

Karyotype analysis of murine macrophages for undergraduate students

L. Millis¹, L. Sigola¹ and A.L. Fuentes²

¹ Biology Department, Faculty of Science and Technology, Douglas College, New Westminster, BC, Canada

² Department of Natural Sciences, LaGuardia Community College, City University of New York, Long Island City, NY, USA

We have developed a karyotype analysis laboratory involving photomicroscopy which is suitable for use by students in biology courses at the undergraduate level. The laboratory activity uses the murine macrophage RAW 264.7 cell line maintained *in vitro*. In this laboratory activity, students benefit from an introduction to conditions required for cell culture (media, growth conditions, sterile technique), and use an inverted microscope to observe live cells in culture.

We describe the use of colchicine to arrest dividing cells in metaphase when chromosomes are most condensed. Cells are then harvested, treated in a hypotonic solution, fixed, and then dropped onto glass microscope slides. The slides are then stained and mounted and sealed. Photomicrographs are taken for analysis. We discuss the significance of cell preparation for microscopic analysis in engaging students as active learners.

Keywords: karyotype; macrophage; photomicroscopy

1. Introduction

Few instruments have been as significant in the advancement of human understanding of the living world as the light microscope. Although most undergraduate biology students may appreciate the light microscope as an essential tool for the elucidation of organisms or structures too small to be visualized with the human eye, in our experience, few undergraduate students have an opportunity to appreciate the significance of microscopy to the advancement of knowledge about such things as inheritance or other phenomena observed at the level of the multicellular organism. Here we will describe student laboratory work in the preparation and analysis of murine macrophage karyotypes, and we will describe how such a laboratory experience may be used to contribute to student understanding of the physical basis of the mechanisms of inheritance as well as aspects of cell biology such as the cell cycle. Although microscopic karyotype analysis is the focus of the student study, the student experiences with mammalian cell culture techniques, staining, photography, and related procedures provide important learning opportunities for students. The discussion of the procedures and techniques associated with the microscopic analysis will include examples of how the procedures can be used to help students reflect on the subject matter under consideration, as well as on other general issues in scientific investigation. Our expectation is that the student laboratory experience will not only enhance what students know about biological mechanisms, but will also help them understand the evidence that was used to give mechanisms or theories validity. We also expect that this experience will allow the student to more confidently consider the meaning and validity of research findings in other areas of scientific research.

Hands-on laboratory experience of karyotype analysis is intended to provide an opportunity for a deeper understanding of the mechanisms behind what is known about the principles of genetics, and may help students better appreciate how information from the microscopic world informs thinking about the more familiar macroscopic world. Having students carry out karyotype analysis using techniques similar to those used to first determine the correct human chromosome complement, allows students to participate in the research methodology in a manner that allows them to relate to research methods and discovery in ways that are not common in undergraduate laboratories.

The karyotype analysis we describe here utilizes equipment and resources typically found in a research laboratory. The equipment available for these studies include general cell culture equipment, including high quality student light microscopes, a phase contrast inverted microscope, laminar flow hoods, a temperature and CO₂ controlled cell culture chamber, centrifuges, and other general lab equipment and materials. This rather specialized equipment became available and justifiable because this equipment was not only used in several Genetics and Cell Biology laboratory courses, but was also used for several research projects, primarily involving the study of the murine macrophage RAW 264.7 cell line. The application of research to student laboratory activities, with the sharing of materials and equipment has been an important consideration in the efficient use of the limited funds available for research and laboratory instruction.

Although light microscopes used in biological studies are primarily designed and utilized to visualize small objects or organisms to elucidate and document their structures, applications of light microscopy have had much broader uses than the documentation of structures. In the field of genetics, for example, the mechanics behind the transmission of genetic information from generation to generation that was first described by Mendel [1], were established by the association of visible heritable characteristics with the transmission of microscopically visualized chromosomes. Although chromosomes had been described in both plant and animal cells before the rediscovery of Mendel's work at the beginning of 20th century, there was little understanding of the importance of chromosomes in heredity. Microscopic

studies had established that female fruit flies of *Drosophila melanogaster* had a different chromosome complement (XX) than male fruit flies (XY), and this observation allowed Morgan, in 1910, to associate the inheritance of sex-linked traits with the inheritance of the X chromosome [2]. The definitive association of genes with chromosomes was established by the observation that unusual inheritance patterns were associated with the transmission of unusual sex chromosome complements [3]. Thus one of the most significant advances in the study of genetics, the association of genes with chromosomes, was made possible by using light microscopy to visualize the chromosome complement in conjunction with phenotypic observations.

Studies of inheritance patterns and genetic linkage also resulted in the first chromosome map identifying the location of 6 genes on the X chromosome of *Drosophila melanogaster* [4]. The light microscope was also the tool that enabled the observation of banding patterns of the large salivary gland chromosomes in *Drosophila melanogaster* which were eventually used to identify the physical location of genes on the X chromosome as well as genes on the autosomes, not because genes are large enough to be visualized, but because abnormal banding patterns such as those caused by deletions, were associated with particular phenotypically observed abnormalities. Microscopic evidence for the locations of genes also provided a mechanism to corroborate explanations of crossing over and the validity of recombination analysis to map chromosomes.

