled probes can typically be eluted in the range from 80 % TEAA solution/20 % methanol to 50 % TEAA solution/50 % methanol over 30 min at a flow rate of 0.5 mL/min (Fig. 1). Fractions of labeled probes are then pooled together and dried in lyophilizer or speed vacuum concentrator.

3.2 RNA-FISH (Fig. 2)

3.2.1 RNA-FISH to RNA Using Nick-Translated Probe (Fig. 2a)

The following protocol describes the hybridization of fixed cells to nick-translated cDNA probes for the localization of individual RNAs or population of RNAs. Conditions for detection of cytoplasmic versus nuclear RNAs may differ slightly, and here we focus only on the detection of nuclear transcripts.

1. Briefly rinse cells grown on coverslips in freshly prepared, RNase-free PBS.
2. Fix cells in freshly made 4 % PFA (FA) in PBS, pH 7.4, for 15 min at room temperature (RT).

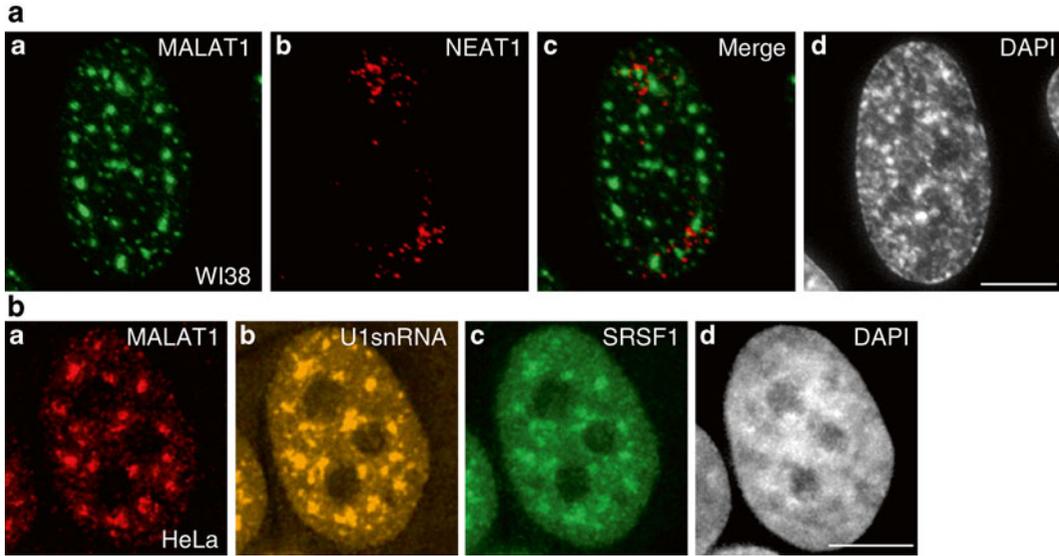


Fig. 2 (a) Simultaneous visualization of MALAT1 (a: green) and NEAT1 (b: red) long noncoding RNAs in human cells by RNA-FISH. Note that MALAT1 (a) and NEAT1 (b) localize to nuclear speckles and paraspeckles respectively. (b) Co-RNA-FISH using probes against MALAT1 (a: red) and U1 snRNA (b: yellow) and immunofluorescence staining of SRSF1 (c: green) shows the intranuclear distribution of these RNAs and protein in human interphase nucleus. The DNA is counterstained with DAPI. The scale bar represents 5 μ m

3. Rinse the cells in PBS thrice for 10 min each. Permeabilize in freshly made 0.2–0.5 % v/v Triton X-100 in PBS containing 2 mM VRC on ice for 5–10 min.

Note: Cells can also be permeabilized in CSK buffer, 0.2–0.5 % v/v Triton X-100, especially when the cells need to be permeabilized prior to fixation. In this case, briefly rinse the cells in CSK buffer, incubate the cells in CSK buffer with Triton X-100 for 2–5 min on ice and fix the cells immediately in freshly made PFA. CSK buffer is better suited for optimal nuclear RNA detection especially for IF combined with FISH, where the fixation step affects the quality of the detection of transcripts and increases the background.

4. Rinse cells in PBS thrice for 10 min each and then in 2 \times SSC once for 10 min.
5. Prepare hybridization solution by mixing 50 % Formamide, 2 \times SSC, dextran sulfate, yeast t-RNA and nick-translated probe. Incubate the mix at 37 $^{\circ}$ C for 10 min (*see Note 3*).
6. Denature the probe by heating at 90 $^{\circ}$ C for 5–10 min. Chill the probe immediately on ice.
7. Place 20 μ L of the hybridization mixture with probe onto a slide as a single drop. Remove the coverslips with cells from the 2 \times SSC buffer (gently remove the excess buffer from the

coverslip by touching one edge of the coverslip onto a 3 mm Whatman sheet. Note: DO NOT dry the coverslip at this stage) and invert them onto the drop of hybridization mixture with cells facing toward the hybridization mix. Seal with rubber cement, and incubate in a dark humid chamber (made using Whatman sheets soaked in 50 % formamide in 2× SSC) at 37 °C for 12–16 h (*see* **Note 1**).

