Chapter 2

Fluorescence In Situ Hybridization on DNA Halo Preparations and Extended Chromatin Fibres

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Abstract

Although many fluorescence in situ hybridisation (FISH) protocols involve the use of intact, fixed nuclei, the resolution achieved is not always sufficient, especially for physical mapping. In light of this, several techniques are commonly used to create extended chromatin fibres or extruded loops of DNA. As a result, it is possible to visualise and distinguish regions of the genome at a resolution higher than that attained with conventional preparations for FISH. Such methodologies include fibre-FISH and the DNA halo preparation. While fibre-FISH involves the stretching of chromatin fibres across a glass slide, the DNA halo preparation is somewhat more complex; whereby DNA loops instead of chromatin fibres are generated from interphase nuclei. Furthermore, the DNA halo preparation coupled with FISH is a useful tool for examining interactions between the inextractable nuclear matrix and the cell’s genome.

In this chapter, we describe how to successfully generate extended chromatin fibres and extruded DNA loops. We will also provide detailed methodologies for coupling either procedure with two distinct FISH procedures; 2D-FISH, which allows for the visualisation of specific chromosomal regions, while telomere peptide nucleic acid (PNA) FISH, enables the detection of all telomeres present within human nuclei.

Key words: 2D FISH, DNA halo preparation, Extended chromatin fibres, Extruded DNA loops, Fibre-FISH, Nuclear matrix, Telomere PNA FISH, Telomeres

1. Introduction

Examining extended chromatin/DNA fibres can be achieved by several procedures: fibre-FISH, the DNA halo preparation, and molecular combing. In this chapter, the latter will not be discussed due to its complexity and its many methodological variations. The release of extended chromatin from interphase nuclei, was first described in 1992 by Heng and co-authors (1), with the possibility of resolving sequences as little as 10 kb apart (2).
Although this group was the first to publish, other laboratories were also utilising similar methodologies at the time (3–5).

In 1993, Parra and Windle described a DNA mapping procedure that involved performing fluorescence in situ hybridisation (FISH) on DNA stretched along a glass microscope slide. This methodology improved resolution by producing DNA that was extended beyond relaxed linear DNA (6). This was achieved by lysing cells with a detergent and allowing the resulting DNA in solution to be stretched by travelling down the microscope slide under gravitational pull. Although this is a relatively straightforward way of producing extended chromatin fibres, the DNA halo preparation can also be used in high resolution mapping (7, 8); however, it is a more time-consuming and less physiological method. The preparation of DNA halos generates elongated extruded strands of linearised DNA as nucleosomes in a halo around the extracted residual interphase nucleus (Fig. 1). As a result, it can be used for physical mapping. However, one of the main problems with employing DNA halo preparations for mapping is that certain regions of DNA remain condensed within the residual nuclei and are thus, inaccessible (9). This DNA is attached to the insoluble nuclear architecture remaining within the residual nucleus, namely the nuclear matrix. These regions of inaccessible DNA may be the regions of interest for the researcher and so preclude DNA halo preparations from being used in mapping. However, this situation has led serendipitously to DNA halos being a platform to study genome interactions with the

Fig. 1. Nucleus following the DNA Halo preparation and counterstained with DAPI. Scale bar = 10 µm.
nuclear matrix (10). The nuclear matrix is a permanent network of core filaments underlying thicker fibres, which run throughout the nucleus (11). The structure is found to interact with the genome via sequences termed matrix attachment regions (MARs) (12). Research shows that whole chromosome and more specifically, telomere, organization is mediated by the nuclear matrix (e.g. (9, 13–15)). The DNA halo procedure, a derivative of a method described by Berezney and Coffey in 1974, involves the removal of all soluble proteins so that only the nuclear matrix, nuclear matrix-associated proteins and chromosomes, remain intact. Since the methodology contains pre-fixation permeabilization, followed by high-salt extraction steps, any DNA not bound to this nuclear structure creates a “halo” of DNA surrounding the residual nucleus. As a result, it can be assumed that regions of DNA found within the residual nucleus have tight associations with the nuclear matrix, while those in the DNA halo have looser attachments.

