Chapter 10

Stellaris® RNA Fluorescence In Situ Hybridization for the Simultaneous Detection of Immature and Mature Long Noncoding RNAs in Adherent Cells

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Abstract

RNA fluorescence in situ hybridization (FISH), long an indispensable tool for the detection and localization of RNA, is becoming an increasingly important complement to other gene expression analysis methods. Especially important for long noncoding RNAs (lncRNAs), RNA FISH adds the ability to distinguish between primary and mature lncRNA transcripts and thus to segregate the site of synthesis from the site of action.

We detail a streamlined RNA FISH protocol for the simultaneous imaging of multiple primary and mature mRNA and lncRNA gene products and RNA variants in fixed mammalian cells. The technique makes use of fluorescently pre-labeled, short DNA oligonucleotides (circa 20 nucleotides in length), pooled into sets of up to 48 individual probes. The overall binding of multiple oligonucleotides to the same RNA target results in fluorescent signals that reveal clusters of RNAs or single RNA molecules as punctate spots without the need for enzymatic signal amplification. Visualization of these punctate signals, through the use of wide-field fluorescence microscopy, enables the counting of single transcripts down to one copy per cell. Additionally, by using probe sets with spectrally distinct fluorophores, multiplex analysis of gene-specific RNAs, or RNA variants, can be achieved. The presented examples illustrate how this method can add temporospatial information between the transcription event and both the location and the endurance of the mature lncRNA. We also briefly discuss post-processing of images and spot counting to demonstrate the capabilities of this method for the statistical analysis of RNA molecules per cell. This information can be utilized to determine both overall gene expression levels and cell-to-cell gene expression variation.

Key words Exon, Intron, Fluorescence, In situ hybridization, FISH, qPCR, Single-molecule detection, lncRNA, mRNA, Gene expression, Transcription burst, Nucleus

1 Introduction

The transcriptome in any given cell at any given time is the result of combinatorial transcription of select genes followed by posttranscriptional processing and modification [1]. The discovery of new coding and noncoding RNA variants continues to add to our understanding of the sophistication of the transcriptome. Long noncoding RNAs (lncRNAs; >200 nucleotides [nts]) are primarily

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restricted to cellular nuclei where they play extensive roles in the central aspects of gene regulation, with wide-ranging effects on numerous cellular functions such as cell cycle progression, cellular differentiation, and metabolism [2–5]. High-throughput approaches to study gene expression, such as RNAseq and qPCR, have been instrumental for the discovery and independent measurements of expression levels of new RNAs [6]. However, such techniques require the extraction of RNA, with concomitant destruction of cellular integrity and loss of cell-specific information. To determine how the transcriptome correlates with the phenotype of individual cells and cell populations, gene expression analysis instead must be performed on intact cells within a population. Ideally, such analyses would facilitate the detection of multiple coding and noncoding RNAs alike, unraveling the intrinsic variation in gene expression and the gene expression networks to which they belong.

In situ hybridization (ISH) is one gene expression analysis method that has been employed to detect and determine the cellular distribution of both DNA and RNA in cells and tissue. ISH long remained a cumbersome process due to the need for long cDNA- or gDNA-derived probes, and signal amplification to detect cellular transcripts, whether it be through colorimetric, radioactive, or fluorometric imaging [7].

Major advances for RNA FISH made in the Singer and Tyagi groups, both using short synthetic and site-specifically labeled oligodeoxynucleotide probes (oligos), enabled the reliable detection of single molecules of RNA in fixed, cultured cells (*summarized in ref.* 8). Major advantages both with Singer's use of three to five multiply labeled 50-mers and with Tyagi's 48 singly labeled 20-mers that bind to the same mRNA target are that they can be designed to avoid repetitive RNA sequences and to hybridize with similar Tm, as well as that they are more efficient at penetrating the cell matrix to reach their target RNAs. Together this leads to a non-amplified, yet strong and specific fluorescent signal with a high signal-to-background ratio. Other advancements such as the generation of an online probe designer and automation in the manufacturing of these probe sets (oligo synthesis, pre-labeling with fluorophores, and purification) have greatly reduced the time and complexity of probe preparation [8, 9].

Thanks to the inherent rapid binding kinetics of these short fluorescently labeled oligos, hybridization protocols have also been much simplified and adapted for single-nucleotide variant detection (SNV FISH) [10], for more rapid detection through TurboFISH [11], and even for combination with immunofluorescence [8, 12]. We expand on aspects of one such application here, intron chromosomal expression (ice) FISH [13]. This method relies on that RNA processing mostly occurs co-transcriptionally in specialized transcription and processing factories [6, 7, 14–18],

such that active transcription can be revealed by using probe sets targeting intron sequences only. We anticipate the continual refinement of ISH methods and novel advances in techniques designed to distinguish highly similar RNAs.

