

## Chromosomal Mosaicism in Neural Stem Cells

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### Summary

Neural stem and progenitor cells (referred to here as NSCs), located in the proliferative zones of embryonic brains, can be seen undergoing mitosis at the ventricular surface. Mitotic NSCs can be arrested in metaphase and chromosome “spreads” produced to reveal their chromosomal complement. Studies in mice and humans have revealed a prominent developmental presence of aneuploid NSCs, whereas other chromosomal defects, such as interchromosomal translocations and partial chromosomal deletions/insertions, are extremely rare (1,2). Aneuploidy is defined as the loss or gain of whole chromosomes, resulting in cells that deviate from the normal diploid number of chromosomes (46 in humans, 40 in mice). In NSCs, aneuploidy can occur as a result of missegregation during mitosis, through events such as lagging chromosomes, supernumerary centrosomes, and nondisjunction events (3). The percentage of aneuploid NSCs can be altered by in vivo and in vitro growth conditions as well as through genetic deletion of genes involved in DNA surveillance and repair (1,4). Aneuploidy can be detected by classical cytogenetic methods such as counting the number of chromosomes visualized by DNA dyes (e.g., 4,6-diamidino-2-phenylindole) by using standard light or fluorescence microscopy. Precise chromosome identification is much more difficult: classical methods using banding patterns or size to assign identity are very time consuming even under ideal conditions, and they are notoriously difficult in mice, which often have ambiguous banding patterns and acrocentric chromosomes. A comparatively new technique that allows the unambiguous identification of chromosomes in mice and humans is “spectral karyotyping” or SKY, developed by Ried et al. at the National Institutes of Health for the study of cancer cells (5). This technique uses chromosomal “paints” that are hybridized to chromosome spreads to produce a distinct spectral output for each chromosome. SKY offers superior speed and sensitivity in its ability to detect many types of chromosomal defects, including deletions, insertions, translocations, and aneuploidy.

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## 1. Introduction

There are four major procedures involved in this protocol. First, neural stem cells (NSCs) must be isolated, cultured, and harvested. Second, metaphase chromosome spreads are made. Third, the slides are pretreated. And finally, “spectral karyotyping” (SKY) is performed. The underlying procedure behind metaphase cell analysis using SKY is straightforward. SkyPaint, which is a combination of fluorescently labeled nucleic acid probes that recognize specific DNA sequences on every chromosome, is hybridized to chromosome spreads to produce a distinct spectral output for each chromosome. To prepare single chromosome paints, individual chromosomes are first separated by flow cytometry. Degenerate-oligonucleotide polymerase chain reaction is then performed using a specific combination of fluorescent nucleotides for each chromosome. SkyPaint is the resulting mixture of these individual chromosome paints.

Individual chromosomes hybridized with SkyPaint emit a unique “spectral signature” upon fluorescence excitation. The spectral identity is detected using an interferometer attached to a standard fluorescent microscope and pseudo-colors assigned via SkyView (Applied Spectral Imaging, Vista, CA). SkyView is a software program that both counts and identifies chromosomes within the examined spread. SkyView analyzes the spectral image in two dimensions, and it displays each chromosome with a distinct classification color from which it creates a karyotype table (*see Fig. 2*).

## 2. Materials

### 2.1. Isolation, Culture, and Harvest of NSCs

1. Timed pregnant female mice or NSC line (*see Note 1*).
2. Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS).
3. Basic fibroblast growth factor (bFGF).
4. Colcemid.
5. 40- $\mu$ m filters.
6. 0.075 M KCl.
7. Fresh fixative (3:1 methanol:glacial acetic acid).

### 2.2. Slide Pretreatment

1. Pepsin (*see Note 8*).
2. 0.01 M HCl.

3. Phosphate-buffered saline (PBS).
4. PBS with 50 mM MgCl<sub>2</sub>.
5. Formaldehyde.
6. 100% ethanol (EtOH).
7. 10× standard saline citrate (SSC) (87.7 g of sodium chloride, 44.1 g of sodium citrate in 1 l of water, pH 7.0).

### 2.3. Spectral Karyotyping

1. SkyPaint (Applied Spectral Imaging).
2. Denaturation solution: (70% formamide, 2× SSC, pH 7.0).
3. 37°C slide warmer or heating block.
4. Formamide solution: (50% formamide, 2× SSC, pH 7.0)
5. 4× SSC, 0.1% Tween 20.
6. Concentrated antibody detection (CAD) kit (Applied Spectral Imaging).
7. DAPI.
8. VECTASHIELD antifade (Vector Laboratories, Burlingame, CA).
9. Microscope equipped with an interferometer and SkyView software (Applied Spectral Imaging).

