Telomeric PNA FISH and COFISH on Metaphase Chromosomes

Required Solutions/Reagents

FISH & COFISH

Colcemid
(Roche 10295892001, 10 ug/mL, stored at 4°C)

Fixative
Make fresh.
3:1 MeOH and glacial acetic acid.

Blocking Reagent
(Roche 11096176001)
Make a 10% stock, dissolved in maleic acid buffer:
100 mM maliec acid
150 mM NaCl
Adjust pH to 7.5 (20°C) with NaOH.
Store at 4ºC.

Hybridizing Solution
Make fresh, but can be stored at 4ºC for 2 weeks if you have excess.
10 mM Tris-HCl pH 7.2
70% formamide (from the deionized stock)
0.5% blocking reagent (from 10% stock)

Hybridization Wash #1
10 mM Tris-HCl pH 7.2
70% formamide
0.1% BSA, must be dissolved before adding formamide.

Hybridization Wash #2
0.1 M Tris-HCl pH 7.2
0.15 M NaCl
0.08% Tween-20
Add DAPI to 2nd of 3 washes:
FISH – 1:3,000 DAPI from 0.5 mg/mL stock
COFISH – 1:750 DAPI from 0.5 mg/mL stock

PNA probes from PNA Bio Inc.
FITC-TelC (F1009)
TelC-FITC: FITC-OO-CCCTAACCCTAACCCTAA 3'
Make 100μM stock in formamide. Heat 10 min at 60˚C and vortex occasionally.
Make 10 μl aliquots and keep at -80˚C for long-term storage.
Store thawed aliquots at 4˚C (in the dark).
Dilute 1:1000* and heat the dilution for 5 min at 60˚C before use.

Cy3-TelG (F1006)
TelG-Cy3: Tam-OO-TTAGGGTTAGGGTTAGGG 3'
Make 50μM stock in formamide. Heat 10 min at 60˚C and vortex occasionally.
Make 10 μl aliquots and keep at -80˚C for long-term storage.
Store thawed aliquots at 4˚C (in the dark).
Dilute 1:1000* and heat the dilution for 5 min at 60˚C before use.

* Adjust dilutions based on signal intensity and/or background.

DAPI
Reconstitute at 0.5 mg/ml, store at -20°C in the dark (Sigma D-9542)

ProLong Gold Antifade Reagent
(Life Technologies P36934)

Frosted Slides
(Fisher Scientific 12-550-11)

Microscope Cover Glass
(Fisherbrand 12-545-M 24x60)

COFISH

BrdU/BrdC
BrdU (MP Biomedicals 100166) stock concentration: 10 mM in H2O
BrdC (Sigma B5002) stock concentration: 10 mM in H2O
Make 1000x working stock solution of BrdU/BrdC by mixing 3 parts BrdU to 1 part BrdC (final concentrations of 7.5 mM of BrdU and 2.5 mM of BrdC.)
Store at -20°C covered in aluminum foil.

RNase A
(Sigma R5000)
Stock solution: 50 mg/mL in 10 mM Tris-HCl pH 7.2
Heat inactive 10 min at 80°C. Store at -20ºC.

Hoechst 33258
(Life Technologies H21491, 100 mg)
Stock solution: 10 mg/ml in H2O. Store at 4°C covered in aluminum foil.

Exonuclease III & buffer
(Promega M1811)

