RNA FISH on cultured cells in interphase (PROT06)

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Last reviewed: 25 Apr 2005 by Edith Heard, Institute Pasteur, Paris

Introduction

Fluorescence *in situ*
hybridization (FISH) has become a widely used method in genome and molecular genetic studies. The technique is highly versatile and has been adapted to carry out genome-wide screenings, microarray quantifications, cancer cytogenetics analysis, and RNA expression and localization studies. The study of intracellular RNA localization using RNA FISH provides insights into the in situ physical characteristics of transcription and intracellular RNA transport in individual cells. In our lab, we use RNA FISH to detect the localization of *Xist*
RNA, a nuclear non-coding transcript that coats the entire chromosome from which it is transcribed.

The RNA FISH technique requires the generation of a labeled probe, hybridization of the probe to a fixed sample, and subsequently, detection of the labeled probe using microscopy.

Procedure

Cell Preparation

With the aim of detecting RNA care must be taken to avoid loss of signal due to sample degradation. Before getting started it is important to establish an RNase free work environment. The reader is referred to standard laboratory manuals such as "Current Protocols in Molecular Biology" for details. Briefly, work surfaces are cleaned thoroughly and wiped with ethanol. All glassware is baked and plastics is either certified RNase free or treated with DEPC water (1% DEPC, Sigma D5758, in deionized water) and autoclaved where appropriate.

Adherent cells can be grown directly onto coverslips. Suspension cells can either simply be placed on to a multiwell slide or they can be cytospun on to glass slides. Cytospin procedure flattens rounded cells, which is not ideal if the cell integrity is critical in the experiment.

Cytospin cells

1. A single cell suspension is prepared according to standard cell culture protocols;
2. 100µl of the cell suspension (10^5 cells/ml) is added to the assembled cytospin cartridge;
3. The cartridge is spun at room temperature for 5 minutes at 1000 rpm.

The slides are ready for cytoplasmic extraction or can be fixed immediately.

Grow cells on multiwell glass slides

Roboz slides are commercially available multiwell glass slides. They have been treated for the attachment of mammalian cells and are suitable for growing a large number of cell types including mouse ES cells. Cells are placed on to a Roboz slide at the proper cell density for 24 hours, and can be subsequently fixed directly or after cytoplasmic extraction.

Cell extraction

In order to detect nuclear transcripts, the nucleus is sufficiently permeabilised to allow the probe to gain access to its target. The cells are then fixed to preserve labile RNAs and the integrity of nuclear structure. Some cell types contain an extensive cytoplasm, which has to be extracted in detergent prior to fixation. Since RNA is prone to degradation during permeabilisation steps prior to fixation, the length of the pre-fixation steps should be kept to a minimum and it might be useful to add RNase inhibitors. The protocol below is optimized for detecting nuclear RNA in mouse ES cells and also works for monolayer cells (such as fibroblasts, epithelial cells or skeletal muscle).

Extraction protocol

1. Rinse slides in 1xPBS in a glass coplin jar;
2. Incubate slides for 30 seconds in CSK buffer at room temperature;
3. Incubate slides for 30 seconds to 10 minutes in CSK buffer + detergent at room temperature. The length of time for detergent extraction depends on the cell type. RNA is vulnerable to degradation at this step, therefore Vanadyl Ribonucleoside Complex (10mM final concentration) can be included in the extraction buffer;
4. Incubate slides for 30 seconds in CSK buffer at room temperature;
5. Fix slides in 4% paraformaldehyde in PBS for 10 minutes at room temperature;
6. Rinse in 70% ethanol.

Slides can be stored in 70% ethanol at 4°C until ready to use. Prolonged storage may cause RNA degradation.

Probe preparation

We have adapted our protocol from commercially available random primer kits which generates specific probes with high sensitivity. The simplicity of the protocol combined with the ease of obtaining reproducible results has led to RNA FISH to becoming a routine procedure in our lab. Fluorescent dye coupled nucleotides are incorporated directly using exonuclease free klenow to generate small probe fragments of less than 200 nucleotides in length. Longer probe fragments tend to self-associate and form aggregates during the hybridization reaction, which can cause significant background problems. The protocol below describes how to generate a cy3, cy5 or fluorescein (FITC) probe.

