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Abstract	The genome has a special relationship with the nuclear envelope in cells. Much of the genome is anchored at the nuclear periphery, tethered by chromatin binding proteins such nuclear lamins and other integral membrane proteins. Even though there are global assays such as DAM-ID or ChIP to assess what parts of the genome are associated with the nuclear envelope, it is also essential to be able to visualize regions of the genome in order to reveal their individual relationships with nuclear structures in single cells. This is executed by fluorescence in situ hybridization (FISH) in 2-dimensional flattened nuclei (2D-FISH) or 3-dimensionally preserved cells (3D-FISH) in combination with indirect immunofluorescence to reveal structural proteins. This chapter explains the protocols for 2D- and 3D-FISH in combination with indirect immunofluorescence and discusses options for image capture and analysis. Due to the nuclear envelope proteins being part of the non-extractable nucleoskeleton, we also describe how to prepare DNA halos through salt extraction and how they can be used to study genome behavior and association when combined with 2D-FISH.

Keywords (separated by " - ")	Fluorescence in situ hybridization - 2D-FISH - 3D-FISH - Genome organization - Chromosome territories - Gene positioning - Nuclear envelope - Nuclear lamins

Chapter 24

Visualizing the Spatial Relationship of the Genome with the Nuclear Envelope Using Fluorescence In Situ Hybridization

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Abstract

The genome has a special relationship with the nuclear envelope in cells. Much of the genome is anchored at the nuclear periphery, tethered by chromatin binding proteins such nuclear lamins and other integral membrane proteins. Even though there are global assays such as DAM-ID or ChIP to assess what parts of the genome are associated with the nuclear envelope, it is also essential to be able to visualize regions of the genome in order to reveal their individual relationships with nuclear structures in single cells. This is executed by fluorescence in situ hybridization (FISH) in 2-dimensional flattened nuclei (2D-FISH) or 3-dimensionally preserved cells (3D-FISH) in combination with indirect immunofluorescence to reveal structural proteins. This chapter explains the protocols for 2D- and 3D-FISH in combination with indirect immunofluorescence and discusses options for image capture and analysis. Due to the nuclear envelope proteins being part of the non-extractable nucleoskeleton, we also describe how to prepare DNA halos through salt extraction and how they can be used to study genome behavior and association when combined with 2D-FISH.

Key words Fluorescence in situ hybridization, 2D-FISH, 3D-FISH, Genome organization, Chromosome territories, Gene positioning, Nuclear envelope, Nuclear lamins

1 Introduction

Chromosomes and genes are spatially organized within interphase nuclei, interacting with proteinaceous nuclear structures which anchor and tether chromatin [1, 2]. Global methodologies, such as DAM-ID, utilizing the ease of sequencing DNA, can assess the spatial relationship of the whole genome with specific nuclear structures such as nuclear lamins [3–5]. These analyses bring useful information to the field, however as studies are data-rich and expensive, they might be excluded as a method of choice if a large number of samples are required (e.g., steps in a differentiation pathway or large numbers of patient samples).

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Furthermore, using cells in mixed populations will result in averaged information and the genome, for example, can be positioned differently in proliferating versus non-proliferating cells, e.g., in a primary cell culture [6]. Real-time imaging in live cells [7, 8] can also be an informative approach to visualize genome interaction with nuclear structures; however, this may present challenges [9]. Therefore, despite global and real-time analyses, there is a necessity to analyze individual cells by fluorescence in situ hybridization (FISH) to reveal specific regions of interest in the genome, and analyze their spatial relationship to specific nuclear structures such as the nuclear envelope [10, 11].

FISH is a cytogenetic technique that utilizes labeled DNA or RNA probes to hybridize to specific DNA sequences on chromosomes, or to RNA [12, 13]. It is possible to delineate whole chromosomes, chromosome arms, chromosome bands, gene loci, specific regions of genes or RNA transcripts [14], and analyze their spatial relationship with other FISH signals or nuclear landmarks such as the geometric center of nuclei, nucleoli, or the nuclear envelope. The nuclear envelope can be revealed by indirect immunofluorescence using antibodies [15–17], but the FISH denaturation process can negatively affect nuclear envelope antigens, especially A-type lamins. The use of formamide for DNA denaturation can also destroy the signal from fluorescently tagged proteins but antibodies that recognize the tag itself can be used [18]. Indirect immunofluorescence can be done prior to the FISH, by fixing the antigen–antibody complexes in place with paraformaldehyde; in this scenario, the nuclear envelope can still be revealed with specific antibodies [18–22]. However, the simplest way of revealing the nuclear edge is by using a DNA stain and where it ends is where the edge of the nucleus is; this is however as long as nuclei have maintained their integrity and shape during the procedure [23–26]. Use of phase contrast microscopy or lipophilic dyes can be useful, in cases where the nuclear envelope is compromised, to reveal the nuclear membranes. Abnormal nuclear structures such as blebs can create issues when defining a nuclear edge since they could be defined as an extension of the nuclear envelope or as a separate entity [27].

