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Quick Fluorescent in situ Hybridization Protocol for Xist RNA Combined with Immunofluorescence of Histone Modification in X-Chromosome Inactivation

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Abstract:	Combining RNA fluorescent in situ hybridization (FISH) with immunofluorescence (immuno-FISH) creates a technique that can be employed at the single cell level to detect the spatial dynamics of RNA localization with simultaneous insight into the localization of proteins, epigenetic modifications and other details which can be highlighted by immunofluorescence. X-chromosome inactivation is a paradigm for long non-coding RNA (lncRNA)-mediated gene silencing. X-inactive specific transcript (Xist) lncRNA accumulation (called an Xist cloud) on one of the two X-chromosomes in mammalian females is a critical step to initiate X-chromosome inactivation. Xist RNA directly or indirectly interacts with various chromatin-modifying enzymes and introduces distinct epigenetic landscapes to the inactive X-chromosome (Xi). One known epigenetic hallmark of the Xi is the Histone H3 trimethyl-lysine 27 (H3K27me3) modification. Here, we describe a simple and quick immuno-FISH protocol for detecting Xist RNA using RNA FISH with multiple oligonucleotide probes coupled with immunofluorescence of H3K27me3 to examine the localization of Xist RNA and associated epigenetic modifications. Using oligonucleotide probes results in a shorter incubation time and more sensitive detection of Xist RNA compared to in vitro transcribed RNA probes (riboprobes). This protocol provides a powerful tool for understanding the dynamics of lncRNAs and its associated epigenetic modification, chromatin structure, nuclear organization and transcriptional regulation.
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RE: revised manuscript 52053_R2_051314, Yue et al.

Dr. Nandita Singh, Senior Science Editor of *JoVE*
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May 22, 2014

Dear Dr. Singh:

I am enclosing the revised version of manuscript 52053_R2_051314 by Yue et al. for publication in *Journal of Visualized Experiments*. The following lists our responses to the editor's comments:

1. We have thoroughly proofread the manuscript.

2. We have highlighted the essential steps for filmable content.

3. We have moved some NOTEs to the corresponding experimental steps and discussion. We think that these NOTEs are helpful for readers to optimize their experimental condition.

We hope that these revisions will render the manuscript publishable in *Journal of Visualized Experiments*. Finally, we thank you for considering our manuscript and look forward to hearing your response.

Sincerely,

Yuya Ogawa

TITLE: Quick Fluorescent *in situ* Hybridization Protocol for Xist RNA Combined with Immunofluorescence of Histone Modification in X-Chromosome Inactivation

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KEYWORDS:
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SHORT ABSTRACT:
We developed an easily customized strand-specific fluorescent *in situ* hybridization (FISH) protocol combined with immunofluorescence. This allows for a detailed examination of RNA dynamics with simultaneous insight into the chromatin structure, nuclear organization, and transcriptional regulation at the single cell level.

LONG ABSTRACT:
Combining RNA fluorescent *in situ* hybridization (FISH) with immunofluorescence (immuno-FISH) creates a technique that can be employed at the single cell level to detect the spatial dynamics of RNA localization with simultaneous insight into the localization of proteins, epigenetic modifications and other details which can be highlighted by immunofluorescence. X-chromosome inactivation is a paradigm for long non-coding RNA (lncRNA)-mediated gene silencing. X-inactive specific transcript (Xist) lncRNA accumulation (called an Xist cloud) on one of the two X-chromosomes in mammalian females is a critical step to initiate X-chromosome inactivation. Xist RNA directly or indirectly interacts with various chromatin-modifying enzymes and introduces distinct epigenetic landscapes to the inactive X-chromosome (Xi). One known epigenetic hallmark of the Xi is the Histone H3 trimethyl-lysine 27 (H3K27me3) modification. Here, we describe a simple and quick immuno-FISH protocol for detecting Xist RNA using RNA FISH with multiple oligonucleotide probes coupled with immunofluorescence of H3K27me3 to examine the localization of Xist RNA and associated epigenetic modifications. Using oligonucleotide probes results in a shorter incubation time and more sensitive detection of Xist RNA compared to *in vitro* transcribed RNA probes (riboprobes). This protocol provides a powerful tool for understanding the dynamics of lncRNAs and its associated epigenetic modification, chromatin structure, nuclear organization and transcriptional regulation.