Random primer direct probe labeling

For probe generation it is important that high quality DNA is used. We routinely use Qiagen plasmid purification systems, which give sufficient yield and purity. We have also used isolated DNA fragments for the same protocol. Random priming of 1µg DNA prepared from plasmids, cosmids or BAC clones serves for at least 20 hybridizations. The probe concentration is adjusted after a titration on test slides to give a 2x probe. This allows simultaneous hybridization by mixing two probes (red and green) in a 1:1 ratio at 1x concentration for each probe. Probes used at too high concentrations may result in non-specific background. If the probe is diluted the sensitivity is also decreased. Therefore it is important to determine the optimal probe concentration for each batch of probe generated.

Incorporation of the probes into DNA fragments - cy3 or cy5 probe

This protocol is based on and uses the reagents from Prime-It II Random Primer Labeling Kit (Stratagene) and cy3 or cy5-dCTP from Amersham Biosciences.

1. Denature 1µg DNA in 23µl H_2O at 95°C for 5 minutes;
2. Add 10µl 9-mer random primer;
3. Denature the reaction mix at 95°C for 5 minutes;
4. Allow to cool on ice for 5 minutes;
5. Add 5µl dCTP buffer, 0.5µl Cy3 or Cy5-dCTP, 1µl (5U) exonuclease free klenow and 9.5µl H_2O;
6. Incubate at 37°C overnight (light protected).

Incorporation of the probes into DNA fragments - Fluorescein (FITC) probe

This protocol uses the reagents from Prime-It Fluor Fluorescence Labeling Kit (Stratagene).

1. Denature 1µg DNA in 29µl H_2O at 95°C for 5 minutes;
2. Add 10µl 9-mer random primer;
3. Denature the reaction mix at 95°C for 5 minutes;
4. Allow to cool on ice for 5 minutes.
5. Add 10µl reaction buffer to 1µl (5U) exonuclease free Klenow.
6. Incubate at 37°C overnight (light protected).

Post-labeling DNA processing and purification

1. Qiagen PCR clean up to get rid of unused oligonucleotides;
2. Add 20µl cot1DNA, 10µl ssDNA to compete for repetitive elements;
3. Precipitate the probe with 1/10 V 3 M NaOAc pH5.2, 2.5 V 100% ethanol at -80°C for 30 minutes;
4. Spin down pellet at 4°C for 20 minutes;
5. Wash pellet with 70% ethanol;
6. Resuspend pellet in 80µl hybridization mix.

The probe is now ready to be used. It can be stored at -20°C for at least a year.

Hybridization to RNA

1. Denature probe at 74°C for 10 minutes and let the probe anneal at 37°C for 30 minutes;
2. Meanwhile, dehydrate cells on slides through an ethanol series, 2 minutes each in 70%, 80%, 95%, 100% and then air dry;
3. Apply 5µl probe to the cells;
4. Place coverslip over probe and seal with rubber cement;
5. Incubate at 37°C overnight in a humidified chamber (light protected);
6. Float off coverslips with 4xSSC;.
7. Wash slides in the following sequence for 5 minutes at each step (unless otherwise stated) with gentle shaking:
    1. 2xSSC, 50% formamide at 39°C for 3 times;
    2. 2xSSC at 39°C for 3 times;
    3. 1xSSC at room temperature once for 10 minutes;
    4. 4xSSC at room temperature.
8. Counterstain cellular DNA with 5 microliter of 2.5mg/ml 4', 6-diamidino-2-phenylindole (DAPI) in 4xSSC, 0.1% TWEEN for 5 minutes at room temperature;
9. Wash with 4xSSC at room temperature for 5 minutes;
10. Mount coverslip with mounting media;
11. Seal the coverslip to the slide with nail polish;.
12. Slides may be visualized immediately or stored at 4°C.

