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Chromosome Painting of Mouse Chromosomes

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Abstract

Chromosome painting enables the visualization of chromosomes and has been used extensively in cytogenetics. Chromosome paint probes, which consist of a pooled composite of DNA-FISH probes, bind to nonrepetitive sequences for individual chromosomes [1, 2]. Here we describe the process of using chromosome paint to study the organization of chromosomes without fragmenting the nucleus. This method can be used to analyze chromosome position, and identify translocations and ploidy within the nucleus. The preservation of nuclear morphology is crucial in understanding interchromosomal interactions and dynamics in the nucleus during the cell cycle.

Keywords

Chromosome paint; DNA-FISH; Fluorescence in situ hybridization; Nuclear organization; Nuclear structure; Chromosomes; Cytogenetics

1. Introduction

The field of nuclear organization is rapidly expanding and many unanswered paradigms have come to light. Increasing research supports that chromosome architecture has significant implications during development [3, 4, 5]. Common methods of chromosome identification such as conventional banding, or karyotyping, are invasive as it requires rupturing nuclear membranes and can only be applied to mitotic cells. Thus, the consequential analysis of metaphase spreads lacks the spatial information necessary to address native chromosome organization.

Chromosome painting is a powerful technique that can be used to study the topological organization of chromosomes at any cell cycle stage, without nuclear fragmentation. Whole chromosome probes are differentially labeled, specific to nonrepetitive sequences, so each mouse chromosome can be identified [1, 2]. Simultaneous staining of multiple chromosome paint probes, each conjugated with a different fluorophore, allows for visualization for up to three chromosomes along with a nuclear counterstain (Fig. 1). The information can then be used to assess the chromosome identity and relative position in many different cell types. In addition to chromosome position in the nucleus, chromosome translocations and changes in ploidy can be identified. For example, supernumerary chromosomes can be stained and identified based on their size (Fig. 2).

Currently there are commercially available probes for all murine chromosomes, which provide a technical convenience to many researchers in the field. Chromosome paint probes can be labeled with various fluorochrome–haptens conjugates and imaged on a microscope with proper filters. Here, we demonstrate an approach to stain individual mouse chromosomes, without comprising nuclear morphology, during multiple stages of the cell cycle. A discussion of critical parameters such as chromosome paint probe accessibility, fixation, and pretreatment methods will be necessary as this will impact the protocol's success. It is also reasonable to discuss denaturation, prehybridization/posthybridization steps in detail, as these are essential steps required for successful chromosome painting.

To date, a detailed understanding of how nuclear organization regulates developmental events are limited. It will be critical to compare nuclear organization and dynamics for embryonic cells undergoing their developmental processes of specification, determination, and differentiation.

2. Materials

Prepare all solutions using double deionized water and molecular biology grade reagents. Prepare all reagents the same day of usage and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

2.1. Harvesting Embryonic Fibroblast Cells (MEFs) from Mouse Embryos

1. 1–2 pregnant female mice.
2. 70% ethanol. Use reagent grade ethanol and dd H₂O.
3. 1× PBS: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄. Add to 800 mL of dd H₂O and use stir bar to help with dissolving. Adjust the pH to 7.4 using HCl. Adjust the final volume to 1 L with additional dd H₂O. Sterilize by autoclaving and store at RT.
4. 1× DPBS (magnesium and calcium free PBS).
5. 0.25% trypsin–EDTA solution.
6. 0.05% trypsin–EDTA solution or Accutase.
7. MEFs culture medium: 10% fetal bovine serum, 0.1 mM β-mercaptoethanol, 50 U of penicillin, 50 µg/mL streptomycin in Dulbecco's modified Eagle's medium (DMEM). Use a bottle top filter vacuum filter system (0.22 µm, Corning) to remove impurities from the solution. This solution can be stored at 4 °C for up to 2 months.
8. Sterile dissecting tools. Forceps and scissors (Dumont #5).
9. 15 mL and 50 mL sterile centrifuge tubes.
10. Cell strainer (100 µm Nylon).
11. 150 mm or 100 mm cell culture dish.