Methanol/acetic acid fixed metaphase spreads

1. Split cells 36-48 hours before harvest to be ~50% confluent at time of harvest.
2. *For COFISH, add BrdU:BrdC 12-16 hours before harvest (for a little less than one cell cycle to avoid double labeling).*
2. Incubate 30 min – 2 hours in regular medium with 0.1 μg/ml of colcemid for human cells and fast-growing mouse cells, and 0.2 μg/ml for slower-growing mouse cells – the effects of the colcemid should be obvious at time of harvest (rounded, refractile cells with blebby membranes).
3. Harvest cells by trypsinization, neutralize trypsin by adding media, and spin down (5 min at 1000 rpm). Be gentle during harvesting, many of the dividing cells tend to lift off. Cells that have floated off during the harvest can be recaptured by spinning down the wash supernatant as well. You can also use the conditioned media to neutralize the trypsin so that you can recover any floating mitotic cells.
4. Remove supernatant completely, resuspend in 5 ml of 0.075 M KCl (pre-warmed to 37ºC), be gentle to prevent lysis, this step swells the cells. (At this stage you can also add a couple of drops of fixative, approx 500μL, if you're having problems with a lot of cell debris when you drop metaphase spreads.)
5. Incubate for 15 to 30 min at 37ºC, invert tubes every 5-10 min to keep cells suspended.
6. Spin the cells down, 5 min at 1000 rpm.
7. Decant the KCl, resuspend cells fully in the small volume of KCl that was left (by tapping).
8. Drop by drop add 500 μl of cold fixative while the cells are slowly and gently mixed on a vortex (<1000 rpm).
9. Add another 500 μl of fixative being less careful.
10. Fill to 10 ml with the fixative and store at 4ºC o/n or longer; cells can be kept at this stage indefinitely. When dealing with few cells, its better to spin them down from the first ml of fixative and then suspend them again in a small volume of new fixative.
11. When ready to drop, spin the cells down (1000rpm).
12. Resuspend cells in 1mL fixative (may vary depending on cell number).
13. Place a few slides in cold water.
14. In the thermotron, drop the resuspended cells from a couple of inches above the end of the wet slide tilted at a 45° angle, wash the nuclei with fresh fixative (drop fixative across the slide with a bulb and Pasteur pipette; the nuclei and chromosomes should not wash off.)
15. **If not using thermotron:** for FISH, place wet paper towels on top of a heating block set to 80ºC. Place slides after dropping metaphases for 1 min on the humidified 80ºC heating block.
16. **If not using thermotron:** for COFISH, place wet paper towels on top of a heating block set to 42°C. Place slides after dropping metaphases for 1 min on humidified 42°C heating block.
17. Check the slides under a regular light microscope for spreading efficiency. You should see many nuclei (all of the cytoplasmic membranes should be washed away or barely visible.) Well spread metaphase chromosomes should look like small black dots at low magnification. The arms of the chromosomes should be visible at higher magnifications. If the nuclei are too crowded or too sparse, you may need to dilute or concentrate your sample and drop the slide again.
18. Let the slides dry o/n, protected from light if intended for COFISH.

COFISH

Degradation of the Newly Synthesized Strand

1. Rehydrate slides in PBS for 5 min at rt. *Perform all steps in coplin jars that are protected from light*.
2. Treat slides with 0.5 mg/ml RNase A (in PBS, DNase free) for 10 min at 37°C.
3. Stain slides with 0.5 μg/ml Hoechst 33258 (Sigma) in 2x SSC for 15 min at rt.
4. Place slides in a shallow plastic tray *(the ones used for DNA gels)* and add enough 2x SSC to just cover the slides. Expose slides to 365 nm UV light at rt (Stratalinker 1800 UV irradiator) for $5.4x10^3 J/m^2$ (choose 'energy' and enter 5400 on Stratalinker display).
5. Digest the BrdU/BrdC-substituted DNA strands using coverslips on the slides with at least 80 μl of 10 U/μl of Exonuclease III (Promega) in buffer supplied by manufacturer (50 mM Tris-HCl, 5 mM MgCl2, and 5 mM DTT, pH 8.0) at 37°C for 30 min.
6. Wash in PBS for 5 min.
7. Dehydrate in ethanol series: 5 min in 70%, 95%, 100% at rt and air dry slides (slides can be stored at rt in the dark for several days).

