

Chapter 14

FISH Analysis of Human Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (PSCs) hold promise for treating a multitude of diseases. These fascinating cells are unique in their ability to both self-renew and differentiate into cells from all three germ layers. However, PSCs, as well as other cultured cells, are prone to genetic instability. Given the possibility that these cells may one day be used clinically, identifying, and perhaps preventing, genetic instability is of particular concern for human PSC researchers. One type of genetic alteration that has been observed in PSCs is aneuploidy. Aneuploidy is defined as any divergence from the normal diploid number of chromosomes. So for human cells, any cell with more or less than 46 chromosomes would be considered aneuploid. Interestingly, there is a tendency for human PSCs, regardless of culture conditions, to gain specific chromosomes. In particular, gains of chromosomes 12, 17, 1, and X have been reported from labs all over the world. Since gains of these specific chromosomes are by far the most common aneuploidy seen in human PSCs, it is relatively easy and inexpensive to screen for these using fluorescent in situ hybridization (FISH). Here we will describe a cytogenetic method for screening human PSCs using FISH.

Key words: ESCs, PSCs, karyotyping, FISH, aneuploidy, chromosomes, genetic instability

1. Introduction

Fluorescent in situ hybridization (FISH) is a molecular cytogenetic technique that uses fluorescently labeled chromosome- or locus-specific sequences to specifically label chromosomes or specific genetic loci. FISH probes hybridize to metaphase chromosomes and interphase nuclei allowing one to assay nondividing cells, which can be a distinct advantage over classical karyotyping by G-banding or spectral karyotyping (SKY).

2. Materials

2.1. Cell Preparation and Harvest

1. Colcemid solution (10 $\mu\text{g}/\text{mL}$).
2. 0.05% Trypsin/EDTA solution.
3. Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS).
4. 0.075 M KCl warmed to 37°C.
5. Transfer pipettes.
6. Fixative: 3:1 Methanol: Glacial Acetic Acid. Make fresh, do not store.

2.2. Metaphase Chromosome Spread Preparation

1. 80°C water bath.
2. Metal plate ~2–4 mm thick that will fit on top of the water bath.
3. Precleaned glass slides.

2.3. Slide Pretreatment and Denaturation

1. *Pepsin solution*: Add 25 μL of 100 mg/mL pepsin (Sigma) to 50 mL of 0.01 M HCl solution that has been prewarmed to 37°C. This gives a final pepsin concentration of 50 $\mu\text{g}/\text{mL}$. Make immediately before use (see Note 1).
2. Dulbecco's Phosphate-Buffered Saline (DPBS, without Mg^{2+} or Ca^{2+}).
3. DPBS with 0.5 mM MgCl_2 .
4. *Formaldehyde solution*: 1% formaldehyde in DPBS with 50 mM MgCl_2 . (see Note 2) Make fresh daily in chemical fume hood.
5. *Ethanol series*: 70, 80, and 100% ethanol diluted in water. Make up fresh ethanol solutions. Discard any unused solutions after 7 days.
6. *20 \times SSC stock solution*: To make 1 L of 20 \times SSC, dissolve 175.3 g of NaCl and 88.2 g of sodium citrate with water to a final volume of 1 L. Dilute the 20 \times SSC stock appropriately to make 1 \times and 4 \times SSC.
7. *Denaturation solution*: 70% formamide in 2 \times SSC pH 7.0. Make 1 mL aliquots and store at -20°C for up to a year.
8. Coplin jars (see Note 3).
9. 75°C heating plate.
10. 24 \times 50 mm coverslips.

2.4. Probe Hybridization

1. FISH probe(s) Store at -20°C and protected from light.
 - (a) Premade probes may be purchased commercially from vendors such as:
 - i. Vysis (<http://www.abbottmolecular.com>).
 - ii. Cambio (<http://www.cambio.co.uk>).
 - (b) Probes may also be made via nick translation of home-made DNA probes.
2. 22×22 mm coverslips.
3. Rubber cement.
4. 37°C hybridization oven.

2.5. Slide Washing and Mounting

1. *Formamide wash solution*: 50% Formamide in $2\times$ SSC pH 7.0. Use Prepare in a chemical fume hood. Pre-warm the wash solution to 45°C . Discard unused formamide wash solution after 2 days.
2. *1 \times SSC pH 7.0*. Store at room temperature, discard unused solution after 6 months.
3. *Tween-20 wash solution*: 0.1% Tween-20 in $4\times$ SSC. Store at room temperature, discard unused solution after 6 months. Ensure the Tween-20 is completely dissolved before use.
4. *DAPI (4', 6-diamidino-2-phenylindole) stain solution*: Dilute 5 μL of 5 mg/mL DAPI into 50 mL of $4\times$ SSC to make a 0.5 $\mu\text{g}/\text{mL}$ solution. Protect solution from light. Discard unused solution after 2 days.
5. Vectashield (Vector Labs).