For 2D-FISH, whereby nuclei are flattened, a standard epifluorescence microscope with a cooled charge coupled device camera will suffice for visualization and imaging of FISH signals. However, for 3D-FISH, microscopy requires optical sections through the z-axis, either using a confocal laser scanning microscope or other microscope systems with a motorized stage or piezo-driven objectives. The 3D datasets then need to be processed through deconvolution packages to remove out of focus fluorescence. There are new opportunities to visualize FISH signals within interphase nuclei using super-resolution microscopy such as 3D-Structural Illumination Microscopy (3D-SIM) [16] or

high-throughput flow imaging [28, 29]. The high power lenses that can be added to imaging flow machines, combined with the extended depth of field, enable software to be developed to perform positional analyses of chromosomes and genes in millions of individual interphase nuclei for one sample in 3D. Individual cells can then be sub-categorized into for example proliferating or not, differentiated or not, transgenic or not, by co-staining with specific markers. A further advancement is in high-throughput / high-resolution imaging, an approach called "Deep Imaging": there, individual cells are imaged at high resolution by automated software and FISH signals positioned by automated analysis software built into the system [30, 32].

Analyzing the position of genes, sub-chromosomal regions or whole chromosomes within interphase nuclei requires the capability to be able to perform measurements and approaches that place FISH signals into specific regions of the nucleus. In 3D, this is usually done using the reconstructed stack of optical sections or a gallery of the 2D optical sections to measure to the nearest point at the nuclear edge in any of the x, y, z axes, or by taking the measurement to the geometric center of the nucleus and expressing this a percentage of the radius. 3D radial analysis packages have been developed [17, 31, 32] to perform measurements between nuclear landmarks and FISH signals, or between FISH signals [33, 34]. Freely available software to position genes and chromosomes includes Smart 3D-FISH [35] and NEMO [36]. However, many laboratories use 2D preparations to perform their spatial positioning analyses since similar nuclear positions for genes and chromosomes are found consistently in 3D, confirming that positional analyses can be performed in 2D [37]. In order to position FISH signals in flattened nuclei different groups have developed their own positional analysis scripts similar to the original created by Paul Perry, MRC Human Genetics Unit, Edinburgh [38], whereby the nucleus is segmented into 5 shells of equal area and the intensity of the FISH signal and DNA signal from the DNA dye DAPI are measured [38–40]. The DNA signal intensity is used to normalize the FISH signal data. Others do not perform any normalization for DNA content across the flattened nuclei but use specific measurements to nuclear landmarks such as the nuclear edge and the nuclear center [41]. With such 2D studies, it is imperative that the data be comparable between samples since they do not give absolute position but an averaged probabilistic position.

By using FISH and delineation of the nuclear envelope or other nuclear structures, it is possible to determine which parts of the genome are colocalized or spatially close to nuclear structures such as the nuclear lamina, nuclear pores and other nuclear components. This chapter outlines methods for 2D- and 3D-FISH in combination with indirect immunofluorescence, and discusses the

value of 2D vs 3D analyses. We also include methods for the DNA halo extraction protocol combined with 2D-FISH, making it possible to gather data about which regions of the genome are embedded or attached to insoluble nuclear structures (Fig. 1).