12. Nitrile gloves.

2.2. Cell Preparation of Adherent Cells

1. 8 mm custom microscope slides (Azerscientific).
2. 150 mm or 100 mm cell culture dish.
3. MEFs culture medium.

2.3. Cell Preparation of Suspension Cells

1. 0.05% trypsin–EDTA solution or Accutase.
2. Cytofunnels, adaptors, and filter paper for Shandon 4 Cytospin.
3. Superfrost slides.
4. MEFs culture medium.

2.4. Fixation and Permeabilization

1. Fixation solution (4% PFA): For a 50 mL solution: 2 g of paraformaldehyde in 50 mL of 1×PBS. Use stir bar and heat (no higher than 65 °C to prevent boiling of PBS) to dissolve paraformaldehyde pellets/powder. Slowly add NaOH until solution is clear. Let solution cool after mixture is clear. Adjust the pH to 7.4 using HCl. Filter with Whatman paper or centrifuge tube filter to remove impurities from the solution. This solution works best if made the day of usage and can be stored at 4 °C for up to 1 week.
2. Permeabilization Solution (1× PBST): 0.5% Tween 20 in 1×PBS. Invert 5–10 times to mix. Make fresh.
3. 70% ethanol. Use reagent grade ethanol and dd H₂O.
4. Coplin jars.
5. Belly Dancer or a general platform rocker.

2.5. Chromosome Paint Protocol

1. Denaturation buffer (2× SSC): 175.3 g NaCl, 88.2 g of sodium citrate in 800 mL of dd H₂O
2. 0.1 N HCl.
3. 1× PBS (prechilled at 4 °C).
4. Ethanol. Use reagent grade ethanol and dd H₂O to make appropriate dilutions for ethanol series (70%, 80%, and 100% and prechill at 4 °C).
5. Denaturation solution (preheated): 70% formamide diluted in 2× SSC. Adjust the pH to 7.0 using HCl. Can be made in bulk and stored at 4 °C.
6. Post-hybridization wash buffer (preheated): 55% formamide, 0.1% NP-40 in 2× SSC. Invert 5–10 times to mix.

7. Coplin jars.
8. Mini coverslips (8 mm di, World Precision Instruments)
9. Large coverslips (12CIR.-1, Fisher Scientific).
10. Slide warmer set at 37–42 °C.
11. Rubber cement (Elmer's).
12. Hybridization oven.
13. Forceps (Dumont #5, Fine Science Tools).
14. Belly Dancer (Denville Scientific) or a general platform rocker.

2.6. Chromosome Paint Probes

1. Chromosome paint probes (Applied Spectral Imaging) conjugated to FITC, Rhodamine, or Aqua.
2. 1.5 microcentrifuge tube.
3. Two Heat blocks set at 80 °C and 37 °C.

2.7. DNA Counterstain and Mount

1. DNA stain: 4',6-diamidino-2-phenylindole (DAPI) or TO-PRO-3 (Thermo Fisher Scientific).
2. Mounting medium: Antifade (Thermo Fisher Scientific) or 9:1 glycerol/1× PBS with pH adjusted to 8.5–9.0.

3. Methods

3.1. Harvesting Primary Mouse Embryonic Fibroblasts

All cell culture solutions and supplies must be sterile and be used under a laminar flow hood. Aseptic technique should be practiced at all times. Cells should be incubated in a humidified 37 °C, 5% CO₂ incubator.

1. Anesthetize the pregnant dam at day E13.5 by CO₂ asphyxiation, followed by manual cervical dislocation, according to your institutional guidelines provided by IACUC.
2. Place pregnant dam ventral/belly side up on a few paper towels. Spray the abdominal area and lower half of the pregnant mouse with 70% ethanol.
3. Use nitrile gloves for the remaining steps.
4. Use dissection forceps to pinch the skin of the abdominal wall and lift up. Use the dissection scissors in your other hand to cut a small incision from the abdominal wall in the direction along the anterior–posterior axis.
5. Use both hands to pull/peel the skin from the abdominal area to expose the uterus containing the embryos.