INTRODUCTION:
Mammalian X-chromosome inactivation (XCI) is a strategy to compensate for the imbalance in X-linked gene dosage between XX and XY, wherein one of the two X-chromosomes in females is transcriptionally inactivated[1]. X-chromosome inactivation is a great model system for long non-coding RNA (lncRNA) research. X-chromosome inactivation is regulated by multiple lncRNA, and

has been extensively studied over the past few decades to uncover the crosstalk mechanisms between lncRNAs, transcription, chromatin structure and nuclear organization[2,3].

The X inactivation center (XIC) located on the X-chromosome is a complex genetic locus comprised of a number of genes producing non-coding RNAs[4]. X-inactive specific transcript (Xist) lncRNA in eutherian mammals is one such lncRNA which plays a crucial role in X-chromosome inactivation[5,6]. Xist transcripts surround the location of the future Xi to initiate X-chromosome inactivation, and appear as a cloud when visualized using RNA FISH; this formation is referred to as the "Xist Cloud" [7]. Since Xist RNA interacts with various chromatin-modifying enzymes, co-localization of the Xist clouds with different epigenetic modifications for silent chromatin and repressive transcription is observed during X-chromosome inactivation[8]. For example, Xist RNA interacts with polycomb repressive complex 2 (PRC2) which is responsible for H3K27me3 and induces a repressive chromatin state[9]. The occurrence of the Xist cloud on the Xi and its co-localization with the intensive H3K27me3 modification represents a facultative heterochromatin landscape of the Xi[10,11].

Cytogenetic techniques, such as DNA/RNA FISH have come a long way from the traditional method using radiolabelled probes[12] to recent and advanced techniques with enhanced sensitivity and fluorescent imaging using multiple oligonucleotide probes[13,14]. DNA/RNA FISH coupled with immunofluorescence has been routinely used as a cytological tool to understand spatiotemporal nuclear organization, RNA localization, chromatin structure and modifications. The most standard probe preparation for RNA FISH involves the use of plasmid or bacterial artificial chromosome (BAC) clones and their subsequent labeling either with nick translation or random priming[15]. However, nearly 70% of genes in mice and 40% of genes in humans show an overlap of sense and antisense transcripts[16], hence requiring a strand-specific FISH method in order to distinguish sense and antisense transcripts. *In vitro* transcribed RNA probes (riboprobe) are often used for strand-specific RNA FISH[17,18]; however, this involves preparing a plasmid clone or PCR product with T7, SP6, or T3 promoter and synthesizing riboprobes. Furthermore, riboprobes derived from genomic DNA or cDNA often contain non-specific regions and repetitive elements, which result in high background noise. Another issue is that riboprobes, which are a few hundred nucleotides in length and contain multiple fluorophores, cannot efficiently penetrate into the nucleus. To circumvent this, multiple shorter oligonucleotide probes labeled with a single fluorophore at the end have been developed that have good sensitivity, uniform signal strength, and ease of purification and handling[14]. In addition, DNA oligonucleotides are generally more stable than RNA. We applied a similar strategy of using oligonucleotides in Xist RNA FISH[19] with immunofluorescence to understand the epigenetic dynamics of the Xi induced by Xist lncRNA during the process of X-chromosome inactivation. This protocol describes creation of the necessary oligonucleotide probes and proper preparation of cells, as well as utilization of immunofluorescence and RNA FISH. Xist RNA FISH using multiple oligonucleotides is cost effective approach in the long-term if Xist RNA FISH is performed routinely in one's laboratory. This technique can be used to identify lncRNA in cells while simultaneously mapping its co-localization with epigenetic modifications or factors. One major advantage of the protocol is the ability to easily modify it to suit one's research interests.

