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Three-Dimensional Fluorescent In Situ Hybridisation in Mouse Embryos

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Abstract

A common problem in research laboratories that study the mammalian embryo is the limited supply of live material. For this reason, new methods are always developed and existing methods for cells in culture are adapted to suit this peculiar experimental model. Three-Dimensional fluorescent in situ hybridisation (3D-FISH) is an important tool to study how genomic sequences are positionned in nuclei without altering their 3D organisation. This provides vital information about the distribution of specific sequences in relation to nuclear substructures such as chromocenters and nucleoli. In this manuscript, we will present a detailed description on how to carry out 3D-FISH in the early preimplantation mouse embryo.

Keywords: 3D; FISH; in situ; embryo; mouse; development.

1. Introduction

Fluorescent in situ hybridisation (FISH) is a cytogenetic technique that provides researchers with a direct way to visualize and map the genetic material in individual cells, including specific genes or parts of specific chromosomes(1). This technique has become important in preimplantation genetic diagnosis (PGD). Often families with known or suspected genetic diseases want to know more about their putative child's conditions before proceeding with full term pregnancy. These concerns can be addressed by FISH to diagnose for a number of diseases such as Prader-Willi syndrome, Angelman syndrome, 22q13 deletion syndrome, chronic myelogenous leukemia, acute lymphoblastic leukemia, Cri-du-chat, Velocardiofacial syndrome, and Down syndrome. On the other hand, many researchers are now studying the non-random spatial distribution of chromosomes and genes within the nuclear space with 3D-FISH approaches as the organization of the genome inside the interphase nucleus seems to be a central determinant of genome functions and that knowing the sequence of a genome is insufficient to understand its physiological function (3). Using interphasic nuclei with preserved 3D shape, one can indeed determine the three-dimensional arrangements of chromosomes, providing detailed information about the compartmentalization of chromosomes into discrete territories (4). It is also possible to locate gene-rich versus gene-poor regions and evaluate the dynamic interactions of specific genomic sequences with other nuclear components (5). For instance, we recently demonstrated using the 3D_FISH method that the organisation of constitutive heterochromatin in the early mouse embryo is associated with their development to term (6).

According to the experimental design, probes used for FISH will bind only to parts of the chromosomes or to specific genomic sequences with which they show a high degree of sequence homology. In this chapter, continuous repetitions of short monomer probes will be used to study two repetitive sequences from the heterochromatic region of the nucleus, centromeric and pericentromeric repeats, also known as minor and major satellites. The probes will be directly tagged with different fluorochromes for simultaneous detection. Thereby, by using multiple probes labeled with different fluorochromes, scientists are able to label each chromosome in its own unique color (the result is known as a spectral karyotype) but also several genomic sequences at the same time, within the same nuclei (7). In all cases, fluorescence microscopy will be used to locate the FISH signal in the sample.

Unlike cells in culture, mouse embryos are surrounded by glycoprotein rich membrane known as the zona pellucida (8) and therefore they do not attach to the plates when cultured in vitro. Moreover, the nuclei of early pre-implantation embryos are larger than those of differentiated cells which makes it more difficult to preserve the three-dimensional structure during the procedure. In this chapter we will describe such multi-color FISH with centromeric and pericentromeric probes in mouse embryos, using both direct and indirect detection.

2. Materials

2.1 *Embryos Preparation*

1. M2 culture medium embryo-tested (Sigma-Aldrich, Saint-Quentin Fallavier, France). Store at 4 °C.

2. Phosphate Buffered Saline (PBS) tablets (Sigma-Aldrich, Saint-Quentin Fallavier, France) dissolved in distilled water according to manufacturer's instructions, autoclaved and then stored at 4 °C.
3. Glass dishes (Electron Microscopy Sciences, Euromedex, Mundolsheim, France)
4. Thin glass manipulation pipettes for embryos, usually home-made with glass capillaries or Pasteur pipettes.
5. Tyrode's solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) stored in single use aliquots of 1 ml at -20 °C.
6. Fixative: 20% paraformaldehyde (PFA) solution, EM grade (Electron Microscopy Sciences, Euromedex, Mundolsheim, France) with adjusted pH (~7.4) and stored at 4 °C with light protection (aluminium foil for example). Working solutions of 4% are prepared by dilution with PBS just before use.
7. SuperFrost Plus slides (VWR, Fontenay-sous-Bois, France).
8. Hydrophobic barrier pen (Sigma-Aldrich, Saint-Quentin Fallavier, France).