Hybridization

1. Place 80 μl of hybridization mix (see recipe) onto coverslips and pick up the coverslip with the metaphase slide (cells down), then turn the slide up.
2. Hybridize for 1.5 – 2 hours with TelG-Cy3 (1:7500), at rt, in the dark, with wet paper towels.
3. Rinse in wash #1 for 2-5 seconds. *Dry slide briefly by dabbing on Kimwipe*.
4. Hybridize for 1.5 – 2 hours with TelC-FITC (1:1000), @ rt, in the dark, with wet paper towels.
5. Take off coverslip, wash slides in hybridization wash #1: 2 times 15 min, on shaker.
6. Wash slides in hybridization wash #2: 3 times 5 min on shaker. To second wash, add 1:750 DAPI from 0.5 mg/ml stock.
7. Dehydrate in ethanol series: 5 min in 70%, 95%, 100% ethanol at rt and air-dry slides.
8. Mount using ProLong Gold, seal with nail polish, store at -20°C protected from light.

FISH

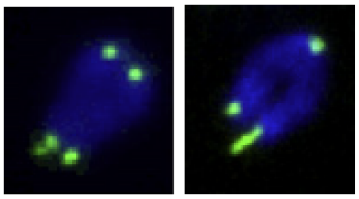
(Lansdorp et.al. Hum Mol Gen, 1996,5, 685-691.)

1. Rehydrate MetOH/Acetic acid spreads in PBS (pH 7.0-7.5) 5 minutes.
2. Perform the following optional steps:
    a. Fix in 4% formaldehyde in PBS, for 2 min (dilute from 37% commercial formaldehyde).
    b. Wash in PBS 3 times, 5 minutes each.
    c. Treat with pepsin (1 mg/ml) @37ºC, 10 min; pepsin prepared fresh in 10 mM glycine pH 2 and warmed up to 37ºC.
    d. Wash in PBS 2 times, 2 min each.
    e. Fix in 4% formaldehyde in PBS, 2 min.
3. Wash in PBS 3 times, 5 min each.
4. Dehydrate in ethanol series: 5 min in 70%, 95%, 100% at rt and air dry slides
5. Place 40 µl of hybridization mix (see recipe) onto coverslips and pick up the coverslip with the metaphase slide (cells down), then turn the slide up.
6. Denature on a 80ºC hot plate, 3 – 10 min.
7. Hybridize for 1 – 2 hours, at rt, in the dark, with wet paper towels, or overnight at 4°C.
8. Take off coverslip, wash slides in hybridization wash #1: 2 times 15 min, on shaker.
9. Wash slides in hybridization wash #2: 3 times 5 min on shaker. To second wash, add 1:3000 DAPI from 0.5 mg/ml stock.
10. Dehydrate in ethanol series: 5 min in 70%, 95%, 100% ethanol at rt and air-dry slides.
11. Mount using ProLong Gold, seal with nail polish, store at -20°C protected from light.

SCORING

Fragile Telomere

score well-separated telomere signals, **only** score long-arms for mouse cells



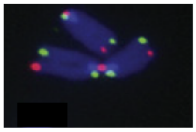
	1 fragile telomere **2** telomeres	**1** fragile telomere **2** telomeres
% fragile telomeres:	50%	50%

Chromosome fusion

8 telomeres fused **12** telomeres

% fused telomeres: 66.7%

Chromatid fusion

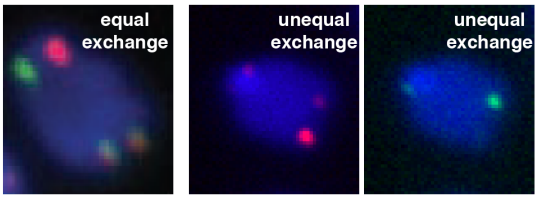


2 telomeres fused **12** telomeres

16.7%

Telomere-Sister Chromatid Exchange

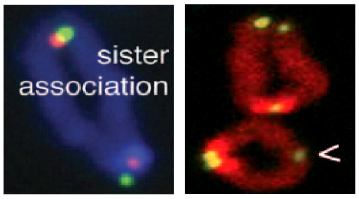
score well-separated telomere signals, **only** score long-arms for mouse cells



	2 exchange **2** telomeres	**2** exchanges **2** ends
% T-SCE per telomere:	100%	100%

Sister Association

score well-separated telomere signals, **only** score long-arms for mouse cells



	2 telomeres 'fused' **2** telomeres	**2** telomeres 'fused' **4** telomeres
% sister association:	100%	50%