Here, we provide a guide for successful RNA FISH assay design for the co-detection of both nascent transcripts and mature RNAs. The human genes for the primarily cytoplasmic MYC mRNA and H19 lncRNA, as well as the nuclear PVT1 and XIST lncRNAs, are used as examples. We present a streamlined RNA FISH protocol where two or more probe sets are employed to generate images that reveal transcription bursts and final localization of mature RNAs, and even single-molecule resolution for mature MYC, H19, and PVT1 RNAs in adherent cells.

These methods are suitable to determine the cell-to-cell distribution of both mRNAs and lncRNAs in fixed cell cultures and tissue sections and, as such, may be applied to interrogate the subcellular (and potentially subnuclear) location of the target RNAs. By comparing signals from exon and intron probe sets it can be determined if the site of action of lncRNAs is associated with or away from its site of transcription (and processing). Overlapping exon and intron signals additionally can provide cross-validation of each probe set. The cell-specific readout that transcription burst analysis provides is also suitable for measurements of dose- and time-dependent gene activation, allele-specific epigenetic control [19, 20], as well as gene-specific karyotyping of (engineered) cell lines. Lastly, we provide a brief overview of RNA spot detection and quantification through the means of image acquisition and post-analysis processes.

2 Equipment and Materials

2.1 Equipment

1. Wide-field fluorescence microscope (Nikon Eclipse Ti and NIS-Elements Ar Imaging Software, or equivalent), with a high numerical aperture (>1.3), 60–100× objective, and XYZ motorized stage for automated *z*-stacking capabilities.
2. Cooled CCD camera (at least −20 °C), ideally optimized for low-light-level imaging rather than for speed (13 μm pixel size or less is preferred) (*see* **Note 1**).
3. Strong light source, such as a mercury or metal-halide lamp over single-line light sources such as LEDs and lasers.
4. Filter sets appropriate for FAM, Quasar® 570, CAL Fluor® Red 610, Quasar 670 fluorophores (e.g., *those from Chroma for* FAM, catalog # 89000; Cy3™, catalog # SP102v1; Cy3.5™, catalog # SP103v2; Cy5.5™, catalog # 49022).
5. Cell culture hood and variable temperature CO_2 incubator.
6. Laboratory oven set at 37 °C.

2.2 Reagents and Cell Culture

All buffers and reagents are made with nuclease-free water.

1. A549 (human lung adenocarcinoma cell line, ATCC, catalog # CRM-CCL-185).
2. SK-BR-3 (human breast adenocarcinoma cell line, ATCC, catalog # HTB-30).
3. F-12K medium (Kaighn's Modification of Ham's F-12 Medium) supplemented with 10 % fetal bovine serum and penicillin-streptomycin A (A549 media).
4. McCoy's 5A medium supplemented with 10 % fetal bovine serum and penicillin-streptomycin (SK-BR-3 media).
5. 12-Well tissue culture plates.
6. Micro Cover Glasses, Round, No. 1 (VWR, catalog # 48380-046).

2.3 Formaldehyde Fixation and Cell Permeabilization

1. Phosphate-buffered saline (1× PBS).
2. Fixation buffer (4 % formaldehyde in 1× PBS) (*see* **Note 2**).
3. Nuclease-free water (not DEPC treated).
4. Ethanol, molecular biology grade, diluted to 70 % in nuclease-free water.

2.4 Alternative Methanol:Acetic Acid Fixation/ Permeabilization

1. Methanol:glacial acetic acid (MeOH:AcOH) fixative (3:1, v/v).

2.5 Hybridization

1. Hybridization buffer (Biosearch Technologies, Inc. catalog # SMF-HBD2-10). Deionized formamide (Life Technologies, catalog #AM9342). Formamide must be added to the hybridization buffer at a 10 % final concentration. The hybridization buffer can be aliquoted and stored at −20 °C (*see* **Note 2**).
2. Stellaris RNA FISH Probes (Biosearch Technologies, catalog numbers; H19 exons Quasar 570: VSMF-2162-5; MYC exons Quasar 570: VSMF-2230-5; PVT1 exons Quasar 670: VSMF-2307-5; XIST exons Quasar 570: VSMF-2430-5; all intron probes were custom designed, catalog numbers MYC FAM: SMF1025-5; H19, and PVT1 CAL Fluor Red 610: SMF-1082-5; and XIST Quasar 670: SMF-1065-5).
3. Tris–EDTA buffer solution (10 mM Tris–HCl, 1 mM disodium EDTA, pH 8.0).
4. Humidified chamber: 150 mm tissue culture plate, Parafilm®.