2.2 *In Situ Hybridization*

1. Phosphate Buffered Saline (PBS) tablets (Sigma-Aldrich, Saint-Quentin Fallavier, France) dissolved in distilled water according to manufacturer's instructions and autoclaved. Store at 4°C.
2. Permeabilization stock solution: 10% (w/v in distilled water) Triton X-100 (Sigma-Aldrich, Saint-Quentin Fallavier, France) solution. Store at 4 °C. The 0.5% working solution is prepared by dilution with PBS just before use.
3. 20X saline-sodium citrate (SSC) buffer (Sigma-Aldrich, Saint-Quentin Fallavier, France) containing 0.3 M sodium citrate and 3 M NaCl at a pH around 7.0. The working solutions of 0.1X and 2X SSC at a pH of 6.3 are prepared by diluting the 20X stock in PBS and lowering the pH with the appropriate volume of HCl.
4. Labelled probe: StarFISH pan-centromeric probe Cy3 conjugated (~200nt; Cambio, Cambridge, UK).
5. Unlabelled probe: consensus 80-nt forward sequence for murine minor satellites biotinylated at its 5' end (Invitrogen, Cergy-Pontoise, France) (9).
6. RNase A (Invitrogen, Cergy-Pontoise, France) stored at 1M in single use aliquots of 100 µl at -20°C.
7. Heating block set at 85°C (Grant, VWR, Fontenay-sous-Bois, France).
8. Deionized formamide (Molecular Biology Grade; Sigma-Aldrich, Saint-Quentin Fallavier, France)
9. Hybridization buffer: 50% formamide, 10% dextran sulphate (from 50% w/v stock solution), 1X Denhardt, 40 mM NaH₂PO₄ in 2X SSC, pH 7 (all products from Sigma-Aldrich, Saint-Quentin Fallavier, France). Store in single use aliquots of 1ml at -20 °C.
10. Hybridisation incubator with humidified loading tray for slides, set at 37 °C (Grant Boekel, VWR, Fontenay-sous-Bois, France).
11. Humidified non-transparent box in which you can keep the slides during the different incubation steps.
12. Detection compound for the unlabelled probe: Streptavidin conjugated with Alexa 633 (Molecular Probes, Invitrogen, Cergy-Pontoise, France)
13. Nuclear counterstain: 10 µM Yo-Pro-1 (Molecular Probes, Invitrogen, Cergy-Pontoise, France).
14. Mounting medium: Citifluor (Biovalley, Marne-la-Vallée, France).

15. Glass cover slips 22 x 40 mm (CML, Nemours, France)

3. Methods

Preimplantation embryos can be collected *in vivo* just before 3D-FISH or cultured according to standard protocols. However, embryos are known to be very sensitive to culture conditions that can affect development (e.g. cleavage rates, fragmentation and quality of post-implantation) and might alter chromosome/gene positioning. We therefore advise to prepare all materials and equipments in advance and perform mounting of the embryos on slides as quickly as possible.

3.1 Mounting the Embryos on Slides

1. Label the slide with name and date, and draw one, maximum two, squares (1 x 1 cm) on your slide with the hydrophobic pen (see **Note 1**). Each square should be allocated to one group of embryos (with 10 to 20 embryos/group) therefore several slides might be required.
2. Prepare culture plates with microdrops (25-50 μ l) of M2 solution to rinse the embryos, and keep at room temperature in open air to equilibrate.
3. Defrost on the bench one aliquot of Tyrode's solution per group of embryos (see **Note 2**).
4. The first step is to denude the embryo by removing the zona pellucida in order to improve access of the probes within the embryos (see **Note 3**). Take the first group of embryos to be processed and transfer it from the collection / culture medium into the M2 medium. Load the manipulation pipette with fresh Tyrode's solution, and then transfer embryos into a glass dish containing 500 μ l of Tyrode's solution. Immediately reload the manipulation pipette with fresh Tyrode and then transfer embryos into a second glass dish containing another 500 μ l of Tyrode's solution. Incubate the embryos for no more than 1.5 minutes (see **Note 4, 5**).
5. Quickly rinse the embryos in multiple drops of the pre-equilibrated M2 and place them inside the drawn square on the glass slide.
6. Aspirate slowly excess M2 medium in order to fix the embryos onto the glass slide (see **Note 6**).
7. As soon as the embryos are fixed and M2 removed, add 10-15 μ l of 4 % PFA
8. Keep slide(s) in a humidified box at 4°C, overnight (see **Note 7**).

