

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 necessary 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 fluorescently 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 sensitivity of RNA FISH. Thus, we have added a brief note to Step 3 - Immunofluorescence part to help others consider using a RNase inhibitor for better detection of RNA FISH signal.