2.6 Washing

1. Wash buffer A (Biosearch Technologies, catalog number SMF-WAD1-60): Add formamide to a 10 % final concentration.

2. Wash buffer B (Biosearch Technologies, catalog number SMF-WBD1-20): Wash buffer B must be diluted to the appropriate concentration with water.
3. DAPI nuclear counterstain (5 ng/ml DAPI in wash buffer A).

2.7 Mounting

1. Vectashield® Mounting Medium (Vector Laboratories, catalog #H-1000).
   Alternative GLOX (GLucose OXidase) anti-fade [21].
   (a) GLOX buffer (0.4 % glucose in 2× SSC and 10 mM Tris–HCl, pH 8.0): 10 % glucose stock solution (powdered glucose [Sigma-Aldrich, catalog # 158968] dissolved in nuclease-free water), 1 M Tris–HCl, pH 8.0.
   (b) GLOX anti-fade: 100 µL GLOX buffer + 1 µL catalase from bovine liver (Sigma-Aldrich, catalog # C3155) + 1 µL glucose oxidase from *Aspergillus niger* (Sigma-Aldrich, catalog # G0543) diluted to 3.7 mg/mL in 50 mM sodium acetate, pH ~5.0.
2. Microscope slides.
3. Clear nail polish.

3 Methods

3.1 Design of Probes

The human RNA targets of focus are from the myelocytomatosis oncogene MYC, the neighboring plasmacytoma variant 1 oncogene PVT1, the oncofetal lncRNA gene H19, and XIST encoding the X inactive specific transcript (Fig. 1). These targets illustrate how alternative transcription initiation, splicing, and polyadenylation [22–24] contribute to the diversity of RNA variants. Note that the 3′ end of some lncRNAs is generated by RNase P cleavage, and that they lack a polyA tails [25].

An advantage of RNA FISH over immunofluorescence is that probe set specificity can be well controlled up front by using available genome and transcriptome information. Probe sets can be chosen to detect single, many, or all RNA variants from a certain gene, from several related genes, or even from several viral serotypes. Probe sets for variant detection are thus referred to as inclusive (many or all) or exclusive (single).

MYC, PVT1, and H19 belong to a triumvirate of aggressive oncogenes [26–30]. As a general effector of transcription and potentiator of cell proliferation and differentiation, MYC protein is stabilized by the PVT1 lncRNA, and as such, the encoding genes on 8q24 are frequently found co-amplified in tumors. In turn, MYC activates the transcription of both PVT1 and H19. Note also that both pre- and mature lncRNAs from PVT1 and H19 also harbor miRNAs. The XIST lncRNA is abundantly expressed in female cells and intimately tied to dosage compensation through inactivation of one of the X-chromosomes [31, 32].

1. Target sequence selection: In this initial pre-design process, RNA variants from each encoding gene are evaluated for differential transcription start site use, splicing, and polyadenylation. By using the sequence common to all variants as the target, the same number of individual oligos in the probe set can be predicted to bind each RNA variant. Such "inclusive" probe sets therefore reveal fluorescent RNA spots with maximal uniform spot intensity, independently of subcellular localization and half-lives, and in turn enable accurate spot counting. "Exclusive" probe sets can be generated by targeting discrete variant-specific exon sequences. When combined, differently labeled inclusive and exclusive probe sets can yield otherwise difficult-to-extract data on the actual number of different splice variants in each cell and their cell-to-cell distribution.

   The genes of interest (Fig. 1) are initially inspected at NCBI's website (www.ncbi.nlm.nih.gov/gene) (*see* **Note 3**). In the case of MYC (gene ID: 4609; 8q24.1), two RefSeq mRNAs are presented with three exons, whereas several shorter (incomplete) mRNAs are found for the same gene at Ensembl (ENSG00000136997). Two transcription start sites can be discerned, but only one true polyadenylation signal and site. Hence, the common CDS (opened by a noncanonical CTG) of the full-length mRNA is chosen for design (nts 526-1890 of

