HUMAN CHROMOSOME METHODOLOGY

EDITED BY

JORGE J. YUNIS

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MEDICAL GENETICS LABORATORY
DEPARTMENT OF LABORATORY MEDICINE
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List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

MURRAY L. BARR, Department of Anatomy, Faculty of Medicine, University of Western Ontario, London, Ontario, Canada (1)

SHEILA BRUNTON, Medical Research Council, Clinical Effects of Radiation Research Unit, Western General Hospital, Edinburgh, Scotland (57)

LEROY P. CHRISTENSON, School of Dentistry, Dental Illustration Laboratory, University of Minnesota, Minneapolis, Minnesota (129)

D. G. HARNDEN, Medical Research Council, Clinical Effects of Radiation Research Unit, Western General Hospital, Edinburgh, Scotland (57)

WILLIAM J. MELLMAN, Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania (21)

SUSUMU OHNO, Department of Biology, City of Hope Medical Center, Duarte, California (75)

KLAUS PATAU, Department of Medical Genetics, University of Wisconsin, Madison, Wisconsin (155)

WALTER J. RUNGE, Department of Medicine, University of Minnesota, Minneapolis, Minnesota (111)

WERNER SCHMID, Section of Cytology, Department of Biology, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas (91)

J. H. TJIO, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland (51)

J. WHANG, National Cancer Institute, Bethesda, Maryland (51)

JORGE J. YUNIS, Medical Genetics Laboratory, Department of Laboratory Medicine, University of Minnesota, Minneapolis, Minnesota (187)

Preface

Recent advances in human cytogenetics have stimulated widespread interest among many investigators in the medical and biological sciences. As a direct result of this interest, a genuine need has been felt for an authoritative and up-to-date treatise which would serve as a text and reference. Readily comprehensible chapters are offered covering each phase of laboratory investigation from the preparation of materials for sex chromatin and chromosome techniques for bone marrow, blood, skin, and gonadal specimens to the subject of autoradiography and chromosome identification. Included also are guides to microscopy and photomicrography as well as an up-to-date treatment of chromosomes in disease. It is hoped that this volume will serve as an adequate guide to laboratory techniques and their applications for research workers, students of genetics, and members of the medical profession involved in setting up a laboratory of cytogenetics.

JORGE J. YUNIS

Minneapolis, Minnesota
March, 1965

Introduction

T. C. HSU

Section of Cytology, Department of Biology,
The University of Texas M. D. Anderson Hospital and Tumor Institute,
Houston, Texas

In the history of scientific research, whenever a new idea, a new material, or a new method was discovered, heavy research activities followed for a period of time. The field of cytogenetics has been no exception. When the genetic importance of polytene chromosomes of Diptera was rediscovered in the early thirties, practically every *Drosophila* geneticist played with salivary glands. Similarly, when the effects of colchicine on mitosis was described in the late thirties, all plant cytogeneticists used colchicine, at least for getting good preparations. However, scientific research, like any other activity, may follow the law of diminishing returns. When a new method or a new idea is extensively explored, progress is retarded or even stops completely. This is what happened to cytogenetics at the beginning of the last decade.

One of the dark alleys of cytogenetics was mammalian karyology. Before the invention of modern technological devices, even determining the diploid number of a mammalian species was considered a difficult accomplishment. The chromosomes were hopelessly crowded in metaphase. An experienced cytologist would have to spend days or months to find a figure that was slightly favorable for a count. No one used photographs because all one could see from a photograph was a cluster of chromosomes, one on top of the other. Drawings were the only sensible way of presenting a figure because one could use lines to indicate overlaps. However, since there were so many overlaps, those pictures rarely meant very much.

Technological improvements during the past 10 years include:

1. Tissue culture. Cells *in vitro* (or in ascites) are either suspended or form a monolayer instead of a tissue block. This allows the full effects of pretreatments as well as fixation.

2. The colchicine pretreatment. Colchicine, or its derivative, Colcemid, interferes with the mitotic spindle so that dividing cells are blocked at metaphase. It also allows the chromosomes to contract further in the blocked metaphase stage. With proper concentration of colchicine and duration of treatment, large numbers of excellent metaphases can be accumulated to facilitate observation and analysis.

