CO-FISH PROTOCOL

Chromosome orientation or CO-FISH is the name given to a FISH technique that uses single-stranded DNA probes to produce strand-specific hybridization. The technique relies on labeling by 5'-bromodeoxyuridine (BrdU) incorporation one strand of the sample cells' DNA during S-phase. Metaphase chromosomes are prepared a number of hours after the BrdU pulse. Following Hoechst staining and UV irradiation, the newly synthesized DNA becomes nicked at the sites of BrdU incorporation. These nicks are enlarged using ExoIII, and the newly replicated DNA is removed, leaving the parental strand as a single-stranded template for the hybridization procedure. Initially, CO-FISH was designed to determine the orientation of tandem repeats within centromeric regions of chromosomes (1,2). This technique had also been useful in assessing aspects of translocated chromosomes, specifically Robertsonian (3,4), and also chromosomal inversions, since an inversion obviously changes the orientation of the involved chromosome segment (5,6). For a review on CO-FISH, see Bailey et al. (7).

PNA CO-FISH Protocol

Note: Use a negative control with non-BrdU treated cells.

Required Solutions

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.

Colcemid (Roche 10295892001)

- Stock solution is 1 mg/ml. Dilute in DMEM to required concentration. Store at -20°C.
- Use 0.2µg/ml for mouse cells and 0.1 µg/ml for human cells.

KCl

0.075 M KCl pre-warmed to 37°C

Fixative

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

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.

- Working solution: Dilute RNase A to 0.5 mg/ml in 1x PBS.

Hoechst (Invitrogen 33258)

- Stock solution: 10 µg/ml in H2O. Store at 4°C covered in aluminum foil.
- Working solution: 0.5 µg/ml in 2xSSC.

Exonuclease III & buffer (Promega M1811)

Store at -20°C

Ethanol: 70%, 95%, and 100%

Blocking reagent (Roche 11096176001)

Stock is 10%, dissolved in maleic acid buffer:

- 100 mM maleic acid
- 150 mM NaCl
- Adjust pH to 7.5 (20°C) with NaOH. Store at 4ºC

Hybridizing Solution

- Make fresh, store at rt. Final concentrations in H2O.
- 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
- 1:750 DAPI from 0.5 mg/ml stock (in H2O)

DAPI

- Dissolve 0.5 mg/ml 4',6-diamino-2-phenylindole (Sigma D-9542) in H2O.
- Stable for at least one year at 4°C.

Embedding medium:

1. Dissolve 20 mg p-phenylene diamine (Sigma P-6001) in 2 ml 10x PBS by vortexing.
2. Immediately add 18 ml glycerol, mix carefully without creating air bubbles. Do not vortex. Store in 1 ml aliquots at -70°C. The solution should be colorless; discard when it turns yellow or brown.* or use ProLong Gold Antifade Reagent (Invitrogen #P36934)

BrdU/C Incorporation & Spreads

1. Incubate cells for 16-20 hours (a little less than one cell cycle; avoid double labeling) in fresh medium containing 5'-BrdU:5'- BrdC (ratio: 3:1, reagents from Sigma). Keep cells covered and reduce their exposure to light as much as possible.
2. 1 1/2 hour before harvesting, add colcemid to the media to accumulate mitotic cells.
3. Take off the medium and save. Harvest cells by trypsinization, suspend in media, and spin them down (5 min at 1000 rpm).
4. Remove supernatant completely, resuspend in 5 ml of 0.075 M KCl (pre-warmed to 37°C), be gentle to prevent lysis, this step swells the cells.
5. Incubate for 30 min at 37°C, invert the tubes to keep the cells suspended. Spin the cells (5 min at 1000 rpm).
6. Decant the KCl, resuspend cells fully in the small volume of KCl that was left (by tapping).
7. Drop by drop add 500 µl of fixative while the cells are slowly and gently mixed on a vortex (<1000 rpm). Add another 500 µl of fixative (you can now be less careful).
8. Fill to 10 ml with the fixative and store at 4°C o/n or longer; cells can be kept at this stage for years. When dealing with few cells, it's better to spin them down from the first ml and then suspend them again in a small volume.
9. When ready to drop, spin the cells down (1000 rpm).
10. Remove 9 ml of fix and resuspend cells in the 1 ml left (may vary depending on cell number).
11. Place a few slides in cold water. Place wet paper towels on top of a heating block set to 42°C (this is essential to prevent denaturation). Drop the resuspended cells from a couple of inches on each end of the wet slide, and wash the nuclei with fresh fixative. Place them for a minute on the humidified 42°C block.
12. Air-dry the slides overnight.

Degradation of the Newly Synthesized Strand

1. Rehydrate slides in PBS for 5 min at rt.
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 and add enough 2x SSC to just cover the slides. Expose slides to 365 nm UV light at rt (Stratalinker 1800 UV irradiator) for 30 min (equivalent to 5.4x103J/m2).
5. Digest the BrdU/BrdC-substituted DNA strands with at least 80 µl of 10 U/µl of Exonuclease III (Promega) in buffer supplied by the manufacturer (50 mM Tris-HCl, 5 mM MgCl2, and 5 mM DTT, pH 8.0) at rt for 10 min.
6. Wash in PBS (5 min).
7. Dehydrate in ethanol series 70%, 95%, 100% at rt and air dry slides (slides can be stored at rt).

FISH

1. Heat up the slide and 80 µl of hybridization mix at 80 ^{o}C for 5 min. Place hybridization mix (see recipe) onto each metaphase spread on the slide, avoid air bubbles. Incubate for 5 min at the same temperature.
2. Hybridize for 2 h TelG-Cy3 (1:250, 50 uM or 250 ug/ml), @ rt, in the dark, with wet paper towels.
3. Rinse in wash #1 for 2-5 seconds.
4. Heat up the slide and hybridization mix TelC-FITC (1:100, 50 uM or 250 ug/ml) at 50 ^{o}C for 5 min. Incubate for 5 min at the same temperature.
5. Hybridize for 2 h, @ rt, in the dark, with wet paper towels.
6. Hybridization wash #1: 2 times, on shaker or stirrer, 30 min in small Coplin jars.
7. Hybridization wash #2: 3 times 5 min in small Coplin jars. To second wash, add 1:750 DAPI from 0.5 mg/ml stock (in H2O).
8. Dehydrate in ethanol series: 5 min in 70%, 5 min in 95%, 5 min in 100% ethanol.
9. Air-dry slides and mount as usual (use embedding medium).

Reference:

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3. **Garagna, S., D. Broccoli, C.A. Redi, J.B. Searle, H.J. Cooke, and E. Capanna.** 1995. Garagna, S., D. Broccoli, C.A. Redi, J.B. Searle, H.J. Cooke, and E. Capanna. 1995. lose telomeric sequences but retain some minor satellite DNA in the pericentromeric area. Chromosoma 103:685-692.
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5. **Bailey, S.M., E.H. Goodwin, J. Meyne, and M.N. Cornforth.** 1996. CO-FISH reveals inversions associated with isochromosome formation. Mutagenesis 11:139-144.
6. **Bailey, S.M., J. Meyne, M.N. Cornforth, T.S. McConnell, and E.H. Goodwin.** 1996. A new method for detecting pericentric inversions using COD-FISH. Cytogenet. Cell Genet. 75:248-253.
7. Bailey, S.M., J. Meyne, M.N. Cornforth, T.S. McConnell, and E.H. Goodwin. 1996. A new method for detecting

pericentric inversions using COD-FISH. Cytogenet. Cell Genet. 75:248-253.