Introduction

Each somatic cell in the human body contains 23 pairs of chromosomes. During the interphase stage of the cell cycle each of these chromosomes is duplicated and consists of two chromatids joined by a common centromere (spindle attachment region). During mitosis the chromatids separate and become independent chromosomes which move to opposite ends of the cell. The subsequent division of the cytoplasm results in the formation of two new daughter cells each containing the same diploid number of chromosomes as the parent cell. Cells grown in tissue culture are used for most human chromosome studies. For this exercise, a human tumor cell line, HeLa, (which is highly aneuploid), is grown in culture and subsequently treated so as to allow for the microscopic examination of the chromosomes. Colchicine, a plant alkaloid, has the unique property of arresting cells in metaphase of the mitotic cycle by interfering with formation of the mitotic spindle which is needed for the movement of chromosome during the metaphase to anaphase progression. This blockage increases the frequency of metaphase cells. Metaphase chromosomes are most readily observed with the light microscope and various chromosomal features such as sister chromatids and centromeres are evident. In summary, the procedure for chromosome visualization (and karyotyping) entails arresting a fraction of a log phase population in metaphase, treating the cells with a hypotonic saline solution to swell the cells and increase their fragility, fixation with acetic acid-methanol, splattering onto slides, and staining. This is followed by a search for ideal chromosome spreads for the study of chromosome number and structure.

This kit contains the following materials:

- 15 tubes each containing 1 ml of metaphase-blocked cell suspension, fixed in acetic acid-methanol fixative.
- Stain #1 and Stain #2.
  (Be careful! Avoid contact with skin and clothes.)
- Permount (mounting medium).
  (Keep in closed container. Avoid prolonged contact with air.)
- Keep in a tightly sealed tube.
- Printed background and procedural information as well as a Glossary of Terms, References, & Further Reading.

If your kit is not complete, contact us immediately.
Teacher Preparation

The following materials will need to be made available to each student or group of students:

⇒ Gloves should be worn while handling stain and cells.
⇒ Binocular or monocular microscopes - magnification of 400X is required to minimally observe the chromosomes while an oil immersion lens (1000X) will ensure the best possible observations.
⇒ Pipettes, microscope slides, coverslips.
⇒ Staining jars or beakers (≈ 250ml) containing room temperature water. Alternatively, the slides used for "splatting" of the chromosomes spreads may be dry and at room temperature.
⇒ Stains #1 & #2 placed at stations around the lab. NOTE: These stains can be reused a number of times provided that caution is taken to avoid excessive carryover from one stain to another.
⇒ Permount distributed at staining stations. NOTE: Benchtops should be protected from Permount with paper towels. Care must be exercised when viewing the slides with a microscope before the Permount dries. If Permount does get on an objective wipe the objective with lens paper and xylene.

Note: You will notice that certain terms are highlighted throughout this manual. The definitions of these terms can be found in the Glossary of Terms, References, and Further Reading supplied with this kit.

Objectives

Technique to be learned

1. How to affix, stain and visualize human chromosomes mounted on microscope slides.

Observations to be made and skills to be developed

1. To use high dry (400X) and oil immersion (1000X) to observe a spread of human chromosomes.
2. To recognize various chromosomal features; sister chromatids, centromeres.
3. To scan the microscope field so as to recognize an ideal chromosome spread void of overlapping chromosomes of the same or adjacent cells.
4. To recognize size differences among the 23 pairs of chromosomes.
5. To recognize the three basic human chromosome morphologies based on centromere position: metacentric, submetacentric, andacrocentric.

