

Fluorescence *In Situ* Hybridization in Cardiovascular Disease

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Summary

Many human diseases are associated with cytogenetic abnormalities or chromosomal disorders including translocations, deletions, duplications, inversions, and other complicated chromosomal changes. Fluorescence *in situ* hybridization (FISH), a technique involving hybridization of labeled probes to chromosomes and detection of hybridization via fluorochromes, has become a popular method for identification and characterization of cytogenetic abnormalities. For FISH analysis, metaphase chromosomes are prepared by mitotic arrest and hypotonic shock, and denatured. Hybridization of digoxigenin- or biotin-labeled probes to these chromosomes is visualized using fluorochromes like fluorescein isothiocyanate and Texas Red. We have successfully applied FISH technology to the characterization of chromosome breakpoints involved in disease-associated cytogenetic abnormalities to identify candidate gene(s) for the disease. FISH is also widely used in clinical diagnosis of chromosomal disorders.

Key Words: Fluorescence *in situ* hybridization; FISH; chromosome painting probes; metaphase chromosomes; cytogenetic abnormality; chromosomal disorder; disease.

1. Introduction

In situ hybridization is a technique for visualizing target nucleic acid sequences on chromosomes by using complementary probe sequences. The technique is called fluorescence *in situ* hybridization (FISH) when fluorescent detection is utilized. This technology has been applied to many clinical and research studies such as identification of chromosomal abnormalities, comparative cytogenetics, gene mapping, characterization of somatic cell hybrids, and positional cloning. FISH involves the cellular preparation of chromosomes, labeling of probes, hybridization of labeled probes to chromosomes, and detection of the hybridization signals. The chromosomal targets for FISH vary from

metaphase chromosomes to extended strands of DNA (1). Hybridized probes can be localized on a highly defined genetic map. High-resolution metaphase chromosomes can be used to resolve two sequences approx 1 Mb apart, whereas interphase mapping allows the resolution of sequences 100 kb apart. Using extended DNA fibers, sequences 1 kb apart can also be resolved (2).

Metaphase chromosomes can be prepared from any population of actively dividing cells by treating cells first with colcemid or vinblastine to arrest mitosis and then with a hypotonic KCl solution to increase cellular volume. The cells are then fixed with methanol/acetic acid to remove water and disrupt cell membranes before being spread onto microscope slides.

A variety of probes targeting repetitive or unique sequences on the chromosomes can be used for FISH. Centromeres and telomeres of mammalian chromosomes usually contain large arrays of simple DNA motifs that produce strong signals with FISH. Unique probes can be prepared from PCR products, cDNAs, cloned genomic DNAs such as cosmids, P1 artificial chromosomes (PACs), bacterial artificial chromosomes (BACs), and yeast artificial chromosomes (YACs). Labeling of YAC DNA is often problematic, and a large amount of probe is required. *Alu*-PCR amplification of total yeast DNA increases the yield of YAC DNA probe and, therefore, hybridization efficiency (3). Whole-chromosome or chromosome-arm painting probes are also available for human chromosomes. These are derived from flow-sorted chromosomes, either as pooled clones from chromosome-specific libraries or by PCR amplification (4). Repetitive sequences in large DNA probes and chromosome paints will result in unspecific hybridization signals all over the genome and should be suppressed with an unlabeled human competitor DNA, such as sonicated total human DNA or *Cot-1* DNA (5).

FISH probes can be labeled directly or indirectly by fluorochromes. They are generally labeled indirectly with digoxigenin (DIG)- or biotin-conjugated to dUTP. Nick translation is the most widely used method for the labeling of probes (6). After hybridization of labeled probes to chromosomes, fluorescent detection of hybridization is carried out using anti-DIG or anti-biotin antibodies conjugated to fluorochromes. The most frequently used dyes are fluorescein isothiocyanate (FITC), which emits a green fluorescence, or dyes with red or orange fluorescence, Texas Red and Cy3 (7). For probes labeled directly using dUTP conjugated with a fluorochrome, there is no need for a further detection procedure. For FISH experiments, chromosomes and cell nuclei are also counterstained by a fluorescent dye that is specific for DNA. The most commonly used dye is 4',6-diamidino-2-phenyl-indol (DAPI), which is excited in the ultraviolet and with which chromosomes and cell nuclei show a bright blue fluorescence. DAPI also gives a G-banded pattern to chromosomes.