## 3. Methods

### 3.1. Isolation, Culture, and Harvest of Cortical NSCs

1. Dissect the cerebral cortex from embryonic day (E)12–14 embryos, and triturate cells with a Pasteur pipette to get a single-cell suspension (*see Note 1*). Keep cells on ice.
2. Spin down cell suspension at 300 × *g* for 5 min at 4°C.
3. Resuspend cells in 3 ml of DMEM with 10% FBS, 40 ng/ml bFGF, and 0.1 µg/ml colcemid, and transfer the cells to one well of a six-well plate (*see Note 2*).
4. Incubate the cells at 37°C for 3–5 h while slowly rotating (70–80 rpm).
5. Triturate cells again briefly to get a single cell suspension. Wash cells with 10 ml of PBS, and filter the suspension through a 40-µm filter.
6. Spin cells down at 300 × *g* for 5 min, aspirate the supernatant, and flick the pellet to resuspend. Add 10 ml of 0.075 M KCl to the cells.
7. Incubate cells in a water bath at 37°C for 15 min (*see Note 3*).
8. Add 3 drops of fixative with a transfer pipette to the cells while flicking the tube between drops.
9. Spin cells at 300 × *g* for 5 min at room temperature. Aspirate most of the supernatant off, and then flick the tube to resuspend the pellet.
10. Add 5 ml of fixative dropwise while slowly vortexing the tube (*see Note 4*).
11. Incubate at 4°C until use (*see Note 5*).

### 3.2. Metaphase Chromosome Spreads

1. Warm cells to room temperature. Wash twice with fixative and resuspend cells in 1 ml of fixative (*see Note 6*).
2. Set up an 80°C water bath with a flat, thin piece of metal positioned horizontally, about 1.3 cm (0.5 in.) above the water level.
3. Flick cell suspension to resuspend. Take 20  $\mu$ l of cell suspension and pipette it onto the slide. Hold the slide level for about 15–20 s. As the fixative evaporates, the center of the slide will become granular.
4. Quickly flip over the slide (cell-side down) and very briefly expose it to the steam from the water bath (about 5 cm [2 in.] above the water level in the bath) (*see Note 7*).
5. Immediately place the slide on the metal heating plate in the water bath. As soon as the liquid on the slide beads up and is almost gone, remove the slide.
6. Use a microscope to check the spreads for (1) spread density and (2) chromosome morphology (*see Fig. 1*).

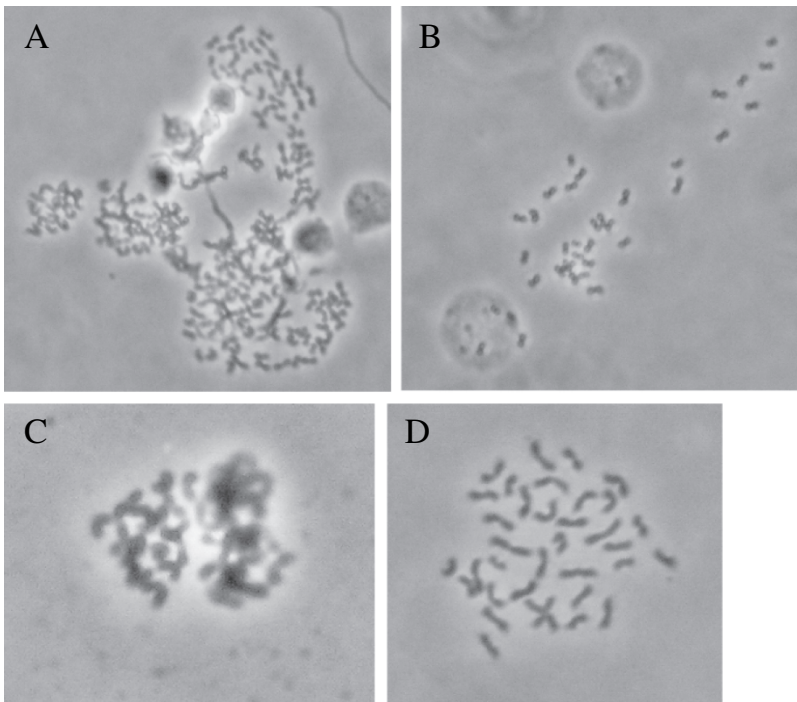


Fig. 1. Examples of metaphase chromosome spreads. (A) Overlapping chromosome spreads. The cell density is too high. (B) Potentially incomplete metaphase spread. (C) Spread containing overlapping chromosomes. (D) Great spread. The chromosomes are well separated, but they are contained in a relatively tight circle.

