Introduction

Techniques routinely used in our lab for analysing the X-inactivation process in differentiating ES cells are described. We focus particularly on chromatin changes (histone modifications, protein association...) during X inactivation, using immunofluorescence (IF) combined with RNA FISH or DNA FISH on interphase nuclei. These should provide a tool for defining potential causal relationships between different events not only during X inactivation but also during the establishment of other patterns of gene activity.

The main purpose of a combined IF and FISH analysis is, on the one hand, to preserve nuclear architecture and the antibody's epitope as far as possible but, on the other hand, to allow the penetration of the FISH probe for detection of nuclear transcripts, gene location or chromosome territories. The optimal conditions for IF are usually poorly compatible with those for FISH. We have therefore tested a variety of methods and conditions and we describe here those that we find optimal for the immuno-detection of histone modifications combined with RNA or DNA FISH on mouse fibroblasts or embryonic stem cells. For more details, the reader is referred to Chaumeil et al., 2002 and Chaumeil et al., 2004.

Procedure

Immunofluorescence

Numerous methods involving a variety of fixation and permeabilization techniques are available for performing IF and the choice depends on cell type, epitope and antibody being used (Spector et al., 1998).

1. Culture undifferentiated or differentiating ES cells on gelatin-coated coverslips for at least 24-48 hours;
2. Wash once in freshly prepared 1X PBS;
3. Fix in freshly made, filter-sterile 3% paraformaldehyde/1X PBS for 10 minutes at RT or 4°C;
4. Wash 3 times in 1X PBS for 5 minutes each;
5. Permeabilize with freshly made 1X PBS/0.5% Triton X-100 (containing the RNAse-inhibitor, 2mM Vanadyl Ribonucleoside Complex, in case of a subsequent RNA-FISH) on ice for 3.5-5 minutes (see note 1);
6. Wash 3 times in 1X PBS for 5 minutes each;
7. Block in 1X PBS/1% BSA (Gibco) for 15 minutes at RT;
8. Incubate with primary antibody diluted in 1X PBS/1% BSA (containing 0.4U/μl RNAGuard in case of a subsequent RNA-FISH) for 45 minutes at RT (or other conditions, see note 2) in dark, humid chamber;
9. Wash at least 3 times in 1X PBS for 5 minutes each;
10. Incubate with secondary antibody (diluted in same solution as aboved) for 40 minutes at RT in dark and humid chamber (see note 3);
11. Wash at least 3 times in 1X PBS for 5 minutes each;
12. DNA counterstaining (2 minutes in 1X PBS containing 0.2mg/ml DAPI);
13. Wash twice in 1X PBS;
14. Mount coverslip on a slide in glycerol based mounting medium.

RNA FISH

For a general description and discussion of RNA FISH protocols, the reader is referred to Spector et al., 1998. Conditions for detection of cytoplasmic versus nuclear RNAs are different, and here we focus only on the detection of nuclear transcripts. To detect the primary transcripts of genes, genomic probes several kilobase pairs long should be used. Probes spanning introns and exons will detect both the processed mRNA and the primary transcript. Oligonucleotides within intronic sequences will be specific for the primary transcript (see Robert Singer's web site for more details on use of oligos as probes: http://singerlab.aecom.yu.edu/). For the detection of Xist RNA coating of the X chromosome in cis, or primary transcripts of X-linked genes, we have used several genomic DNA probes, spanning a minimum of 3 kb, labelled by nick translation or random priming, with success. An example of RNA FISH is shown in Figure 1.

Preparation of FISH probes

1. DNA probes, to be used for RNA or DNA FISH, are labeled by nick translation using 1 to 2µg of DNA per 50µl of reaction and following manufacturer's instructions (see note 4);
2. Approximately 0.1µg of probe (usually 5µl of a standard nick translation reaction of 50µl) is ethanol precipitated together with 10µg of salmon sperm per 18x18mm coverslip (see comment 1);
3. Perform two washes of the pellet in 70% ethanol (to remove unincorporated nucleotides);
4. The pellet is resuspended thoroughly in formamide (5µl per coverslip), by pipetting and incubating at 37°C if necessary;
5. Denature the probe for 7 minutes at 75°C;
6. Add 5µl of 2X hybridization buffer per coverslip (see stock solution section). Mix well and keep on ice (probe can be kept on ice for up to 30 minutes), while coverslips are being prepared for the FISH step (see comment 2).