3. The hypotonic solution pretreatment. The osmotic pressure causes the cells to expand and the chromosomes to scatter.

4. The squash or air-dried method. Even though the chromosomes are spread apart by the hypotonic solution treatment, they are distributed over the entire expanded cell. The squash or air-dried method forces the chromosomes to lie in one plane of focus, so that errors in chromosome characterization can be minimized.

Because of these technical advances, mammalian cytology suddenly became an important part of medical research. The discovery of the correct diploid number for man and aneuploidy in some congenital syndromes set the foundation of human cytogenetics which has invaded practically every branch of medicine. The demand for qualified cytogeneticists during recent years has been so high that it is possible to fill only a fraction of the positions available. There has been no training center or enrichment course of this type. Those who desire to learn the techniques could not find a pertinent manual or handbook to follow. Therefore, a book describing all the modern procedures used by active investigators in this field is indeed most timely and appropriate.

I must mention at this point a fallacious notion held by noncytogeneticists concerning chromosome study: Who can't count to 46? Indeed, anyone, after learning in a cytogenetic laboratory for a week or two, is able to make leucocyte cultures and good chromosome preparations. But cytogenetics does not mean just "chromosome counts." Mastering techniques alone does not make one a competent cytologist, although a competent cytologist must master as many techniques as possible. A vast background in genetics and cytogenetics, in cell biology, and in molecular biology is necessary for carrying out a good project as well as extending research frontiers.

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Sex Chromatin Techniques

MURRAY L. BARR

Department of Anatomy, Faculty of Medicine, University of Western Ontario, London, Ontario, Canada

I. INTRODUCTION

The sex chromatin of interphase nuclei has proved a useful marker, in spite of its diminutive size, in several practical and theoretical problems of biology and medicine. It is widely used as a diagnostic aid in errors of sex development, especially in hermaphroditism, Turner's syndrome, and Klinefelter's syndrome. As a companion technique to chromosome analysis, study of the sex chromatin pattern allows one to determine the number of X chromosomes in the complement, according to the rule that the maximum number of sex chromatin masses is one less than the number of X chromosomes. This is a helpful, indeed a crucial, rule in certain chromosome abnormalities, because the X chromosome is almost identical, in size and the more obvious structural characteristics, with autosomes 6 and 7. The sex chomatin test has also been used in a number of studies where an indication of the cells' sex chromosome complex was needed. To cite a few examples, the sex chromatin has been a useful marker in determining the success of homologous tissue transplants when donor and recipient were of unlike sex (Peer, 1958; Woodruff and Lennox, 1959);

using the neutrophiles' drumstick as a marker, the same principle was applied in studying the results of bone marrow replacement (Davidson *et al.,* 1958). Attempts have been made to study the sex factor in spontaneous abortions (Bohle *et al.,* 1957; Schultze, 1958; Wagner, 1958; Serr *et al.,* 1959; Moore and Hyrniuk, 1960), and there have been provocative findings, whose significance is not yet understood, in teratomas in males (Hunter and Lennox, 1954; Tavares, 1955; Myers, 1959; Theiss *et al.,* 1960). In more recent work, evidence for genetic inertness of the X chromosome that forms the sex chromatin, and the probability of an X chromosome mosaicism in females, have opened new avenues in research that are likely to have an important influence on genetic theory (Ohno, 1961; Lyon, 1961, 1962; Beutler *et al.,* 1962; Grumbach and Morishima, 1962).

The sex chromatin principle is thus basic to a rather wide range of studies. This chapter deals, however, with sex chromatin tests that are useful as a diagnostic aid in developmental anomalies caused by an abnormality of the sex chromosome complex.

II. HISTORICAL SKETCH

A brief account of the development of the sex chromatin tests will perhaps be of interest to those who use them as a diagnostic aid.