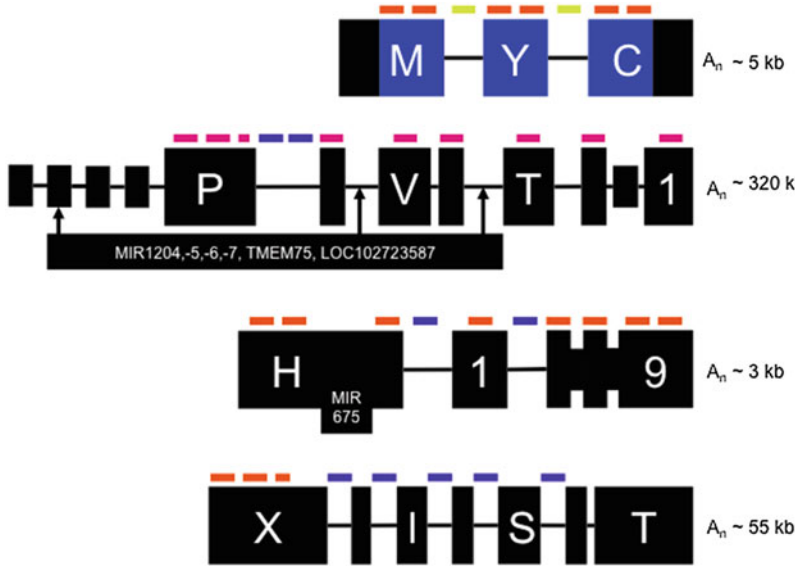

Fig. 1 A schematic of the design of inclusive probe sets to detect primary and mature and MYC mRNAs, as well as primary and mature H19, PVT1, and XIST lncRNAs. Noncoding sequences are represented in *black* and coding sequences in *blue*. Exons common to all variants are shown as *thick lines*, alternatively spliced exons as *medium thick lines*, and introns as *thin lines*. All four exon probe sets are inclusive and target the common regions of the mature RNA variants. The probe sets are color coded according to the dye label: MYC, H19, and XIST exons with Quasar 570: *orange*; PVT1 exons with CAL Fluor Red 610: *purple*; MYC introns with FAM: *green*; and H19, PVT1, and XIST introns with Quasar 670: *dark blue*

NM_002467.4). (XM_005250922.1 excludes a single CAG codon at the 5′ end of exon 2.) To further ensure specificity of this probe set to MYC mRNAs, the common RefSeq sequence is used to BLAST the human genome/transcriptome at NCBI (www.ncbi.nlm.nih.gov/blast/). This exercise reveals segments of NM_000389.4 with homology to the mRNAs from the MYCN and MYCL1 genes. In addition to information about potential cross-hybridization, the presence of potentially transcribed pseudogenes may also be uncovered by the BLAST process. The final target sequence for the generation of an inclusive exon probe set that will detect both NM_000389.4 and XM_005250922.1 mRNAs was maintained. Both introns (NG_007161.1, nts 5555-7178, 7951-9326) were chosen for the intron probe set.

The PVT1 locus spans 350 kb (gene ID: 5280; 8q24.1), and harbors in addition to the PVT1 lncRNA RefSeq another lncRNA and four miRNAs within the introns of the PVT1 gene. At Ensembl several PVT1 variants are presented at varying support levels. In the absence of a published comprehensive overview of PVT1 lncRNA variants and their expression, the RefSeq lncRNA (NR_003367.2, nts 1-1221, 1426-1699), excluding one frequently skipped exon, was chosen as a target for the exon probe set. At the same time, ≥15 unique exons leav19.7 pte room for the design of exclusive probe sets. The first 8 kb of the intron common to the major variants was chosen for the intron probe set (NC_000008.11 nts 127,890,999-127,898,944). (8 kb is the maximal target sequence length accepted by the probe designer.)

For H19 (gene ID: 283120; 11p15.5), one RefSeq lncRNA and two miRNAs are presented over five major exons. Alternatively spliced transcripts at varying support levels are shown at Ensembl. The gene and exon structure is well conserved in mouse, supporting the choice of the full-length lncRNA (NR_002196.1 nts 1-2322), excluding the miRNA sequences, as a target. For design of the intron probe set, 10 nts from each flanking exon were added to the target introns (NG_016165.1, nts 6307-6426, 6538-6656, 6746-6849, 6949-7053).

For XIST (gene ID: 7503; Xq13.2), one six-exon RefSeq lncRNA is presented. Alternative transcripts at varying support levels are shown at Ensembl. The full-length RNA is the major variant [33], and so the first 8 kb of mature lncRNA NR_001564.2 and up to 2 kb of introns 1–5 (NG_016172.1 nts 16373-18374, 20315-22316, 24517-26356, 26385-28286) were chosen as targets.

As a final step, differentially spliced exons in target sequences for inclusive exon and intron probe sets are each replaced by a single "n" before entry into the designer. This last step prevents the design of oligos across the splice site, which would otherwise only bind certain or nonexistent variants (*see* **Note 4**).

2. Probe set design: The Stellaris probe set name, gene name, and selected target sequence (with or without FASTA header) are entered into the designated fields in the Stellaris Probe Designer (http://www.biosearchtech.com/stellarisdesigner/). Choosing the organism (in this case human) allows the designer to utilize genome-specific information to mask against repetitive sequences, such as Alu elements. To start, a masking level of 5 (the highest) is chosen. The maximum number of oligos chosen for an exon probe sets is 48, and for intron sets 32. The output for all chosen targets exceeded the recommended 25 minimum number of oligos, except for the H19 introns (12 oligos) (*see* **Note 4**).

