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Immunofluorescence protects RNA signals in simultaneous RNA-DNA FISH

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Abstract

Cell research often requires combinational detection of RNA and DNA by fluorescence *in situ* hybridization (RNA-DNA FISH). However, it is difficult to preserve the fragile RNA signals through the harsh conditions used to denature the DNA template in DNA FISH. The current protocols of RNA-DNA FISH still cannot work robustly in all experiments. RNA-DNA FISH remains as a technically challenging and tedious experiment. By incorporating protein components into the signal detection steps of RNA FISH, which is then followed by a post-fixation step, we established an improved protocol of RNA-DNA FISH. The established method worked satisfyingly and robustly in our studies on *Xist* (inactivated X chromosome specific transcript) RNA and *Terra* (telomeric repeat-containing RNA). Our results provided the direct evidence to show that, not all the telomeres are associated with *Terra*, and a significant fraction of *Terra* foci do not overlap with telomere DNA in interphase cell nuclei. The improved method of simultaneous RNA-DNA FISH is reliable and time-efficient. It can be used in a variety of biological studies.

Keywords

Fluorescence *in situ* hybridization (FISH)

Simultaneous RNA-DNA FISH

X chromosome inactivation (XCI)

Xist

Terra

Introduction

Fluorescence *in situ* hybridization (FISH) is commonly used to detect RNA and DNA signals in cells. Established protocols for RNA FISH or DNA FISH are widely available. Cell research, however, often requires simultaneous detection of RNA, DNA and proteins. One example would be the study on X-chromosome inactivation (XCI). In mammals, one of the two X chromosomes in each female cell is transcriptionally inactivated to balance the X-linked gene dosage between males (one X) and females (two Xs). A 18 kb long non-coding RNA, called *Xist* (inactivated X chromosome specific transcript) plays critical roles in XCI [1]. The *Xist* gene is located along the X chromosome within a DNA region (about 100 kb long) called the X chromosome inactivation centre (*Xic*). The X-linked *Xist* gene is allele-specifically transcribed from the inactive X (Xi). The *Xist* RNA transcripts coat the X chromosome territory *in cis*, which can be visualized in RNA FISH as an intriguing “cloud” signal (the “*Xist* cloud”). Coating of the *Xist* RNA further establishes specific enrichment of multiple layers of histone modifications on the Xi chromosome territory. The chromosome-wide gene silencing on Xi is then achieved. Studies on XCI often require combinational stains on *Xist* clouds (RNA FISH), all the Xs within a cell (DNA FISH), and histone modifications (immunostain on proteins).

Simultaneous detection of RNA and DNA (RNA-DNA FISH) is technically challenging. The established methods of RNA-DNA FISH still cannot work robustly. The fragility of RNA molecules causes the difficulty in RNA-DNA FISH, as the RNA targets or the RNA signals of RNA FISH are often damaged during the harsh treatments (high temperature, low pH) involved in DNA FISH to denature the target DNA in cells. Three different experimental approaches can be found in available reports on RNA-DNA FISH. The first one is the simultaneous hybridization approach[2-5], in which the probe hybridization steps of DNA and RNA FISH are

carried out simultaneously. This approach is difficult to be applied to all RNA targets, because it requires that the original RNA targets (not the RNA FISH signals) remain stable during DNA FISH. The second one is the superimposition approach [4, 6-8]. In this approach, RNA and DNA FISH are carried out sequentially, and two rounds of image collection are involved. The RNA FISH is carried out first; and microscope images of RNA FISH are collected. The position (x-y coordinates) of each collected image has to be marked for reference. Subsequently, the slide is subjected to DNA FISH, in which the signals from the previous round of RNA FISH are lost. After DNA FISH, a second image of DNA FISH is taken, which is then manually overlapped (superimposed) with the first image of RNA FISH. This is a tedious and time-consuming approach. The third approach is the post-fixation approach. A few successful attempts of RNA-DNA FISH were done in this approach [2, 4, 9-12]. During a post-fixation step, the RNA FISH signals are fixed by fixatives before the slide is subjected to DNA FISH. It is believed that post-fixation can protect RNA FISH signals from being damaged during DNA FISH. However, as what our results show in this paper, a simple post-fixation step still cannot provide reliable results.