6. Use clean forceps with one hand to lift up the bottom of the uterus, and use clean scissors, with the other hand, to remove the entire uterus containing the embryos.
7. Transfer the uterus containing embryos into a clean 100 mm culture dish containing 1× PBS.
8. Use forceps and scissors to separate individual embryos in the uterus. Transfer the individual embryos into a new 100 mm culture dish containing 1× PBS.
9. Under a dissecting/stereomicroscope, working with one embryo at a time, open the yolk sac and dissect out the embryos. Using forceps, remove and discard the liver, heart, and brain. If embryos have different genotypes, transfer each embryo to individual 15 mL centrifuge tubes. Keep them on ice until all embryos are dissected. Transfer all yolk sacs to 1.5 mL centrifuge tubes for DNA extraction. These will be used for genotyping by PCR [10] or Southern blot [11].
10. Use forceps to fragment embryos into 2–3 pieces each. Transfer embryos to a 50 mL centrifuge tube with up to 30 mL of cold 0.25% trypsin–EDTA (or 3 mL per embryo) on ice.
11. Incubate the 50 mL centrifuge tube containing embryos at 4 °C overnight allowing for trypsin-EDTA to permeate through the embryo with minimal enzymatic activity.
12. The next day, without contacting the embryos at the bottom of the centrifuge tube, aspirate the trypsin–EDTA solution until approximately double the volume of embryonic tissue remains.
13. Incubate the centrifuge tube in a 37 °C water bath for 30–45 min. Vortex the tube every 5–10 min to fragment the tissue and help disassociate cells.
14. In a laminar flow hood, add up to 20–30 mL of MEF culture medium to the 50 mL centrifuge tube containing fragmented embryos, and pipette up and down with a serological pipet repeatedly to aid in disassociating the cells from the tissue.
15. Use a serological pipet to transfer your tissue suspension through a cell strainer fitted over a new 50 mL centrifuge tube. Collect the supernatant as this is your cell suspension. Note: Transfer 1–3 mL at a time as to not overflow the cell strainer. Additionally not fitting the cell strainer tightly on the 50 mL centrifuge tube, rather at a slant, will help speed up the filtration process.
16. Split the cell suspension in the supernatant into 150 mm culture dishes. Divide total supernatant by number of embryos. The ratio is one embryo per 150 mm dish containing 16–18 mL of MEF culture medium.
17. Incubate the MEF cells overnight in a humidified 37 °C, 5% CO₂ incubator.
18. Remove MEF medium and wash cells twice with 1× DPBS to remove nonadherent cells and debris. Note: DPBS, rather than PBS, must be used as Mg²⁺ and Ca²⁺ can inhibit trypsin/Accutase activity.

19. Add enough 0.05% Trypsin-EDTA solution to cover all cells (typically 2–3 mL for a 150 mm cell culture dish) and place in a 37 °C, 5% CO₂ incubator for approximately 5–10 min, or until most cells detach from cell culture plate. Cell morphology will change from spindle-like to a more rounded, shrunken shape. Alternatively, Accutase can be used instead of trypsin. Cells with Accutase can be incubated at RT for up to an hour.
20. Add 20 mL of MEF media to culture dish to stop neutralize reaction. Pipette the media-containing cells to a new 50 mL centrifuge tube and centrifuge at 300 × *g* for 3–5 min to pellet cells.
21. Aspirate media while not perturbing the cell pellet at bottom. Resuspend cell pellet with 1–5 mL of media and add to cell culture dish containing 30–40 mL of media with submerged slides (flame-sterilized). Additionally, cells can be further expanded in culture or cryopreserved in liquid nitrogen.

3.2. Cell Preparation of Adherent Cells

1. Flame-sterilize (with option of additional UV sterilization) 1–5 slides.
2. Place sterilized slides in cell culture dish and submerge with appropriate medium.
3. Culture adherent cells (*see above* for primary MEFs) on 8 mm slides until they are about 75%–80% confluent.