2.6. Viewing and Interpretation

1. Fluorescence microscope with filters that match the fluorophore used on probe(s) and DAPI.
2. Laboratory counter with at least 4 channels.

3. Methods

Molecular cytogenetic analysis by FISH involves 5 distinct steps. First, the PSCs are arrested in metaphase with colcemid. While FISH can be performed on intact, nonmitotic nuclei, the colcemid treatment is often useful. Next, cells are trypsinized to a single cell suspension, swollen in a hypotonic solution, and fixed. Fixed cells are then dropped onto slides to make metaphase chromosome spreads. Acceptable slides are then pretreated with a

set of solutions that open the chromatin and denature it. The slide is then hybridized overnight with a fluorescently labeled, chromosome-specific FISH probe. The next day, nonhybridized probe is removed through a series of washes and the slide is visualized with a fluorescence microscope and levels of aneuploidy are quantified.

3.1. Cell Preparation and Harvest

1. Add colcemid to cells in their culture medium at a final concentration of 0.1 $\mu\text{g}/\text{mL}$ and incubate at 37°C for 3–4 h (see Notes 4 and 5). Two 70% confluent wells of a six-well plate should provide plenty of cells for this analysis.
2. Trypsinize the cells with 0.05% trypsin/EDTA solution, transfer the cells to a 15 mL conical tube, and gently triturate the cells until you have a single cell suspension. Add an equal volume of DMEM, 10% FBS medium to inactivate the trypsin.
3. Centrifuge the cells at 200 $\times g$ for 3 min and aspirate the supernatant. Flick the tube resuspend the cell pellet and add 10 mL of prewarmed 0.075 M KCl. Incubate at 37°C for 10–15 min.
4. Add 3 drops of fresh fixative to the cells and centrifuge at 200 $\times g$ for 3 min (see Note 6).
5. Aspirate the supernatant and flick tube to resuspend the pellet. Using a transfer pipette, add fixative slowly in drops while vortexing the cells at the lowest speed possible (see Note 7). Add approximately 5–10 mL of fixative. Store fixed cells at 4°C, for up to 6 months.

3.2. Metaphase Chromosome Spread Preparation

1. Prepare an 80°C water bath with a thin metal plate across part of the top of the water bath. Ensure the water is only 1–2 in. below the metal plate (see Fig. 1).
2. Centrifuge the fixed cell suspension at 200 $\times g$ for 3 min. Aspirate the supernatant and wash two times with 5 mL of fresh fixative. Resuspend the pellet in 1 mL of fixative. Depending on the number of cells, you may have to spin it down again and resuspend in a smaller or larger volume to achieve the best concentration.
3. Flick the tube to resuspend the cell pellet and take 20–30 μL of your cell suspension and load it onto the slide. Hold the slide level for 10–30 s or until the center of the slide becomes granular due to evaporation of the fixative.
4. Immediately, flip over the slide and expose it to the steam for 5–10 s (see Note 8).
5. Quickly place the slide, cell side up, on the metal plate and leave it there until the fixative solution has almost completely evaporated.

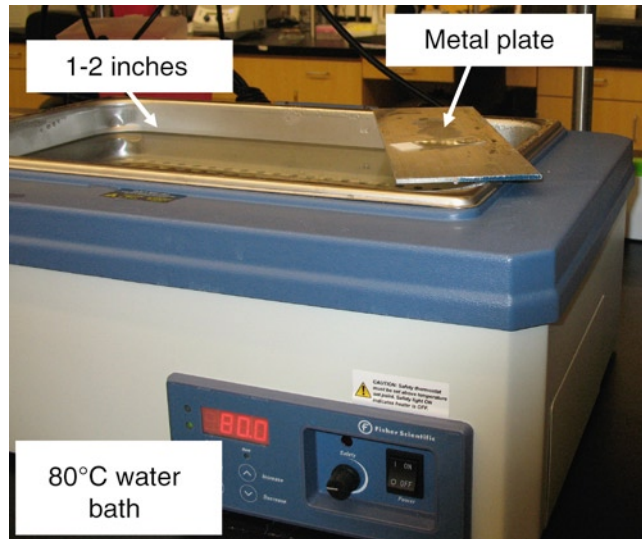


Fig. 1. Metaphase chromosome spread setup. Fill water bath to 1–2 below the top and set the temperature to 80°C. Set a metal plate across the top of the water bath to warm. After dropping cells onto the slide, expose the slide to steam and then place it on the hot metal plate just until all the liquid is evaporated.