PROTOCOL:

1. Probe preparation

1.1) Obtain multiple unique oligonucleotides in Xist (20-30 nucleotide-length oligonucleotides, 63-65 ºC melting temperature, Table 1) with a 5'-amine modification and suspend in water.

1.2) Pool equimolar amounts of oligonucleotide with 5'-amine (total 4.5 μg) and label with amino-reactive fluorescent dye following the manufacturer's instruction.

1.2.1) Dissolve oligonucleotide in final 5 μl of nuclease-free water and add 3 μl of 1 M sodium bicarbonate to the oligonucleotide solution.

1.2.2) Add 2 μl DMSO to the vial of amino-reactive dye and vortex briefly.

1.2.3) Mix the oligonucleotide solution with the reactive dye in DMSO and incubate the mixture at room temperature for 1 hour in the dark.

1.3) Purify the fluorescently labeled oligonucleotide probes by G-25 column followed by ethanol precipitation.

1.3.1) Centrifuge G-25 column at 750 x g for 1 min to remove excess storage buffer.

1.3.2) Add 40 μl nuclease-free water to the labeling mixture produced in Step 1.2.3 and apply to the G-25 column. Centrifuge at 750 x g for 2 min.

1.3.3) Add 50 μl nuclease-free water, 10 μl 3 M sodium acetate (pH 5.2) and 250 μl ethanol to the purified probe from Step 1.3.2. Store at -80 °C for 30 min and centrifuge at 21,000 x g for 15 min at 4 °C.

1.3.4) Wash the precipitated oligonucleotides once with 1 ml of 70% ethanol.

1.3.5) Re-suspend in 50 μl nuclease-free water or TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) for a final concentration of approximately 10 μM.

1.4) Dilute the fluorescently labeled oligonucleotide probes to a final concentration of 250 nM with hybridization buffer (10% formamide; 2x SSC [300 mM NaCl; 30 mM sodium citrate]; 2 mg/ml BSA; 10% dextran sulfate).

NOTE: With this quick FISH protocol, oligonucleotide probes in Xist were designed and used at a higher concentration than the normal FISH protocol using oligonucleotide probes in order to shorten the incubation time for hybridization.

2. Slide preparation

NOTE: Slides can be prepared for immuno-FISH using mouse embryonic stem (ES) or embryoid body (EB) differentiating cells[20,21] by either growing them directly on a poly-lysine-treated slide or using cytospin with cell suspension. Here, slide preparation by cytospin is shown.

2.1) Aspirate the culture medium in a 6-well dish. Wash with PBS (room temperature), and aspirate thoroughly.

2.2) Add 0.5 ml of trypsin to the 6-well dish, incubate for 5-10 min at 37 °C.

2.3) Add 5 ml of medium and disperse cells by gently pipetting up and down several times.

2.4) Transfer cells to a 15 ml tube, centrifuge for 5 min at 200 x g at room temperature.

2.5) Discard the medium and gently suspend the cells in 2 ml PBS.

2.6) Count cells using hemocytometer, and dilute cell suspension at $4x10^5$ cells/ml by PBS.

2.7) Assemble a slide, a filter card and a chamber with a clip and place them into the appropriate slots in the rotor of the cytospin. Add 250 µl of each sample prepared in step 2.6 into each chamber of the assembled units in the cytospin. Centrifuge at 1500 rpm for 10 min at room temperature.

2.8) Fill 3 coplin jars on ice with 40 ml each of ice-cold PBS, CSK buffer (10 mM PIPES, pH 6.8; 100 mM NaCl; 300 mM sucrose; 3 mM $MgCl_2$), and CSKT buffer (CSK buffer with 0.5 % Triton X-100), respectively.