3.3. Cell Preparation of Suspension Cells

1. Culture cells until they are about 75%–80% confluent.
2. Collect cells in centrifuge tube and spin at appropriate time/speed (optimal centrifuge differs by cell type) to form cell pellet.
3. Aspirate supernatant and resuspend pellet in 500–1000 µL of medium. Keep cells on ice.
4. Add 30–250 µL of cells to each Cytotunnel and use a Cytospin to spin down cells at 800 RPM, or 139 × *g*, for 3 min on Superfrost slides.

3.4. Fixation and Permeabilization

1. Submerge slides in a Coplin jar containing 50 mL of freshly made fixation solution: 4% paraformaldehyde for 10 min at RT. Note: PFA is the recommended fixative as it preserves the nuclear structure the best [12].
2. Remove fixation solution. Wash slides with permeabilization solution (PBST 0.5% Tween 20) in Coplin jar while rocking on a Belly Dancer or a general platform rocker for 10 min at RT.
3. Dehydrate slides with 70% ethanol.
4. Place Coplin jar with slides in ethanol at 4 °C overnight to age slides (Slides can be stored for up to 3 months at –20 °C).

3.5. Chromosome Painting

1. Probe preparation: Heat-denature Whole Chromosome Paint in a 1.5 microcentrifuge tube in a heat block set at 80 °C for 10 min, then cool to 37 °C for ~60 min until use in **step 10**. Note: Multiple chromosome probes can be combined for a single application in the hybridization step.
2. Rehydrate slides in 2× SSC for 10 min on ice, on a Belly Dancer or a general platform rocker.
3. Depurinate slides with 0.1 N HCl for 5 min at RT to fragment DNA. Note: Depurination is an essential step to increase accessibility for chromosome paint probes to their target chromosome.
4. Wash slides with cold 1× PBS for 2–5 min each, with rocking. Repeat wash two additional times.
5. Dehydrate slides in a cold ethanol series: 70%, 80%, and 100% for 2–5 min each on ice, with rocking.
6. Pour out ethanol and air-dry slides in Coplin jar for 1 min at RT.
7. 7. Place slides on slide warmer until slides are dry (~4 min). Note: Glass slides will absorb a large amount of heat of the denaturation solution immediately after submersion and thus decreases the temperature of denaturation solution. To minimize this temperature change, slides must be prewarmed.
8. Emerge slides in prewarmed denaturation solution at 80 °C for 7.5 min in a hybridization oven (temperature and time will vary and cell-type specific).
9. Following heat denaturation, immediately dehydrate slides with a second series of ice cold ethanol washes: 70%, 80%, and 100% for 2–5 min each on ice, while rocking.
10. Pour out ethanol and air dry slides in Coplin jar for 1 min at RT.
11. Place slides on slide warmer until slides are dry (~4 min).
12. Apply 2.5 µL of the Whole Chromosome Paint mixture to the center of each 8 mm well on slide, 2 wells per slide without any air bubbles. Immediately use a pair of forceps to carefully place a mini 8 mm coverslip over each well to seal the sample. Note: If there are air bubbles, or insufficient covering of the cells with chromosome paint, there will be areas devoid of staining when slide is imaged.
13. Overlay a large coverslip over the mini 8 mm coverslips and seal the edges with 700 µL of rubber cement with a trimmed pipette tip. Note: Cut the bottom of a 1000 µL tip to pipette rubber cement as it is a viscous aqueous solution.)
14. Place coverslipped slides at 80 °C for 7.5 min in a hybridization oven (temperature and time will cell-type dependent) to codenature both chromosome probe and sample.

15. Place denatured slides in a humidified chamber and incubate at 37 °C overnight. Note: In situ humidified chambers can be made by using pipette tip boxes or slide boxes that can be sealed/closed containing moistened paper towels in a 50% formamide, diluted in dd H₂O, solution.
16. The following day, carefully remove both large/small coverslip by breaking the sealed edge of rubber cement with forceps. Slowly and carefully peel off, with the forcep tip slightly underneath the large coverslip edge, both coverslips simultaneously. Note: the small coverslip will be adhered to the large coverslip. If the small coverslip remains after the large coverslip is peeled off, carefully submerge the slide in wash solution a few times until it comes off or remove with coverslip with forceps.
17. Immediately following coverslip removal, submerge slides in a Coplin jar containing preheated posthybridization Wash buffer for at 42 °C for 10 min in a hybridization oven, while rocking. Repeat two times.
18. Incubate slides with a nuclear counterstain: DAPI or TO-PRO3 for 5 min at RT.
19. Washed slides in Coplin jar containing 1× PBS for 5 min at RT, while rocking.
20. Use forceps to remove any trace amounts of rubber cement. Dry areas around the wells with Kim Wipes. Mount wells on the slide with Prolong Antifade and large coverslips.
21. Seal coverslips with nail polish.