8. Peel off the rubber cement carefully. Remove the coverslips carefully with forceps and wash them three times in freshly made 50 % formamide, 2× SSC (adjusted to pH 7.2) for 5 min each at 37 or 42 °C (*see* **Note 2**).
9. Wash three times in 2× SSC, pH 7.2 for 5 min each at 37 or 42 °C.
10. Wash three times in 1× SSC, pH 7.2 for 5 min each at 37 or 42 °C.
11. Wash twice in 4× SSC for 10 min at RT.
12. Counterstain DNA with DAPI prepared in 4× SSC.
13. Wash coverslips in 4× SSC for 5–10 min.
14. Mount the coverslips on a slide and fix in place with a minimal amount of nail polish.

3.2.2 RNA-FISH to Poly (A⁺) RNA Using an Oligonucleotide Probes

The procedure is similar to the standard RNA-FISH protocol (described above) except the hybridization conditions.

1. The cells are processed following **steps 1–4** from Subheading **3.2.1**.
2. Cells are hybridized using a hybridization buffer (20 μL) containing 5 μL of formamide, 2 μL of 20× SSC, 2 μL yeast t-RNA, 4 μL of 50 % dextran sulfate along with 1 μL of oligo dT probe (100 ng/μL) and incubated at 42 °C for 12–16 h.
3. Follow **steps 8–13** from Subheading **3.2.1**.

3.2.3 smRNA-FISH

The protocol described in Subheading **3.2.1** for the hybridization of fixed cells with nick-translated DNA probes can in general be applied to sample preparation for smFISH. The major difference is that in smFISH hybridization solution, 10 % formamide rather than 50 % is used. Detailed description is as below.

1. Aspirate the growth media from the imaging chamber. Wash the cells once with 1× PBS. Fix the cells in fixation solution for 10 min at RT. Wash the cells as described in Subheading **3.2.1**.
2. Cells can be permeabilized with Triton X-100 solution, by incubating the cells on ice for 10 min as in Subheading **3.2.1**. Alternatively, 70 % ethanol is also very frequently used for permeabilization in smFISH. If 70 % ethanol is used, the cells are incubated at 4 °C for at least overnight. After permeabilization, wash the cells three times with 1× PBS at room temperature.

3. smFISH hybridization.

- (a) Wash cells with $2\times$ SSC one or two times, with each time incubating for 5–10 min at room temperature.
- (b) Add in smFISH wash solution, and incubate the cells at room temperature for 5–10 min.
- (c) Clean some 18×18 cm coverslips by washing with ethanol, drying with nitrogen and then briefly burning with flame.
- (d) Dilute the fluorescent probes into 100 μ l smFISH hybridization solution to a final concentration of 1–10 nM of each probe (*see Note 6*). Make sure the added volume of probes is smaller than 3 μ l. Mix the final solution by gentle vortexing.
- (e) Aspirate the FISH wash solution. Drop the mixed solution to the center of the imaging chamber, and cover the chamber with the cleaned coverslips. Incubate the hybridization reaction at 37 °C overnight.
- (f) In the morning, add 1 ml of FISH wash solution to the imaging chamber and take out the coverslip. Incubate the cells at 37 °C for 30 min. This wash step is performed twice.
- (g) Wash the cells with $2\times$ SSC and incubate at room temperature for 5–10 min. Sample is then saved in fresh $2\times$ SSC (or $4\times$ SSC) at 4 °C before imaging.

Imaging and Data Analysis

Cells are washed with FISH imaging solution (without gloxy) first by incubating at room temperature for 1–2 min. Fresh FISH imaging solution with gloxy is then exchanged into imaging chamber for imaging (*see Note 7*). Images can be captured using a wide-field fluorescence microscope. In our lab, we use Zeiss Axiovert 200 M fluorescent microscope. Images are captured using a $100\times/1.46$ oil immersion objective and CCD camera. Thirty z-stacks are usually taken with step size of 0.3 μ m.

Compared to conventional FISH, in smFISH, mRNA transcripts appear as spatially separated individual spots. Therefore, by directly counting the number of these spots, the absolute copy number of certain transcripts can be determined. For this purpose, computer-assisted identification and counting of these spots in a three-dimensional image is required. Relatively detailed description on computational identification of mRNA spots has been described in [21], and therefore will not be detailed in this section.