The first human autosomal gene (the Duffy blood group gene) was found to be associated with a particular chromosome by using pedigree analysis and associating the inheritance pattern of the gene with the familial transmission of an unusual form of chromosome 1, characterized by an unusual microscopically observable loose and extended region near the centromere [5]. Associating inheritance patterns with observed chromosome anomalies, was the basis of the determination of the chromosomal location of many genes in humans and other organisms, and established the chromosomal basis of genetic disorders such as Down Syndrome [6]. Similar examples of the significance of microscopy to our understanding of the world might well be drawn from other areas of study, such as geology, physics, or chemistry, and we think that the student experience and reflection on the use of microscopy in the preparation of the murine macrophage karyotypes would add meaning to student learning and understanding in those studies too.

2. Laboratory Procedures

The major steps utilized in our laboratory to prepare macrophage karyotypes include the basic procedures described by Tjio and Levan in 1956 when 46 was established as the normal human chromosome diploid number [7]. Our procedures include culturing murine macrophages using an environmentally controlled growth chamber, and treating the cells with colchicine to increase the number of cells at metaphase. Cells are then harvested and concentrated using a clinical centrifuge. The collected cells are treated with a hypotonic solution to swell the cells. The cells are then fixed, applied to microscope slides, stained, viewed microscopically and photographed. The implications of several stages of the procedure to student learning will be discussed.

2.1 Growth of cells and pre-treatment with colchicine

RAW 264.7 murine macrophages (ATTC TIB-71) were grown in DMEM medium supplemented with fetal calf serum, HEPES buffer, glutamate, and penicillin/streptomycin. Growth was in 25 ml. Corning vials in a temperature (37^o) and CO₂ (5%) controlled incubator (Fig. 1). Dividing cells at approximately 60 % confluence were treated with 10⁻⁶ M colchicine for 3 to 15 hr. Growth of cells was monitored visually using a Nikon Eclipse TE2000S inverted microscope with phase-contrast optics (Fig 2.).



Fig. 1 Temperature and CO₂ controlled incubator.

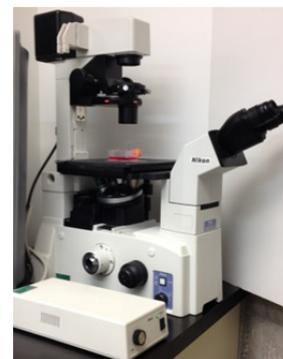


Fig. 2 Nikon Eclipse TE2000S inverted microscope.

2.2 Harvesting Cells and Hypotonic treatment

Cells were harvested by placing the culture vials on ice for 5 min. and dislodging cells by vigorous pipetting. Students worked in pairs and each student group was provided with approximately 5 ml of suspended cells. Cells were centrifuged in a plastic 15 ml. centrifuge tube for 5 min. at 1000 rpm using a clinical benchtop centrifuge. The supernatant was removed and the cells were gently re-suspended in 8 ml of warm 0.75 M KCl (hypotonic solution) for 5 min.

2.3 Fixation

Cells were centrifuged, and the supernatant hypotonic solution was removed. Approximately 8 ml. of freshly prepared Carnoy's fixative (3 parts ethanol: 1 part glacial acetic acid) was added, and the cells were re-suspended in the fixative and kept in fixative for 5 min. The fixation step was repeated by centrifugation, removal of supernatant, and re-suspension of the cells in 5 ml. of fresh Carnoy's fixative.

2.4 Slide preparation and staining

Three drops of cell suspension were pipetted onto clean microscope slides by letting each drop fall from a distance of about 30 cm. onto different regions of the slide. Slides were then passed through an open flame to allow the fixative to catch fire and burn off the alcohol. The slides were allowed to dry and stained by submersing in Giemsa stain for 8 min., followed by a rinse in distilled water and then followed by a rinse in tap water. Slides were then air dried, and Permount was used to seal the slides. Slides were kept at 37° until the next laboratory period (one week) and then observed and photographed.

2.5 Photomicrography

Students examined their slides using Nikon Eclipse E200 microscopes, and photographs for karyotype analysis were taken using a Nikon Eclipse 50i microscopes equipped with 100 X oil immersion lenses and Nikon DS-Fi1 Digital Sight camera with monitor (Fig. 3). Students printed photographs of metaphase cells, selected for having chromosomes spread well enough to accurately count the chromosomes. Ideograms were not prepared from the photographs.



Fig. 3 Nikon Eclipse 50i microscope with Nikon DS-Fi Digital Sight camera and monitor.