Both DNA halo preparation and fibre FISH protocols can be coupled with either two-dimensional fluorescence in situ hybridisation (2D-FISH) or telomere PNA (peptide nucleic acid) FISH, in order to visualise specific regions of the genome. While 2D-FISH allows the detection of specific chromosomes, telomere PNA FISH theoretically paints all telomeres present in the human nucleus.

2. Materials

2.1. DNA Halo Preparation

2.1.1. Cell Culture

1. Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco/BRL) supplemented with either 15% (v/v) foetal bovine serum (FBS) or 10% (v/v) newborn calf serum (NCS), depending on the cell line. The medium also needs to contain 2% (v/v) penicillin/streptomycin and 1% (v/v) L-glutamine.

2. Versene (phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; pH 7.4) with 0.2% (w/v) ethylenediaminetetraacetic acid; EDTA) kept refrigerated at 4°C, pre-warmed to 37°C.

3. 0.25% trypsin produced from a 2.5% stock solution (Gibco/BRL), which has been diluted (1:10 v/v) in Versene.

4. Haemocytometer.


6. 10% (v/v) HCl.

7. QuadriPERM chambers (Greiner Bio One) for growing cells directly upon glass microscope slides.
2.1.2. DNA Halo Preparation

1. CSK buffer (made up in ddH₂O): 100 mM NaCl, 3 mM MgCl₂, 0.3 M Sucrose, 10 mM 1,4-Piperazinediethanesulfonic acid (PIPES; pH 7.8), 0.5% (v/v) Triton X-100. Store at 4°C or on ice.

2. 10× phosphate buffered saline (10× PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄; pH 7.4. Dilute accordingly for 1×, 2× and 5× PBS.

3. Extraction buffer (made up in ddH₂O): 2 M NaCl, 10 mM PIPES (pH 6.8), 10 mM EDTA, 0.05 mM (v/v) spermine (Sigma Aldrich), 0.125 mM (v/v) spermidine (Sigma Aldrich), 0.1% (w/v) digitonin (Sigma Aldrich; see Notes 1 and 2).

4. Ethanol series comprising of 10, 30, 70 and 95% (v/v) ethanol. Store at room temperature.

5. Glass Coplin jars.

2.2. Slide Preparation for Fibre-FISH

2.2.1. Cell Culture

1. As per Subheadings 2.1.1 and 2.1.2.

2. Plastic cell culture flasks/dishes.


4. Phosphate buffered saline (PBS, 1×).

2.2.2. Slide Preparation

1. SuperFrost™ microscope slides.

2. Lysing buffer: 0.5% (w/v) sodium dodecyl sulphate (SDS), 5 mM EDTA, 100 mM Tris–HCl (pH 7).


4. Ethanol row comprising of 70, 90 and 100% (v/v) ethanol at room temperature.

2.3. Two-Dimensional Fluorescence In Situ Hybridisation

2.3.1. Slide Denaturation

1. 20× saline sodium citrate (SSC): 3 M NaCl, 0.3 M tri-sodium citrate; pH 7.0. This buffer can then be diluted as necessary.

2. 70% formamide: 70% (v/v) formamide, 2× SSC; pH 7.0. See Note 3 for safety instructions.

3. Ethanol series comprising of 70, 90 and 100% (v/v) ethanol at room temperature.


5. Warming plate.

6. Oven that can bake slides at 70°C.

2.3.2. Probe Denaturation and Hybridisation

1. Directly labelled total human chromosome DNA probes (Cambio or see Chapter 1 for how to make DOP-PCR chromosome painting probes).

2. Rubber cement (Halfords).

3. Humid hybridization chamber; ensure that this is moistened so that the slides hybridizing do not dry out (see Note 4).