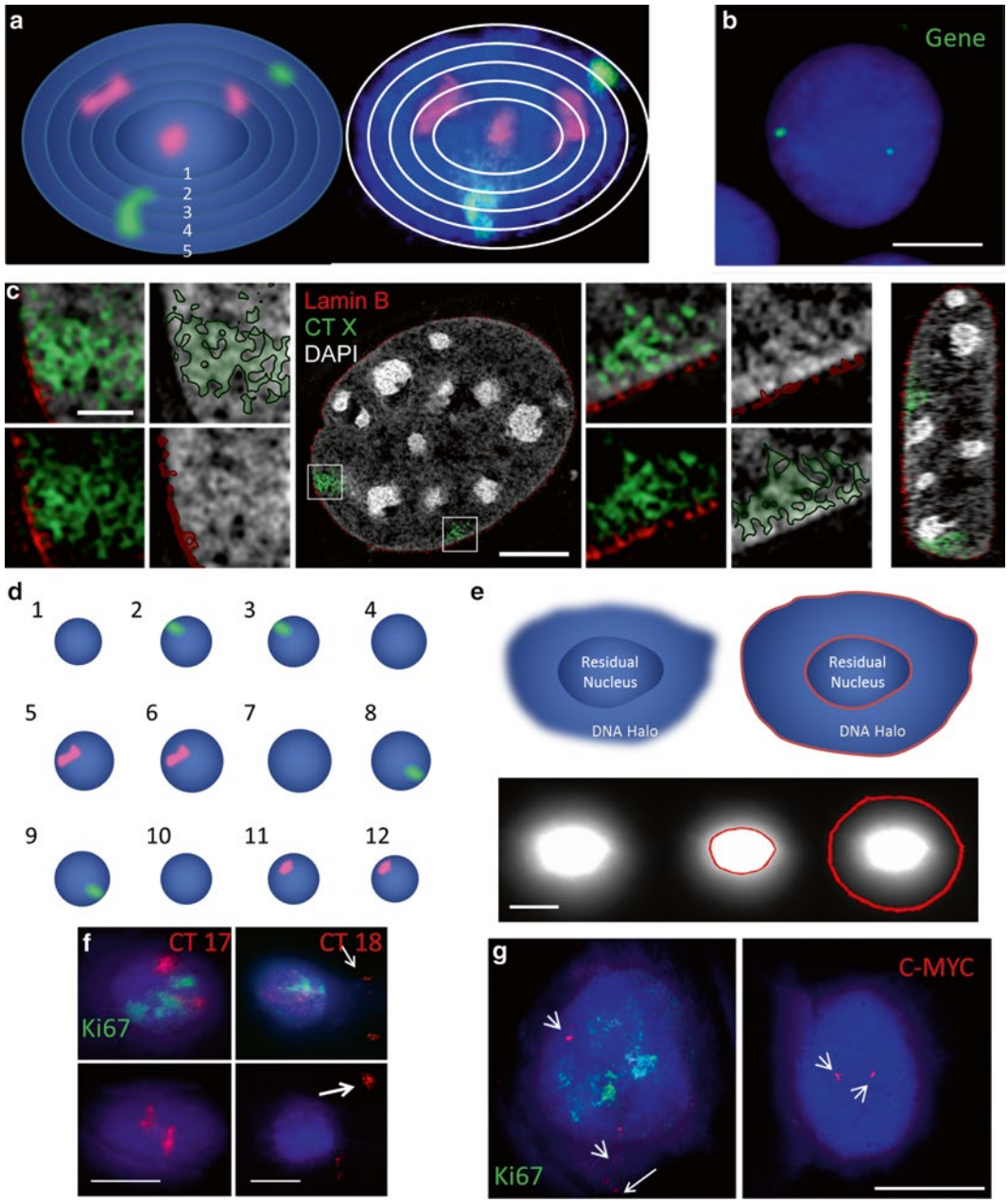

Fig. 1 The composite figure displays images from 2D-FISH protocol to visualize chromosome territories in panel A (*red* and *green*) and gene loci in panel B (*green*). The nucleus is counterstained with DAPI to reveal DNA. This allows the edge of the nucleus to be delineated easily allowing for erosion scripts (*panel A*) to define the edge of the nucleus, as is shown by the cartoon in *panel A. Panel B* displays gene loci FISH signals using a

2 Materials

Careful selection of probe sequence and type is required when asking questions about which parts of the genome are associated with nuclear structures. Chromosome painting probes are commercially available and ready labeled containing *Cot*1 DNA for suppression of repetitive sequences [42] (*see* **Note 1**). Templates of individual chromosomes, that have been either flow-sorted [43] by size or microdissected are available [44]. Labeling of original template is normally performed by degenerate oligo primer-PCR [45]. Specific centromere and telomere probes are also commercially available as are many gene probes. When performing gene positioning studies we use commercially available bacterial artificial chromosomes (BAC) containing the sequence of interest (*see* **Note 2**). The BAC DNA is released from the bacteria using a commercial kit for DNA extraction, following measurement of the amount of DNA generated. A nick-translation reaction is performed to label the BAC DNA with either biotin or digoxigenin conjugated nucleotides, performed through commercially available kits. Other methods of labeling include random priming and end labeling through PCR methods. The method for labeling probes described here is the DOP-PCR method for whole chromosome painting probes.

2.1 Two-Dimensional Fluorescence In Situ Hybridization

2.1.1 Cell Culture and Fixation

1. Specific cell medium supplemented with 10 % (v/v) fetal bovine serum (FBS) and 2 % (v/v) penicillin/streptomycin.
2. Versene (phosphate buffered saline (137 mM NaCl, 2.7 mM, KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) with 0.2 % (w/v) ethylenediaminetetraacetic acid; EDTA) kept refrigerated at 4 °C, pre-warmed to 37 °C.

Fig. 1 (continued) BAC probe labeled with biotin and fluorescently tagged streptavidin. One of the gene loci is localized at the nuclear edge, whereas the other maybe at the edge but it is not possible to tell with 2D-FISH and simple measurements. This is why the erosion scripts can be used with a large number of nuclei. Using 3D-FISH the positioning of a FISH signal in the *x*, *y* or *z* coordinates can be revealed by confocal laser scanning microscopy reconstruction or analysis of a gallery of 2D images as shown in the cartoon in *panel D*. Chromosome territories are drawn, that can be seen to touch the nuclear edge in the *x* and *y* planes but also the *z* plane as one of the territories in *red* is at the bottom of the nucleus. *Panel C* was kindly provided by Dr. Marion Cremer, Ludwig-Maximillian University, Munich; it shows the relationship of chromosome *X* territories (*green*) at the nuclear edge with lamin B1 (*red*) in great detail due to the use of super-resolution microscopy (3D-SIM) [16, 51]. *Panels E, F* and *G* display DNA halo images and how to delineate the residual nucleus and DNA halo (*panel E*). *Panel F* displays territories of chromosomes 17 and 18 (*red*) in proliferating primary fibroblasts (Ki67 in *green*) and in senescent fibroblasts (negative for Ki67). Chromosome 18 is seen at some distance from the residual nucleus in the DNA halo in both proliferating and senescent DNA halos. In *panel G* DNA halos are also displayed subjected to 2D-FISH with labeled BAC probes revealing gene loci signals (*red*), again Ki67 is revealed in *green*. Scale bar, 10 μm

3. 0.25 % trypsin produced from a 2.5 % stock solution, which has been diluted (1:10 v/v) in Versene.
4. Fixative: methanol–acetic acid (3:1 v/v), made fresh and made ice-cold.
5. Hypotonic solution: 0.075 M KCl (*see* **Note 3**).

2.1.2 Slide preparation and Denaturation

1. Glass microscope slides that can withstand high and low temperatures.
2. 20× sodium citrate saline (SSC, 300 mM sodium citrate hydrate, 3 M NaCl).
3. Denaturation solution: 70 % (v/v) formamide, 2× SSC, pH 7.0 (*see* **Note 4**).
4. Water baths.
5. Ethanol series comprising of 70, 90, and 100 % (v/v) high quality ethanol at room temperature.
6. Glass Coplin jars that can withstand heat.
7. Oven that can be set at 70 °C.