We recognized that an important reason for the successes of some of the previous attempts on RNA-DNA FISH [9-12] is that haptens, such as biotin, were chosen in the signal detection steps of RNA FISH. This is a critical point, which has not been clearly acknowledged in the current protocols [2, 4]. Actually, the crucial component is not the hapten itself. Rather, the proteins involved in the hapten signal detection, for example streptavidin, are the crucial components, which enable the post-fixation step to protect the RNA FISH signals efficiently. Paraformaldehyde (PFA) is commonly used in fixative solution for FISH experiments. PFA is a solid polymer that is hydrolyzed into formaldehyde when heated to 60°C in the presence of hydroxide ions. Formaldehyde acts as a fixative largely by cross-linking the proteins

leading to a formation of an insolubilized protein matrix that is relatively resistant to harsh conditions (Figure 1). One of the most reactive sites in formaldehyde-mediated cross-linking is the amine group of lysine [13]. The probes used in RNA FISH are usually DNA or RNA fragments labeled with nucleotide analogs conjugated with fluorescent dyes. Therefore, both the original RNA target and the probe are nucleotides. We argue that, different from proteins, the nucleotide probes for RNA FISH cannot be fixed by PFA efficiently enough to survive the subsequent DNA FISH. Such a problem could be solved by introducing protein components into the signal detection steps of RNA FISH.

Here, we present our experimental results to show that introducing protein components into the signal detection steps of RNA FISH is critical for the post-fixation step to work efficiently. We also show that significant RNA signal loss still occurred in the biotin-streptavidin signal detection approach, which was used in previous studies, although the method did preserve the majority of the RNA signals. By introducing immunofluorescence into RNA FISH, we further improved the current protocol of simultaneous RNA-DNA FISH. The established method generated RNA signals with satisfying quantity and quality after DNA FISH. Furthermore, we solved the additional problems that the “well-preserved” RNA FISH signals generated in the subsequent DNA FISH. The established protocol worked robustly and provided satisfying results in our studies on *Xist* and *Terra*.

Methods

Cell lines and culture

A transformed female mouse fibroblast cell line, named 129T, primary mouse embryonic fibroblast (MEF) cells derived from embryonic day 13.5 (E13.5) female mouse embryos, and a male mouse embryonic stem cell line, named J1, were cultured in DMEM medium with 10% FBS at 37°C in a 5% CO₂ incubator.

Cell harvest and slide preparation

In the cytopspin approach, after trypsinization, cells were resuspended in a concentration of 8×10^5 cells per ml in PBS. The cells suspended were then cytopspun onto Superfrost/Plus microscope slides (Fisher Scientific, Cat# 12-550-15) using cytofunnels in a Shandon Cytospin 4 centrifuge (Fisher Scientific) at 1500 rpm for 10 minutes. The slides were air dried and rinsed in ice-cold PBS for 5 minutes and the cells were permeabilized in ice-cold cytoskeletal (CSK) buffer (100mM NaCl, 300mM sucrose, 10mM PIPES pH 6.8, 3mM MgCl₂) plus 0.5% Triton X-100 for 5 minutes. The slides were then fixed in 4% paraformaldehyde (PFA) at room temperature for 10 minutes. After fixation, the slides were stored in 70% ethanol at 4°C until use.

For the cells directly cultured on cover slips, the samples were washed with PBS before the cells were fixed in 4% PFA at room temperature for 10 minutes. After fixation, the samples were stored in 70% ethanol at 4 °C until use. Before the samples were subjected to FISH experiments, the cells were permeabilized in ice-cold PBS containing 0.5% Triton X-100 for 30 minutes.

Fluorescence *in situ* hybridization (FISH) probes

DNA probes were used in both DNA FISH and RNA FISH. The probes were prepared using nick translation kit (Roche, Cat# 10976776001). Mouse *Xist* RNA was detected with Sx9 probe, a PI DNA construct containing a 40 kb genomic fragment covering the mouse X inactivation centre (*Xic*) region. The DNA region of the mouse *Xic* locus was detected with DNA probe of BAC clone Rp23-11p22 (Invitrogen). A X-linked DNA region (~200kb) outside of the *Xic* locus was detected with DNA probe of BAC clone Rp23-280L7 (Invitrogen). Mouse X chromosomes were detected with chromosome X paint (Cambio, Cat# 1189-XMF-02). Telomere DNA and *Terra* were detected with PNA probes (Panagene, Cat# F1002, F1009 and F1010). The probes were denatured at 75°C for 10 minutes and pre-annealed at 42°C for 15 minutes before being applied onto slides for hybridization. Nucleotide analogs used in probe labeling were Cy3-dUTP (Amersham, Cat# PA53022), Fitc-dUTP (Stratagene, Cat# 300380-54) or biotin-dUTP (Roche, Cat# 11093070910).