Time requirement: 40-60 minutes.
Background Information

The 46 chromosomes and mitochondria located in each somatic cell of the human body contain the entire human genetic complement (genome). Located within the nucleus, these 23 pairs of homologous chromosomes are comprised of 22 pairs of autosomes (non-sex chromosomes) and 1 pair of sex chromosomes (XX or XY). The genetic material, or DNA (deoxyribonucleic acid), exists within the chromosomes and contains the entire genetic blueprint for development of an individual. It exists in a highly coiled and condensed state, due in part to the action of a class of DNA binding proteins called histones. All normal human cells contain identical numbers and types of chromosomes. Aberrations in the chromosomal number and/or structure will most likely result in some type of genetic defect. The analysis of human chromosomes has allowed researchers to identify specific genetic diseases and abnormalities which are attributed to this disruption in the normal complement and structure of the chromosomes. Each chromosome pair contains unique physical attributes which distinguishes them from all others. The three main criteria used to distinguish and identify individual chromosomes are:

⇒ length of the chromosome

⇒ position of the centromere (the primary constriction)

⇒ staining/banding pattern of a chromosome when exposed to certain chemical conditions

Using these criteria, cytogeneticists (individuals who analyze and research chromosome structure and function) have set up a classification system for chromosomes which labels each chromosome with a number, or for the sex chromosomes, as X or Y. This system of standardization allows for accurate communication among scientists.

Many genetic diseases have been associated with a specific change or abnormality within the chromosomes. These abnormalities can include: an increase or decrease in the amount of; chromosome material or the translocation of one piece of a chromosome to another chromosome. Several kinds of cancer are associated with chromosomal abnormalities. Some examples of genetic diseases and their respective chromosomal aberrations are:

1. **Down's Syndrome** - characterized by an extra chromosome #21 (trisomy 21).

2. **Cri du Chat** - characterized by a deletion of the short arm of chromosome #5.

3. **Turner's Syndrome** - characterized by the absence of one X chromosome (one of the sex chromosomes); these females only have 45 chromosomes.
On the other hand, there are many genetic diseases which result from a defect within a particular gene. The abnormal genotype may result in an abnormal phenotype. Such defects may be more subtle and more difficult to analyze. Recent advances in recombinant DNA technology and genetics, however, have allowed researchers to identify specific locations of genes on chromosomes. This information is useful for researchers from around the world who are constructing a genetic map of the human genome. Continued advancement in this field may ultimately lead to the eradication of diseases such as diabetes, muscular dystrophy, cystic fibrosis, and hundreds more.

In order to analyze an individual's chromosomes, or prepare a karyotype, the chromosomes must be in a state in which they can be easily observed. This is accomplished by treating the cells with a chemical called colchicine. The action of colchicine causes the arrest of mitosis in the metaphase stage of the mitotic cycle. It is during this stage that the chromosomes are in their most condensed state and the most visible with the light microscope. Once the cells have been "arrested" in metaphase, the cells are placed in a hypotonic solution. Since the osmotic pressure is greater inside the cell as compared to the outside, water will enter the cell until a state of equilibrium between the cell and its environment has been reached. Movement of water into the cells causes the cells to swell in size. The hypotonic solution is then replaced with a fixative which preserves the existing cell architecture. The cells are now ready to be "splatted" onto microscope slides, stained and observed. When preparing a karyotype the investigator will take a photograph of a chromosome spread which shows clear and distinct chromosomes. The photograph is enlarged and the individual chromosomes are cut out and arranged based on the physical criteria stated earlier (i.e., size, centromere location, banding patterns). This representation of an individual's chromosomes is called an idiogram and is pictured below.

![Chromosome Idiogram]

One practical application of karyotype analysis is in the early detection of genetic defects through amniocentesis. In this process some of the amniotic fluid surrounding the fetus is removed by a physician. This fluid contains fetal cells which will propagate under very specific laboratory
conditions. Once the cells have increased in number a karyotype can be performed on these fetal cells. The results of the karyotype analysis may alert the physician to potential problems or abnormalities of the fetus. In karyotyping not involving fetuses the cell type most often used for analysis are lymphocytes. As with fetal cells these cells are grown in culture, treated with hypotonic solution, and fixed prior to performing a karyotype.

