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Metaphase_spread_and_DNA_FISH_cell_lines

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We use this protocol and it's working

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Abstract

Cytogenetically detect single-cell ecDNAs in cell lines via staining/homologous DNA hybridization and fluorescence microscopy -- fluorescence in situ hybridization (FISH).

Troubleshooting

Introduction

1 Cytogenetically profile single-cell DNAs through metaphase karyotyping by DNA FISH.

Material

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- Methanol/glacial acetic acid 3:1 -- Prepare fresh in a fume hood prior to use
- Hypotonic stock solution: 0.075M KCl (gibco, ref. 10575-0821)
- 2 X SSC (Saline-Sodium Citrate) stock solution (/0.05% TWEEN20)
- 0.4 X SSC stock solution
- Total probes : hybridization buffer = 1 : 4 (e.g. for a master mix of 10 uL from 2 different probes, add 1 uL probe 1 + 1 uL probe 2 to 8 uL hyb buffer) -- prepare fresh

Procedure

3 **Prepare metaphase spreads**

3.1 Grow cells to 60-80% confluency.

3.2 Arresting cells at metaphase**:** Replace old media with media with 0.1 µg/mL Colcemid diluted from 10 µg/mL stock solution (Karyomax from Gibco. Ref#15212-012), (e.g. 100 µL of 10 µg/mL Colcemid/10 ml medium) and incubate O/N (depending on the cell doubling time).

3.3 Warm up 0.075M KCl in 37°C water/bead bath.

3.4 Examine if cells are mostly mitotic that round up under the inverted microscope.

3.5 Transfer the colcemid-spiked media to a 15 ml conical tube. Wash cells with 5 ml PBS. Transfer the PBS wash to conical.

3.6 Add 1-2 ml trypsin (depends on cell density) to the cells in a 10 cm dish and digest for 3 min at 37°C.

3.7 Examine cells under the inverted microscope (optional). Quench the digestion by adding 3-5 ml colcemid-spiked media when most cells start to lift off (media should be at least 3 times the volume of trypsin). Gently pipette up and down 10x to flush off adhered cells.

3.8 Transfer the collected cells to the remaining colcemid-spiked media and centrifuge at 400x G for 4 min. Aspirate the supe.

3.9 Wash cells with PBS: If cells <6 million, add 1 ml PBS; if cells ≥ 6 million, add n mL PBS, *n = #cell/6 million rounded up* (e.g. 2 tubes for 8 million). Gently pipette 10x to resuspend. Transfer the suspension to one or n 1.5 ml microcentrifuge tube(s). Centrifuge at 5000 rpm for 2 min and aspirate the supe.

3.10 Swelling cells: Add 600 µl pre-warmed 0.075M KCl dropwise down the side and resuspend by tapping/very gentle pipetting → incubate for 15 min in 37°C water/bead bath (15 min is the optimal time for swelling. Swollen cells are brittle – *no vortex or pipetting hereafter*. Cells must be sufficiently swollen to burst on microscope slides, whereas with excessive swelling, cells break in fixative and release cytoplasmic contaminants). Prepare fresh fixative in a fume food while waiting: ≥ 2.4 ml/pellet. Optional: place fixative on ice.

3.11 Add 600 µl Carnoy's fixative (use 100-300 and 800-1000 µl for hundreds of K cells and ≥ 7 million cells, respectively) dropwise down the side to quench the reaction and *immediately* centrifuge at 5000 rpm for 2 min and aspirate/pipette the supe till a few drops (100-200 µL) left.

3.12 Fixing cells: Resuspend pellets in remaining solution by gently tapping/flicking the bottom of the tube till no clumps. Add (pre-chilled) 600 µl Carnoy's fixative dropwise down the side and flick to mix well. Centrifuge at 5000 rpm for 2 min. Aspirate/Pipette until a few drops left.

3.13 Repeat step 11 twice.

3.14 Centrifuge and resuspend pellets in the remaining fixative. Add 300-1000 µl fixative depending on pellet size for a slightly milky suspension (over-concentrated cells are hard to profile individually and impair image quality). Cells can be stored in fixative at -20°C for an extended time.