Analysis of the FISH results is carried out under a fluorescence microscope connected with a cooled charge-coupled device (CCD) camera.

In our laboratory, we use metaphase chromosomes for the characterization of chromosomes of the somatic hybrids and localization of chromosomal breakpoints to identify candidate gene(s) for several human disorders (8,9). Metaphase chromosome spreads are prepared from mouse–human hybrid cells and/or human lymphoblastoid cells. YAC and BAC DNAs containing our target sequences are used to prepare probes for FISH. Probes are generally labeled with DIG and hybridization signals are detected using an anti-DIG antibody conjugated with FITC.

2. Materials

2.1. Preparation of Metaphase Chromosomes

1. Suspension or attached cell cultures (*see Note 1*).
2. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X antibiotic-antimycotic solution (Gibco BRL), prewarmed to 37°C (*see Note 2*).
3. Phosphate-buffered saline (PBS) solution, prewarmed to 37°C (*see Note 3*).
4. Trypsin/EDTA solution: 5% (w/v) trypsin, 2% (w/v) EDTA, prewarmed to 37°C.
5. Vinblastine (Sigma) (*see Note 4*).
6. Hypotonic solution: 0.075 M KCl, prewarmed to 37°C (*see Note 5*).
7. Fixative: 3:1 (v/v) methanol/glacial acetic acid, ice-cold (*see Note 6*).
8. 15-mL conical-bottom plastic tubes.

2.2. Preparation of Chromosome Slides

1. Fixative: 3:1 (v/v) methanol/glacial acetic acid, ice-cold.
2. Microscope slides (one end frosted) (*see Note 7*).
3. Micropipet.
4. Lint-free tissue (Kimwipes).
5. Standard phase-contrast microscope.

2.3. Preparation of Probes

1. 500 ng of probe DNAs (*see Note 8*).
2. DIG- and/or biotin-nick translation mix (Roche) (*see Note 9*).
3. Human *Cot-1* DNA (Invitrogen) and sonicated human placental DNA (Sigma) (*see Note 10*).
4. 5 M NaCl.
5. Ice-cold absolute ethanol.
6. 70% ethanol.
7. Hybridization buffer: 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate, 1% (v/v) Tween-20, 2X SSC, pH 7.0 (*see Note 11*).

8. Chromosome painting probes (optional) (*see Note 12*).
9. Heat block or PCR machine.

2.4. In Situ Hybridization

1. 2X SSC, pH 7.0, prewarmed to 37°C.
2. 70, 85, and 100% ethanol.
3. Coplin jars.
4. Water bath.
5. HYBrite (Vysis) denaturation/hybridization unit (*see Note 13*).
6. Cover slips.

2.5. Detection of Hybridization

1. Solution I: 50% (v/v) formamide, 2X SSC, pH 7.0, prewarmed to 43°C (*see Note 14*).
2. Solution II: 0.6X SSC, pH 7.0, prewarmed to 50°C.
3. Solution III: 4X SSC, 0.1% (v/v) Tween-20, pH 7.0.
4. Solution IV: 5% (w/v) nonfat dry milk (*see Note 15*).
5. Solution V: 2X SSC, pH 7.0, room temperature.
6. Anti-DIG or Anti-biotin antibodies conjugated with fluorochromes (*see Note 16*).
7. Mounting medium with DAPI (VECTASHIELD, Vector laboratories).
8. Light protective Eppendorf tubes.
9. Parafilm.