2.1.3 Degenerate Oligo Primer-PCR

1. PCR machine.
2. 5× GC Buffer containing 2 mM $MgCl_2$ when at 1×.
3. dACGTP mix made up in water at a concentration of 2 mM.
4. dTTP at a concentration of 2 mM.
5. Forward and reverse DOP primers at a concentration of 20 μM (CCGACTCGAGNNNNNNATGGG).
6. Taq DNA polymerase.
7. PCR grade water.
8. Template DNA—chromosome paint.
9. Biotin-16-dUTP or digoxigenin-11-dUTP.
10. Agarose.
11. Agarose mini gel kit.
12. DNA ladder—containing bands at 500 bp and lower.
13. TAE buffer.

2.1.4 Probe Preparation and Hybridization

1. Labeled total human chromosome or BAC DNA probes.
2. *Cot*1 DNA.
3. Herring sperm DNA.
4. 3 M sodium acetate.
5. 4× SSC.
6. High quality ethanol.
7. Hybridization mixture: 50 % formamide, 10 % dextran sulfate, 2× SSC, and 1 % Tween 20.

8. Heating block.
9. 70 % formamide and 2× SSC solution, pH 7.0.
10. Humidified hybridization chamber.
11. Rubber cement.
12. Glass coverslips (22 × 22 mm).
13. Glass Coplin jars (*see* **Notes 5** and **6**).
14. Bench top centrifuge.

2.1.5 Washing 2D FISH and Visualization of Probe

1. Buffer A: 50 % (v/v) formamide, 2× SSC; pH 7.0.
2. Buffer B: 0.1× SSC; pH 7.0.
3. Blocking solution: 4 % bovine serum albumin (BSA).
4. Streptavidin conjugated to a fluorochrome.
5. Antibody reacting with digoxigenin conjugated to a fluorochrome.
6. 4× SSC solution containing 0.05 % Tween 20.
7. Vectashield containing 6-diamidino-2-phenylindole (DAPI).

2.1.6 Ki67 Antigen Marker for Proliferation in Primary Cells

We have found that chromosome positioning changes according to whether cells are proliferating, quiescent or senescent [6, 46–48]. It is possible to identify replicative senescent cells within a proliferating culture grown in high serum since they are anti-Ki67 negative. It is also possible to work with quiescent cells using Ki67 as a marker in a young culture containing fewer senescent cells. After 4 days, the Ki67 is no longer visible. It is also possible to observe three different stages of G1 by Ki67 patterns type Ia, type Ib, and type II [49].

1. 1× PBS.
2. 1× PBS containing 1 % new born calf serum.
3. Anti-Ki67 antigen antibody.
4. Fluorochrome-conjugated secondary antibody raised against the appropriate species.
5. Glass coverslips.

2.1.7 Microscopy and Analysis

1. Epifluorescence microscope equipped with a 100× lens and filter sets to permit the capture of at least fluorochromes emitting photons in the blue, green, and red spectra.
2. Cooled charged coupled device camera.
3. Computer and program to run microscope and camera.
4. Hardware and software to visualize captured images.
5. Image analysis to perform measurements from FISH signal to nearest edge the nucleus or geometric center of the nucleus or erosion analyses as discussed in the Introduction.
6. Programs for data handling, analysis, and presentation.

2.2 Three-Dimensional Fluorescence In Situ Hybridization

2.2.1 Cell Culture and Fixation

1. Cell medium of choice for specific cell type containing 10 % (v/v) fetal bovine serum (FBS) and 2 % (v/v) penicillin/streptomycin.
2. Versene (phosphate buffered saline (137 mM NaCl, 2.7 mM, KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) with 0.2 % (w/v) ethylenediaminetetraacetic acid; EDTA) kept at 4 °C, and pre-warmed to 37 °C.
3. 0.25 % trypsin from a 2.5 % stock solution, which has been diluted (1:10 v/v) in Versene.
4. Chambered tissue culture dishes to hold slides.
5. Glass microscope slides that can withstand high temperatures.
6. 1× phosphate buffered saline.
7. 4 % paraformaldehyde (w/v) (*see* **Note 7**).
8. Permeabilization solution: 0.5 % Triton X-100 (v/v) and 0.5 % saponin (w/v) in 1× PBS.
9. 20 % glycerol in 1× PBS solution.
10. Liquid nitrogen.
11. Glass Coplin jars.

2.2.2 Probe Preparation and Hybridization

See Subheading 2.1.4 for probe preparation for 2D FISH.

2.2.3 Slide Preparation and Denaturation

1. Denaturation buffer A: 70 % formamide, 2× SSC, pH 7.0.
2. Denaturation buffer B: 50 % formamide, 2× SSC, pH 7.0.
3. Glass coverslips (22 × 32 mm).
4. 0.1 N HCl.
5. 2× SSC.
6. Coplin jars.
7. Rubber cement.

2.2.4 Washing and Visualization of Probe in 3D-FISH

See Subheading 2.1.5 for washing and probe visualization for 2D FISH.

2.2.5 Using Ki67 as a Proliferation Marker

See Subheading 2.1.6 for using Ki67 antibody.