RNA FISH

Slides were dehydrated through 80%, 90% and 100% ethanol sequentially for 2 minute each and dried at room temperature for 3 minutes. Denatured probes were added onto slides for hybridization. Hybridization was carried out overnight at 42°C in a dark and humid environment. After hybridization, the slides were washed three times in 50% formamide, 2xSSC at 45°C for 5 minutes each, followed by three washes in 2xSSC at 45°C for 5 minutes each. The washing steps were done in a shaker-waterbath with slight agitation.

Immunofluorescence (IF) and biotin-streptavidin detection

For both IF and biotin-streptavidin detection, antibodies or streptavidin were

diluted 1:200 in PBS plus 0.2% Tween-20 and 0.5% Bovine Serum Albumin (BSA). Incubation was carried out in darkness at room temperature for 1 hour. After incubation, the slides were washed for 5 minutes, three times, at room temperature with PBS plus 0.2% Tween-20. Antibodies used were anti-Fitc IgG conjugated with alexa-488 (Invitrogen, Cat# A-11090) and anti-Cy3 IgG conjugated with biotin (Sigma, Cat# C3117). Streptavidin conjugates used were streptavidin Cy3 conjugates (Caltag Laboratories, Cat# SA1010) and streptavidin alexa-555 conjugates (Invitrogen, Cat# S-21381).

Post-fixation

Slides were post-fixed prior to DNA FISH in 4 % PFA for 10 minutes at room temperature and rinsed twice in PBS.

DNA FISH

Slides were treated with 400 μ g/ml RNase A (Fermentas, Cat# EN0531) at 37°C for 30 minutes. The slides were then treated with freshly made 0.2M HCl plus 0.5% Tween-20 at room temperature for 10 minutes and briefly rinsed twice in PBS plus 0.2% Tween-20. The slides were subsequently denatured in 70% formamide, 2xSSC at 80°C for 10 minutes. Before hybridization, the denatured slides were dehydrated in ice-cold 80%, 90% and 100% ethanol series for 2 minutes each. Denatured probes were then applied for an overnight hybridization at 42°C in a dark humid environment. After hybridization, the slides were washed three times in 50% formamide, 2xSSC at 45°C for 5 minutes each, followed by three washes in 2xSSC at 45°C for 5 minutes each. The washing steps were done in a shaker-waterbath with slight agitation.

For DNA FISH using the chromosome paint in a RNA-DNA FISH experiment,

two adjustments were made on the DNA FISH procedure in order to overcome the side effects generated by the well-preserved RNA FISH signals to the DNA FISH. The slides were treated with freshly made 0.2M HCl plus 0.5% Tween-20 at room temperature for 15 minutes. The slides were denatured in 70% formamide, 2xSSC at 85°C for 10 minutes.

Microscopy and image collection

Slides were mounted with Dapi-containing mounting medium with antifade (Vector Laboratories Inc, Cat#H-1200). Fluorescence images were collected on Eclipse Ti microscope (Nikon) using digital camera Clara Series model C01 (Andor) and NIS Elements AR imaging software (Nikon).