3. Methods

3.1. Preparation of Metaphase Chromosomes From Suspension and Attached Cells

1. Cells are split at 60–80% confluence 1 d before the preparation of metaphase chromosomes. The next day, 10 mL of fresh medium is added to the suspension cells. For attached cells, 5 mL of old medium in a tissue-culture plate is replaced with 10 mL of fresh medium. Cells are incubated for 5–6 h in a humidified 37°C, 10% CO₂ incubator (*see Note 17*).
2. Vinblastine is directly added to the cell media to a final concentration of 1 µg/mL and cells are returned to the incubator for a further 30 min (*see Note 18*).
3. Suspension cells are directly transferred into a 15-mL conical-bottom plastic tube. For attached cells, a modest trypsinization combined with a sharp tap on the culture plate is carried out to collect the rounded, mitotic cells.
4. Cells are centrifuged for 5 min at 400g. Supernatant (medium) is removed completely except for about 300 µL. Cell pellet is resuspended in this volume by flicking.
5. 10 mL of 0.075 M KCl is added and cells are incubated in a 37°C water bath for 4 min for the human lymphoblastoid cells and for 15 min for the mouse–human hybrids (*see Note 19*).
6. Cells are centrifuged for 5 min at 400g.

7. Supernatant is removed except for about 300 μ L. Cell pellet is resuspended in this volume by flicking.
8. A few drops of ice-cold fixative (methanol:acetic acid, 3:1) is added to the cells and flicked gently several times. Then, another 8 mL of the fixative solution is added.
9. Cells are centrifuged for 5 min at 400g.
10. Supernatant is aspirated and cell pellet is resuspended in 5–8 mL of the methanol-acetic acid solution. Cells should be restored at -20°C at least for 1–2 h before use (see **Note 20**).

3.2. Preparation of Chromosome Spreads

1. Microscope slides kept in a container filled with 70% ethanol are dried up.
2. By tilting the slide to $30\text{--}45^{\circ}$, 5–10 μ L of cell suspension is dropped from a height of about 5–7 in. onto the part close to the frosted end of the slide, then the slide is immediately tilted more to allow the cell solution spread down through the slide surface. Slides are left to air-dry and chromosome spreads are examined under a microscope (see **Notes 21** and **22**).

3.3. Labeling and Denaturation of Probes

1. Probe labeling is carried out on ice in a 10- μ L reaction volume. Water is used to complete the volume. 500 ng of probe DNA is mixed with 2 μ L of DIG- or biotin-nick translation mix (Roche, Applied Science) on ice.
2. Reaction mixture is incubated at 15°C for 90 min.
3. Reaction is stopped by heating the mixture to 65°C for 10 min.
4. Whole reaction volume (10 μ L) is mixed with 5 μ g of *Cot-1* DNA, 1 μ g of human placental DNA, and 0.02 vol 5 M NaCl in a volume of 50 μ L completed with water. 125 μ L of ice-cold ethanol (2.5 vol) is added and mixture is kept at -20°C for 30 min.
5. Mixture is centrifuged for 25 min at the maximum speed in a microcentrifuge.
6. 100 μ L of 70% ethanol is added to the pellet and centrifuged for 10 min at the maximum speed.
7. Supernatant is removed very carefully and 15 μ L of hybridization buffer:water (7:3) is immediately added without letting the pellet to dry up. Allow DNA to dissolve at room temperature for 30 min to 1 h (see **Note 23**).
8. Chromosome painting probes can be added to the precipitated, labeled probe at this stage (see **Note 24**).
9. The labeled probe is denatured at 70°C for 10 min and reannealed at 37°C for 30 min, and kept on ice until ready to add chromosome slides.

3.4. Chromosome Denaturation and Hybridization

1. Chromosome slides are placed in coplin jars and 2X SSC, pH 7.0, prewarmed to 37°C is added. They are incubated in 37°C water bath for 30–45 min.
2. Slides are dehydrated through ethanol series of 70, 85, and 100% for 1 min each and air-dried.

3. 15 μL of hybridization buffer:water (7:3) mixture is placed longitudinally onto the slide following the mid-line; put a cover slip on it by avoiding air bubbles between cover slip and slide (*see Note 25*).
4. Slides are placed onto the plate of HYBrite; chromosomes are denatured at 70°C for 1 min (*see Note 26*). They are immediately put on ice (*see Note 27*).
5. The cover slip is removed carefully, the previously labeled probe is placed longitudinally on the slide following the mid-line, and a cover slip is placed on it, with no air bubbles allowed between the cover slip and slide.
6. Slides are placed onto the HYBrite plate and left for incubation at 37°C overnight (*see Note 28*).