3. Discussion

In the context of studying genetics, students review mitotic and meiotic cell division with a focus on the behavior of chromosomes. Because students are often informed that the cell cycle applies to all dividing cells, it is not surprising that students often do not appreciate that interphase chromosomes generally have a visually unhelpful microscopic appearance (Fig. 4), as compared to chromosomes visualized at metaphase of mitosis (Fig. 5)

Given the challenge of finding cells at metaphase in typical human or other mammalian tissues, culturing cells *in vitro* provides a means of obtaining numerous dividing cells and thus increasing the opportunity to observe chromosomes at metaphase. Colchicine treatment, although not a requirement for murine macrophage karyotype analysis, increases the proportion of cells at metaphase. We have used varying lengths (3 to 15 hr.) of treatment with colchicine, the varying times depending on convenience with respect to the timing of labs. Equivalent success was obtained with both short and long colchicine treatment times (data not shown).

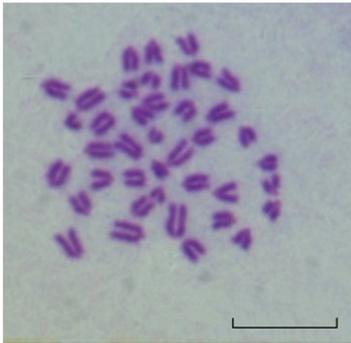


Fig. 4 Murine macrophages at metaphase. Size bar is 10 μm .

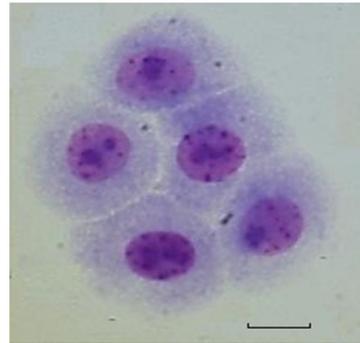


Fig. 5 Murine macrophages at interphase. Size bar is 10 μm .

This particular karyotype analysis methodology has been used over three different semesters with a total of about 130 students, and virtually every student was successful in finding cells with metaphase chromosomes. Moreover, most students had enough cells with well spread chromosomes that they could confidently count the chromosomes in at least one cell (data not shown).

Each stage of karyotype preparation provides an opportunity to highlight the importance of the procedure or technique to the process of investigation and analysis. A discussion of the implications of tissue culture provides students with an opportunity to consider the implications of cell culture for research as well as the importance of procedures such as sterile techniques. For example, using laminar flow hoods, sterilizing materials and instruments, and generally maintaining sterile conditions can provide an opportunity for students to appreciate that good laboratory work requires both knowledge and expertise to perform effective experiments. In experiments described here, *in vitro* cell culture involved growing cells in growth medium and in a temperature, and CO_2 controlled growth chamber. Students readily appreciate the significance of temperature control, however a consideration of the significance of the control of CO_2 concentration provides an opportunity to consider such things as the relationship between CO_2 , energy metabolism, physiological pH, and physiological control of pH. The focus and extent of such discussions would necessarily depend on the context of the laboratory work in relation to the students' general studies.

Before our students do karyotype analysis, they spend a considerable amount of time on practical studies of microscopy, with an emphasis on understanding how to achieve critical illumination, how to determine the sizes and magnification of specimens, and how to achieve optimal resolution and contrast. One consideration in this process is the use of stains to increase contrast of specimens. It is interesting to note that staining played a rather important role in the history of human karyotype analysis. In 1923, Painter determined that 48 was the number of chromosomes present in human diploid cells [8]. That number went unchallenged until 1956, when Tjio and Levan determined that human cells typically had 46 rather than 48 chromosomes [7]. An analysis of Painter's work by Ruddle in 2004 suggests that the mis-counting was likely the result of a staining artifact resulting from the choice of stain (Haemalum acid) that leaves the central region of chromosome 1, the largest human chromosome, poorly stained [9]. The two arms of each copy of chromosome 1 were likely identified as separate chromosomes rather than as one large chromosome. That a staining anomaly could mislead geneticists for thirty years is likely to stimulate considerable and useful discussions about microscopy.

3. Summary

Including karyotype analysis in a student genetics laboratory experience creates a unique opportunity for students to gain an understanding of how the microscopic view of cellular structures can help elucidate genetic mechanisms at the phenotypic level. Karyotype analysis using techniques similar to the ones described here could be done using human peripheral blood and commercially available kits. Because educational institutions may have policies or restrictions that make working with human tissues impractical or impossible, using cultured macrophages provides a practical alternative to using human tissues. We believe that the student experience in working with cells cultured *in vitro*, and the manipulation of those cells to create visible images of chromosomes introduces students to engagement that is not easily duplicated with readings, videos, virtual laboratory experiences or discussions of experiments. The instrument at the core of this particular experience is the microscope, however; the associated technical procedures and manipulations such as cell culture, colchicine treatments, hypotonic treatments, fixation, staining and photography, add additional opportunities for students learning.

It is our experience that having laboratory activities such as karyotype analysis, makes scientific research and knowledge less intimidating to students, and enhances their ability to consider important questions such as: "What is the evidence that genes are on chromosomes?" or "Why did it take more than 30 years for scientists to discover that 48 was the wrong diploid chromosome number for humans?"

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