Materials & Reagents

CSK buffer	100mM NaCl 300mM sucrose 3mM $MgCl_2$ 10mM PIPES pH 6.8 Store at 4°C.
hybridization mix	1 part 20xSSC 2 parts 10mg/ml BSA 2 parts 50% dextran sulfate (mix very well, store at -20°C) 5 parts formamide
reaction buffer	8µl of fluor-12 dUTP nucleotide 92µl of 5 x nucleotide buffer
CSK buffer + detergent	**CSK buffer** 0.5% Triton X-100 Store at 4°C.

1. Superfrost Plus slides - Sigma
2. Roboz slides - CellPoint Scientific, Gaithersburg, MD 20898-0757, order no: F107-HTC
3. 200mM vanadyl ribonucleoside complex (VRC, Invitrogen)
4. Paraformaldehyde extra pure - Merck 104005; 4% paraformaldehyde in PBS, pH 7.0 - made fresh from powder before each fixation.
5. Prime-IT ® II Random Primer Labeling kit - Strategene, 300385
6. Prime-It ® Fluor Fluorescence Labeling Kit - Strategene, 300380
7. Cy3-dCTP - Amersham Biosciences, PA53021, 25 nmol

8. Cy5-dCTP - Amersham Biosciences, PA55021, 25 nmol
9. Qiagen PCR Clean-up Kit
10. Cot1 DNA - Invitrogen
11. Salmon sperm DNA - Gibco
12. Formamide Fluka, 47470
13. Dextran sulphate - American Bioanlytical, Mg 500 000, ultra pure; to make 50% dextran sulphate in H_20: mix at 4°C overnight.
14. BSA 10mg/ml NEB
15. 20xSSC stock: 3M NaCl, 0.3 M sodium citrate pH 7.4
16. Vectashield ® Mounting Medium - Vector Laboratories, H-1000
17. 4',6-diamidino-2-phenylindole (DAPI) - Molecular probes D-1306
18. 10mg made up as a 2.5mg/ml solution in methanol

Figures

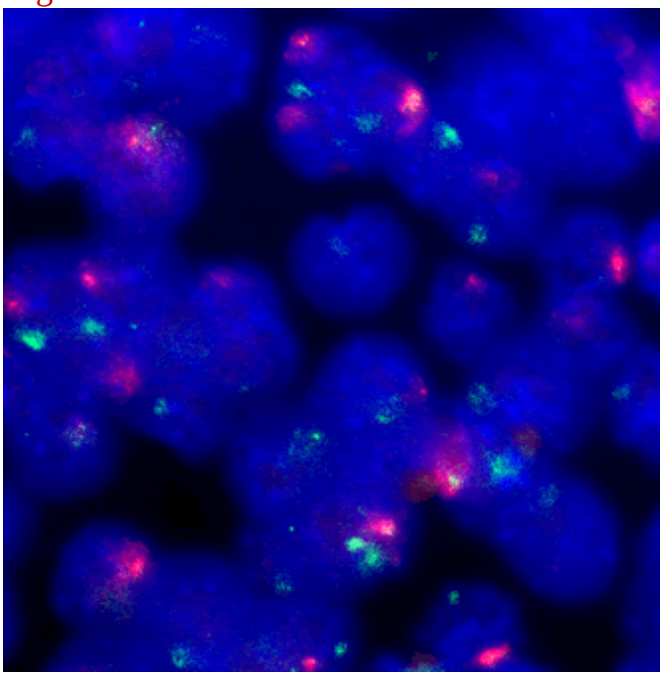

Figure 1. *Xist* RNA FISH in transgenic XXz mouse ES cells. The *in situ* hybridization is performed as described in the protocol. XXz cells express *Xist* RNA from 2 different loci. One expresses the full 17 kb *Xist* RNA, whilst the other site expresses a transgenic version, where the *Xist* 3' end is replaced by a LacZ sequence. Using DNA probes specific for the 3' end of *Xist* (Cy3, red) and LacZ (fluorescine, green), both *Xist*
transcripts can be visualized simultanously in the cells. DNA is stained in blue (DAPI).

References

1. Beatty B, Mai S, Squire J (eds.). (2002). FISH – A Practical Approach. Oxford University Press.