RNA FISH following immunofluorescence

When IF and FISH are to be combined, we prefer to perform IF (under RNAse-free conditions) prior to the FISH, as the formamide treatment during the FISH procedure is sometimes incompatible with preservation of the epitopes detected by some antibodies. An example of Xist RNA FISH combined with IF is shown in Figure 2.
Preparation of FISH probes (see RNA FISH section above)

IF - RNA FISH

1. Follow IF protocol up to PBS washes after secondary antibody (no DAPI coloration);
2. Post-fixation for 10 minutes at RT, using filter-sterile, freshly made 3% PFA/1X PBS;
3. Wash twice in 2X SSC (freshly made from a sterile 20X stock) for 5 minutes;
4. Hybridization and washes: see RNA FISH section above.

DNA FISH

For a general description and discussion of DNA FISH protocols, the reader is referred to Spector et al., 1998.

Preparation of FISH probes

- Preparation of the nick translation probe:
  1. FISH probe is labeled overnight (see RNA FISH section);
  2. Precipitate 0.1µg of probe with 10µg of salmon sperm and 5µg of Cot-1 DNA per 18x18mm coverslip;
  3. Two washes of the pellet in 70% ethanol;
  4. Resuspension of the pellet in 5µl of formamide per coverslip at 37°C;
  5. Denaturation for 7 minutes at 75°C;
  6. Competition for 30 minutes to 1 hour at 37°C;
  7. Addition of 5µl of hybridation buffer per coverslip (see RNA FISH section).

- For chromosome paint probes, we follow the supplier's recommendations (Cambio).

DNA FISH

1. Culture ES cells on gelatin-coated glass coverslips or slides;
2. Wash once in freshly made 1X PBS;
3. Fix in filter-sterile, freshly made 3% Paraformaldehyde/1X PBS for 10 minutes at RT (see comment 7);
4. Wash twice in 1X PBS for 5 minutes each;
5. Permeabilize in freshly made 1X PBS/0.5% Triton X-100 on ice for 5-7 minutes (see note 1);
6. Dehydrate the slides in 80%, 95%, 100% ethanol, for 3 minutes each;
7. Air dry;
8. Denature in 50% formamide/2X SSC (adjusted to pH 7.2) for 30 minutes at 80°C (see comment 8);
9. Wash twice in ice-cold 2X SSC;
10. Deposit denatured probe solution onto cells on slide (or place coverslip cell-side down onto probe on slide);
11. Hybridize with the probe overnight at 42°C in a dark and humid chamber (paper tissues soaked in 50% formamide/2X SSC) (see note 7);
12. Wash 3 times in 50% formamide/2X SSC (adjusted to pH 7.2) for 5 minutes each at 42°C;
13. Wash 3 times in 2X SSC for 5 minutes each at 42°C;
14. If a biotin-labelled probe (e.g. chromosome paint) is used, a detection step has to be included;
15. Block in 4X SSC/0.1% Tween/5% BSA (Gibco) for 15 minutes at RT;
16. Incubate in fluorescently labelled streptavidin or avidin diluted in blocking buffer for 40 minutes at RT in humid chamber;
17. Wash 3 times in 2X SSC;
18. DNA counterstaining (2 minutes in 2X SSC containing 0.2mg/ml DAPI);
19. Wash twice in 2X SSC for 5 minutes each;
20. Mount coverslip on a slide in glycerol based mounting medium.