2.9) After the cytospin is finished, quickly transfer the slide into ice-cold PBS and incubate for 5 min.

2.10) Transfer the slide into ice-cold CSK buffer and incubate for 1 min.

2.11) Transfer the slide into ice-cold CSKT buffer and incubate for 5 min.

2.12) Transfer the slide back into ice-cold CSK buffer and incubate for 1 min.

2.13) Transfer the slide into 4% paraformaldehyde in PBS and incubate for 10 min at room temperature.

3. Immunofluorescence

3.1) Place the slide in a coplin jar with PBST (1x PBS with 0.1 % Tween-20) for several seconds.

3.2) Place the slides at a slant allowing remaining buffer to flow down the slide. Carefully wipe excess liquid off the slide and place it in an empty pipet-tip box with water. Overlay the slide with 40 µl of blocking solution (1x PBS, 1 % BSA, 0.1 % Tween-20), gently place a coverslip, and incubate for 10 min at room temperature.

3.3) Remove the coverslip, remove the blocking solution, and add 40 µl of the Histone H3K27me3 primary antibody diluted to a concentration of 1:500 in blocking solution. Place the coverslip back on the slide and incubate for 30 min at room temperature.
NOTE: An RNase inhibitor in the blocking solution might be helpful to preserve RNA in this RNA FISH protocol when polyclonal antibodies are used.

3.4) Wash slide 2 times in coplin jars filled with PBST, for 5 min each, gently shaking while washing.

3.5) Place the slides at a slant allowing remaining buffer to flow down the slide. Carefully wipe excess liquid and place the slide back into the tip box. Overlay with 40 µl of the secondary antibody (1:500) diluted in blocking solution, place a coverslip, and incubate for 15 min in the dark. For the following steps, keep the slide in the dark at all times.

3.6) Wash 2 times in coplin jars with PBST as in step 3.4.

4. RNA FISH with oligonucleotide probes

4.1) Pre-warm an empty pipet-tip box with water at the bottom to prevent slides from drying at 37 °C for RNA FISH in step 4.3.

4.2) Place the slides at a slant allowing remaining buffer to flow down the slide. Carefully wipe excess liquid off the slide from step 3.6 and place the slide into the pipet-tip box with water at the bottom. Add 500 µl of FISH wash buffer (10% formamide in 2 x SSC) on the slide and incubate for 5 min at room temperature.

4.3) Remove FISH wash solution, add 8 µl of oligonucleotide probes onto the slide, place a cover slip, and transfer the slide to a pre-warmed pipet-tip box with water at the bottom; incubate at 37 °C for 1-2 hrs.

4.4) Place 3 pre-warmed coplin jars in a water bath at 37 °C with FISH wash buffer, FISH wash buffer with DAPI, and 2 x SSC.

4.5) After 1-2 hrs incubation for RNA FISH, put the slide into the pre-warmed FISH wash buffer.

4.6) Incubate the slide for a few minutes until the coverslip falls off of the slide.

4.7) Place the slide back into the wash buffer, and continue incubation for 5 min.

4.8) Transfer the slide to another coplin jar with pre-warmed FISH wash buffer with 0.5 ng/ml DAPI and incubate for 5 min.

4.9) Transfer the slide to another coplin jar with pre-warmed 2 x SSC and incubate for 5 min.

4.10) Place the slides at a slant allowing remaining buffer to flow down the slide. Carefully wipe excess liquid and place the slide back into a dry tip box. Add 4 µl of antifade with DAPI onto the slide and place a coverslip.

4.11) Seal the coverslip with nail polish and observe under a microscope. The slide can be stored at 4 °C in the dark for a few months.