2.2.6 Microscopy and Analysis

1. Fluorescence microscope capable of acquiring optical Z-Stacks with high powered lens such as a confocal laser scanning microscope (with at least two lasers), Deltavision, 3D-simulation illumination microscope, 3D-STORM [50].
2. Software for wide-field deconvolution, 3D reconstruction, measurements in 3D.

2.3 FISH in Combination with Indirect Immunofluorescence

The best way to visualize the nuclear envelope is by indirect immunofluorescence using antibodies to nuclear lamins—A-types and B-types, nuclear pores or integral membrane proteins such as emerin, LAP2β, SUN1, SUN 2, MAN1, or other nuclear envelope transmembrane proteins [18] and even nuclear pore proteins. The issue with combining FISH with visualization of proteinaceous structures is that the fixation, and specifically the formamide, can affect the ability of specific antibodies to bind to their antigen post-FISH. We have found this especially difficult for nuclear envelope proteins such as A-type lamins, which for mapping associations with genomes and the nuclear envelope are the presumed first choice. It is not possible to just use an integrated GFP fused protein since GFP is also affected by the FISH process and is no longer visible post-FISH.

There are two ways to get around this problem: either use the primary and fluorescently tagged secondary antibodies and fix them prior to the FISH protocol, or use the primary antibody prior to the FISH protocol, fix them in place with paraformaldehyde and then carry out the FISH protocol, following the hapten visualization step, or concomitant with it, use the fluorescently labeled secondary antibody to reveal the primary antibody bound to the antigen. As mentioned above, GFP fluorescence does not survive the FISH denaturation process but it possible to use an antibody to GFP to reveal the exogenous protein.

The 2D-FISH protocol could, given that it uses acetic acid, alter structure of proteins. Therefore, it would be necessary to perform a pilot experiment to determine if the antigen of choice at the nuclear envelope survives fixation and the FISH denaturation. With respect to analyzing genome interaction with the nuclear envelope, it is more appropriate to use 3D preserved cells or nuclei.

1. Reagents for 2D or 3D FISH (*see* Subheadings 2.1 and 2.2).
2. Primary antibodies to specific nuclear envelope antigen, Ki67 or GFP.
3. Secondary antibodies reacting with species primary antibodies are made in 4 % paraformaldehyde.
4. 1× phosphate buffered saline.
5. Glass Coplin jars.
6. New born calf serum.
7. Mounting medium containing a DNA counterstain.
8. Humid incubation chamber.
9. Glass coverslips.

2.4 DNA Halo Preparations

Although this chapter concerns visualizing the interaction of the genome with the nuclear envelope we have decided to include a protocol to look at DNA halos which is a technique we have been

using in our laboratory to give a further insight into the behavior and binding of the genome to the nucleoskeleton (also known as the nuclear matrix). Many of the nuclear envelope proteins and complexes at the nuclear edge are part of the nuclear matrix and indeed, a number of nuclear envelope proteins are also found deep within the nucleoplasm as part of the unextractable nucleoskeleton, such as the nuclear lamins.

1. Temperature resistant glass microscope slides or poly-L-lysine coated slides.
2. Chambered tissue culture dishes.
3. Glass Coplin jars.
4. CSK buffer: 10 mM Pipes pH 7.8; 100 mM NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, 0.5 % Triton X-100.
5. Phosphate buffered saline 10×, 5×, 2×, and 1×.
6. Extraction buffer: 2 M NaCl, 10 mM Pipes pH 6.8, 10 mM EDTA, 0.1 % digitonin, (*see* **Note 8**) 0.05 mM spermine, 0.125 mM spermidine.
7. Ethanol series 10, 30, 70, and 95 % made up in water
8. *See* Subheadings 2.1.4 and 2.1.5 for reagents required for 2D-FISH
9. Epifluorescence microscope capable of taking 2D images.
10. CCD camera with hardware and software to control microscope and camera.
11. Image analysis software.

3 Methods

3.1 Two-Dimensional Fluorescence In Situ Hybridization

3.1.1 Cell Culture and Fixation

1. Culture adherent cells for at least 1 week after recovering from liquid nitrogen storage (*see* **Note 9** for suspension cells).
2. Remove medium and wash the cells once with Versene.
3. Harvest cells by incubation 0.25 % trypsin in Versene; neutralize the trypsin by adding at least an equal volume of medium.
4. Centrifuge at 400×*g* for 5 min; remove the supernatant. Resuspend cells in a small amount of remaining medium.
5. Treat harvested cells with a hypotonic solution (0.075 M KCl) for 15 min at room temperature to swell the cells (*see* **Note 10**).
6. Centrifuge the cell suspension at 300×*g* for 5 min using a bench top centrifuge.
7. To fix the cells, remove the supernatant and resuspend the cell pellet in a small amount of buffer left to avoid cell clumping.

8. Add ice-cold methanol: acetic acid (3:1 v/v) drop-wise to the cells with gentle shaking.
9. Incubate cells at 4 °C or on ice for at least 1 h, up to 18 h (*see* **Note 11**).
10. Centrifuge cells at 300×*g* at 4 °C for 5 min and repeat the fixing steps 8, 9 and 10 four to five times, but only leaving the cells in the fix solution on ice for 5–15 min until more than 90 % of the cells have lost their cytoplasm. Use phase contrast with X40 lens to view the slides prior to use. If there are still cells with cytoplasm, keep repeating the fixation steps until there are fewer than 10 %.
11. Store fixed cell suspensions at –20 °C until required.

