01-07-13

Culture and Metaphase Spread Preparations of Mouse Epithelial Tumors

Reagents:

Acetic acid, glacial
MACRON Fine Chemicals, Cat. V193-05
Antibiotic-Antimycotic (Anti-Anti) (100X)
Life Technologies, Cat. 15240-062
Collagenase Type I (for epithelial cell tumors, lung and liver tumors)
Life Technologies, Cat. 17100
Collagenase Type III (for mammary tumors)
Life Technologies, Cat 17102
Colcemid
Roche, (10 µg/ml), Cat. 10 295 892 001
1X DMEM/F12 medium without phenol-red, (1:1)
Life Technologies, Cat. 11039-021
Fetal bovine serum (FBS)
Life Technologies, Cat. 16140-022
Fungizone (Antimycotic)
Life Technologies, Cat. 15290-018
Methyl Alcohol (MeOH), Anhydrous (Absolute)
MACRON Fine Chemicals, Cat. 3016-02
100X Penicillin-Streptomycin (Penn/Strep)
Life Technologies, Cat. 15070-063
1X Phosphate Buffered Saline (PBS), pH 7.4, w/o calcium or magnesium
Life Technologies, Cat. 10010-023
Potassium chloride (KCl)
Mallinckrodt, Cat. 6858
BD BIOCOAT™ Cellware (Mouse Collagen Type IV 6-well plates)
BD Biosciences, Cat. 354428
BD BIOCOAT™ Cellware (Mouse Collagen Type IV 25 cm_2 flasks)
BD Biosciences, Cat. 354428

Preparation of Tumor Isolation Media

DMEM/F12 media	480 ml
FBS	5 ml
Pen/Strep (100X)	5 ml
Anti-Anti (100X)	5 ml
Fungizone	5 ml

Filter-sterilize and store at 4°C.

Preparation of Tumor Culture Media

DMEM/F12 media	460 ml
FBS	25 ml
Pen/Strep (100X)	5 ml
Anti-Anti (100X)	5 ml
Fungizone	5 ml

Filter-sterilize and store at 4°C.

Collagenase Solution (type I or type III)

Working solution = 10mg/ml
15 mg collagenase
10 ml complete medium

Culture of Tumor Cells

1. Dissect out the tumor from the mouse, keeping the operating field as sterile as possible
Note: Douse the skin of the mouse with 70% ethanol prior to first incision to reduce contamination of the tumor tissue.

2. Place the tumor in a sterile 10 cm petri dish containing 2 ml of tumor isolation media. Wash the tumor by transferring it to a new 10 cm petri dish (containing 2ml isolation media). Remove fat and extraneous non-tumor tissue using sterile forceps/tweezers. Cut and mince the tumor with sterile scalpel blades. Add 1 ml of collagenase type III for 30 min-1 hr (monitor culture during this process. Stop digestion of the tumor tissue when single cells are visible under the tissue culture microscope).

3. Add 5 ml isolation media to the dish and aspirate up and down 2-3 times using a sterile pipette to further disassociate the cells.

4. Transfer cells with medium to a 15 ml screw-cap centrifuge tube.

5. Centrifuge tube at 1000 rpm for 10 min.

6. Discard supernatant.

7. Re-suspend in 10 ml of fresh medium and repeat centrifugation (see step 5).

8. Discard supernatant.

9. Add 12 ml of tumor culture medium, and re-suspend cells using a 5 ml pipette.

10. Transfer cell suspension to three wells (starting with a 6-well sterile collagen plate, dispense 4 ml of cell suspension per well).

11. Incubate overnight at 37°C in a CO_2 incubator and check the next morning for cell attachment. Once the culture becomes ~70% confluent, the cultures can be passaged (1:2 split) and the contents of each individual well transferred to T25 collagen-coated flasks.

12. Use a cell scraper to remove the cells from the bottom of each well. Transfer the contents to a sterile 50 ml centrifuge tube. **Note:** Trypsin is sometimes toxic to primary cells and therefore not recommended for passaging primary cell cultures.

13. Remove any remaining residual cells by adding another 5 ml tumor culture media to each well and transfer contents to each tube.

14. Centrifuge tube at 1000 rpm for 10 min.

15. Discard supernatant.

16. Add 15 ml of culture medium, and re-suspend cells using a 5 ml pipette.

17. Transfer the cell mixture to three T25 flasks (5 ml per flask) and incubate overnight at 37°C in a CO_2 incubator.

1**8.** Check the cultures daily and when the culture become ~70% confluent, the cultures can be processed for chromosome metaphase preparations following the protocol outlined next.
Note: This method is empirical and will depend upon how large the tumor is and how large a cell suspension was obtained. How well and how quickly the tumor cells grow will determine when the cells are ready to harvest for chromosome preparations. Epithelial cells will grow as small foci (cuboidal-shaped cells spreading out from a central mass).