7. Make 5–10 good slides/sample and age them at room temperature for 1 to 7 days.
8. Add 5 ml of fixative to the remaining cell suspension and store at 4°C for up to 1 year.

### 3.3. Slide Pretreatment

1. Wash aged slides in 2× SSC for 5 min at room temperature.
2. Add 25 µl of 100 mg/ml pepsin to 50 ml of prewarmed (to 37°C) 0.01 M HCl. This results in a final pepsin concentration of 50 µg/ml. Incubate slides in the pepsin solution for 5 min at 37°C (*see Note 8*).
3. Wash slides two times with PBS at room temperature for 5 min.
4. Incubate slides in PBS with 50 mM MgCl<sub>2</sub> for 5 min at room temperature.
5. Incubate slides in PBS containing 1% formaldehyde plus 50 mM MgCl<sub>2</sub> for 10 min at room temperature.
6. Wash slides in PBS for 5 min at room temperature.
7. Dehydrate slides in increasing EtOH concentrations of 70, 80, and 100% for 1 min each.
8. Air dry slides (*see Note 9*).

### 3.4. Spectral Karyotyping

1. Place 10 µl of SkyPaint in a small microfuge tube and incubate at 37°C for 10 min (*see Note 10*).
2. Denature SkyPaint for 10 min at 80°C (using water bath or thermocycler) and then incubate for 60 min at 37°C.
3. Dehydrate slides in increasing EtOH concentrations of 70, 80, and 100% for 1 min each. Air dry slides.
4. Denature slides for 1.5 min in denaturation solution at 73°C.
5. Immediately repeat dehydration in 70, 80, and 100% EtOH sequence for 1 min each. Air dry slides.
6. Warm slides on a 37°C slide warmer for 5 min before adding the SkyPaint.
7. Apply 10 µl of SkyPaint to a 24- × 24-mm coverslip. Immediately put coverslips on slides and use rubber cement to seal the edges.
8. Place slides in a humidified box prewarmed to 37°C and allow hybridization to proceed overnight in the dark (*see Note 11*).
9. Prepare the wash solutions for the next day and leave at 45°C until use.
10. Remove the rubber cement seal carefully, using forceps, and place slides in 2× SSC until the coverslip comes off.
11. Wash slides three times for 5 min in formamide solution (at 45°C).
12. Wash slides three times for 5 min in 1× SSC (at 45°C).
13. Wash slides once in 4× SSC + 0.1% Tween 20 for 5 min.
14. Prepare staining solution: add 10 µl of reagent 3 and 5 µl of reagent 4 (from the CAD kit, Applied Spectral Imaging) to 1 ml of 4× SSC. Vortex mixture for 10 s, then spin for 2 min in a microfuge to pellet any unwanted fluorescent aggregates.

15. Remove as much moisture as possible from the slides without allowing them to dry out. Add 100  $\mu$ l of staining solution to the slides and cover with a coverslip.
16. Incubate slides in a dark 37°C humidified chamber for 30 min.
17. Carefully remove coverslips in 4 $\times$  SSC and wash slides at 45°C in 4 $\times$  SSC + 0.1% Tween 20, three times for 5 min.
18. Incubate slides at room temperature in 4 $\times$  SSC with 0.5  $\mu$ g/ml DAPI for 5 min.
19. Immediately dehydrate slides in increasing EtOH concentrations of 70, 80, and 100% for 1 min each.
20. Air dry slides in the dark.
21. Add coverslip (24  $\times$  50 mm) and VECTASHIELD (antifade) to slides.
22. Use a microscope equipped with an interferometer and SKY software to view and analyze slides (*see Fig. 2*).
23. Analyze at least 20, preferably 40, metaphase spreads for each sample.