6. Look at the slide under a light microscope and check the density of the metaphase spreads. Chromosome spreads should not be so dense that they are overlapping, but do get as many spreads on the slide as reasonably possible. If the cells are too sparse, spin your cell suspension down and resuspend in a smaller volume. If they are too dense, resuspend in a larger volume.
7. After the cell density is acceptable, check the morphology of the spreads. Chromosomes should not be overlapping but they should be contained within a reasonably tight circle. If the chromosome morphology is not acceptable, experiment with different fixative drying times and different steam exposure times (see Fig. 2a–d).
8. Also, check the color of the chromosomes in the spreads. Ideally, they should not be too bright nor too dark. Good chromosome spreads are typically light gray in color.
9. Make 5–10 slides. Pick the best one to hybridize with the FISH probes. Slides should be stored in the dark at room temperature for no more than a week before hybridization.

3.3. Slide Pretreatment and Denaturation

1. Wash slide in a coplin jar with room temperature 2× SSC for 5 min (see Note 9).
2. Incubate slide in prewarmed pepsin solution for 5 min.

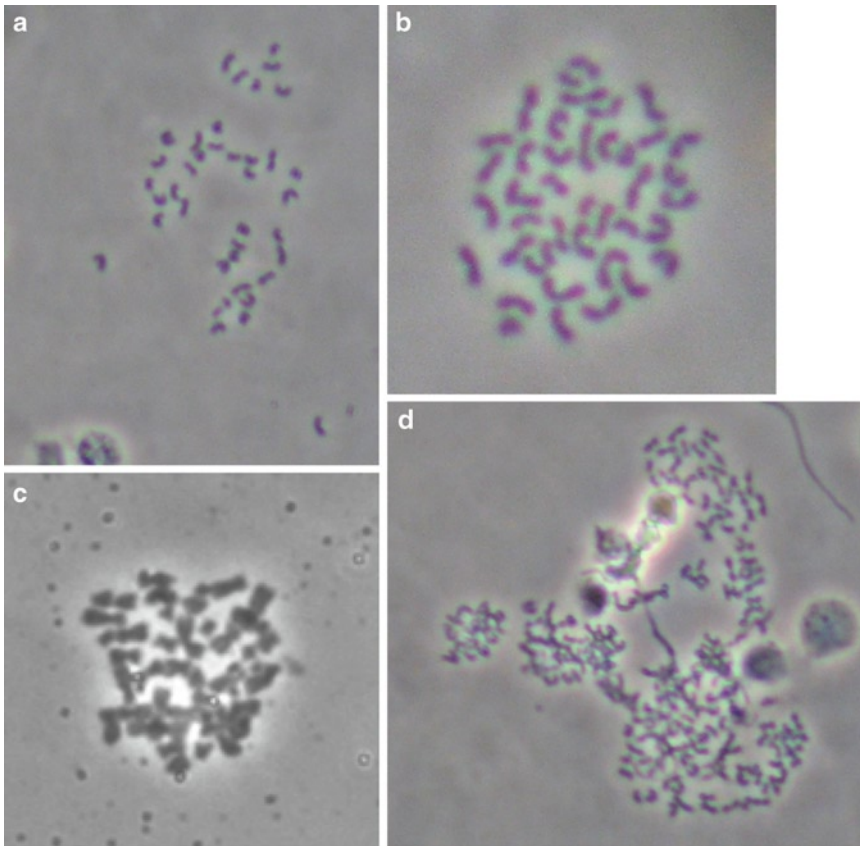


Fig. 2. Metaphase chromosome spread morphology. (a) The chromosome spread is too spread out. Chromosomes may be lost from the spread. (b) Perfect chromosome spread. Chromosomes are in a tight circle but are not overlapping. (c) Chromosomes in this spread are clumping and overlapping too much. (d) Too many chromosome spreads in the same area. It is too hard to tell which chromosomes belong to which spread.