4 Prepare microscope slides

4.1 Prepare slides on a clean, dust-free surface (e.g. a clean slide box). Keep the sample side clean -- NO TOUCHING/SCRATCHING. Dropped metaphase spreads must be processed within 2 days to prevent chromosome degradation.

4.2 Addditional washing steps for pre-made metaphase samples:

1. Thaw -20℃ metaphase samples at RT.
2. Prepare fresh Carnoy's fixative in a fume hood while waiting.
3. Repeat step 10 twice or more till the supernatant is clear.

4.3 Prepare 200-250 ml H_20 in a 400 ml beaker. Microwave for 1-1.5 min for heated vapor.

4.4 Mix the suspension well by flicking/tapping. Pipette up 10 µL.

4.5 Place the slide over the hot water for humidity until tiny droplets are formed. *Immediately* drop the 10 µl suspension from height (an arm's stretch) to the moist slide. Re-humidify if the slide dries before drops hit.

4.6 Briefly hover the slide above the beaker for 2-3 s to further spread chrs by humidity.

4.7 Age/Dry at room temp (20-25℃) 1h - overnight in a closed environment (e.g. slide box/drawer...).

5 DNA FISH

5.1 Minimize exposure of fluo probes to light to limit photobleaching. Photobleaching impairs fluorescence and image contrast.

5.2 Preparations**:** Prepare 4 Coplin jars for 2X SSC, 70%, 85%, and 100% ethanol, enough to submerge metaphase spreads. Pre-warm DNA FISH probes in 37℃ water/bead bath; Set heat block to 70℃ based on *the physical thermometer*. Fill grooves in humidified chamber with dH_2O and set to 37℃ → close the lid.

5.3 **Dehydration:** briefly equilibrate spreads in 2X SSC (~3 sec) and *immediately* transfer to ascending alcohol gradients, 2 mins w/ lid on: 70% → 85% → 100% ethanol → air-dry. While air-dry, prepare 10n µL of 1:5 probe master mix in hybridization buffer, e.g. In a colored PCR tube, add 2 µl of pre-warmed probes (Empire Genomics, stored in -20℃ protected from light), including all probes types, to 8 µl hybridization buffer (e.g. 1 µl each for 2 types of probes) and mix well by 3 pulses of vortex followed by a brief spin. *For just DAPI staining, start from 28.*

5.4 Add 5 µL of probe mix/slide onto the middle of the chromosome spread (NO TOUCHING/SCRATCHING!). Cover the spread with a 22×22 mm coverslip, taking care not to have bubbles or press too hard or slip. Push bubbles out using a pipette tip.

5.5 **Co-denaturation:** *Immediately* incubate the slide in the dark on a 70-72℃ heat block for 2 min (Heat exceeding 3 mins and/or 75℃ will damage chromosome morphology).

5.6 **Probe hybridization:** Incubate the slides at 37℃ O/N (16-20 h) in the pre-set dark humidified chamber. Humidity keeps the probe mix from drying out. Elongated hybridization leads to non-specific binding of FISH probes.

5.7 After hybridization: Prepare 3 Coplin jars containing fresh 0.4X SSC, 2X SSC + 0.05% tween20, and 2X SSC.

5.8 Post-hybridization wash: Gently lift the coverslip tweezing a corner -- no scraping! Wash slides in 0.4X SSC and 2X SSC/0.05% Tween20 for 2 min each with shaking for 10-15 s. Wash briefly in 2X SSC.

5.9 **DAPI counterstaining:** Remove excess buffer by wicking slides and wipe back of the slide clean using a paper towel. Add 1 drop of SlowFade Diamond Antifade Mountant with DAPI and seamlessly seal a 24×60 mm coverslip with nail polish.

5.10 Alternative DAPI staining: add 10 µl of 1:1000 or 200 µl of 1:10000 DAPI solution per slide and stain for 2 min in the dark. Agitate briefly in 2X SSC to remove excess DAPI. Remove the buffer as much as possible and wipe the back with a paper towel (watery liquid mixed with mounting media changes optimal refractive index for imaging).

5.11 Add 30 µl of VectaShield mounting medium (non-hardening with anti-fade) (Optional for just DAPI staining). Seal a 24 X 60 mm coverslip with nail polish.