3.2 Cell Culture

The procedure for the culture [33] and fixation detailed here is for the male human lung adenocarcinoma cells (A549) and the mammary gland adenocarcinoma SK-BR-3 cell lines in 12-well cell culture plates, but can be adapted to other cell lines with minor modifications. A549 is haplotriploid [34] with the relevant karyotype: 8,8,8; 11,11,11; A10 [t(11;8) (q13;q8.24)]. SK-BR-3 is hypertriploid [35] with three X chromosomes.

Volumes should be adjusted accordingly when adapting this protocol for use in cell culture dishes or multi-well plates of a different size.

1. Seed cells on sterile, 18 mm round #1 cover glass in a 12-well plate. Plating density should range from 30,000 to 50,000 for A549 cells and 70,000 to 90,000 for SK-BR-3 cells per well.
2. Incubate cells at 37 °C and 5 % CO_2.
3. Allow cells to grow to approximately 80 % confluency prior to fixation, usually 2–3 days.

3.3 Formaldehyde Fixation and Cell Permeabilization

1. Aspirate the growth medium from each well, and wash with 1 mL of 1× PBS.
2. Aspirate the 1× PBS, and add 1 mL of fixation buffer. Allow the cells to incubate in the fixation buffer for 10 min at room temperature. Aspirate the fixation buffer, and wash twice with 1 mL of 1× PBS, aspirating between washes.
3. To permeabilize the cells, immerse the cells in 1 mL of 70 % ethanol for at least 1 h at 4 °C.
4. The cells can be stored submerged in 70 % ethanol at 4 °C for up to a week prior to hybridization.

3.4 Methanol–Acetic Acid Fixation/ Permeabilization

This alternative method of fixation and permeabilization provides faster results, but may alter the overall cellular morphology and may not be compatible with simultaneous immunofluorescence [8, 10–12, 36].

1. Aspirate the growth medium from each well, and wash with 1 mL of 1× PBS.
2. Aspirate the 1× PBS, and add 1 mL of MeOH:AcOH solution. Allow the cells to incubate in the fixative solution for 10 min at room temperature.
3. The cells can be stored submerged in MeOH:AcOH at 4 °C and should be used within 48 h.

3.5 Hybridization

Reconstitute the Stellaris RNA FISH Probes in Tris–EDTA buffer solution to create a probe stock of 12.5 μM. To ensure that the probes are completely resuspended, thoroughly pipette up and down, then vortex, and centrifuge briefly. Make sure that the hybridization buffer and wash buffers are properly diluted and supplemented with formamide. To prepare the hybridization solution, add 0.5 μL of probe stock solution to 50 μL of hybridization buffer, then vortex, and centrifuge. This creates a working probe solution of 125 nM.

1. Aspirate the 70 % ethanol from the cover glass containing adherent cells within the 12-well plate. Add 1 mL of wash buffer A, and allow the cells to incubate at room temperature for 2–5 min.
   (a) For cells fixed and permeabilized with MeOH:AcOH, aspirate the MeOH:AcOH from the cover glass containing adherent cells within the 12-well plate. Add 1 mL of wash buffer A, and allow the cells to incubate at room temperature for 2–5 min.
2. Create a humidified chamber using a 150 mm tissue culture plate. Evenly line the bottom of the tissue culture plate with a flat, water-saturated paper towel. Place a 10 × 10 cm piece of Parafilm on top of the water-saturated paper towel. This chamber will help prevent evaporation of the probe solution from under the cover glass.
3. Within the humidified chamber, dispense 50 μL of the hybridization solution (containing probe) onto the Parafilm. Use forceps to gently transfer the cover glass, cell side down, onto the 50 μL droplet of hybridization solution. It is important that both the paper towel and Parafilm are completely level so that the hybridization solution will disperse evenly under the cover glass. Avoid the formation of bubbles.
4. Cover the humidified chamber with the tissue culture lid, and seal it with Parafilm.
5. Place the humidified chamber in a dark 37 °C oven for at least 4 h. The incubation can be continued overnight up to 16 h, thus allowing for an entire day of imaging, if necessary, on the next day (*see* **Note 5**).

3.6 Washing

1. Add 1 mL of wash buffer A to a fresh 12-well plate. Remove the humidified chamber from 37 °C, and gently transfer the cover glass (cells side up) to the 12-well plate containing wash buffer. Allow the cells to incubate in the dark at 37 °C for 30 min.
2. Aspirate the wash buffer A, and then add 1 mL of DAPI nuclear counterstain. Allow the cells to incubate in the dark at 37 °C for 30 min.
3. Aspirate the DAPI counterstain solution, and then add 1 mL of wash buffer B. Allow the cells to incubate at room temperature for 2–5 min.