3. Transfer the slide to room temperature DPBS for 5 min.
4. Wash the slide in DPBS with 50 mM $MgCl_2$ at room temperature for 5 min.
5. Incubate the slide in formaldehyde solution for 10 min at room temperature.
6. Dehydrate the slide in the 70, 80, and 100% ethanol series, 1 min in each solution.
7. Air-dry the slides. The slides can now be denatured and hybridized with the FISH probe or they can be stored in a desiccator at $-20^{\circ}C$ for at least 1 year.
8. Add 100 μL of denaturation solution to the slide and cover with a 24×50 mm coverslip (see Note 10).
9. Place the slide on a $75^{\circ}C$ heating block for 1.5 min.

- Carefully remove the coverslip and immediately dehydrate in the 70, 80, and 100% ethanol series, 1 min in each solution (see Note 11).
- Air-dry the slide.

3.4. Probe Hybridization

- Thaw the probe, if necessary, then vortex vigorously. Protect it from light.
- Place the manufacturer recommended volume, typically 10 μ L, into an PCR or microfuge tube.
- Denature the probe for 10 min at 80°C, then at 37°C for 60 min in a thermocycler or water bath.
- When the probe is ready, place slide and a 22 \times 22 mm coverslip on a 37°C heating block.
- Add the probe to the area of the slide that contains the spreads. Add the coverslip to the slide and quickly seal the edges with rubber cement (see Note 12).
- Incubate overnight, in the dark, at 37°C.

3.5. Slide Washing and Mounting

- The next day, carefully remove the rubber cement from the slide with a forceps and by rubbing across it with your fingertips.
- Incubate the slide in 2 \times SSC until the coverslip lifts off by itself. Try to keep the slide protected from light for this and all subsequent steps.
- Wash the slide in formamide wash solution for 5 min at 45°C.
- Wash in 1 \times SSC for 5 min at 45°C.
- Wash in Tween-20 solution for 5 min at 45°C.
- Wash in DAPI stain solution for 5 min at room temperature.
- Dehydrate the slide in a 70, 80, and 100% ethanol series, 1 min per solution.
- Air-dry the slide.
- Add 1 drop of vectashield to the slide and cover with a 24 \times 50 mm coverslip.

3.6. Viewing and Interpretation

- Observe the slide using a fluorescence microscope equipped with filters that are appropriate for the fluorophore used to label your probe as well as DAPI (see Note 13).
- In diploid interphase nuclei, probes should show two signals (see Fig. 3a). In metaphase chromosome spreads, the signal may appear as 1 signal per chromosome (see Fig. 3b) or as two closely spaced signals per chromosome (see Fig. 3c). Two closely spaced signals on a chromosome spread should be counted as one signal.

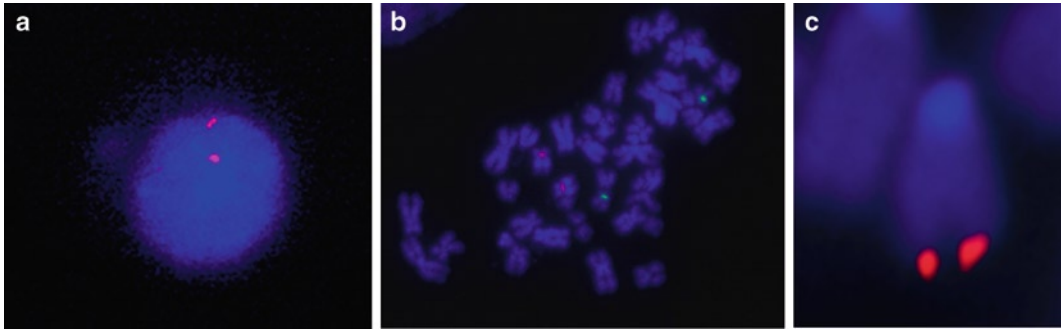


Fig. 3. Nuclei and chromosome spreads hybridized with FISH probes. (a) Interphase nuclei hybridized with chromosome 12 probe and stained with DAPI. Disomic cells should show two fluorescent spots. (b) Metaphase chromosome spread hybridized with chromosome 12 (*red*) and chromosome 17 (*green*) FISH probes. The probes show up as a single dot on the chromosome. This spread is disomic for both chromosome 12 and 17. (c) Example of a chromosome hybridized with a probe that shows up as two dots on a single chromosome. Either morphology – single dot or double dot per chromosome – can be observed.