Results and discussion

RNA FISH signals can be “protein” preserved to survive the subsequent DNA FISH

We attempted different experimental designs to introduce immunofluorescence into RNA FISH (Figure 2). In RNA FISH design I, the *Xist* RNA FISH was carried out with Cy3 (red) labeled DNA probes. In this design, the DNA probe is directly labeled with the fluorescent dye. Therefore, there is no immunofluorescence involved in RNA FISH of this design. Although the RNA FISH signals were post-fixed, the RNA FISH signals were completely lost after DNA FISH. In design II, the *Xist* RNA FISH was carried out with Fitc (green) labeled DNA probes. The slide was then immunostained by anti-Fitc antibodies conjugated with alexa-488 (green). After post-fixation, the slide was subjected to DNA FISH. In this experimental design, the *Xist* RNA signals survived after DNA FISH, although the RNA signal's intensity decreased after DNA FISH (Figure 2, white arrowhead). In design III, the *Xist* RNA FISH was carried out using DNA probes labeled by biotin. The slide was then treated with streptavidin conjugated with Cy3. Streptavidin is a protein containing 8 lysine residues. In this design, the RNA FISH signals also survived after DNA FISH, and the signals were in a better quality compared with the signals from RNA FISH design II, although signal intensity drop still occurred in some cells (Figure 2, white arrowhead). In design IV, *Xist* RNA FISH was carried out using Cy3 labeled DNA probes. The slide was then stained with anti-Cy3 antibody conjugated with biotin. After anti-Cy3 antibody stain, the slide was treated with streptavidin conjugated with alexa-555 (red). Different from the other experimental designs, two protein components were involved in design IV. We achieved the best result in this design. The *Xist* RNA signals were bright and clear after DNA FISH. The experiment was successfully repeated a number of times by different pairs of hands.

The established method provided satisfying results in protecting RNA signal quantities after DNA FISH

To test on the efficiency of the established RNA-DNA FISH method, we used the method to detect the low level expression of the *Xist/Tsix* RNAs in undifferentiated ES cells. The *Xist* gene is expressed at low level in undifferentiated ES cells, in which XCI has not been established and the *Xist* RNAs have not spread out to coat the X chromosome territory. At this stage, the *Xist* RNA and its antisense RNA transcripts, called *Tsix*, form a small pinpoint RNA signal covering the *Xic* regions of all X alleles in both male and female cells. A male ES cell line was chose in this experiment, because roughly 85% of the cell population in this cell line carries the perfect 40XY karyotype. The small populations of aneuploid cells are all tetraploid. In contrast, the aneuploid cell population in a female mouse ES cell line is a mixture of “XO” cells and tetraploid cells, which complicates the data analysis in this experiment. The RNA FISH was carried out in two different experimental designs (design III and IV). The RNA FISH signals were post-fixed before a mock DNA FISH (DNA FISH without using labeled probes) was carried out. The surviving RNA signals after DNA FISH are shown in Figure 3A. The profile of RNA pinpoint number per cell is shown in Figure 3B. Comparing the RNA FISH results before and after DNA FISH, it can be found that although the RNA FISH of both experimental designs successfully detected the majority of the RNA signals after DNA FISH, RNA FISH signal loss did occur after DNA FISH in both experimental designs. About a quarter of the diploid cells lost the RNA signals in design III. In design IV, signal loss occurred in about 6% of the diploid cells. Therefore, RNA FISH design IV provided a significantly better protection on RNA FISH signals than design III.

The established method is compatible with cells cultured directly on cover slips and provided satisfying protection on RNA signal quality after DNA FISH

To further validate the established method, we test the method's compatibility with samples prepared in a different way for microscopy works. In the foregoing experiments, the cells were harvested using the cytopsin method, in which the cells were trypsinized, permeabilized and deposited onto the microscope slides by cytopsin centrifuge, before the cells were subjected to fixatives. Since cell clusters are dissociated into single cells by trypsinization, this method provides significant advantages in dealing with small cells forming clusters in culture, such as the ES cells. It also works well with nuclear RNA targets, such as the *Xist* RNA, which prefer an efficient cell membrane permeabilization before the sample is subjected to fixation. For RNA FISH on different RNA targets in different cell types, cells can be directly cultured on cover slips. Since the cells directly cultured on cover slips can be directly fixed without trypsinization and membrane permeabilization, this method provides better protection on the target RNA compared to the cytopsin method. We, therefore, carried out simultaneous *Xist* RNA-DNA FISH on primary female MEFs directly cultured on cover slips.

In order for the RNA FISH probes to penetrate into the nuclei to detect the *Xist* RNA, cells were permeabilized before RNA FISH using PBS containing 0.5% Triton X-100. The permeabilization step was carried out in an RNase-free condition after the fixation step. The RNA FISH experiment detected bright *Xist* RNA cloud signals (data not shown). However, without the preservation by proteins, the RNA signals were completely lost after DNA FISH (Figure 4A). This result shows that whether RNA signals can survive DNA FISH does not depend on how well the RNA

targets or the RNA signals are protected from RNase during the sample preparation and the probe hybridization steps of RNA FISH.