In this exercise, the human tumor cell line, HeLa, is used for karyotyping. The HeLa cell line originated in the early-1950's from the cancerous cervical cells of a woman named Henrietta Lacks. Because the cells are of tumor origin they have continued to divide and multiply over the last 50 years and will continue to do so for an indefinite period of time. Furthermore, since these cells are of tumor origin, they will not contain the normal diploid number of chromosomes characteristic of human beings (46). Instead, these cells are considered aneuploid and will possess a chromosome number greater than the diploid number, with three, four, five, or more copies of a particular chromosome being present in some cells. When you make a chromosome count of your spreads you may find chromosome numbers of 50 to 60/cell, or more even greater than 100!

Note: See Appendix 1 for a complete description of the methods used to prepare the cells.

Student Procedures

1. The slides used can be either:
   a. dry and at room temperature, or
   b. placed in room temperature water prior to use.
2. Place the wet or dry slide vertically at a 45° angle.
3. **Put on gloves.** With a pipette, *Gently Resuspend* the cells in the tube provided. Remove a small sample of cell suspension with a pipette and hold the pipette 2 feet above the slide. Allow one drop of cell suspension to "splat" onto the slide about 3/4 inch from the upper end and tumble down the slide. Carefully apply 6-8 more drops from various heights, *One drop at a time*, onto the same region of the slide. **It is important to release the cell suspension one drop-at-a-time. Do not expell all of your cell suspension in one squirt, or you will obtain poor results.** Gently blow across the slide for 2-3 seconds. The drying will help "spread" the chromosomes.
4. Allow the cells to AIR DRY COMPLETELY.
5. Dip the slide into the tube containing STAIN #1 for 1 Second only.
   Remove the slide and dip into STAIN #1 again for 1 Second only.
   Remove the slide and dip into STAIN #1 again for 1 Second only.
6. Drain off stain and dip the slide into tube containing STAIN #2 for 1 Second only. Remove the slide and dip into STAIN #2 again for 1 Second only. Remove the slide and dip into STAIN #2 again for 1 Second only. Caution should be taken to avoid carryover of stains (wipe the bottom of slide with a paper towel before transferring).
7. Remove slide from stain and thoroughly rinse with distilled water.
8. Allow slide to AIR DRY COMPLETELY. A stream of warm air or blowing may help speed up the drying process. Incomplete drying will result in very poor resolution when the mounting medium (Permount) is added.

9. Place 2 drops of Permount on the stained area of your slide and place a #1 coverslip over the Permount. Apply gentle pressure to the coverslip to spread the Permount evenly under the coverslip. You may wish to place 2 coverslips side by side so as to allow viewing of the entire microscope slide. Once the Permount has dried the slide is ready for viewing.

10. Low and high dry observations can be made immediately. Observations with the oil immersion objective should await complete drying of the Permount (48-72 hours). Under low power scan your spread for cells which appear to have ruptured and released their chromosomes. Shift to high power (400X) to examine your spread more carefully. An ideal chromosome spread will contain chromosomes which appear distinct, do not overlap with adjacent chromosomes, and whose sister chromatids are separate and distinct (see Figure 1). This exercise requires careful observation so take your time when viewing. Once you have found what appears to be a clear and distinct set of chromosomes place a small drop
of immersion oil (if available) on the coverslip over that area and switch to 1000X. **Note:** When using the oil immersion lens you will have to increase the amount of light passing through the specimen. This can be accomplished by increasing the light intensity (if available), and by raising the condenser. Adjustment of the iris diaphragm can also enhance resolution. The diaphragm opening should be sufficient to just flood the field with light. **Caution:** Only use immersion oil on those areas of the slide containing a coverslip.

11. Try to count the number of chromosomes present in 10 different cells on the slide. Remember that this cell line is aneuploid and each cell will probably contain a different number of chromosomes, each greater than the diploid number (46). In addition, try to identify and locate the three characteristic chromosomes based on the location of the centromere.