4. Buffer A: 50% (v/v) formamide, 2× SSC; pH 7.0.
5. Buffer B: 0.1× SSC; pH 7.0.
6. 4× SSC.
7. Vectashield self-sealing mountant containing 4',6-diamidino-2-phenylindole (DAPI), (Vectorlaboratories; see Note 5).
8. Glass Coplin jars.
9. Glass Coverslips: 22×22 mm and 50×22 mm.

**2.4. Telomere PNA FISH**

1. Telomere PNA FISH kit/FITC, available from Dako (see Note 6). Included in this kit is a PNA telomere probe which contains formamide. See Note 3 for advice regarding work with formamide.
2. Glass Coplin jars.
3. 3.7% (v/v) formaldehyde (see Note 7).
4. Ethanol series comprising of 70, 85 and 96% ethanol. Store at 4°C.
5. Glass Coverslips: 22×22 mm and 50×22 mm.
7. Oven for baking slides.

**3. Methods**

As mentioned in the introduction, both the DNA halo preparation and fibre FISH can be combined with either 2D FISH or telomere PNA FISH. Therefore, this section has been divided into four parts; with the first two, Subheadings 3.1 and 3.2, detailing distinct methodologies for producing extended DNA loops and chromatin fibres (DNA halo preparation and fibre FISH, respectively), while Subheadings 3.3 and 3.4 explain the methodology involved in performing two different FISH procedures (2D FISH and telomere PNA-FISH).

**3.1. DNA Halo Preparation**

**3.1.1. Cell Culture**

1. SuperFrost slides should be thoroughly cleaned in 10% (v/v) HCl for an hour; slides are dropped individually into the acid. Following this, the slides are washed in tap water ten times and then in double distilled water, ten times. Next, the slides are rinsed in methanol twice and then, remain in methanol until flaming. Once the flaming is complete, the slide is placed in the QuadriPERM chamber (see Note 8).
2. The respective cells are harvested and then, after using the haemocytometer to determine cell density, 1×10⁵ cells are seeded per slide.
3. Cells are grown for at least 48 h at 37°C in 5% CO₂ (see Note 9).
Once the CSK buffer is made, it is placed on ice or refrigerated at 4°C (see Note 10). Following this, the QuadriPERM chambers, containing the cells are removed from the incubator; the media is then discarded and the slides are labelled accordingly, using a pencil. The slides are placed in an ice-cold Coplin jar, containing CSK buffer and are left on ice for a period of 15 min. This whole process is performed as quickly as possible.

After this incubation, the CSK buffer is thrown away and the slides are rinsed in 1× PBS, three times (straight-in, straight-out).

The slides are then placed in a fresh Coplin jar containing extraction buffer for an incubation time of 4 min at room temperature (see Note 11).

Using the same Coplin jar, slides are rinsed consecutively in 10×, 5×, 2× and 1× PBS (for 1 min each).

Slides are taken through an ethanol series comprising of 10, 30, 70 and 95% ethanol (consecutively; straight-in, straight-out).

Cells are placed in the same medium described in item 1, Subheading 2.1.1. However, the cells are grown in flasks or dishes (not in QuadriPERM chambers, as per Subheading 3.1), at 37°C in 5% CO₂, for 2–4 days (depending on the cell line used). At this point, the cells are harvested (using solutions detailed in Subheadings 2.1.1 and 2.1.2).

1. Harvest and then calculate the number of cells using a haemocytometer.
2. Resuspend the cell pellet in 1× PBS (40,000 cells/mL).
3. Using a pipette, place 2 µL from the cell suspension onto one end of the microscope slide. Then, allow the cell suspension to air-dry.
4. Following this, add 30 µL of lysing buffer to the cell area and incubate for 7 min at room temperature.
5. The slide is tilted to a 30° angle; at this point, the chromatin released by cell lysis, will slowly move down the slide.
6. The slide is then air-dried and fixed in ice-cold fixative (methanol: acetic acid; 3:1 v/v) for 2 min.
7. After allowing the slide to air-dry once again, it is taken through an ethanol row (70, 80 and 100%), 5 min in each solution.
8. The slide is then air-dried before it is baked for 1 h at 70°C. The slide is now ready for hybridisation.
1. Pre-warm waterbaths to 70°C and 37°C. Previously prepared formamide solution is heated to 70°C.