REPRESENTATIVE RESULTS:

Representative images of quick immuno-FISH are shown in Figure 1A. Co-localization of the Xist RNA cloud and H3K27me3 signal on the Xi was detected in differentiating female cells. At day 12 upon differentiation, more than 90% of EB cells had an Xist cloud (Figure 1B). Short oligonucleotide probes efficiently penetrated into the nuclei, leading to a visualization of almost all H3K27me3 signals co-localized with Xist RNA (Figure 1C; 97%, n=150).

Figure 1: Xist RNA and H3K27me3 co-localize on the Xi in differentiating mouse EB.

A) Immuno-FISH for Xist RNA with H3K27me3 modification in differentiating female EB cells at day 12 upon differentiation. Xist probes were labeled with Alexa Fluor 488, and anti-H3K27me3 primary antibody was detected by anti-mouse IgG Alexa Fluor 555-conjugated secondary antibody. Nuclei were counterstained with DAPI. The boxed region is magnified in the bottom panels. Scale bars: 10 µm. B) Frequency of Xist cloud- and H3K27me3-positive cells in EB at day 12 upon differentiation. C) Frequency of co-localization of Xist cloud with H3K27me3 signal in EB at day 12 upon differentiation.

Table 1: Sequence of fluorescently labeled oligonucleotides for Xist RNA FISH

The 48 oligonucleotides were synthesized with a 5'-amine modification to prepare fluorescently labeled oligonucleotide probes for Xist RNA FISH.

DISCUSSION:

In this paper, we have presented a quick immuno-FISH protocol that takes less than 5 hours to complete slide preparation, Xist RNA FISH, and immunofluorescence for H3K27me3. In comparison with the general immuno-FISH approach for Xist RNA detection, which usually needs overnight incubation for RNA FISH, this protocol not only significantly reduces time spent but also improves the sensitivity of immune-FISH using oligonucleotide probes.

Since Xist is highly transcribed during X-chromosome inactivation and its transcript is found to intensely accumulate on the Xi *in cis*, it is easy to detect *Xist* RNA signal in differentiating and differentiated cells by RNA FISH utilizing a relatively brief incubation time. This immuno-FISH protocol could be applied for other proteins of interest and abundant RNA, although optimization in immunofluorescence and RNA FISH may be required. In order to apply the

protocol to other RNA, the number of possible oligonucleotide probes would need to be considered, followed by trial-and-error optimization of the probe's concentration, incubation time, and temperature for RNA FISH. One example of other applications is our previous success in detecting telomeric RNA using single LNA probes[22]. For probe design and preparation for the detection of low abundant RNA targets, refer to the article by Raj and Tyagi[23]. Another issue that must be considered is permeabilization step. Careful consideration regarding different permeabilization condition is needed; for example, milder effects than what is described in our protocol can occur with permeabilization after fixation[15]. Various permeabilization procedures also need to be considered when carrying out immuno-FISH using different cell types. The order of immunofluorescence and RNA FISH may also affect the detection of target proteins, modifications, and RNA by immuno-FISH. If RNA FISH is performed prior to immunofluorescence, one hour incubation during the RNA FISH segment is enough to detect Xist RNA signal. However, since the formamide contained in the hybridization buffer and FISH wash buffer negatively affects H3K27me3 immunofluorescence, we always carry out H3K27me3 immunofluorescence prior to Xist RNA FISH in this protocol. For other applications, the order of procedures would depend on the target proteins and epigenetic modifications to be observed by immunofluorescence. As the primary antibody we used for H3K27me3 immunofluorescence has a high titer and specificity to H3K27me3[24], we are able to use a shorter incubation time for immunofluorescence. However, reaction time and temperature for immunostaining are dependent on the antibody used and thus optimization is needed for each antibody.