3.1.2 Slide Preparation and Denaturation

1. Drop the cells onto humid or damp slides from a height, air dry the slides and then age the cells at 70 °C for 1 h or for 2 days at room temperature.
2. Pass the aged slides through an ethanol row of 70, 90 and 100 % ethanol for 5 min in each solution, followed by air drying.
3. Pre-warm the slides at 70 °C for 5 min.

[AU1]

4. Incubate in denaturating solution (70 % (v/v) formamide, 2× SSC, pH 7.0 at 70 °C for 2 min.
5. Immediately plunge the slides into ice-cold 70 % ethanol for 5 min and then again pass through the ethanol row (90 and 100 % ethanol).
6. Air-dry the slides and keep them warm on a warm plate until hybridization with the probe.

3.1.3 DOP-PCR Protocol

1. To make 50 µl of secondary template from the primary template, add together in a small Eppendorf tube that fits the PCR machine to be used: 10 µl DOP-PCR buffer, 5 µl 2 mM dACGTP, 5 µl 2 mM dTTP, 5 µl 20 µM DOP primers, 23 µl PCR grade water, 1 µl of chromosome paint template and 1 U of *Taq* polymerase (to be added last).
2. Place into PCR machine.
3. For amplification of primary product into secondary template use the following temperature and times: 1 cycle of 95 °C for 3 min followed by 98 °C for 20 s; 31 cycles of 62 °C for 1 min followed by 72 °C for 30 s; 1 cycle of 72 °C for 5 min; 4 °C hold.
4. To check the amplification and length of the secondary template, run 3 µl of the PCR product in a 1 % agarose gel with markers that give you the ability to assess smears of PCR product between 200 and 500 bp.

5. Use the unlabeled secondary template DOP-PCR product as the next template DNA and add 5 µl to a plastic PCR tube the correct size for your machine chambers.
6. To the secondary template, add 10 ml of DOP-PCR buffer, 5 ml dACGTP (2 mM), 2 µl dTTP (2 mM) and 10 µl of biotin-16-dUTP or digoxigenin-11-dUTP, 5 µl of DOP-PCR primers, and 1 unit of *Taq* polymerase.
7. For amplification of primary product into secondary template use the following temperature and times: 1 cycle of 95 °C for 3 min followed by 98 °C for 20 s; 34 cycles of 62 °C for 1 min followed by 72 °C for 30 s; 1 cycle of 72 °C for 5 min; 4 °C hold.
8. Using a small aliquot check that there are PCR bands between 200 and 500 bp in an agarose gel.

3.1.4 Probe Denaturation and Hybridization

1. Prepare the probe mixture by ethanol precipitation of the labeled chromosome paint (8 µl per slide) with the addition of *Cot*-1 DNA (7 µl per slide), herring sperm (3 µl per slide), 1/20th volume of 3 M sodium acetate, and 2× volume of high quality ethanol (ice-cold).
2. Incubate the mixture at –80 °C for at least 30 min; centrifuge at full speed in a standard microfuge for 30 min at 4 °C.
3. Wash the DNA pellet with ice-cold 70 % ethanol and centrifuge at full speed in a standard microfuge for 15 min at 4 °C.
4. Dry the pellets at 50 °C on a hot block (*see* **Note 11**).
5. Dissolve the probes in 12 µl (per slide) of hybridization mix overnight at room temperature.
6. Denature the probes at 75 °C for 5 min; allow the *Cot*-1 DNA to anneal to the repetitive sequences at 37 °C for at least 10 min but no longer than 2 h.
7. Incubate slides at 70 °C in 70 % formamide, 2× SSC, pH 7.0 for 2 min.
8. Slides are plunged into ice-cold 70 % ethanol and then placed in 90 and 100 % ethanol for 5 min each at room temperature.
9. Apply 12 µl of the probe to the slide and cover with pre-warmed 22 × 22 mm coverslip and seal with rubber cement.
10. Allow the slides to hybridize with the probe in an humidified hybridization chamber at 37 °C for 2 days.

3.1.5 Washing 2D-FISH Slides

1. Post hybridization, gently remove the rubber cement and wash the slides three times for 5 min each in buffer A (50 % (v/v) formamide, 2× SSC, pH 7.0) preheated to 45 °C.
2. Wash the slides in buffer B (0.1× SSC, pH 7.0) pre-warmed at 60 °C but used in the 45 °C water bath, three times for 5 min each before transferring to 4× SSC at room temperature.

3. Incubate the slides with a 100 µl blocking solution (4 % bovine serum albumin; BSA, w/v) for 10 min at room temperature.
4. Incubate the slides in 100 µl of fluorescently tagged streptavidin or anti-digoxigenin conjugated to a fluorochrome diluted in 1 % BSA for 1 h at room temperature.
5. Wash the slides in a 4× SSC solution containing 0.5 % Tween 20 in the dark at 42 °C with three changes of the solution every 5 min.
6. Mount the slides in mounting medium containing DAPI.

3.1.6 Ki67 Antigen Marker for Proliferation in Primary Cells

1. Once cells have been washed, hapten visualized and slides checked positive for the FISH signals, soak the coverslips in 1× PBS for 30 min at room temperature.
2. Dilute primary Ki67 antibody in 1× PBS containing 1 % new born calf serum and add 100 µl to the slide and cover with a 22×40 mm coverslip.
3. Incubate for 1 h at room temperature or 30 min at 37 °C or overnight at 4 °C.
4. Wash in 1× PBS for 15 min with 3 changes.
5. Place 100 µl of appropriate secondary antibody on the slides, cover with a 22×40 mm coverslip and incubate 1 h at room temperature, or 30 min at 37 °C, or at 4 °C for 4 h.
6. Mount in suitable mounting medium containing DAPI (*see* **Note 12**).