2. Take slides through a 70, 90, 100% ethanol series; 5 min in each solution. Following this, the slides are air-dried using a warming plate and then, baked in an oven at 70°C for 5 min.

3. For denaturation, the slides are placed in 70% formamide for 2 min at 70°C.

4. Next, the slides are taken through the same ethanol series as before, however, with one exception; the 70% ethanol is ice cold. They are then dried on the warming plate.

1. Probes are denatured at 75°C for 10 min in a waterbath or on a heat-block.

2. Probes are heated at 37°C for 30 min; following this, 10 µL of whole chromosome probe is added to the appropriate slide, covered by a coverslip secured using rubber glue.

3. Next, the slides are left at 37°C, in a humid hybridization chamber for at least 18 h.

4. After this incubation period, slides are removed from the hybridization chamber and the rubber glue is carefully removed using forceps.

5. The slides are then washed in buffer A, pre-warmed to 45°C, for 15 min, with three changes of buffer.

6. Subsequently, the same procedure is performed with buffer B but instead the buffer is incubated at 60°C.

7. The slides are then plunged into 4× SSC at room temperature.

8. At this point, slides can be mounted in Vectashield mountant containing DAPI. They are now ready for visualisation.

1. Pre-heat the oven to 80°C. Also, place a Coplin jar containing Wash solution in a waterbath and allow it to heat to 65°C. Ideally, this should be done a couple of hours before step 3.

2. Fill four Coplin jars with TBS and label TBS1–TBS4 accordingly. In addition, pour 100 mL of Rinse solution into a Coplin jar. These steps should be performed an hour before step 3 in order to allow the solutions to warm to room temperature.

3. Place the slides in TBS1 for approximately 2 min.

4. Following this, the slides are removed and placed in a Coplin jar containing 3.7% formaldehyde for 2 min exactly. This is performed in the fume hood.
5. The slides are washed twice; once in TBS1 for 5 min and then in TBS2 for 5 min.
6. The slides are incubated for 10 min in a Coplin jar containing Pre-Treatment solution.
7. Again, the slides are washed in TBS3 and TBS4, for 5 min each. They are then taken through an ice cold ethanol series (70, 85 and 96%) for 2 min per concentration.
8. Air-dry the slides.
9. When dry, add 10 µL of PNA probe onto each slide and then place a coverslip on top. Incubate at 80°C for 5 min (see Note 12).
10. Remove the slide(s) from the oven and incubate in the dark at room temperature for 40 min at least (see Note 13).
11. Following this incubation period, the slides are placed in Rinse solution for 1 min at room temperature.
12. Slides are then incubated in the Wash solution for 5 min at a temperature of 65°C (see Note 14).
13. Next, the slides go through an ice cold ethanol series (70, 85 and 96%) for 2 min per concentration. After this, they are air-dried vertically and then mounted in Vectashield DAPI mountant.
14. The slides are now ready for visualisation (Fig. 2) and are routinely stored at 4°C (see Note 15).

Fig. 2. Nucleus following the DNA halo preparation and Telomere-PNA FISH. Nucleus is counterstained with DAPI (light grey) while telomeres are detected in FITC (bright punctate foci). Scale bar = 10 µm.
4. Notes

1. Spermine, spermidine, and digitonin are added to the extraction buffer at the end of preparation; by doing this, their biological activity is preserved.

2. When weighing-out digitonin, great care must be taken since it is extremely dangerous; a lab coat, mask, doubled gloves, and safety glasses should be worn. The powder is dissolved separately using heat (60–70°C) and then added to the extraction buffer once cooled. Store at room temperature.