3.7 Mounting

1. Place a small drop (approximately 15 μL) of Vectashield Mounting Medium onto a microscope slide. Gently lift the cover glass, and briefly touch the bottom edge to a Kimwipe® (or equivalent tissue) to remove excess buffer. Then place the cover glass, with the cells facing down, onto the drop of mounting medium. If necessary, GLOX anti-fade may be used as an alternative (*see* **Note 6**).
2. Gently wick away excess mounting media from the perimeter of the cover glass. Seal the cover glass perimeter with a thin coat of clear nail polish and allow it to dry. As needed, gently wash away any dried salt off the cover glass with water.
3. For best results, image the samples on the same day.

3.8 Imaging

1. Use a wide-field fluorescence microscope with a 60 or 100× oil objective to obtain single-molecule resolution. Acquire z-sections with 0.3 μm spacing that span the entire thickness of the cell. This ensures that each individual RNA spot is captured (*see* **Note 7**).
2. The exposure times can range from 1 to 2 s (*see* **Notes 8** and **9**).

3.9 Image Processing

1. ImageJ (http://rsbweb.nih.gov/ij/) is a widely accessible and useful collection of software for post-processing image analysis. Each three-dimensional stack is exported out of the Nikon software and imported into ImageJ. The three-dimensional stack is then merged into a two-dimensional image using the Maximum Intensity Projection feature. Here, images can be overlaid with DAPI images and/or another RNA target image from the same field of view (Figs. 2, 3, and 4). There are a variety of free or proprietary software to help facilitate quantification of single-molecule RNA FISH. We use the software developed by the Arjun Raj lab at University of Pennsylvania rajlab.seas.upenn.edu/StarSearch/launch.html [10, 13] (*see* **Note 10**). Alternate software has been discussed elsewhere [8].

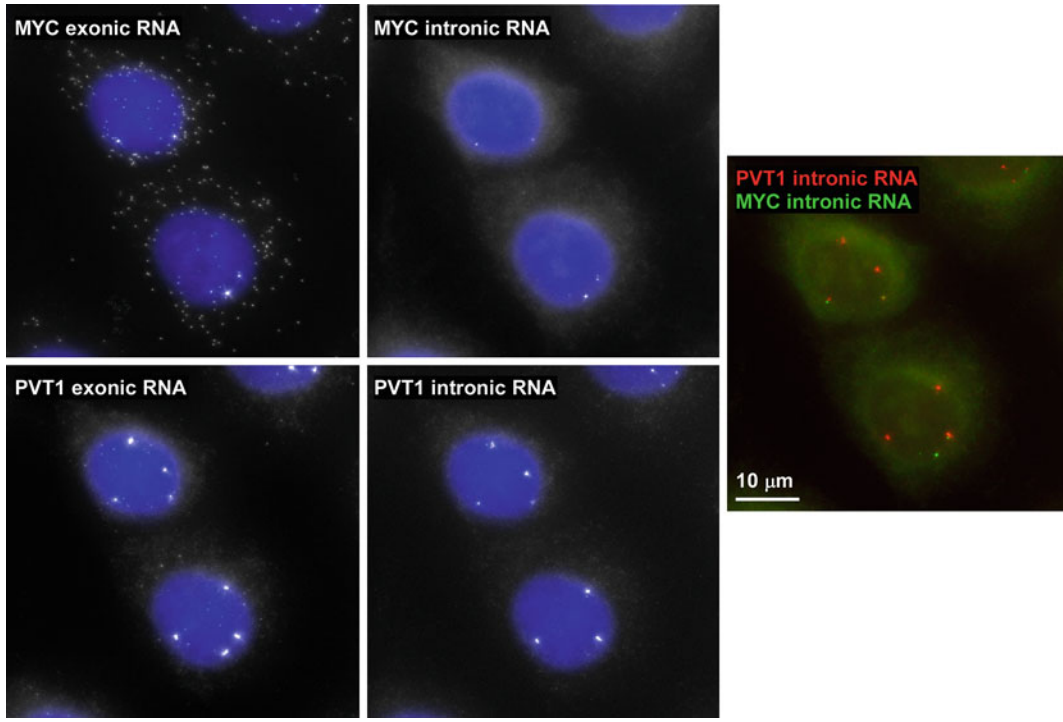

Fig. 2 Quadruplex Stellaris RNA FISH detection of MYC and PVT1 RNAs. Fixed A549 cells with DAPI-stained nuclei (*blue*) were probed simultaneously for mature MYC mRNAs, MYC introns, mature PVT1 lncRNAs, and PVT1 introns, and imaged with a 60×/1.4 NA oil objective. The MYC exon probe set revealed ~100 cytoplasmic mRNA spots. The exon and intron MYC probe sets both showed two bright and two weak (longer exposures) co-localized nuclear foci indicating variable activity at the MYC loci. The PVT1 exon set revealed predominantly nuclear single-molecule spots as well as four bright nuclear foci, and the PVT1 intron set revealed three to four bright nuclear foci. When superimposed (*right panel*), the MYC (*pseudo-colored green*) and PVT1 (*pseudo-colored red*) intron signals are adjacent and overlapping, consistent with the active expression of two neighboring genes. The difference in signal between MYC and PVT1 indicates independent control. The two types of signal by the PVT1 exon set also indicates that this lncRNA may have a clustered *cis*-function (or delayed miRNA processing), in addition to a nuclear single-molecule *trans*-function. Addition of probe sets targeting unique exons could add detail to the function of subsets of PVT1 lncRNAs