**CAUTION: All attempts should be made to avoid getting Permount on the objectives of the microscope. If Permount does get onto an objective it can be removed by wiping it with lens paper and xylene.**
HeLa cells, a human tumor cell, shows the typical aneuploidy condition common to transformed cells. Normal human diploid cells will contain 46 chromosomes but it is evident that this cell contains more than that number. Note the typical chromosome structure with the centromere evident in each chromosome. The sister chromatids are also evident. The position of the centromere is used to classify chromosomes as either: metacentric, submetacentric, acrocentric. A chromosome of a fourth category, telocentric chromosome, has the centromere terminally situated. However, there are no human telocentric chromosomes. Close examination will show the presence of all three types in this photograph.
Chromosome Classification

1. Metacentric Chromosome: centromere is centrally situated.
2. Submetacentric: centromere is moderately off center.
3. Acrocentric: centromere is grossly off-center.
4. Telocentric: centromere is terminally situated.

Figure 2. Centromere location
Student Assessment

1. Specify the location and function of the centromere.

2. State an example of a genetic disease (mentioned in this exercise) which is caused by an abnormal chromosomal condition.

3. Why is it necessary to expose the cells to a hypotonic solution when preparing cells for karyotyping?

4. Define the role of histones in the chromosomes.

5. Explain the difference between an aneuploid cell and a diploid cell.

6. During which stage of mitosis are the chromosomes in their most condensed state and thus best suited for karyotyping?

7. Describe the role of amniocentesis?

8. Name the three types of chromosomes found in human cells based on the location of the centromere.
Further Study

1. Identify a genetic disease which is associated with a numerical chromosome abnormality. What factors are believed to be responsible for the origins of the numerical aberration?

2. Explain how a numerical or structural chromosome aberration may cause a genetic disease.

3. The first experiments involving gene therapy were recently performed. Describe the nature of gene therapy and what current therapies are being performed. Also, discuss the ethical problems this raises with regard to the ability of scientists to overcome nature’s mistakes and correct them.

4. The gene responsible for Cystic Fibrosis was recently identified. Research this disease, describe its symptoms and frequency, and discuss how knowledge of the location of this gene may lead to the eradication or control of the disease.

Appendix 1

The following procedures were used to prepare the cells and chromosomes prior to shipping. The cell suspension will be stable for a few days but should be Refrigerated when not in use.

1. A log phase culture of HeLa cells was incubated for 3.5 hours in EMEM (complete) containing colchicine at a final concentration of 0.06µg/ml.

2. The medium was decanted and rinsed twice with CMF-PBS. This was followed by the addition of 3 ml 0.05% trypsin.

3. After cells were released from the bottom of the tissue culture flask and collected, the cell suspension was centrifuged at 200 g's for 3 minutes.

4. The supernatant was decanted and 5 ml of a hypotonic solution (one part EMEM and two parts distilled water or alternatively 0.075M KCl can be used as the hypotonic solution) was added. This was mixed, the cells allowed to settle for 10 minutes, and centrifuged at 200 g's for 3 minutes.

5. The supernatant was poured off, 5 ml of fixative (three parts methyl alcohol and one part glacial acetic acid) was added and the preparation was left undisturbed for 20 minutes.

6. The cells were resuspended with a pipette and recentrifuged.

7. The old fixative was decanted and 5 ml of new fixative was added; the cells were then resuspended and recentrifuged. The volume of fixative was reduced and readied for shipment.
Teacher Evaluation - Kit #4

In order to provide the best instructional material possible, we would greatly appreciate your comments. Please answer the following questions, and do not hesitate to include comments and suggestions that may not be covered. In order for us to improve and expand our program, please be frank in your comments. Please use back of sheet if necessary.

1. Was the information provided you adequate enough to cover this material with your class?

2. Was the material and information provided to the students easily understood and straightforward?

3. Did this material meet your expectations? Please indicate other cell culture materials that you may be interested in introducing to your students. (Keep in mind the stability of materials as well as the transportability of materials).

4. Were the student assessment questions appropriate for the level at which you would like to teach this section. (Were they too difficult/easy)? Were the further study problems adequately stated and useful for future study by your students? If you feel inclined please provide sample questions which you feel would be appropriate for students using these kits.

THANK YOU

Please return this sheet to:

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