3.3 Hybridization with the Probes

1. On day 2, gently aspirate the PFA and replace with PBS for 30 minutes to rinse the embryos.
2. Permeabilise the embryos for 30 minutes with 0.5 % Triton X-100 (diluted in PBS just before use).
3. Rinse embryos in PBS then in 2x SSC for 5 minutes each time.
4. To decrease unspecific background, RNAs are digested by incubation in 200 μ M of RNase diluted in 2x SSC (pH 6.3) for 30 minutes at 37 °C.
5. Rinse embryos in 2x SSC (pH 6.3) then in PBS for 5 minutes each time.
6. Equilibrate in hybridization buffer for 1-2 hr (see **Note 8**).
7. In a 0.5 ml tube, mix 1 μ l of each of the probe solutions with 12 μ l of hybridization buffer and denature at 85 °C for 10 minutes, and transfer the tube into ice straight after.
8. At the same time, put your glass slides with the embryos (still in the hybridization buffer) on the heating block to denature the DNA at 85 °C for 10 minutes (see **Note 9**).

9. Replace the buffer on the embryos with the hybridization mix and place the slides in the humidified loading tray of the hybridization incubator at 37 °C for 24 hr (see **Note 10**).
10. On day 3, several washing steps are required to remove the remaining unbound probes and decrease the unspecific background. First, rinse embryos in pre-warmed (37 °C) 2x SSC for 3x 5 minutes at 37 °C. Then in pre-warmed (60 °C) 0.1x SSC for 3x 5 minutes at 60 °C.
11. Further rinse the embryos at RT in 0.1x SSC then in PBS for 5 minutes each time.
12. The DNA is counterstained with 10 μ M of Yo-Pro-1 in PBS for 15 minutes (see **Note 11**).
13. Rinse the embryos for 2x 5 minutes and post-fix with 2 % PFA for another 5 minutes (post-fixation helps maintaining the fluorescent signal until observation).
14. After removal of the PFA, slowly add one drop of mounting medium, and gently place the coverslip.
15. Seal the borders of the coverslip with nail varnish and keep slide at 4 °C in the dark until observation with a microscope (see **Note 12**).
16. An example of the results produced is shown in **Fig. 1** (see **Note 13**).

4. Notes

1. Due to the nature of the solutions that are used in this method and that can easily dilute / erase ink (even permanent one), a diamond pencil is usually used to label the slides.
2. Do not use Tyrode's solution more than 15 minutes after defrosting as it loses its activity and becomes ineffective on embryos.
3. When dealing with several groups, we advise to carry out the denudation process for one group at a time as it is a very critical step in this experiment. An extended incubation with the acid will damage the embryos and their nuclei, while a short incubation time might not be enough to remove the zona pellucida completely.
4. This step and all subsequent ones are carried out at room temperature (RT) unless otherwise stated.
5. Under the dissection microscope, you can observe the zona pellucida being degraded by the acid activity of the Tyrode's solution.
6. Even though this step is carried out at room temperature, the light from the microscope produces enough heat to dehydrate the embryos. This must be avoided to preserve the 3D structure of the nuclei. Rehydration can be accomplished by adding M2 medium if the embryos are not yet fixed on the slide .
7. Alternatively, incubate in 4 % PFA for 30 minutes at room temperature and proceed to the next step.