4 Notes

1. In general, spots observed in a wide-field fluorescence microscope are too dim to view through the eyepiece. This emphasizes the need for a cooled CCD camera, which greatly minimizes background noise. Furthermore, we discourage using a confocal microscope as the primary means of imaging; results tend to be inconsistent.
2. Formaldehyde and formamide are teratogens that are easily absorbed through the skin and should be used in a chemical fume hood. Be sure to warm the formamide to room temperature before opening the bottle.

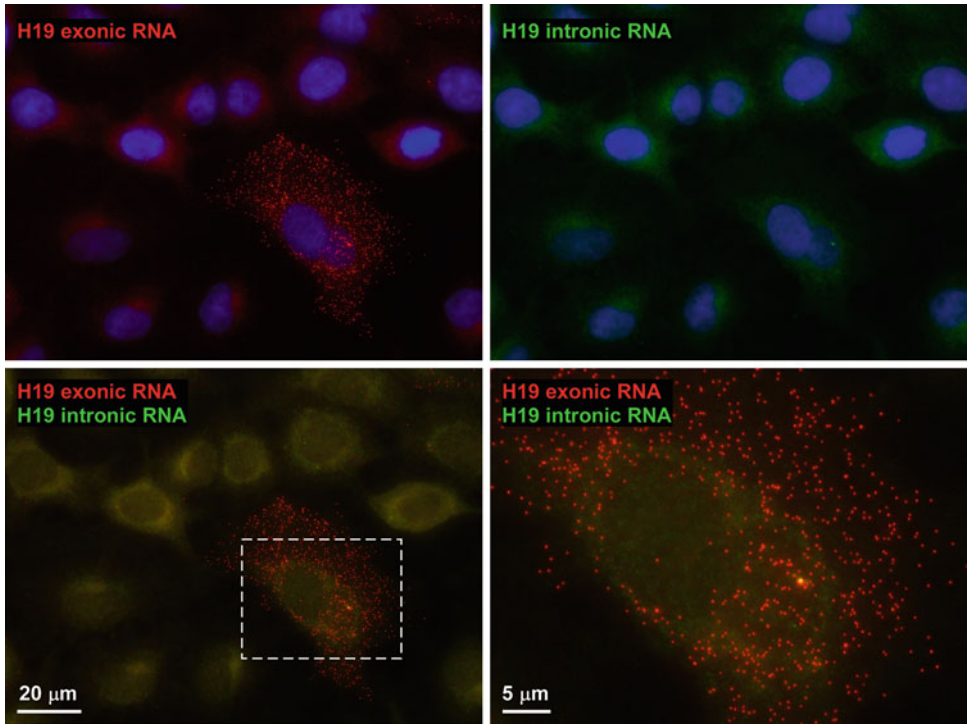

Fig. 3 Duplex Stellaris RNA FISH to detect H19 lncRNA introns and exons. Fixed A549 cells with DAPI-stained nuclei (*blue*) were probed simultaneously for mature H19 lncRNA (*upper left panel*; *pseudo-colored red*) and introns (*upper right panel*; *pseudo-colored green*), and were imaged with a 60×/1.4 NA oil objective. Single nuclear transcription bursts are revealed by both H19 probe sets (*lower panels*; *yellow*) as expected for the paternally imprinted gene [19]. The cytoplasmic punctate spots revealed by the exon probe set are consistent with the reported localization of the mature H19 lncRNA [37]

3. Similar sequence information can be obtained from the UCSC genome browser (www.genome.ucsc.edu) and Ensembl (www.ensembl.org). Benefits with the Ensembl annotation are modifiers for the quality confidence and completeness of annotated transcripts. Variant specific lncRNA information is also available elsewhere (www.lncipedia.org, lncrnadb.org).
4. To prevent the faulty design of probes, any nucleotide redundancy must be represented as "n" in the sequence. Other IUPAC letters are not allowed in the designer. It is also recommended that output oligo sequences from lower masking levels (3 and below) are BLASTed against the human transcriptome at NCBI. By furnishing the oligos with individual FASTA headers (>1, >2, etc.), the batch BLAST option can be utilized. Both the oligos that produce hits with <4 mismatches and the hit transcripts are tabulated. Transcript hits that are represented more than once are identified and the oligos responsible for the hits should be excluded from the final