DNA-FISH following immunofluorescence

The detection of DNA requires a DNA denaturation step which can destroy the immunofluorescence signal in some cases. Therefore, if the combination doesn't work properly, images should be recorded prior to the DNA FISH experiment using squared coverslips.
An example of IF combined with DNA FISH is shown in Figure 3.

Preparation of FISH probes - see DNA FISH section above

**IF - DNA FISH**

1. Follow IF protocol up to PBS washes following secondary antibody detection (no DAPI coloration);
2. Postfix in freshly made, sterile 3% PFA/1X PBS for 10 minutes at RT;
3. Wash twice in 2X SSC (freshly made from a 20X sterile stock) for 5 minutes;
4. Permeabilize in freshly made 0.1M HCl/0.7% Triton for 10 minutes on ice;
5. Wash twice in 2X SSC for 5 minutes each;
6. Denature in 50% formamide/2X SSC (adjusted to pH 7.2) for 30 minutes at 80°C (see note 8);
7. Wash several times in ice-cold 2X SSC;
8. Hybridize with denatured probe O/N etc: see DNA FISH section.

**DNA FISH following RNA FISH**

The detection of DNA requires a DNA denaturation step which can destroy the RNA FISH signal in some cases, rendering the simultaneous combination (on coverslips) impossible. Post-fixation (3% PFA / 1X PBS for 10 min at RT) of the RNA signal prior to the DNA-FISH can be performed; however, this post-fixation step can dramatically affect the efficiency of DNA denaturation. In these cases, it is possible to perform the RNA-FISH procedure first and to record the RNA FISH images prior to performing DNA FISH (on slides), using a microscope capable of tracking nucleus coordinates.

**Simultaneous RNA - DNA FISH (on coverslips)**

1. Follow RNA FISH and DNA FISH sections for the preparation of the FISH probes (see note 9).
2. Follow DNA FISH section for preparation of coverslips, denaturation and hybridization (see note 10).

**Sequential RNA - DNA FISH (on slides)**

1. Follow RNA FISH section for the preparation of the FISH probe and preparation of slides (as coverslips).
2. Record images and coordinates of nuclei on an appropriate microscope.
3. Scratch off the nail polish.
4. Wash off the mounting medium in 4X SSC / 0.2% Tween, 3 times at 42°C.
5. Follow the DNA FISH section for preparation of FISH probes.
6. RNase treat the samples at 37°C for one hour (1 U/ml RNase A (fermentas) + 10 U/ml (NEB) in 2X SSC).
7. Denature in 70% formamide / 2X SSC (adjusted to pH 7.2) for 2-4 min at 75°C.
8. Follow the end of the DNA FISH section (steps 9 to 20).

**Materials & Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2X hybridization buffer</td>
<td>(Storage at -20°C)</td>
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<tr>
<td>4X SSC</td>
<td></td>
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<tr>
<td>20% dextran sulfate</td>
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<tr>
<td>2mg/ml BSA (Biolabs)</td>
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<tr>
<td>40mM Vanadyl Ribonucleoside Complex (VRC)</td>
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<tr>
<td>90% glycerol</td>
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<tr>
<td>0.1X PBS</td>
<td></td>
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<tr>
<td>0.1% p-phenylenediamine, pH 9.</td>
<td>(Should be &quot;straw&quot; colored - if it veers to purple or yellow, discard.)</td>
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</tbody>
</table>
Author Notes

1. The exact time of permeabilization depends on the cell type and antibody, but a shorter time (less than 3 minutes) usually results in less efficient RNA FISH using probes labelled by nick translation.

2. The coverslips are placed cell-side down, avoiding the formation of air bubbles, onto a drop of antibody solution on a sterile glass slide. The volume depends on the size of coverslip used (we routinely use 18x18mm coverslips and 40µl of antibody solution). The temperature and length of incubation can vary between antibodies (we routinely perform a 45 minute incubation at RT). The coverslips are then carefully removed with forceps and put back into PBS for washing. If resistance is encountered when removing the coverslip, it should be flooded with PBS so that it floats, in order to avoid damaging the cells.