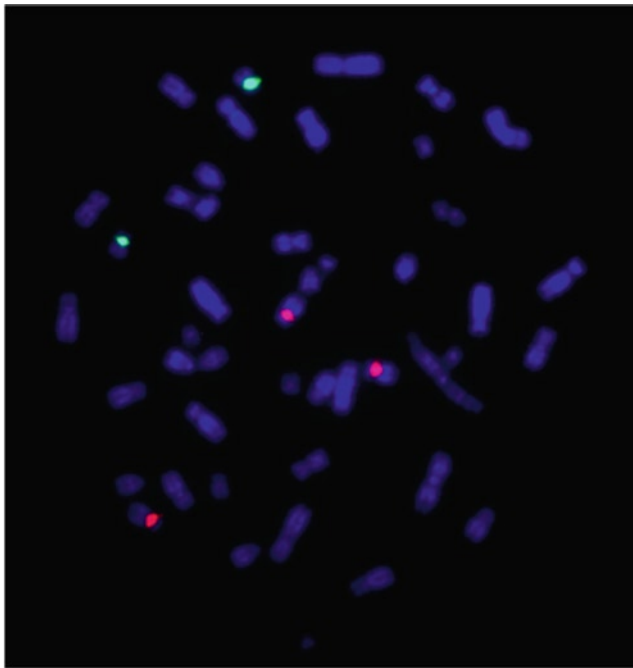


Fig. 4. Metaphase chromosome spread hybridized with probes for chromosome 12 (*red*) and 17 (*green*). Note that this cell is disomic for chromosome 17 but trisomic for chromosome 12 so this cell is considered aneuploid. Photograph courtesy of Dr. Zoltan Simandi.

3. Using a multichannel laboratory cell counter, count the number of signals from 200–500 nuclei or chromosome spreads. Record the number of monosomic and trisomic chromosome events in the culture.
4. Figure 4 shows a metaphase chromosome spread from human ESCs hybridized with probes for chromosome 12 in red and 17 in green. The cell is trisomic for chromosome 12 and disomic for chromosome 17, making the cell aneuploid.

4. Notes

1. Ensure the 25 μL of pepsin is thoroughly distributed in the 0.01 M HCl solution by swirling the coplin jar before adding the slide.
2. Stock formaldehyde solutions are 37%.
3. If coplin jars are not available, 50 mL conicals can be used.
4. If the PSCs were cultured on inactivated human fibroblasts, the fibroblasts will be present on your slide and indistinguishable from human PSCs that are not in metaphase. This typically is not a problem due to the much lower number of fibroblasts compared to PSCs in the culture. However, if this is a concern, only analyze metaphase chromosome spreads as these are derived from actively dividing cells and can only be PSCs, assuming the feeder layer is fully inactivated.
5. FISH can be done on intact, nonmitotic nuclei, so the colcemid treatment is optional, but it is often useful. Longer colcemid incubation times can be used but this will result in shorter, more condensed chromosomes. Incubations much longer than ~ 8 h can be toxic to the cells.
6. Treatment with hypotonic 0.075 M KCl solution leads to cell swelling. When the cells are centrifuged after treatment with 0.075 KCl, the cell pellet should be visibly larger.
7. It is very important that the cells are moving (slowly) and not in clumps when the fixative is added. If not, the cells will become “fixed” together in the clumps, rendering those cells uninformative when analyzed.
8. The quality of metaphase chromosome spreads is dependent on drying time. Steam is used to slow down the evaporation process. Depending on the atmospheric conditions in the lab on that particular day, it may or may not be necessary to slow the evaporation process with steam. One must empirically determine the best drying procedure for any particular day. In addition to manipulating the drying time with steam, the fixative can be altered so that it has more or less methanol or glacial acetic acid. A fixative with more methanol (e.g., 6:1 methanol to glacial acetic acid) would dry faster than one with more glacial acetic acid (e.g., 1:1 methanol to glacial acetic acid). Getting good metaphase chromosome spreads can be very, very difficult some days but, fortunately, FISH can be done on intact, nonmitotic nuclei as well.
9. From this point on, slides must not dry out until step 7.
10. From this point on, slides must not dry out until step 11.

11. Often the easiest way to remove the coverslip is to turn the slide perpendicular to the floor (preferably over a sharps container) and quickly flick your wrist in a downward motion.
12. The rubber cement seal does not have to be pretty – just make sure that all the edges of the coverslip are sealed.
13. Typically, it is easiest to find the cells first in the DAPI channel then look at your probe in the appropriate channel.

References

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