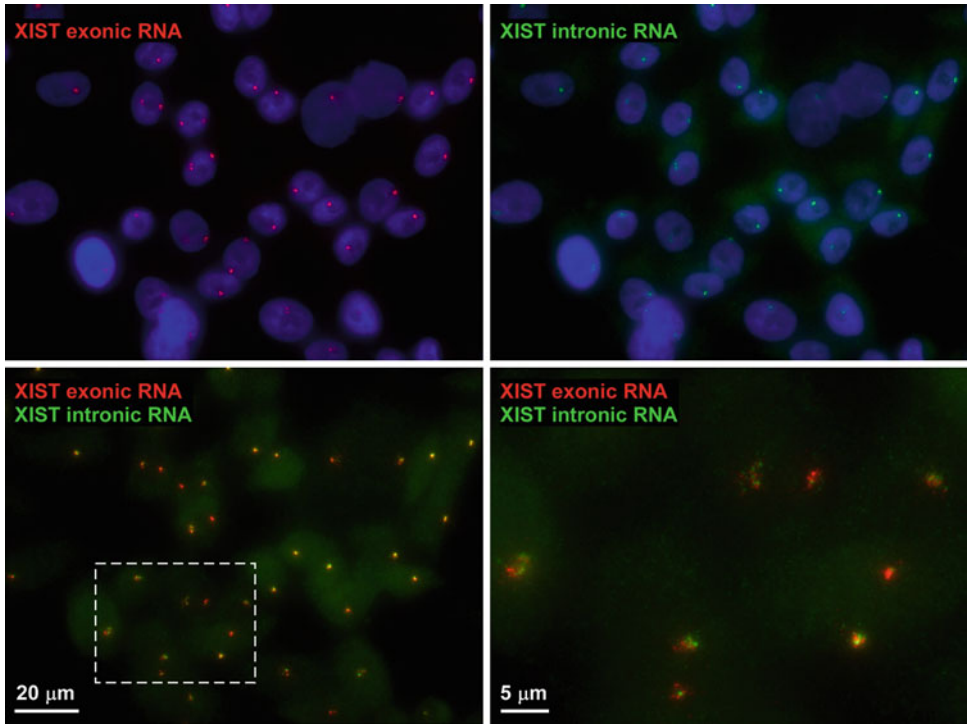

Fig. 4 Duplex Stellaris RNA FISH to detect XIST lncRNA introns and exons. Fixed SK-BR-3 cells with DAPI-stained nuclei (*blue*) were probed simultaneously for mature XIST lncRNA (*upper left panel*; *pseudo-colored red*) and introns (*upper right panel*; *pseudo-colored green*), and cells were imaged with a 60×/1.4 NA oil objective. The partially overlapping location (*lower two panels*) of the nuclear foci indicates that XIST gene transcription and X-chromosome inactivation in SK-BR-3 cells occur in *cis* on two of the three X-chromosomes

probe set to prevent off-target binding (Fig. 1). For the eight probe sets herein, all designed oligos were retained. Probe sets with <25 oligos generally are only recommended for detection of RNAs found in clusters, such as in transcription bursts or in Cajal bodies, and then in cells with minimal autofluorescence.

5. In general, hybridization times of 4–16 h are sufficient for quality results with Stellaris FISH Probe sets. However, for probe sets that initially demonstrate a low signal-to-background ratio, or that contain fewer that the recommended 25 oligos, both the optimal hybridization time and probe concentration must be determined experimentally.
6. The GLOX anti-fade is incompatible with the fluorescein (FAM) dye.
7. Our microscope is equipped with a motorized stage that enables the automatic capture of a series of z-sections at 0.3 μm. For each field of view, each different RNA target can be imaged sequentially. The ability to multiplex is dependent on the availability of filter sets with minimal spectral overlap.

8. In general, probe sets labeled with higher wavelength emitting fluorophores (e.g., Quasar 670) allow for the observation of reduced autofluorescence. However, these fluorophores generally require longer exposure times, ranging from 2 to 5 s, due to less efficient excitation light and emission detection.
9. Imaging via an unused filter at lower wavelengths (e.g., FAM/FITC) allows for the visualization of inherent cellular autofluorescence. This can greatly aid in the discrimination of true spots of single-RNA molecules versus false spots of autofluorescence.
10. The RNA quantification program processes a single TIFF *z*-stack and applies a rough linear Laplacian filter to assess the spatial derivation of the image with regard to intensity. A Gaussian smoothing filter helps subtract background and enhance spots of appropriate size and intensity. The convolved filter generates a graphical output that displays the relative signal-to-background ratio of every spot detected. The threshold automatically defined can also be manually adjusted, if necessary (i.e., low signal to background).

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