3. We commonly use secondary antibodies from Molecular Probes™ (e.g. Alexa Fluor 488, 568 and 680). For combined IF and RNA or DNA FISH, the choice of fluorochrome to which the secondary antibody is conjugated will depend on the fluorochrome with which the FISH probe is labelled, and on the filter sets available on the microscope. In the case of a double IF experiment, high affinity purified secondary antibodies should be used (e.g. molecular probes, highly cross-absorbed antibodies) to minimise cross-species reactivity. Even then, appropriate controls (i.e. each primary with both secondary antibodies) should be performed systematically to confirm specificity. In the case of a double IF combined with a DNA FISH, we find that infra-red fluorophores are very sensitive to the denaturation step, so in this case detection of the epitope with the infra-red secondary antibody should be performed following the DNA FISH.

4. We routinely use nick translation (requires 1-2µg DNA) to label genomic probes (plasmids, lambda clones, or BACs) for FISH; random prime labelling of DNA probes is sometimes used (particularly if starting DNA quantity is limiting as only 50ng is required). We also sometimes use fluorescently tagged oligonucleotides. The latter avoids the labelling step and also enables discrimination between sense or antisense transcripts (double stranded DNA probes will of course detect both) but is costly. When nick translation reaction is used, the size-range of the labelled DNA must be checked by electrophoresis on an 1% agarose gel. The optimal size range of the FISH probe is between 50-200 bp, short enough to enter the nucleus and long enough to be specific. Fluorescently labelled probes of this kind can be directly stored at -20°C for a few weeks.

5. Permeabilization in PBS/0.5% Triton can also be used (especially for IF combined with FISH), but CSK
buffer is best suited for visualising RNAs.

6. Slides can be stored in 70% ethanol at -20°C for several months prior to use.
7. The 10µl of denatured probe solution is deposited onto a sterile glass slide; the coverslip is removed from the 2xSSC solution with forceps and carefully lowered cell-side down avoiding the formation of air bubbles. Once the coverslip has made contact with the probe solution it should not be moved, to avoid damaging the cells.
8. The exact time and temperature of this denaturation step is highly variable (depending on cell type and differentiation status, as well as on the degree of fixation and the IF step that preceded denaturation). Different conditions should therefore be tested, to ensure the best compromise is made between denaturation and detectability of DNA on the one hand, and nuclear structure on the other.
9. RNA FISH and DNA FISH probes are precipitated separately, and resuspended in a half volume of a simple RNA or DNA FISH e.g., 2.5µl per coverslip. A competition is performed for the DNA FISH probe, and the 2 probes are mixed just prior to the overnight hybridization.
10. The time of denaturation is highly variable. Different conditions should be tested in order to determine the best compromise between the detectability of DNA and the RNA signal preservation. Note that the temperature of the overnight hybridization depends on samples and probes: 42°C or higher is usually best for DNA FISH, but can lead to loss of the RNA FISH signal - in this case, use 37°C.
11. The length of denaturation can vary between cell types. We usually perform a 3 min denaturation.

Reviewer Comments

Reviewed by: Joost Gribnau, Erasmus University, Rotterdam.