3.5. Detection of Hybridization Signals

1. Cover slips are removed gently by dipping the slides in solution I once, and then slides are washed three times in solution I for 5 min each in a 43°C water bath (*see Note 29*).
2. Slides are washed three times in solution II for 7 min each in a 50°C water bath.
3. Slides are equilibrated in solution III for 3 min at room temperature.
4. Long sheets of Parafilm are cut to cover the bottom of a container. 120 μL of solution IV per slide is placed for each slide on these Parafilm sheets.
5. Slides from the solution III are drained by touching the short edge of the slide to a lint-free tissue and covered onto each 120 μL solution IV, which was placed on the Parafilm. The container is humidified (*see Note 28*) and slides are incubated for 10 min at room temperature.
6. While slides are incubating, antibodies conjugated with fluorochromes are diluted with solution IV in sufficient amounts (120 μL of the diluted antibodies per slide) (*see Note 30*). Dilution is carried out in a light-protective Eppendorf tube and centrifuge for 5 min at the maximum speed in a microcentrifuge to eliminate some participation particles.
7. Slides are removed from the chamber and placed upside down in a safe place, and the old Parafilm sheets are replaced with the new ones. 120 μL of the antibody-conjugated fluorochrome solution is placed for each slide on these new Parafilm sheets.
8. Slides are covered onto each 120 μL antibody-conjugated fluorochrome solution that was placed on the Parafilm. The container is humidified (*see Note 28*) and wrapped with foil. Slides are incubated 30 min at a 37°C incubator.
9. Slides are washed in solution III three times for 5 min each in dark at room temperature.
10. Slides are washed finally in solution V once for 5 min in the dark at room temperature.
11. Slides are lined up on a lint-free tissue at an angle to let them drain. Two drops of DAPI are placed on the middle of each slide, and the slides are covered with cover slips. Excess DAPI is removed by placing the slide between towel sheets and gently pressing on top. They can be kept in slide boxes for several weeks at 4 or -20°C . Now, the slides are ready to be examined under a fluorescence microscope. Because there are two sister chromatids, hybridization signals are gener-

