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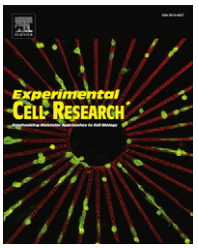

Research Article

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.

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 center (*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

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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 (Fig. 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% CO_2 incubator.

Cell harvest and slide preparation

In the cytospin approach, after trypsinization, cells were resuspended in a concentration of 8×10^5 cells per ml in PBS. The cells suspended were then cytospun 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 min. The slides were air dried and rinsed in ice-cold PBS for 5 min and the cells were permeabilized in ice-cold cytoskeletal (CSK) buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$) plus 0.5% Triton X-100 for 5 min. The slides were then fixed in 4% paraformaldehyde (PFA) at room

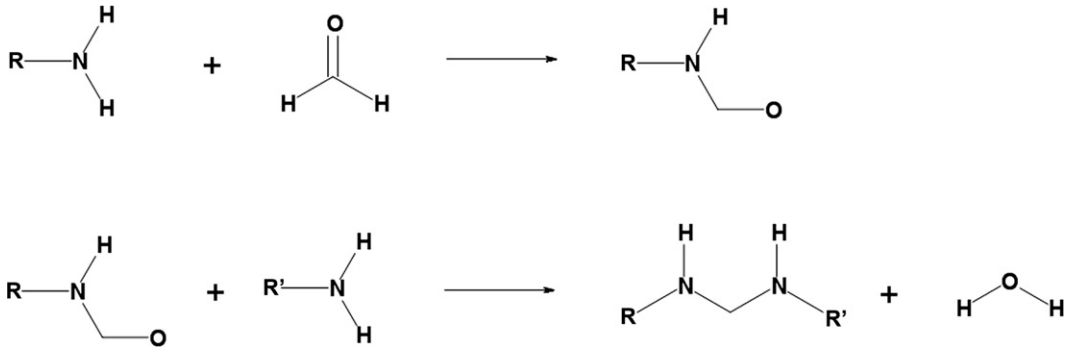

Fig. 1 – The chemistry of fixation by formaldehyde. The diagram shows that formaldehyde acts as a fixative to generate the cross-link between two amine groups (for example the amine group of lysine) of two proteins R and R′.