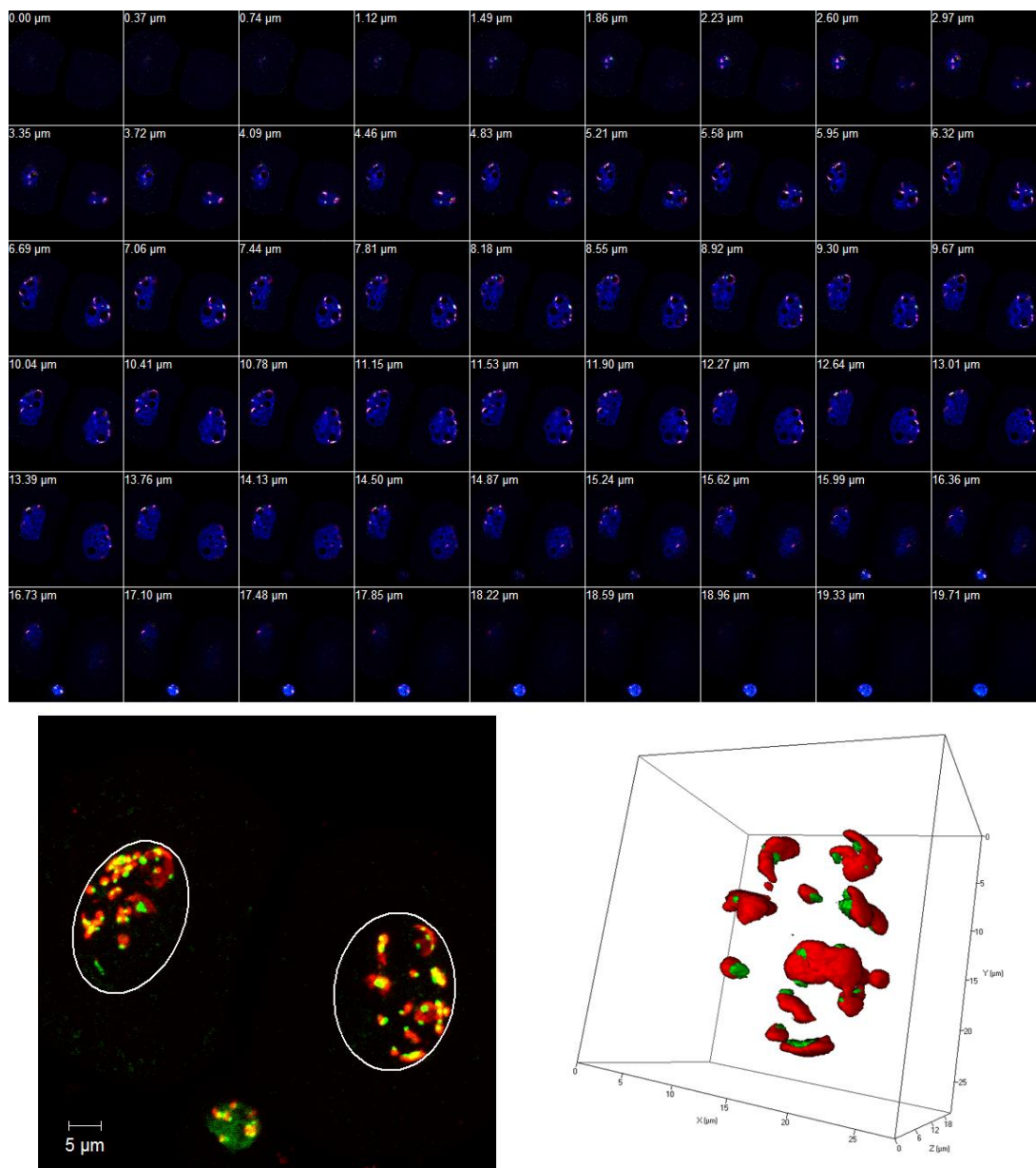


Fig. 1. 3D-FISH on a two-cell stage mouse embryo with the pan-centromeric and centromeric probes counterstained with Yo-Pro-1. The z-series (upper panel, z-step of $0.37\mu\text{m}$) was acquired on a Zeiss LSM510 confocal with a plan-apochromat 63x/1.4 oil DIC objective at 488, 543 and 633 nm wavelengths sequentially. Blue color was assigned to DNA, red to the pan-centromeric signal and green to the centromeric one to allow better signal vs. background contrast for the biologist. With the LSM browser software the z-series can be merged to give a single image with the two probes (lower panel, left) on which each nuclei is underlined by a white circle. 3D reconstruction of one nucleus from this embryo (lower panel, right) clearly shows the conserved shape of the nucleus and the so-called « chromocenters » formed by aggregations of major (pan-centromeric probe) and minor (centromeric probe) satellites.

8. It is recommended to switch-on the heating block when equilibration starts in order to reach the desired temperature for the subsequent steps.
9. To prevent the microdrops from evaporating during that step filter paper humidified with formamide 50% should be placed in the bottom of the tray.

10. All subsequent steps are carried out in a non-transparent humidified chamber.
11. Other nuclear counterstain, such as propidium iodide, can be used according to the choice of fluorochromes used to label the probes.
12. Confocal or grid microscopy is necessary for three-dimensional imaging. The optimal parameters to obtain the best 3D reconstruction is to perform Z-sectionning at no more than 0.5 μm between sections and to take 2-3 additional sections on top and below the limits of the nucleus.
13. If the fluorescent signal is too weak, amplification of the signal may be necessary. Secondary components such as fluorescently-tagged secondary antibodies can be used to provide a more pronounced signal.

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