Cytologists studying insect material have known for some time that the X chromosome may have the property of heteropycnosis in interphase nuclei and form a distinctive chromatin mass or chromocenter (Geitler, 1937). The first intimation that the same principle applies in mammals came with the demonstration of a female-specific chromocenter in nerve cells of the cat (Barr and Bertram, 1949). This initial disclosure was followed by nuclear studies on a variety of mammals, including man (Moore and Barr, 1954), which showed that sex chromatin could usually be demonstrated, in the various tissues of animals examined, in females but not in males. The possibility that nuclear sexual dimorphism might be useful diagnostically was first explored in hermaphroditism, by use of skin biopsy specimens (Moore *et al.,* 1953). Davidson and Robertson Smith (1954) showed that neutrophiles had a sex characteristic, in the form of a drumstick-shaped nuclear lobule, in a small proportion of neutrophiles of females. A search for a simpler technique for demonstration of the sex chromatin pattern resulted in the buccal smear test, which is now in general use (Marberger *et al.,* 1955; Moore and Barr, 1955). The

tests were applied promptly to patients with puzzling anomalies of sex development, and it was found that most females with gonadal agenesis or Turner's syndrome had chromatin-negative nuclei (Polani *et al.,* 1954; Grumbach *et al.,* 1955), whereas the majority of males with seminiferous tubule dysgenesis or Klinefelter's syndrome had chromatin-positive nuclei (Bradbury *et al.,* 1956; Plunkett and Barr, 1956). The chromosomes of similar patients were studied soon after the appropriate techniques became available, resulting in the crucial demonstration of an XO sex chromosome complex in patients with Turner's syndrome and chromatin-negative nuclei (Ford *et al.,* 1959a) and an XXY sex chromosome complex in patients with Klinefelter's syndrome and chromatin-positive nuclei (Ford *et al.,* 1959b; Jacobs and Strong, 1959). The relationship between sex chromatin and the sex chromosome complex (the derivation of the sex chromatin from a single X chromosome) was established largely through the work of Ohno and his collaborators (e.g., Ohno and Hauschka, 1960; Ohno and Makino, 1961). The most recent notable development concerns the genetic inertness of the heteropycnotic X chromosome that forms the sex chromatin and the possibility of a mosaicism in mammalian females, the paternal X being active in some cells and the maternal X in others (Lyon, 1961, 1962). The foregoing items are only a few of a long series of developments, during a period of a little over a decade, that saw hundreds of papers published dealing, in one way or another, with the sex chromatin.

III. MORPHOLOGICAL CHARACTERISTICS OF THE SEX CHROMATIN

With the exception of the opossum, where a sex chromatin mass occurs in the cell nuclei of both sexes (Graham and Barr, 1959), this chromocenter is normally a female characteristic, and the following description applies to cells of females. There seem to be no important differences in the intrinsic morphology of the sex chromatin from one mammalian species to another or in different tissues, although the position of the sex chromatin in the nucleus does vary to some extent with the species and cell type. Nuclei of representatives of the orders Rodentia and Lagomorpha, except in embryonic material and a few mature cells such as those of mesothelium, are unsuitable for sex chromatin studies because of the coarse chromatin pattern and multiple large chromocenters.

For reasons of intranuclear dynamics that are not clear, the sex chromatin is usually situated against the inner surface of the nuclear membrane

(Fig. 1). Exceptions to this generalization are found in nerve cells. For example, the sex chromatin is usually adjacent to the large nucleolus in neurons of carnivores (Moore and Barr, 1953) and free from any nuclear surface in neurons of the opossum (Graham and Barr, 1959). The proportion of nuclei in which sex chromatin can be seen varies with the type