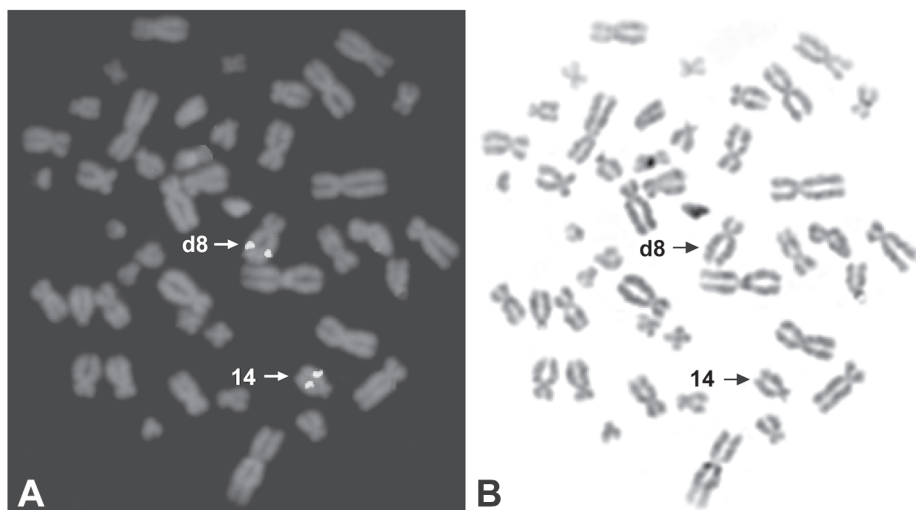


Fig. 1. Localization of translocation $t(8;14)$ (q22.3;q13) breakpoints by fluorescence *in situ* hybridization (FISH) analysis. (A) *Alu*-PCR product of yeast artificial chromosome (YAC) 964b11 (from chromosome 14) DNA was labeled by digoxigenin (DIG)-nick translation and hybridized to metaphase chromosome spreads prepared from the lymphoblastoid cells of a patient with Klippel-Trenaunay syndrome (KTS). This patient with KTS carries a balanced translocation involving chromosomes 8 and 14 (8). Hybridization signals were detected using an anti-DIG antibody conjugated with FITC, which gives a green fluorescence as pointed by arrows. Biotin-labeled chromosome 14q arm-specific painting probe was used as a marker and hybridization was detected by using Texas red, which gives a pinkish to red color. Metaphase chromosomes were counterstained by DAPI showing blue fluorescence. Because green hybridization signals were detected only on the normal chromosome 14 and derivative chromosome 8 (d8), the chromosome 14 translocation breakpoint should be located above the position of this YAC clone. (B) The same image as in A was processed for G-banding patterns.

ally seen as two dots on the metaphase chromosomes. An example of the FISH data is shown in **Fig. 1**.

4. Notes

1. In our laboratory, metaphase chromosomes are prepared from the suspension cultures of human lymphoblastoid cells and attached cultures of mouse-human hybrid cells. Lymphoblastoid cells are maintained in 25-cm² cell-culture flasks and hybrid cells are grown in 100-mm tissue-culture plates.
2. For antibiotic-antimycotic, 100X solution is purchased from Invitrogen. It contains 10,000 U/mL penicillin G sodium, 10,000 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B.
3. All PBS solutions mentioned here contain 137 mM of NaCl, 2.7 mM of KCl,

8.0 mM of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 1.5 mM of KH_2PO_4 .

4. Vinblastine can be purchased from Sigma as 5 mg powder. It is dissolved in 5 mL of PBS to a stock solution of 1 mg/mL and kept at 4°C in dark.
5. KCl can be prepared as 0.75 M stock solution and, prior to the experiment, can be diluted to 0.075 M with sterile water.
6. Fixative solution should be prepared fresh each time and kept at -20°C.
7. Microscope slides are placed on slide racks, and the racks are placed in a big container filled with water and a detergent suitable for glassware. The container is placed on a shaker for 15 min; then, the slides are rinsed with distilled water several times following a final 15-min rinse on the shaker. Finally, racks are kept in a big container filled with 70% ethanol.
8. Our probes for FISH are prepared from YAC and BAC DNAs. YAC probes are prepared by *Alu*-PCR specifically using 200–400 ng of YAC DNA, 0.5 μM of *Alu*-specific ALE1 primer (5'-GCCTCCCAAAGTGCTGGGATTACAG-3') and ALE3 primer (5'-CCA[C/T]TGCACTCCAGCCTGGG-3'), and 4 mM of MgCl_2 . PCR products are purified using QIAquick spin columns (QIAquick PCR purification kit, Qiagen Inc.).
9. We generally label our probes with DIG and use chromosome painting probes labeled with Biotin.
10. Human *Cot-1* DNA and total human placental DNA is used to suppress cross-hybridization of large human DNA probes to the human repetitive DNA sequences.
11. Dextran sulfate used in the preparation of hybridization buffer is difficult to dissolve. After adding all ingredients, the preparation should be kept in a 70°C water bath for several hours and then allowed to cool down. The pH of the solution is adjusted to 7.0 and its volume is brought to the desired value. It is sterilized by using 0.45- μm filters, aliquoted as 500- μL vials, and stored at -20°C. Hybridization buffer should be always used as a 7:3 ratio of hybridization buffer and any other solution, such as water or painting probe, respectively.
12. We purchase whole-chromosome, chromosome-arm, or centromere painting probes from the American Laboratory Technologies and Vysis.
13. We strongly recommend the HYBrite denaturation/hybridization unit for the automated denaturation and hybridization of the chromosomes. It works as a hot plate for the microscope slides. It can be programmed to different temperatures and times, as can PCR machines. Otherwise, chromosomes should be denatured manually.
14. pH is adjusted by 1 N citric acid.
15. Nonfat dry milk is dissolved in solution III, and sterilized using a 0.45- μm filter.
16. We generally use anti-DIG-FITC (Roche, Applied Science) for the detection of probe hybridization and anti-biotin-Texas Red (Rockland Immunochemicals, Inc.) for the detection of biotin-labeled chromosome painting probes.
17. Established cell cultures should be subcultured the day before metaphase chromosome preparation, because it is important to obtain actively proliferating cells. Generally five to six mitotic cells per view are enough to proceed.
18. Vinblastine binds to microtubular proteins of the mitotic spindle, leading to