However, this counting method does not apply to certain cases, for example, noncoding RNAs (ncRNAs) localized to specific subnuclear domains, as they are often packed in relatively high density such that individual RNAs are not resolvable by smFISH. To solve this problem, signal from smFISH on spatially well-separated mRNA can usually be used as a standard for calibration [24]. Here, we describe an example of such case, using smFISH signal to

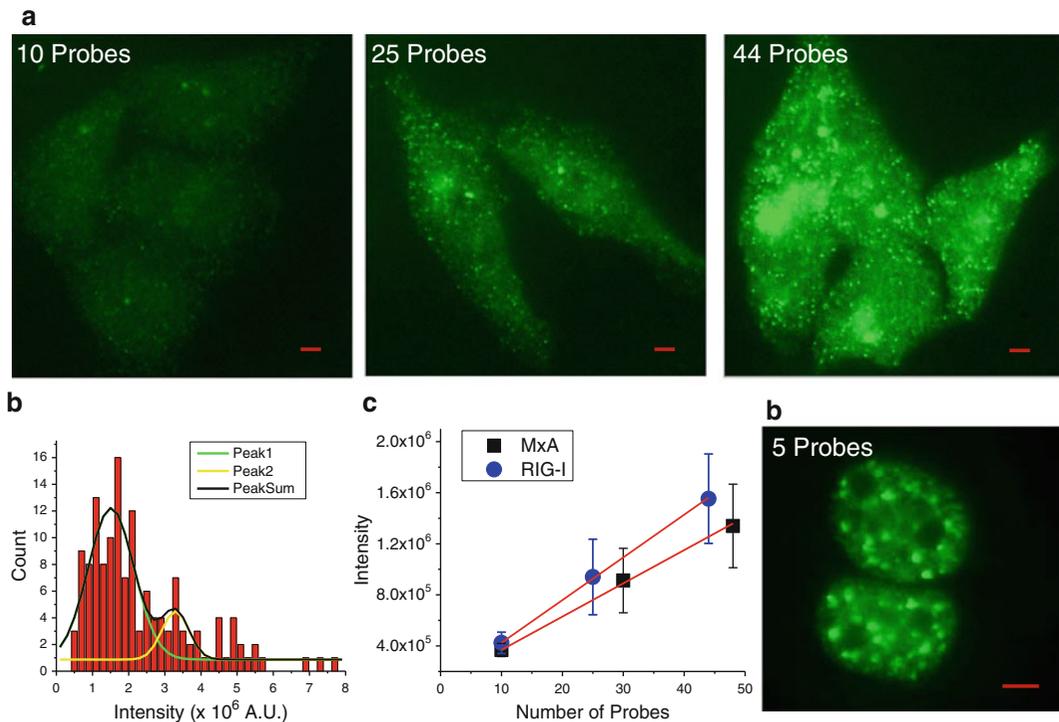


Fig. 3 Estimation of copy number of ncRNA localized in nuclear speckles based on smFISH. **(a)** Various numbers of probes are used to label certain RNA transcripts. Individual transcripts show as diffraction-limited spots with different brightness depending on the number of probes used (*scale bar*: 5 μm). **(b)** Intensities of spots are extracted in each case of **(c)**, plotted as population vs. intensity histogram, and fit with Gaussian distributions. **(c)** Intensity corresponding to single RNA as a function of number of probes is treated with linear equation. **(d)** Intensity from individual nuclear speckle can be extracted and by comparing with the linear equation from **(c)**, copy number of ncRNA can be estimated

estimate the copy number of MALAT1 ncRNA localized within nuclear speckles (Fig. 3). smFISH is first performed on regular mRNA transcripts of certain gene using various number of labeled FISH probes under the same imaging condition (Fig. 3a). Using commercial software, intensity from single spot is extracted under each condition. As at high expression level, transcription sites are often observed as large aggregations, oversized spots are discarded from further analysis. Population vs. Intensity histogram of individual mRNA spots is fit with Gaussian distributions, and the first peak is considered to be the average intensity coming from single RNAs using specific number of FISH probes (Fig. 3b). Relationship between intensity from single RNA and the number of probes can be empirically treated as linear function (Fig. 3c). Even though for different mRNAs, the absolute intensities are slightly different, due to the fact that different mRNA can fold into different secondary or tertiary structures that affect the hybridization efficiency, when extrapolating to the case of using very few probes, the difference

becomes very small. By comparing total intensity from each speckle to the calibration curved built on single RNA, we can estimate the copy number of RNA in each speckle (Fig. 3d).