3.1.7 Microscopy and Analysis

1. Examine the slides using 100× oil immersion lens.
2. Cells can be imaged according to their Ki67 (proliferative status).
3. Select nuclei randomly by following a rectangular scan pattern, and capture gray-scale images of these nuclei.
4. Capture at least 50–60 images per slide and convert into TIFF or PICT format.
5. Pass the images through an erosion analysis script (see Introduction). These scripts are devised to divide each captured nuclei in five concentric shells of equal area, the first shell starting from the periphery of the nucleus going to the interior of the nucleus (fifth shell). The script measures the pixel intensity of DAPI and the chromosome probe in these five shells and puts the data obtained into a table.
6. Normalize the probe signal by dividing the percentage of the probe by the percentage of DAPI signal in each shell. Thus, the normalized proportion of probe is calculated in all five shells for at least 50 nuclei.

7. Plot histograms using these data, with standard error bars (+/− SEM).
8. Alternatively, make measurements to the nearest edge or geometric center of the nucleus to position genes/chromosomes. These types of analyses can be meaningful in 2D especially when studying peripherally located FISH signals.

3.2 Three-Dimensional Fluorescence In Situ Hybridization (3D-FISH)

3.2.1 Cell Culture and Fixation

1. Culture adherent cells on sterile glass slides placed in a chambered tissue culture dish for at least 2 days at 37 °C, 5 % CO_2 at a starting density of 1×10^5 cells per slide.
2. Wash slides three times in 1× PBS.
3. Fix in 4 % paraformaldehyde (w/v) for 10 min at room temperature.
4. Wash slides three times in 1× PBS.
5. Permeabilize cells with 0.5 % Triton X-100 (v/v) and 0.5 % saponin (w/v) in 1× PBS solution for 20 min at room temperature.
6. Wash slides three times in 1× PBS.
7. Incubate slides in a solution of 20 % glycerol in 1× PBS solution for at least 30 min at 4 °C
8. Snap-freeze slides in liquid nitrogen for 15–30 s.
9. Repeat step 8–9 another 4–5 times without the 30 min incubation in glycerol.
10. Place slides in boxes and store at −80 °C until required.

3.2.2 Probe Preparation and Hybridization

See Subheading 3.1.4

3.2.3 Slide Preparation and Denaturation

1. Thaw slides taken out of −80 °C storage at room temperature.
2. Repeat the freeze–thaw process in liquid nitrogen for another 4–5 times, soaking the slides in 20 % glycerol between each freeze–thaw if this has not been done prior to freezing.
3. Wash excess glycerol from the slides using three changes of 1× PBS for 10 min each, followed by depurination in 0.1 N HCl for 5 min at room temperature with shaking (*see* **Note 13**).
4. Wash off excess acid with 2× SSC for 15 min with three changes of the buffer and then incubate slides in 50 % formamide, 2× SSC, pH 7.0 solution overnight.
5. Denature the slides by incubation in denaturation buffer A (70 % formamide, 2× SSC, pH 7.0) pre-warmed at 73 °C for precisely 3 min.
6. Rapidly transfer the slides to denaturation buffer B (50 % formamide, 2× SSC, pH 7.0) pre-warmed at 73 °C for 1 min.

During this time, add 10 μl of previously denatured probe that has been allowed to partially re-anneal at 37 °C to a pre-warmed 22 × 22 mm coverslip.

7. Once the slide is ready after denaturation, remove excess denaturing solution with a tissue without disturbing the cells.
8. Present the slide immediately to the probe on the coverslip.
9. Seal the coverslip on the slide using rubber cement and leave to hybridize on the slide in a pre-warmed humidified chamber at 37 °C for 2 days.

3.2.4 Washing 3-D FISH

Same as for 2D-FISH; *see* Subheading 3.1.5.

3.2.5 Using Ki67 as a Marker for Proliferation

Same as for 2D-FISH; *see* Subheading 3.1.6.

3.2.6 Microscopy and Analysis

1. Capture the images of nuclei using a microscope capable of capturing in three dimensions.
2. Collect stacks of optical sections with an axial distance of 0.2 μm for high resolution from random nuclei (*see* **Note 14**)
3. For confocal images you could obtain stacks of 8-bit gray-scale 2D images with eight averages from each optical image.
4. Assess the positioning of chromosomes in relation to the nuclear edge by performing measurements using image analysis packages as described in the introduction, whereby the distance between the geometric center of each chromosome territory/gene signal and the nearest nuclear edge can be measured.
5. Perform measurements for at least 20 nuclei for each sample.
6. Plot frequency distribution curves with the distance between the center of chromosome territory and the nearest nuclear periphery on the x-axis and the frequency on the y-axis.