4. Notes

4.1. Critical Parameters and Troubleshooting

A common problem in chromosome painting is low/or weak signal detected over background staining. This can be caused by the inaccessibility of the probe to its target chromosome by insufficient denaturation. To correct for this, the user must optimize denaturation temperature and time. A general practice would be to start with the recommended times used in this protocol for MEFs then increase either time or temperature to find the optimal conditions for the cells. Certain cells, such as murine cell lines or cardiomyocytes, display robust cell membranes and cytoskeletal elements that require longer denaturation times for probe accessibility to the nucleus. However, longer hybridization times will affect the recovery of the sample as many cells will be lost during this process. Annealing temperatures have been reported at temperature of 72 °C from manufacturer (Applied Spectral Imaging); therefore the user can balance increasing hybridization duration with lowering temperatures for maximum retention of sample.

Another solution to correct for insufficient denaturation is to increase hybridization temperature (up to 85 °C). This will greatly compromise the cellular and nuclear membrane architecture making it easier for the probes to gain access to their targets. However, this can lead to overdenaturation of the nucleus where nuclear morphology is distorted and DNA fragmentation will occur. Consequently loss of cells will also occur.

It is extremely important to prewarm your slides as glass slides absorb heat from the denaturation solution and can lower its temperature, therefore compromising denaturation.

Increasing concentration of HCl can also aid in increasing signal strength as this will depurinate a larger amount of DNA and strip more proteins to allow for easier accessibility for probe to DNA. However this can lead to overfragmentation of chromosomes and provide inconclusive results.

A cause for weak or inconsistent signals could be due to the age of the cell samples on the slide. Older slides can sometimes require longer denaturation times for good signal recovery. However if samples were not kept cold (-20°C) or in proper conditions, DNA degradation could occur therefore making it inadequate for chromosome painting. Slides made from fresh samples prepared overnight are optimal for chromosome paint protocols.

If there is too much background staining in your nucleus from your chromosome paint, you can alter the stringency of the posthybridization wash solution by decreasing the concentration of SSC and increasing the concentration of formamide and NP-40. This will increase the stringency of your probe to target association and remove other nonspecific DNA binding. Alternatively, too much background staining in the cytoplasm can be removed by pepsin/trypsin/proteinase K digestion as pretreatment for slides.

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Fig. 1. Chromosome paint labeling in mouse C212 tetraploid myoblasts. Top view of stacked confocal optical sections in spun down nuclei show three sets of duplicated homologous chromosome pairs (chromosome 3 in green, 6 in yellow, and 1 in red) localized in the nucleus (TO-PRO3, a nuclear stain for DNA presented in blue). Chromosome paint probes hybridize to the majority of each chromosome by sequence specificity. During anaphase (arrowhead), the homologs chromosome replicate and each daughter nucleus contain two copies of chromosome 3, 1, and 6. Note: Condensed chromosomes at anaphase stain more intensely than interphase nuclei, which exhibit more diffuse staining throughout the nucleus. Scale bar: 10 μ m

Fig. 2. Supernumerary translocated chromosome in male Ts65Dn [6, 7] mouse can be detected based on size [8, 9]. Top view of stacked confocal optical sections of nuclei (TO-PRO3, a nuclear stain for DNA presented in blue) of primary MEFs of male Ts65Dn mice painted for chromosome X (red), 16 (green), and 17 (yellow) in **(a)** interphase and **(b)** anaphase nuclei. The translocated chromosome 17 (green, arrowhead) can be visualized based on its size. Scale bar: 3 μ m