We then carried out *Xist* RNA FISH using experimental design III and IV (Figure 2). As shown in Figure 4A, with the preservation by proteins, the post-fixed RNA signals survived DNA FISH. Analysis of *Xist* cloud number per nucleus showed that DNA FISH did not generate significant *Xist* cloud signal loss (Figure 4B). However, DNA FISH still caused damages on the *Xist* RNA signals detected in experimental design III. Such damages can be revealed by the *Xist* cloud volume (Figure 4C). We measured the *Xist* cloud volume by manually selecting an appropriate fluorescent signal intensity threshold for each individual *Xist* cloud. The selected signal intensity threshold enables the image analysis software to distinguish the RNA signal from its surrounding background. Once a *Xist* cloud signal is digitally recognized by the software, its volume is computationally calculated. Analysis of the *Xist* RNA cloud volume showed that DNA FISH caused volume reduction on the *Xist* clouds. The volume reduction of *Xist* clouds is statistically significant on the RNA signals detected by experimental design III, but not design IV. This result shows that design IV provides better protection on RNA signal quality in simultaneous RNA-DNA FISH experiments. It should be noted that, in theory, the secondary signal detection/amplification step involved in hapten-labeled probes could artificially increase the signal volume. Therefore, directly labeled probes should provide more accurate results on RNA signal volume. In this study, we only use the RNA signal volume measurement to reveal the damages that DNA FISH generates on the RNA FISH signals.

The DNA FISH procedure needs to be adjusted to eliminate the side effects generated by the well-preserved RNA FISH signals

Interestingly, the well-preserved RNA FISH signals generated additional concerns in the subsequent DNA FISH. We made two observations (Figure 5). First, In a DNA FISH to detect the *Xic* DNA region, which is about 100 kb long and normally shows up as a pinpoint signal in DNA FISH, “cloud” *Xic* signals were detected (Figure 5A). These “cloud” *Xic* DNA FISH signals specifically associated with the Xi chromosome territories coated by the *Xist* RNA clouds (Figure 5A, white arrow head), while the *Xic* region from the Xa (active X chromosome) could be correctly detected as a pinpoint signal (Figure 5A, white arrow). We believe that the well-preserved probes of the RNA FISH experiment caused this problem. In this experiment, the *Xist* RNA FISH probe and *Xic* DNA FISH probe share a common DNA region, therefore, the RNA FISH probes were detected as “cloud” signals in the subsequent DNA FISH. The second observation was made in DNA FISH experiments using chromosome X paint (Figure 5B). The chromosome paint probes are used in DNA FISH to detect the entire chromosome territory. As a DNA FISH probe, the chromosome paint should not be able to distinguish the chromosome territory of Xa and Xi. However, we noticed that in many cells the chromosome X paint probe failed to detect the chromosome territories of Xi, but not Xa (Figure 5B, yellow arrow). We believe that, in this case, the Xi chromosome territory was “well-preserved” by the RNA FISH so that the chromosome X paint probes in DNA FISH could not easily access it.

To eliminate the side effects of the preserved RNA FISH signals on the subsequent DNA FISH, we modified the DNA FISH protocol. Figure 5C shows that a ~200kb X-linked DNA region was successfully detected as a pinpoint signal within

the *Xist* RNA coated Xi chromosome territory in the RNA-DNA FISH. In this case, we eliminated the side effect by selecting a X-linked bacterial artificial chromosome (BAC) as the probe for DNA FISH. The BAC probe is not homologous with the *Xist* RNA FISH probe. Figure 5D shows that the Xi chromosome territory was successfully detected by chromosome X paint in RNA-DNA FISH. In this case, we eliminated the side effect by including stronger conditions for denaturing the template DNA during DNA FISH. These conditions include, RNase treatment, HCl treatment. Our established protocol is included in the Methods section.

Simultaneous detection of telomeric repeat-containing RNA (*Terra*) and telomere DNA

To demonstrate that the established method can be applied to RNA targets other than the *Xist* RNA, we performed simultaneous RNA-DNA FISH on *Terra* and telomere DNA. Mouse telomere DNA contains simple repetitive DNA sequence of “TTAGGG”. *Terra* is transcribed from the telomeric DNA region and carries the G-rich telomeric sequence [14-16]. Biochemistry assay revealed the inhibitory effect of *Terra* on telomerase activity *in vitro* [17]. This result, together with the assumption that *Terra*, similar with other non-coding RNA, may directly bind with the DNA region from which it is transcribed, lead to the conclusion that *Terra* is involved in regulating telomerase-mediated elongation of telomere *in vivo*. However, no direct evidence is available to show the co-localization pattern of *Terra* and telomere DNA.