3.3 3D-FISH Combined with Indirect Immunofluorescence

1. Place slides with fixed cells into a Coplin jar full of 1× PBS.
2. Place slides in an humidified chamber
3. Pipette 20–100 μl of diluted primary antibody (in PBS 1 % new born calf serum or similar) and cover with a glass coverslip.
4. Incubate at room temperature for 1 h, at 37 °C for 30 min or overnight at 4 °C.
5. Remove coverslips gently and place slides in glass Coplin jar in 1× PBS. Place on a rocker at a gentle speed changing buffer three times for a total wash time of 15 min.
6. Carefully wipe away PBS from the back of slides and replace in humidified chamber and repeat steps 3–5 but with the appropriate fluorochrome-conjugated secondary antibody. Incubate at room temperature for 1 h, 37 °C for 30 min or 4 °C for no longer than 4 h.

7. After the final wash, incubate slides in 4 % paraformaldehyde made up in 1× PBS for 10 min at room temperature.
8. Place slides in 2× SSC to prevent drying out before proceeding to the 3D FISH protocol (*see* **Note 15**) for description of doing primary antibody staining before FISH and secondary antibody after FISH.

3.4 DNA Halo Preparations

1. Either grow adherent cells directly onto glass microscope slides in culture or allow suspension cells to settle and attach to poly-L-lysine coated slides.
2. Rinse slides twice in 1× PBS.
3. Add CSK buffer for 15 min.
4. Replace CSK buffer with extraction buffer and leave for 4 min.
5. Rinse slides consecutively in 10×, 5×, 2×, and 1× PBS for 1 min each.
6. Take slides through an ethanol series comprising 10, 30, 70, and 95 % ethanol.
7. Air-dry slides and bake for 2 h at 70 °C. The slides are now ready to be subjected to 2D-FISH.

3.4.1 Measuring the Ratio of the Residual Nucleus to the Maximum Extent of the DNA Halo

1. At 100× magnification, select at least 50 images of extracted cell nuclei per sample at random.
2. Capture of 8-bit gray-scale 2D images of each DNA halo in TIFF format using a high resolution digital camera.
3. Open the image in NIH ImageJ software or similar.
4. Configure ImageJ to analyze area (Analyse > Set Measurements, tick the area checkbox).
5. Using the adjust threshold tool (Image > Adjust > Threshold), move the top slider to cover only the residual nucleus.
6. Measure the area using analyze particles (Analyze > Analyse Particles) and record the measurement.
7. Using the adjust threshold tool again, move the top slider to cover the entire area of the DNA halo, noting that the DNA halo will likely be very pale at its greatest extent.
8. Repeat step 6 and open the next image.
9. The ratio of the residual nucleus to the maximum extent of the DNA halo is calculated by dividing the total area of the DNA halo by the area of the residual nucleus.
10. Plot histograms using these data and standard error bars representing +/− SEM.

3.4.2 Analyzing Positioning of Whole Chromosomes Within a DNA Halo

1. Using an image analysis program the images are cropped and split into individual color channels, i.e., red, blue, and green.
2. Save each channel separately.
3. Identify and select the residual nucleus in an imaging software program to perform density threshold and binary functions to reveal not only the edge of the nucleus but also the center.
4. The distance from the nuclear center to each furthest chromosome territory edge (ctE) can then be determined.
5. These distances are divided by the distance from the nuclear center to each respective nuclear edge (NE).

4 Notes

1. Sometimes commercial paints scrimp on the Cot-1 DNA and it may be necessary to add more Cot1 to avoid repetitive elements in the genome from being hybridized to.
2. The BAC gives you your gene of interest but be aware that BACs may contain other sequence adjacent to the gene of interest.
3. This concentration of KCl in the hypotonic solution maybe too high for some cells; in some instances, this may need to be reduced to 0.05 M KCl (e.g., with snail cells).
4. Formamide is a teratogen and its use should be forbidden to pregnant women. Use in a fumehood otherwise.
5. Use glass Coplin jars that can withstand high heat, i.e., >75 °C: otherwise they crack.
6. To avoid large amounts of formamide in the denaturing step, it is possible to use a heating block and place the probe and sample together and co-denature them. Alternatively it is possible to purchase a programmable temperature controlled slide processing system that will perform the denaturing steps and the hybridization step as well.
7. The Cremer laboratory recommends 2 % paraformaldehyde as it preserved 3D structure better when using super-resolution microscopy (53).
8. Digitonin is a dangerous reagent and requires a careful and comprehensive risk assessment.
9. Suspension cells can be used for any FISH methodologies. They need to be placed on a glass microscope slide. This can be done by cytospinning but this disturbs three-dimensionality. Cells can also be placed on slides coated with poly-L-lysine and allowed to stick. These cells can then be subjected to 3D protocols.

10. It is very important that cells are fully suspended and triturated so that they are separated and not clumped together.
11. The DNA must not be over-dried as it will not be possible to resuspend it in the hybridization mix. It is best to keep an eye on the probe as it dries and take it off the heating block when the liquid has evaporated but the DNA looks "glassy."
12. At this stage it is also possible to improve the FISH process by using an RNAseA treatment for 30 min. Only do this if you are not detecting RNA obviously but also bear in mind that RNA is part of the nuclear structure and a component of chromosomes themselves.
13. For protocols specifically for super-resolution microscopy [18].
14. The Ki67 antibody can disassociate from its antigen after a few hours. 4 % paraformaldehyde (5 min at room temperature) can be used to fix the antibody in place after washing off excess antibody.
15. It is also possible to perform the primary antibody step first prior to the FISH procedure and fix it into place with 4 % paraformaldehyde for 10 min at room temperature. The diluted secondary antibody should be mixed with the streptavidin or anti-digoxigenin antibody after the FISH washing step, and as part of the visualization step.

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