crystallization of the microtubule and mitotic arrest. Some protocols use colcemid, a colchicine analog, to a final concentration of 0.1 $\mu\text{g/mL}$ for the same purpose. Vinblastine is known to be more effective and gentler on mitotic arrest.

19. Hypotonic treatment causes swelling of the cells. The optimal time of KCl treatment varies from cell type to cell type and must be determined empirically. But we recommend 4 min for the human lymphoblastoid cells and 15 min for the mouse–human hybrid cells.
20. Fixed cells can be kept at -20°C for a maximum of 1 yr. Before they are used again, a freshly prepared fixative solution is added to the cells to a volume of 10 mL and cells are centrifuged for 5 min at 400g and dissolved in an appropriate volume of fixative according to the cell density. According to our protocol, about 50 μL of the fixative results in an adequate number of cell spreads when 5–10 μL of this solution is dropped onto the microscope slides.
21. After dropping the cells onto microscope slides, cell density, the number of metaphase spreads, and the quality of chromosome preparation should be examined under the microscope. Chromosomes should be dark gray when viewed under phase contrast. If the cell number is too low, the cell solution volume to be dropped can be increased or the cells can be centrifuged and dissolved in less fixative solution. If the cell number is too high, cells can be further diluted with fixative solution. To a certain degree, this can also be a solution if the number of metaphase spreads is low. Sometimes, the number of intact cells can be too high and there can be some granules in the cell environment. Repetition of fixative treatment (fixative addition and centrifugation) can solve the problem. Duration of slide drying is also critical. It should not be too fast or too slow. To dry them, blowing gently can be helpful. Another widely used strategy is to drop the cells on steam-warmed slides. There are many reported ways of chromosome slide preparation. It is best to try different strategies and use the most efficient one.
22. Slides can be used for hybridization the same day as they are made. However, they can be stored several weeks in a dark, clean environment at room temperature or frozen at -80°C for more than 1 yr. Once thawed, the slides should not be frozen again.
23. Precipitated, labeled probes can be kept at -20°C for several years.
24. Manufacturers' suggested amounts of the chromosome painting probes are added to the precipitated labeled probe.
25. Small air bubbles can be squeezed out from the sides by gentle pressing.
26. If chromosomes are denatured manually, then the slides can be incubated in a denaturing solution (70% [v/v] deionized formamide, 2X SSC, 0.1 mM EDTA, pH 7.0) in a fume hood in a 70°C water bath for 2 min. They are quickly dipped in ice-cold 2X SSC and kept in ice-cold 70% ethanol for 1 min. Slides are dehydrated through ethanol series of 70, 85, and 100% for 1 min each and air-dried (not longer than 10 min). The probe is added, and slides are covered by a cover slip and left to incubate in a moist environment at 37°C overnight.
27. A glass plate can be placed on ice in a container, and denatured chromosome

slides can be placed on it.

28. A humid environment is necessary to avoid evaporation and slide drying. Paper towels are soaked in water and placed on the empty spaces of HYBrite. If hybridization is carried out in an incubator, a moist environment can be created in a conveniently sized container or chamber with a lid. Slides are lined up on the bottom of container (not on top of one another). Soaked towels are folded up into rolls and placed on the empty spaces, and the container covered with the lid. For use with light-sensitive reagents, the moist chamber is wrapped with aluminum foil.
29. Wash stringency (temperature and salt concentration) can be adjusted for the optimum hybridization results.
30. Antibody dilution should be optimized for each new batch of antibody. As a reference, we use 1:100 dilution of Anti-DIG-FITC and 1:25 dilution of anti-biotin-Texas Red.

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