We carried out RNA FISH on *Terra* using peptide nucleic acid (PNA) probes carrying the C-rich telomeric sequence. Consistent with the previous report [16], the PNA probe detected bright *Terra* signals in undifferentiated male mouse ES cells (Figure 6A). In a majority of cells, there are two major *Terra* signals in each nucleus

(Figure 6A, white arrow). A major *Terra* signal can be clearly distinguished from other signals by its size and signal intensity. Besides the major *Terra* signals, there are small pinpoint signals of *Terra* detected in the nuclei (Figure 6A, yellow arrow). The number of *Terra* pinpoint signals per nucleus varies from cell to cell. We carried out DNA FISH to detect telomere DNA using a PNA probe with the G-rich telomeric sequence. In corroboration with the forgoing experiments, with the protein preservation, *Terra* signals were well protected after DNA FISH (Figure 6A, B). Without the protein preservation, however, the RNA signals, including the major *Terra* signals, were completely lost after DNA FISH (Figure 6A). This result shows that PNA probes with a higher binding affinity to the target RNA compared to regular nucleotide probes still cannot protect RNA signals from the damage of DNA FISH.

The simultaneous detection of *Terra* and telomere DNA provides the direct evidence on the co-localization pattern of the two identities. It can be immediately recognized from these results that not all the telomeres are associated with *Terra* in interphase nuclei. Furthermore, three types of co-localization pattern of *Terra* and telomere DNA can be recognized (Figure 6C). Some *Terra* signals are overlapping (or partially overlapping) with telomere DNA. Some *Terra* signals are not overlapping with telomere DNA. The non-overlapping *Terra* signals include the RNA signals adjacent to one or more DNA signals and the RNA signals separated from all the nearby DNA signals. We examined the co-localization pattern of *Terra* and telomere DNA on 104 selected *Terra* signals (Figure 6D). Surprisingly, the result shows that, in interphase nuclei, a significant fraction of *Terra* signals do not overlap with telomere DNA (Figure 6C). It is possible that the non-overlapping *Terra* signals associate with the sub-telomeric regions (DNA regions upstream of telomeres). However, it is unlikely that the non-overlapping *Terra* signals are directly involved in the

telomerase-mediated elongation of telomeres. Besides a regulatory factor of telomerase-mediated telomere elongation, *Terra* may have other possible functional roles.

In summary, our data show that including protein components into the signal detection steps of RNA FISH is crucial for the post-fixation step to succeed in the RNA-DNA FISH. This critical information was not clearly acknowledged in current protocols. Nowadays, DNA probes directly conjugated with fluorescent dyes are widely used in RNA FISH. Unfortunately, the post-fixation approach is unreliable in preservation of nucleotide signals. For an efficient post-fixation effect, it is crucial to include protein components into RNA FISH. Our results supported this idea. Following this principle, we have further improved the current protocol of RNA-DNA FISH.

Besides the post-fixation approach, another approach exists for RNA-DNA FISH, the simultaneous hybridization approach [4]. In this approach, the cells are denatured before RNA FISH and the hybridization steps of RNA and DNA FISH are carried out simultaneously. Interestingly, based on our experience, this approach is never successful with fibroblast cells (a somatic cell type), but it could give occasional success in differentiating embryonic stem (ES) cells (data not shown). XCI occurs in early embryos. *In vitro* differentiation of female ES cells is used in experiments to recapitulate XCI. The observation that the simultaneous hybridization approach could give occasional success in differentiating ES cells suggests that the *Xist* RNA may interact heavily with protein factors during the onset of XCI, which may help to preserve the *Xist* RNA during the fixation step of slide preparation. However, caution needs to be taken for the simultaneous hybridization method. For any

differentiating ES cell population, there are always cells, which have undergone XCI (*Xist* cloud positive), and cells, which have not initiated XCI (*Xist* cloud negative). In a cell where no RNA signal was detected, false negative may be concluded, as it is not sure if the RNA signal was lost due to the DNA FISH procedure.