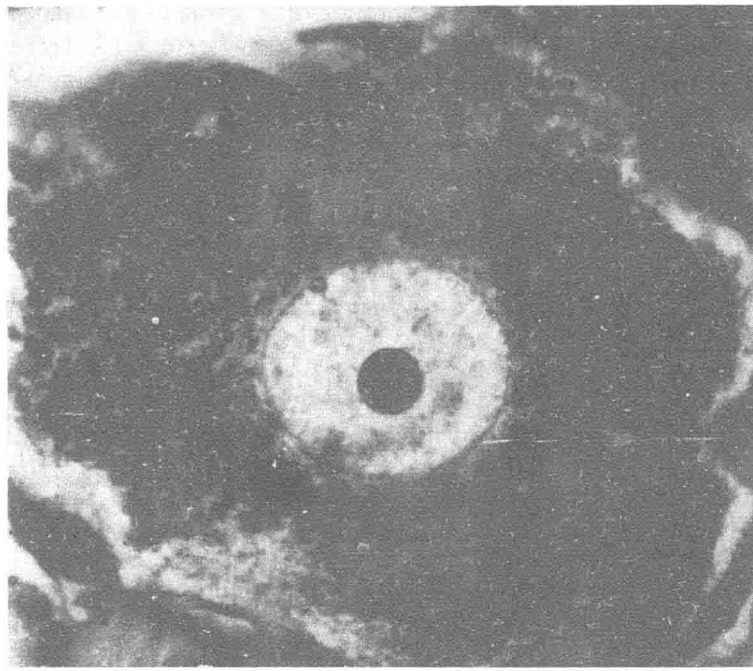

FIG. 1. Dorsal root ganglion cell, with typical sex chromatin at the nuclear membrane (Barr *et al.*, 1950).

of preparation and the tissue being studied. Counts approaching 100% are obtained in whole mounts of amniotic membrane and in relatively thick sections of nervous tissue. But the count is usually of the order of 60%, and often lower in buccal smears, in which many nuclei are not suitable for the study of fine, intrinsic detail. The frequency of sex chromatin in a preparation may be influenced by the mitotic activity in the cell population being studied, because the sex chromatin probably disappears during the period of replication of deoxyribonucleic acid (DNA) and chromosomes in preparation for the next mitotic division.

The sex chromatin is of the order of 1 μ in diameter; measurements of large numbers of sex chromatin masses in human tissues give a mean value of 0.7 μ × 1.2 μ. Its shape is variable, but a planoconvex or triangular outline is seen most frequently because one surface adheres to the nuclear membrane. Except for an occasional bipartite appearance, little structural detail can be made out in the usual preparations. But the bipartite appearance is enhanced, regions of differing density are seen, and there may be a suggestion of strands intertwining in a complicated manner in preparations from which ribonucleic acid (RNA) has been removed by mild acid hydrolysis (Klinger and Ludwig, 1957) or if the sex chromatin of living cells growing *in vitro* is examined with phase contrast. As is to be expected, fixation tends to condense the sex chromatin and obscure its structural detail.

The reaction of the sex chromatin to stains is that of chromatin, chromocenters, and chromosomes generally. It has an affinity for basic dyes such as cresylecht violet, fuchsin, gallocyanin, hematoxylin, pinacyanole, and thionine, and is stained well by orcein, a dye much favored by chromosome cytologists. Because of the sex chromatin's DNA content, it is Feulgen-positive, has an affinity for methyl green in the methyl green-pyronine staining procedure, and persists after mild acid hydrolysis or treatment with ribonuclease.

IV. SEX CHROMATIN TECHNIQUES

A. Buccal Smears

The study of a smear preparation from the buccal mucosa is by far the most convenient method of applying the sex chromatin test in clinical investigation. Certainly no other form of the test is as applicable to surveys (of newborns or institutionalized retardates, for example) or as simple to do in a hospital laboratory. From the strictly cytological point of view, buccal smears are inferior to carefully prepared sections of some tissues, to monolayers of cells growing *in vitro,* or even to smears taken from the vulva or the urethral orifice. But cytological disadvantages are outweighed by the universal availability of buccal smears, and errors of interpretation should not occur if the necessary precautions are taken. The procedure is as follows.