3.3 Combination of Immunofluorescence and RNA-FISH (Fig. 2b)

Various methods involving a variety of fixation and permeabilization techniques can be used for IF applications, and the choice depends on cell type, epitope, and antibody being used. The following protocol is optimized for the detection of nuclear proteins in mammalian primary and cancerous cell lines. When FISH is combined with IF, we prefer to perform IF (under RNase-free conditions) prior to FISH, because the formamide treatment during the FISH procedure is sometimes incompatible with preservation of the epitopes detected by some antibodies.

1. For the preparation of the RNA-FISH probe, follow Subheading 3.1.
2. Briefly rinse cells cultured on coverslips in PBS.
3. Fix in freshly made 2–4 % PFA for 15 min at RT.
4. Wash three times in PBS for 5–10 min each.
5. Permeabilize with freshly made 0.5 % v/v Triton X-100 in PBS (add an RNase inhibitor, 2 mM VRC) on ice for 5–10 min. The exact time of permeabilization depends on the cell type.
6. Wash three times in PBS for 5 min each.
7. Block in PBS + 1 % BSA for 30 min with three changes at RT.
8. Incubate with primary antibody diluted in 1 % BSA (containing 0.4 U/mL RNAGuard) for 45 min at RT in a humid chamber. The temperature and length of incubation can vary between antibodies (*see Note 4*).
9. Wash at least three times in PBS + 1 % BSA for 10 min each.
10. Incubate with secondary antibody (diluted in the same solution as in **step 8**) for 40 min at RT in a dark and humid chamber (*see Note 5*).
11. Wash at least three times in PBS for 5 min each.
12. Postfix in freshly made 4 % PFA for 5 min at room temperature.
13. Wash twice in 2× SSC (freshly made from a sterile 20× stock) for 5 min.
14. For RNA-FISH, follow **steps 5–13** of Subheading 3.2.1.

4 Notes

1. A mock hybridization should be performed as a control with each experiment. Depending on the experiment following parameters could serve as appropriate control:

- (a) Mock hybridization reaction without labeled probe.
 - (b) Hybridization reaction with a nonspecific probe, i.e., vector DNA or sense oligonucleotide probe.
 - (c) Pretreatment of a sample with RNase A prior to hybridization.
2. High background, often caused by staining derived from non-specific interactions between labeled probes and cellular structures, decreases the signal-to-noise ratio. To improve that following points should be considered:
 - (a) Titration of the probe concentration to reach the maximal signal-to-noise ratio.
 - (b) The size of the probe should be optimal (e.g., 50–500 bp for nick-translated DNA probes).
 - (c) The probe should be purified away from nonincorporated fluorochrome-conjugated free nucleotides.
 - (d) Additional prehybridization step prior to hybridization limits the nonspecific binding of the probe.
 - (e) Increasing the concentration of blocking reagents can help blocking nonspecific interactions.
 - (f) Higher stringency achieved by decreasing the formamide concentration or increasing the hybridization or washing temperature or reducing the SSC concentration in washing buffers limits nonspecific interaction.
 - (g) Decreasing the hybridization time also helps in reduction of nonspecific stickiness.
 3. When nick translation is used for labeling, the size range of the labeled DNA must be checked by electrophoresis on a 1 % agarose gel. The most critical factor in the hybridization efficiency and background is the size of the probe. The probe must be small enough to enter the cell efficiently, and it must not be so big that it gives a high nonspecific signal by sticking to random sites. Fluorescently labeled probes of this kind can be stored at -20°C for months as small aliquots.
 4. The coverslips are placed cell-side down, avoiding the formation of air bubbles, onto a drop of antibody solution on a sterile glass slide. The volume depends on the size of coverslip used (we routinely use 18×18 -mm coverslips with 50 μL of antibody solution). Following incubation, the coverslips are carefully removed with forceps and put back into PBS for washing. If resistance is encountered when removing the coverslip, it should be flooded with PBS so that it floats.
 5. For combined IF and RNA-FISH, the choice of fluorochrome to which the secondary antibody is conjugated will depend on the fluorochrome with which the FISH probe is labeled, and on the filter sets available on the microscope. In the case of a

double IF experiment, high-affinity purified secondary antibodies should be used to minimize cross-species reactivity. Even then, appropriate controls (e.g., each primary with both secondary antibodies) should be performed systematically to confirm specificity.

6. The concentration of smFISH probes used in the experiments is usually between 1 and 10 nM. In the first set of experiments, testing a series of dilutions of probes is recommended to find a best condition for the experiments.
7. smFISH imaging solution is usually used for smFISH detection other than commercial mounting medium. We find commercial mounting media usually reduces the fluorescent intensity, and therefore causes the loss of single-molecule sensitivity.

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