Using oligonucleotide probes, Xist RNA detection by RNA FISH was significantly improved. In our previous studies, we found that a subset of differentiating cells with H3K27me3 staining on the Xi are not associated with Xist RNA signal[25]. Since H3K27me3 modification on the Xi is associated with PRC2 recruitment by Xist RNA[9-11,26] , we speculated that this was due to the inefficient penetration of riboprobes into the nuclei compared to the antibody for immunofluorescence. By using oligonucleotide probes in this protocol, we observed that H3-K27me3 signal in differentiating female cells nearly always co-localized with Xist RNA (Fig. 1).

This quick immuno-FISH protocol allows us to gain a snapshot of the dynamic changes revolving around Xist lncRNA and offers a new tool to help gain a better understanding of the way lncRNA works. lncRNAs affect various cellular functions such as genomic imprinting, gene regulation, RNA translation, and RNA maturation[27]. Developing an increased understanding of the diverse roles played by lncRNA would help to propel the field forward and allow for future discoveries in the regulation of vital cellular processes within the human body, as well as contribute to the development of potential targets for disease treatments. Since this protocol can be easily optimized with respect to other markers and lncRNAs of interest, it is a great tool to help tackle the mysteries of various lncRNAs in cells.

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DISCLOSURES:
No conflicts of interest declared.

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Figure
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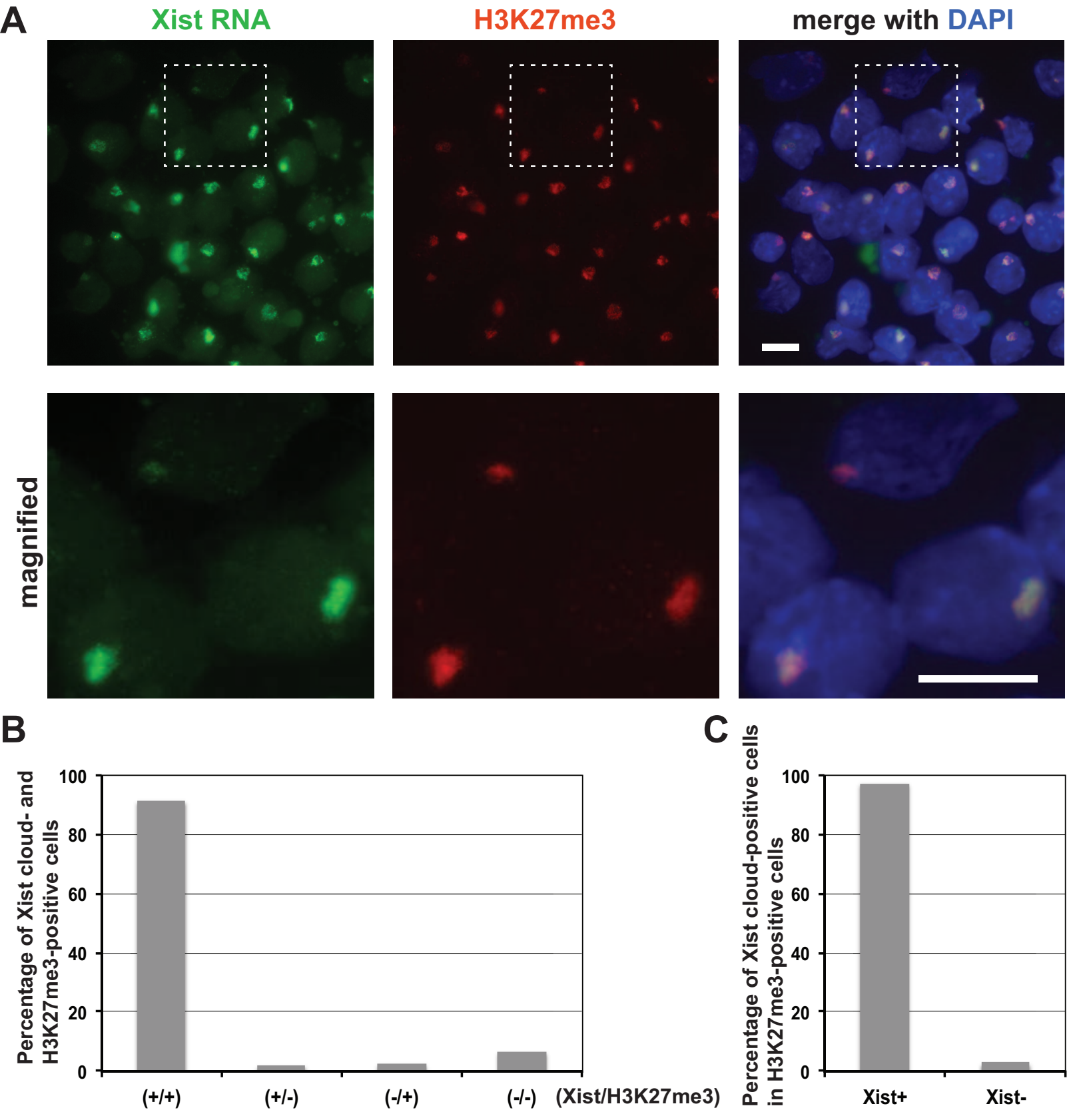

