EXERCISE 9

Chromosome Analysis Lab

To observe chromosomes, cells must be in mitotic phase, during which time the chromatin has condensed into distinct chromosomes. Only a small percentage of the total culture will be in this phase at any given time. To increase this percentage, we treat cells with colchicine for 4-6 hours. This perturbs microtubule assembly, and, therefore, will eventually kill the cells. However, treatment for this length of time is not lethal. Since microtubules are required to form the mitotic spindle and pull the chromosomes apart, cells become trapped in mitotic phase. They continue to replicate their DNA and condense chromosomes, but then become "stuck." Since they are unable to complete division, they do not have the opportunity to "de-condense" their chromosomes.

The other major task is to cause the cells to "swell," accomplished by incubating them in a slightly hypotonic buffer (therefore, water rushes into them), and to cause them to break open on a glass slide. We stain the chromosomes and observe them under high-powered microscopy.

The karyotype of cells in culture changes over time. Continuous cell lines are particularly susceptible to this as "better-fit" mutations eventually take over the culture. Periodic karyotype analyses are required to confirm that you are still working with the cells that you think you are working with. On many occasions, other cell types have drifted into heterologous cultures maintained in the same lab from sloppy techniques (e.g., having 2 cells types in the hood at once; using the same pipette or media bottle for both, etc).

In this exercise, it is useful if possible to use a relatively stable line, such as 3T3 fibroblasts, which will yield a routine karyotype with relatively long chromosomes, and a transformed line, such as a neuroblastoma, glioma, hepatoma, etc., which will exhibit an uneven distribution of chromosomes, many of which will be small and appear fragmented.

PROTOCOL

1. Make "hypotonic buffer" (1 mL serum-free medium + 2 mL distilled water).

2. Incubate cells with 10^{-6}M colchicine (this was started earlier).

3. Rinse cultures with serum-free medium.

4. Trypsinize with 1mL of trypsin; wait until most cells detach.

5. Transfer the detached cells to a centrifuge tube, pellet the cells (3-5 mins. at 1,000 x g).

6. Carefully decant the supernatant.

7. Gently, down the side of the tube, add 2 mL of the hypotonic buffer.

8. YOUR CELLS WILL SWELL AND BE QUITE FRAGILE NOW; HANDLE GENTLY.

9. Let the suspension incubate on ice for 7 minutes.

10. Centrifuge and carefully decant the supernatant.

11. GENTLY resuspend pellet in 2 mL of fixative solution.

12. Repeat Steps 10 and 11.

13. While doing the last spin, clean 3 glass slides with alcohol and immerse them in an ICE-COLD mixture of 40% methanol/60% distilled water in Colpin jars.

14. Remove one slide at a time and touch its edge to a paper towel to remove excess solvent (but do not dry it off).

15. Tilt the slide on the bench at approximately 45ºC (e.g., use a book under one edge of the slide). Drop ONE drop of the resuspended cells onto the chilled, wet slide. The brittle cells will crack on impact, and their chromosomes will spread.

16. Try several heights, such as eye-level to the floor. Too short a distance and the cells may not break open or the chromosomes will not spread out; too long a distance and the chromosomes will spread too much, confounding determination of karyotype.

17. Dry the slides by waving them through the air.

18. Stain the cells:
    a. Flood the slide for 30 seconds with the already-prepared Giemsa stain.
    b. Rinse with distilled water and examine under the microscope.

Generate a histogram for each cell type, counting as many discrete “smears” as you can.

It is initially very difficult to discern chromosomes from debris (which can be similar in size and staining intensity). We recommend searching first for intact nuclei, which also stain, and some of which maintain their “in situ” appearance without chromosome condensation. This will help orient you for magnification, and it should then be easier to locate chromosomes.

MATERIALS

Slides (3 per person)
Colpin jars
Fixative solution
Sterile centrifuge tubes
Transfer pipettes
Colchicine
Trypsin
Serum-free medium
Distilled water
Giemsa stain
40/60 mixture of methanol and water (to be kept on ice)

- Colchicine:
  - Make a 10^{-3} M stock solution to be kept in 4ºC.
  - Only 2 mL needs to be added per plate for a final concentration of 10^{-6} M.

- Fixative solution:
  - 3 parts methanol + 1 part glacial acetic acid.

- Giemsa stain:
  - Follow manufacturer's instructions (Sigma-Aldrich); filter before use to reduce debris that looks and stains like chromosomes.

INTRODUCTION TO PLANT CELL CULTURE

So far, we have considered only mammalian cell culture. However, there are a wide variety of uses for plant culture, such as creating genetically modified crops. While there are several common features of both mammalian cells and plants, there are some key differences between them that must be considered when conducting an experiment. The following is a basic overview of plant cell culture to introduce you to the topic.

First, the cellular organization in plants is markedly different from mammalian cells. Plant cells contain a cell wall, which is made up of cellulose and lignin, and functions to provide structural strength, limit water absorption, and protect the inner cell structures; a central vacuole, which stores nutrients and water, and is involved in metabolism by retaining and degrading waste products and also some color; and plastids, which gather and store nutrients, pigments, and perform photosynthesis. Plant cells are also more totipotent than animal cells. When culturing plant cells, any cell is capable of creating an entirely new, genetically-identical plant.

There are many different ways of generating plant cells from a plant, including inducing callus formation; culturing meristems, roots, anthers, or embryos; and making protoplasts. Unlike dealing with animal cell cultures, plant cells are not judged on confluency.

Plant cells are more tolerant as far as their environment is concerned; they do not require the rigid conditions that animal cells require. This is true for all aspects of their environment: pH, temperature, nutrient availability, soil type, and water and light availability.

In addition to being extremely tolerant, these cells are also easier to manipulate. This characteristic is due to several factors, including their ability to generate new parts, such as a new leaf or flower, anywhere at any time, and their ability to clone themselves, producing genetically identical offspring. Plant cell manipulation has made use of these characteristics in order to propagate new varieties and cultivars of ornamental plants. Some ways to propagate plants include tip culture, cuttings, grafts, twig in water, and chopped up bits of roots.

Making a plant graft is much different from making an animal graft. Plants lack the ability to distinguish their own parts from parts derived from another plant. Therefore, there are no tissue rejection issues to consider for the plant.

Like animal cells, plants require hormones/growth regulators to maintain proper growth. Auxin, refers to a group of compounds including indole-3-acetic acid, IAA, IBA, NAA, and 2,4-D. This group of hormones is involved in several key processes, such as differentiating vascular bundles, opening tree buds, developing fruit from ovaries, and growing young shoots. Moreover, they stimulate "secondary thickening," which is the increased cellular division within the cambium that adds girth to the stem and maintains apical dominance. The concentration of auxins has been shown to affect elongation of roots and shoots: the lower the concentration means elongation continues unabated; the higher the concentration results in elongation inhibition.

Cytokinins are another type of plant growth regulators and include compounds such as kinetin, zeatin, BA, and coconut milk. The function of these substances increases cell division and thereby increases DNA replication, RNA, and protein synthesis. Cytokinins also reduce senescence and stimulate light-dependent seeds to germinate in the dark. This would be useful in plants such as tobacco or maple. Often, cytokinins are used together with auxins.