Chromosome Metaphase Spread Preparations

If the cells are to be analyzed by **Spectral karyotyping (SKY)**, or conventional banding methods, it is ideal to harvest cells for metaphase spread preparations within the first 48-72 hr of culture, as (1) the cells may be chromosomally unstable; or (2) the karyotypes may no longer reflect the original status of the tumor cells if the cultures are maintained for longer periods.

1. Harvest time is dependent upon condition of culture. The criteria for determining the best time to prepare metaphase spreads from the tumor cultures is (a) when the culture is 60-70% confluent, after the last passaging of the tumor cells, and (b) using the inverted tissue culture microscope, the number of cells in mitosis is approximately 10 per field of vision.

2. Add colcemid (0.1 g/ml, 100 µl for every 10 ml media) to cell cultures.

3. Incubate cells for 1 h. Monitor the cultures following addition of colcemid, and if the number of cells in mitosis has not changed significantly after 1 hr, the time can be extended. **Note:** The longer the cells are exposed to colcemid, the shorter the chromosomes become.

4. Remove the media from each flask with a sterile 10 ml pipette, save entire contents into a 50 ml conical centrifuge tube.

5. Add 5 ml sterile 1X PBS (pH 7.4) to each flask, then transfer contents to 50 ml tube (the same tube as described above). **Note:** Monitor cells under inverted microscope continuously, as some cultures are sensitive to trypsin and cells will not survive treatment, i.e. you may not end up with any metaphase spreads as the cells have been totally digested.

6. Add 0.5 ml sterile trypsin to each flask and incubate until cells slough off. Time will vary with each cell culture as some cells adhere to the bottom of the flask more than others, but is approximately 1-2 min. Note: The FBS (fetal bovine serum) present in the media used to grow the cells inactivates the trypsin.

7. Spin cells in a clinical centrifuge at 1000 rpm, for 5 min at RT.

8. Remove the supernatant carefully by vacuum aspiration, leaving 0.5 ml in the tube and flick the tube with finger to loosen the pellet.

9. Add 10-50 ml hypotonic solution to this same conical tube and incubate at RT for 25 min. **Note:** The volume of hypotonic solution added to each tube is variable and determined specifically by final amount of cells in pellet. One does not need to measure the number of cells but generally speaking, if the cell pellet is just coating the bottom of the tube, add no more than 2ml hypotonic solution, if there appears to be 0.5-1.0 ml of cells at the bottom of the tube, add approximately 50 ml hypotonic solution. Adherent cells typically require 20-25 min exposure. Chromosomes will have excess cytoplasm if exposure to the hypotonic time is too short.

10. Add 1 drop of fresh methanol (using a 1 ml pipette): acetic acid fixative (3:1/vol: vol) per each ml of cell/hypotonic mixture and mix well.

11. Spin cell suspension using a clinical centrifuge at 1200 rpm for 5 min at RT.

12. Remove the supernatant carefully, leave 0.5 ml liquid, and flick the tube to loosen the pellet and break up all clumps of cells.

13. Add 5 ml fresh fixative down side of the tube very slowly, gently flicking pellet all the time. **Note:** To ensure that the final metaphase cell pellet is fully re-suspended, it is crucial to add fixative very slowly down the side of the centrifuge tube, otherwise the cell pellet will remain full of clumps, metaphase spreads will become trapped in the clumps and spreading will be compromised.

14. Spin at 1200 rpm for 5 min at RT.

15. Remove the supernatant and flick the tube to loosen the pellet.

16. Add 5 ml fresh fixative.

17. Spin at 1200 rpm for 5 min at RT.

18. Remove the supernatant and flick the tube to loosen the pellet.

19. Add 1-5 ml fresh fixative along the wall of the tube. **Note:** The final volume of fixative is determined by the desired concentration of cells. This requires a visual examination of the final solution so that it appears slightly cloudy yet translucent when held up to the light. If too much fixative is added, the SKY analysis will be hampered by low cell density, if the fixative volume is too small, chromosomes do not spread well as there will be too much cellular debris in close proximity to the spreads.

20. Pipette 15 µl of the fixed cell suspension onto a clean glass microscope slide in a humidity chamber or Thermotron set at 45-49 % humidity at RT and allow the slide to dry by evaporation (approximately 1-2 min).

21. View the dried slide with a 16x high-dry phase objective on a light microscope to determine the appropriate cell density, and extent of chromosome spreading. If there are too many cells on the slide, the amount of fixative can be re-adjusted to obtain the optimum cell density. **Note:** The spreading of chromosomes and the integrity of chromosome structure is dependent on the evaporation rate of the fixative as determined by the percent humidity, temperature, and success of the hypotonic procedure. If the chromosomes are light grey in color, increase the humidity, if the chromosomes appear shiny or have a bright halo around them, decrease the humidity in the chamber. If there is still excess cytoplasm surrounding the chromosomes, pepsin treatment can be used prior to hybridization methods. If this does not resolve the problem, redo the entire procedure and during the hypotonic solution step use a greater volume and or increase the time.

Storage: To store, tighten cap and wrap cap with laboratory film (Parafilm®), maintain cell pellets at -20 °C for short-term storage or at -80 °C for one year.