3. Care must be taken when using formamide; if women suspect that they might be or could be pregnant, they should avoid using formamide since it is a teratogen. All applications using formamide should be performed in a fume hood.

4. We construct humid chambers out of sandwich boxes that are covered in foil to exclude light. The floor of the box has three to four layers of tissue that is moistened during a hybridization reaction. We cut plastic pipettes to size and create a platform for the hybridizing slides to rest upon. The chambers will float in a waterbath or can be placed in an oven.

5. DAPI is a DNA intercalator dye and so care should be taken when using – wear gloves when mounting slides.

6. Telomere PNA FISH kit/FITC, available from Dako. This kit is also available in Cy3. These kits provide the majority of reagents required for the procedure. Included is a PNA telomere probe which hybridises to repetitive sequences within human telomeres. Theoretically, all 184 human telomeres should be detected with this assay. Furthermore, signal intensity correlates with telomere length. In addition, each kit provides five foil packages, each of which contains the required components to make 1 L of Tris-Buffered Saline (TBS) pH 7.5 in distilled water. This solution needs to be stored at 4°C. Also included is a Pre-Treatment solution (composed of proteinase K), which needs to be diluted 1:2,000 in TBS. The manufacturers suggest adding 40 µL of Pre-Treatment solution to 80 mL of TBS. This needs to be diluted freshly before each experiment. Concentrated Rinse and Wash solutions are also provided in the kit; the 20 mL bottles for each need to be diluted (1:50) in 980 mL of distilled water. Once diluted, these are kept at 4°C until use and are stable for 1.5 years.

7. Use gloves, safety glasses and a fume hood when using formaldehyde. Do not ingest, inhale, or spill on skin. If this happens, rinse off with copious amounts of water.
8. Flaming slides is a great way to obtain sterile slides immediately prior to use. However, there are hazards associated with this method. Have the QuadriPERM dish close to the beaker containing the slides in methanol; the Bunsen burner should be positioned slightly further away. Use long forceps but make sure that they grip the slides well. Do not have the methanol too deep, just covering the slides is best and then the methanol is not a long way down the forceps when they are dipped into the beaker. Use a large beaker. Always ensure the methanol has evaporated from the forceps and that the forceps have cooled slightly before placing them back into the beaker and the methanol. The beaker should be covered by a piece of aluminium foil; this will starve oxygen from any methanol that catches fire. DO NOT flame slides in any kind of hood where the air is circulated.

9. Ensure that the cell density on the slides is not too high; if the cells are too confluent, then loops of DNA from different nuclei will cross over each other, making the analysis difficult and/or inaccurate. You should aim for 60–70% confluence of cells. If early passage human dermal fibroblasts are to be used and have been set up at a density of $1 \times 10^3 / \text{cm}^2$, then 1–2 days growth should be sufficient to obtain 60–70% confluence. Other cells, such as transformed cells may achieve this level of confluency earlier and primary cells with increased numbers of senescent cells may take longer.

10. Both the CSK and extraction buffers are made freshly on the day of use.

11. Ensure that the extraction buffer is at room temperature before use with an accurate thermometer.

12. This temperature should be a minimum of 80°C and a maximum of 90°C. Temperatures below 75°C will significantly impair the FISH signals.

13. The kit’s manufacturers, DakoCytomation, suggest that slides can be left for 30 min – 4 h.

14. The manufacturers state that the washing conditions must not deviate from this temperature by more than ±3°C. The temperature must be monitored throughout using an accurate thermometer.

15. Once the slides have been mounted in DAPI, the nuclei can be visualised using a fluorescent microscope. The telomeres can be seen as small fluorescent spots within the interphase nucleus. Theoretically, 184 telomere signals should be present; however, in practice, it is extremely rare to record this number. Furthermore, the signal intensity is correlated with the length of the telomere; as a result, the kit can be used to
measure and compare the telomere lengths of different cell lines. When coupled with the DNA halo preparation, telomere signals should be detected both within the residual nucleus as well as in the surrounding DNA halo.

References

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