Table
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TABLE 1: Sequence of fluorescently labeled oligonucleotides for Xist RNA FISH

Xp1, ggtaagtatccaaaaccccgttgg
Xp2, cgatcagcagcaacagtacacg
Xp3, gcaattggttgcttttatccagtcc
Xp4, cgcaacaccgcacactaatacg
Xp5, gccatcttagacacattcaagagcat
Xp6, cacacgtgaagtaccaagcgaaac
Xp7, gccacgtatagagcactgtaagagactatg
Xp8, caaagcacactatcagacgtgtcg
Xp9, ggagaatctagatgccataaaggcaag
Xp10, tgatggacactgcattttagcactg
Xp11, ggacactgcattttagcaatacgattc
Xp12, gtgatgggcactgcattttagc
Xp13, agatgggctatctcagtcttataggct
Xp14, ggaagtcagtatggaggggtatg
Xp15, tgggactgtgactactacagcaatga
Xp16, aagactcaattcctagtcaggattatccac
Xp17, gggctgtagctctatgacagtgcttt
Xp18, gtcttcaccagatgcagattactacagtg
Xp19, aatagtttgaggaaggggtttcaagtg
Xp20, gctgttcaggtttccttctgtagtga
Xp21, gcaagagatacaatggtccgaaaagt
Xp22, agcacttcgtacaaccctctttctg
Xp23, gaagagagcaggtcattcgtcagag
Xp24, gcaactgagacactgtagccatatgaag
Xp25, ttcctggaggaagaacggaaaga
Xp26, tgattagaaggcttaggtcatcttcca
Xp27, ttttgttcagagtagcgaggacttga
Xp28, aatagagcagaatggcttcctcgaa
Xp29, acattgcttgatcacgctgaagac
Xp30, gcaaggaagaaatagacacacaaagc
Xp31, ggaagaaatagatgtaacaaagaattagacaca
Xp32, cacttcagagccacttgaatcctg
Xp33, agtcacaggtgtcctgtagaaacagttc
Xp34, cctttatgggcaatggcaacaat
Xp35, ggcacatctgcatattgcttgtcta
Xp36, gcaactaagaccatgaacccacaa
Xp37, aaacacactggccttaagtatatggactg
Xp38, cattcatttgcacacatggaacaat
Xp39, tgggagacaatatttagcctccaggt
Xp40, cctagcaagggcactgttttgtaataa
Xp41, taacatttagcacactgccttgcac
Xp42, cagtgatctacactaggtccacctcaca
Xp43, ttatgttgaaggaatcttggccttg
Xp44, aagtgagagctgtagtctcaaggtgtga

Xp45, gtattcaacctctgaggcaaactgtg
Xp46, agattgtggaacttagatggctgtca
Xp47, tggaactgcattaaagtcccaacttag
Xp48, gaactcccagacctcttcaacctg