Conclusions

The established protocol of simultaneous RNA-DNA FISH provides better protection on RNA signals compared to the current protocols. It is also more reliable and time-efficient. This method can be used in the research of XCI and easily adapted into other research fields.

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Figure legends

Figure 1 The chemistry of fixation by formaldehyde. The diagram shows that formaldehyde acts as a fixative to generate the cross-link between two amine groups (for example the amine group of lysine) of two proteins R and R'.

Figure 2 *Xist* RNA FISH experiments of four different experimental designs.

The *Xist* RNA FISH experiments were carried out using four different experimental designs. The DNA FISH was carried out without using any probes (mock DNA FISH).

The *Xist* RNA signals before and after DNA FISH are compared. The white arrowheads show the *Xist* RNA signals with low signal intensity after DNA FISH.

Note: A transformed female mouse fibroblast cell line was used in this study. The cell line contains a mixed cell population of diploid, tetraploid and aneuploid cells.

Therefore, some cells showed more than one *Xist* cloud signals.

Figure 3 Detection of *Xist/Tsix* RNA signals in undifferentiated male ES cells.

The *Xist/Tsix* RNA FISH experiments were carried out using a double strand DNA probe. The RNA FISH were carried out in two different designs (design III & IV). One round of mock DNA FISH was carried out after RNA FISH. The surviving RNA signals after DNA FISH are shown in **(A)**. The number of pinpoint RNA signals per cell was analyzed. The results are shown in **(B)**.

Figure 4 The compatibility of the established method with cells directly cultured on cover slips and the quality of the surviving RNA signals after DNA FISH. (A) Simultaneous RNA-DNA FISH on *Xist* RNA. The RNA FISH experiments were carried out in three different designs (design I, III, and IV). The *Xist* RNA was

detected with the red fluorescence. The nuclei were stained with Dapi (blue). One round of mock DNA FISH was carried out after RNA FISH. Only the surviving RNA signals after DNA FISH are shown. The images of design III & IV were taken using the same exposure time and settings. **(B)** Analysis of *Xist* RNA clouds number per nucleus. **(C)** Analysis of *Xist* clouds volumes. Data shown are in box and whisker plot format (n = 50 for each data set). The asterisk indicates p-value < 0.01 with the Student *t* test.

Figure 5 Simultaneous RNA-DNA FISH with modified DNA FISH procedures to eliminate the side effects generated by the well-preserved *Xist* RNA signals.

Xist RNA FISH followed by DNA FISH to detect **(A)** the *Xic* DNA region using a Fitc-labeled BAC probe and **(B)** the X chromosome territory using Fitc-labeled chromosome X paint. The white arrow shows that a *Xic* region from an active X allele was successfully detected as a pinpoint signal in DNA FISH. The white arrowhead shows that a *Xic* region from a Xi allele was abnormally detected as a cloud signal in DNA FISH. The yellow arrow shows a Xi chromosome territory, which the DNA FISH failed to detect. **(C)** RNA-DNA FISH detecting the *Xist* RNA and a ~200 kb X-linked DNA region using a BAC probe. **(D)** RNA-DNA FISH detecting the *Xist* RNA and the X chromosome territory.

Figure 6 Simultaneous RNA-DNA FISH on *Terra* and telomere DNA. (A) *Terra* RNA FISH was performed with experimental design I using a Fitc-labeled PNA probe (green) carrying the C-rich telomeric sequence. The yellow arrow points to a pinpoint *Terra* signal. The white arrow points to a major *Terra* signal, which can be clearly distinguished from the pinpoint *Terra* signals by its brightness and its size. **(B) *Terra*** RNA FISH was performed with experimental design IV using a Cy3-labeled PNA

probe (red) carrying the C-rich telomeric sequence. The RNA signals were post-fixed, before DNA FISH was performed using a Fitc-labeled PNA probe (green) carrying the G-rich telomeric sequence. Images of merged z-series are shown. The images were not deconvolved. **(C)** A deconvolved image of a selected single focal plane is shown. The enlarged views of 6 selected *Terra* signals are shown on the right hand side of the image together with each signal's corresponding number and its surrounding telomere DNA signals. **(D)** The co-localization patterns of *Terra* and telomere DNA of 104 selected *Terra* signals. The co-localization pattern of each *Terra* signal was determined by examining the signal through all the collected focal planes in a deconvolved z-series.