1. In general PFA fixation after CSK buffer treatment works well for the detection of abundant RNA's or RNA's fixed to chromatin like Xist. However, the triton treatment may wash out RNA and therefore result in loss of signal. If higher sensitivity is needed formaldehyde/acetic acid fixation as described by van de Corput and Grosveld, 2001, is the preferred technique. This fixation technique can also easily be combined with IF and DNA FISH analysis.
2. Several different hybridization mixes are used for RNA and DNA FISH. Besides the mix mentioned in this protocol hybridization mixes with formamide are routinely used. An example of a commonly used hybridization mix is: 50% formamide, 2xSSC, 50mM phosphate buffer pH 7.0, 10% dextran sulphate.
3. Besides RNase inhibitors DEPC treatment of solutions also works well to protect RNA from degradation.
4. Dehydration though 70%, 90% and 100% ethanol steps works well too.
5. To minimize the background all our genomic probes used for RNA and DNA FISH analysis are prehybridized with unlabeled repetitive Cot DNA (Cot DNA/probe ratio is 50:1).
6. When hybridizing with a formamide based hybridization mixture, as mentioned in comment B, a 37°C incubation temperature overnight is recommended. Washes are three times 10 minutes in 50% formamide/2xSSC and once 10 minutes in 2xSSC at 37°C.
7. DNA FISH can also be performed by using methanol/acetic acid fixed cells as described by Selig et al. 1992. The advantage of this technique is that no pretreatment is required and that the target sequences are better accessible for the probe than target sequences prepared with crosslinking (para) formaldehyde fixation methods. A problem with this technique is that it can not be combined with either IF or RNA FISH. In addition, for many cell types, including ES cells and MEF's, methanol/acetic acid treatment results in a complete loss of the nuclear architecture.
8. The denaturation step should be as short as possible. Incubating the cells too long at 80°C will damage or even completely destroy the structure of the cell.

Figures
Figure 1: Example of Xist RNA and MeCP2 primary transcript detection by RNA FISH on interphase nuclei of differentiating female ES cells.

RNA FISH was performed, following IF, to detect Xist (Spectrum Green labelled probe) and MeCP2 (Spectrum red labelled probe). An accumulation of Xist RNA (green) can be seen on the X chromosome undergoing inactivation (arrowhead) while only a punctate Xist signal (the primary transcript) can be seen on the active X, reflecting the low level of transcription and the instability of the transcript on the active X chromosome (asterisk). The top panel shows a Xist RNA accumulation (arrowhead), while the MeCP2 gene is still active, as can see by the presence of two MeCP2 primary transcript signals, one at the site of the Xist RNA domain (arrow) and one on the active X chromosome (asterisk). The bottom panel shows that the MeCP2 gene on the inactive X chromosome is repressed. Only the MeCP2 primary transcript of the active X chromosome can be seen (asterisk). DNA is stained with DAPI (grey).

Figure 2. Examples of IF combined with RNA FISH on interphase nuclei: Modifications of the N-terminal tails
of histone H3 on the inactive X chromosome in differentiating female ES cells.

IF with two antibodies, detecting H3 lys-27 tri-methylation (gift from D. Reinberg, see Sarma et al. 2002; top panel) and H3 lys-4 di-methylation (Upstate Biotechnology 07-030; bottom panel) was performed. The secondary antibody used in both cases was the Alexa 568 goat anti-rabbit antibody (red, column 2). Xist RNA FISH (Spectrum Green labelled probe, green, column 3) was performed following IF. An accumulation of Xist RNA (green) can be seen on the inactive X chromosome. The histone modifications are shown in red. In merge images of IF and RNA FISH (column 4), yellow coloration shows the enrichment of H3 Lys-27 tri-methylation on the Xist domain (arrowhead) and green coloration shows the exclusion of H3 Lys-4 di-methylation (arrowhead). DNA is stained with DAPI (grey, column 1).

Figure 3. Example of IF combined with DNA FISH on interphase nuclei: H3 lys-27 tri-methylation combined with an X chromosome paint and X-linked gene probe.

Immunodetection of H3 lys-27 tri-methylation (Upstate Biotechnology 07-449) with Alexa GAR 680-conjugated secondary antibody (pseudo-red, column 2), was combined with X chromosome DNA FISH (mouse X-chromosome paint detected with Alexa avidin fluorescein-conjugated secondary antibody, green, column 3) and Chic-1 DNA FISH (Spectrum Red labelled probe, red, column 5). In merged images of IF and X chromosome DNA FISH (column 4), yellow coloration shows the enrichment of H3 Lys-27 tri-methylation on the inactive X chromosome (arrowhead) and green coloration shows no enrichment on the active X (asterisk). DNA is stained with DAPI (grey, column 1).

References

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http://www.epigenome-noe.net/researchtools/protocol.php?protid=3