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Yue_JoVE_Material_v3.xlsx

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
oligonucleotides with 5’-amine modificatior	IDT		
Alexa Fluor 488 Reactive Dye	Life Technologies	A32750	
MicroSpin G-25 columns	GE Healthcare	27-5325-01	
Cytospin 2	Shandon	59900102	
Bovine Serum Albumin, Molecular Biology Grade (for hybridization buffer)	Roche	10715859103	
Histone H3K27me3 antibody	Active Motif	61017	
Accutase	Innovative Cell Technologies	AT104	
Alexa Fluor 555 Goat Anti-Mouse, highly cross-adsorbed	Life Technologies	A21424	
ProLong Gold antifade reagent	Life Technologies	P36931	

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Title of Article: Quick Fluorescent in situ Hybridization Protocol for Xist RNA Combined with Immunofluorescence of Histone Modification in X Chromosome Inactivation

Author(s): Minghui Yue, Charles Richard John Lalith, Norishige Yamada, Akiyo Ogawa and Yuya Ogawa

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Response to Reviewer 1

1. We agree that fluorescently labeled probes by nick translation using plasmid or fosmid is more common for Xist RNA FISH. The introduction now includes this information. However, since labeling via nick translation is not strand-specific, and we want to specifically detect Xist RNA but not its antisense transcript, Tsix RNA, we choose to focus mainly on strand-specific RNA FISH analysis. The technique with a ribo-probe is used in several articles such as PMID: 12667455, 14975321, 17512404, 18535243 and 21852535.

2 & 3. All reagent information is listed in the Material table and we have added details for the G-25 spin column to the table. Additionally, we have included further information in Steps 1.2 and 1.3 regarding the use of the amino-reactive dye labeling kit and G-25 spin column.

4. We have added NOTE2 at the beginning of the Step2 - Slide preparation section to mention the effect of permeabilization on our results.

5. We did not specify the exact time required for the coverslip to fall off in Step 4.6 because it varies in each experiment.

6. We have modified Step 4.10 to clarify the procedure of wiping off excess liquid.

Response to Reviewer 2

1 & 2. We have revised the introduction section to describe the stability of DNA oligonucleotide probes and the cost effectiveness of this approach.

3. Basically, either combination of Alexa 488, 555, 657 works in our immuno-Xist RNA FISH, therefor it is not necessacy to discuss the choice of fluorophor in this protocol.

4. We have added to the discussion the reason for doing H3K27me3 immunofluorescence prior to Xist RNA FISH.

5. To clarify Figure 1, we have modified Figure 1A.

6. Yield of the fluorescetly labeled oligonucleotide is now shown in Step 1.3.5. We have not determined the efficiency of fluorescent labeling.

Response to Reviewer 3

1. All reagents that were used in this protocol are listed in the Material table.

2. We have added to Figure 1B the percentage of cells with Xist RNA and H3K27me3 signals, and Xist cloud-positive cells in H3K27me3-positive cells.

3. We decided to show only one representative image of immuno-FISH of Xist RNA and H3K27me3 for the sake of clarity and brevity.

Response to Reviewer 4

Instead of adding a trouble-shooting section as the reviewer suggested, we added several NOTEs to the beginning of each experimental step. These NOTEs will be helpful to others in optimizing the experimental conditions while clearly describing certain problems to be aware of in each pertinent section.

Response to Reviewer 5

1. We have corrected the typo reviewer 5 pointed out.

2. At least for Xist RNA FISH, we successfully detected strong and clear Xist RNA signals by immuno-RNA FISH without a RNase inhibitor. However, we agree that polyclonal antibodies might contain RNase contamination which may affect the sensitivety of RNA FISH. Thus, we have added a brief note to Step 3 - Immunofluorescense part to help others consider using a RNase inhibitor for better detection of RNA FISH signal.