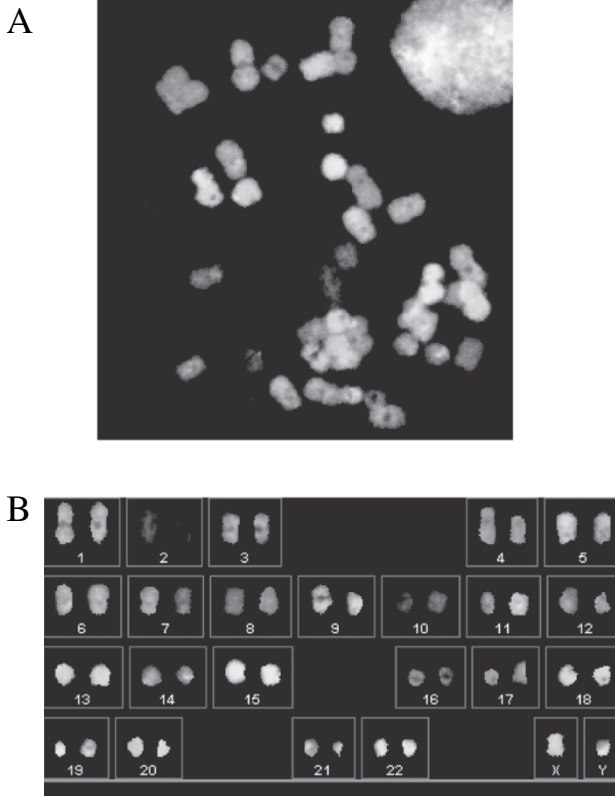


Fig. 2. SKY analysis of a human NSC line. **(A)** Chromosome spread hybridized with SkyPaint. **(B)** Corresponding karyotype table for spread shown in **A**. The karyotype of the cell is 46,XY.

#### 4. Notes

1. Cells must be dividing to be karyotyped via SKY or other methods such as Giemsa banding (G banding). Cortices must be taken from embryos at E12–14 to get a sufficient number of dividing cells. To karyotype cultured NSC lines, make sure a large percentage of cells are dividing at the time of harvest. Treat cells with 0.1  $\mu\text{g/ml}$  colcemid for 3–5 h, and then trypsinize cells to get a single-cell suspension. Proceed to **step 6**.
2. Colcemid arrests dividing cells in metaphase. If few cells are dividing or the colcemid step is omitted, there will likely be an insufficient number of metaphase chromosome spreads for karyotyping. Longer colcemid treatments can be toxic to the cells, and they can cause the chromosomes to become short, making analysis more difficult.
3. This hypotonic KCl solution causes cells to swell, so they will break open when dropped on a slide.
4. It is very important to have a single-cell suspension when the fixative is added. In addition, the cells should be in motion as the fixative is added. If not, the cells will form clumps, making analysis impossible.
5. It is often possible to use cells that have been stored in fixative at 4°C for more than a year.
6. Addition of 1.0 ml of fixative to resuspend the cells is an estimate. Depending on the number of cells in solution, it may be necessary to resuspend them in a larger or smaller volume of fixative. Test with 1.0 ml, and if the spreads on the slide are too sparse or dense, adjust the volume appropriately and then repeat the procedure (*see Fig. 1*).
7. Making chromosome spreads is an art, and it is dependent on many uncontrollable variables, such as humidity. If the cells are not spreading well, try some of the following variations in the procedure:
  - a. Do not place the slides on the heated metal plate, just allow them to air dry slowly.
  - b. Do not expose the slides to steam.
  - c. Expose the slides to steam before adding the cell suspension.
  - d. After the drying fixative becomes granular, add a few drops of glacial acetic acid to the slide and continue with the procedure.
  - e. Try a different fixative, such as 1:1 methanol:glacial acetic acid. If all else fails, try making chromosome spreads on a different day when the atmospheric conditions have changed. For more advice, see <http://info.med.yale.edu/genetics/ward/tavi/FISH.html>.
8. Pepsin treatment of the slides is crucial, and it may vary with cell type. Some cells contain more cytoplasm than others, and they will need longer pepsin treatments. Thus, pepsin concentration and incubation time should be determined empirically. It is important not to expose the cells to pepsin for too long, because this will make the chromosomes difficult to hybridize. Insufficient

pepsin treatment may cause fluorescent green background haze on the slide after it is hybridized with SkyPaint.

9. At this point, slides can either be hybridized immediately or stored in a desiccator at  $-20^{\circ}\text{C}$  for several months.
10. The first time the SkyPaint is thawed it should be incubated at  $37^{\circ}\text{C}$  and vortexed every 3–5 min for 30 min to mix it thoroughly. The paint should then be aliquoted and stored at  $-20^{\circ}\text{C}$ . In addition, the paint is light-sensitive, so keep it protected from light as much as possible.
11. The SkyPaint hybridization can be performed overnight or for as long as 2 days.

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