Obtain epithelial cells by drawing the edge of a metal spatula firmly over the buccal mucosa. The long narrow spatula used for analytical weigh-

ing is recommended and is superior to the wooden tongue depressor that was recommended when the test was introduced. Discard the material first obtained, since gentle scraping of the mucosa a second time secures deeper and healthier epithelial cells. Spread the material over a small area of an albuminized slide; a common mistake is to make a smear that is too thin. Fix the smear promptly, i.e., with no opportunity for drying, for 15–30 minutes in 95% ethyl alcohol. Longer fixation, although unnecessary, does no harm up to 24 hours. Immerse the slide in absolute alcohol for 3 minutes, followed by 2 minutes in a 0.2% solution of Parlodion in equal parts of absolute alcohol and ether. (The last step attaches the cells firmly to the slide.) Dry in air for 15 seconds, pass through 70% alcohol for 5 minutes and two changes of distilled water for 5 minutes each. The preparation is now ready for staining.

A variety of staining methods are in use. All of them give satisfactory results and the choice is usually made on the basis of previous experience. Suitable dyes are, among others, cresylecht violet, thionine, acetoorcein, and carbolfuchsin. Feulgen staining of buccal smears has been advocated but is perhaps inadvisable as a routine method. Acetoorcein squash preparations, a technique that has been popular for many years with chromosome cytologists, give good detail of the nuclear chromatin, and the method is rapid (Sanderson, 1960). Guard (1959) introduced a staining method that is potentially very useful because a large chromocenter, such as the sex chromatin, is stained differently from small chromatin particles. In the Guard method, biebrich scarlet in the presence of phosphotungstic acid has a strong affinity for nuclear chomatin generally. Fast green has a slow differentiating action and can be made to replace the biebrich scarlet in the general chromatin while the sex chromatin retains the dye. Thus, in successful preparations, the sex chromatin is red against a green background. The method needs refinement because it is difficult to obtain consistently good results.

We have used carbolfuchsin as a routine stain for buccal smears for several years in our laboratory. Our attention was attracted to this dye by illustrations in a paper by Eskelund (1956), and it was found later that carbolfuchsin is also an excellent stain for human chromosomes (Carr and Walker, 1961). It is simple to apply in the staining of mucosal smears, the results are uniform, there is no objectionable staining of bacteria, and the preparations are permanent.

The staining solution is prepared as follows:

Stock solution

Basic fuchsin (CF-41, Coleman & Bell)	3 gm
70% Ethyl alcohol	100 ml

(Solution will keep indefinitely.)

Working solution

Stock solution	10 ml
5% Carbolic acid in distilled water	90 ml
Glacial acetic acid	10 ml
37% Formaldehyde	10 ml

(Let stand for 24 hours before using. The solution should be freshly prepared monthly, or more frequently if many smears are being stained.)

Stain for 5–10 minutes in the working solution of carbolfuchsin, followed by differentiation for about 1 minute in 95% alcohol and about 1 minute in absolute alcohol. The exact time for differentiation varies a little, depending mainly on the thickness of the smear. Clear in xylol and mount in a neutral medium.

The smear should be examined with the oil immersion objective of a good binocular microscope and, to obtain maximum contrast, a green filter should be used when studying preparations stained with carbolfuchsin. If the chromatin is not finely particulate in a substantial number of nuclei, the test should be repeated to obtain deeper and healthier cells. It is good practice to examine 100 healthy looking nuclei and record the number containing sex chromatin. The number of chromatin-positive nuclei with sex chromatin is often lower in buccal smears than in other kinds of preparations and there is a wide range (from 30% to 80%). However, this is compensated for by the virtual absence, in chromatin-negative smears, of a chromatin mass simulating sex chromatin. One must be on the alert for nuclei that have more than one mass of sex chromatin, which indicates the presence of more than two X chromosomes (*vide infra*).

In the main, the sex chromatin of buccal smears conforms to the general description that has been outlined (Fig. 2). It is probably always at the nuclear membrane, but whether this is obvious depends on the orientation of the nucleus. The sex chromatin is often more flattened against the nuclear membrane than it is in tissue sections and other preparations. In some buccal smears, the nuclei of both sexes contain a chromocenter that is not in contact with the membrane. This is probably nucleolar-associated chromatin and, because of its position, need not be confused with sex chromatin. Any error that occurs in the interpretation of a buccal smear