ADVANCES IN STRUCTURAL BIOLOGY

Editor: SUDARSHAN K. MALHOTRA

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PREFACE

In keeping with the broad objectives set for the serial publication of *Advances in Structural Biology*, Volume 6 contains exhaustive articles from experts in diverse areas of biomedical research. The common thread among the various articles is their relevance to the applications of cell biology to human health.

I am most appreciative of the interest and efforts of the contributors to this volume. I would also like to acknowledge others involved with the volume. Theordor K. Shnitka, M.D., has been most helpful in reading several of the articles included in this volume. Ms. Brenda Metherell has provided invaluable and efficient secretarial help in compilation of this volume. It is a pleasure to acknowledge here her contribution in the preparation of the volume. The cooperation of the production staff at JAI Press, particularly that of Mr. Christian Costeines, production editor, is gratefully acknowledged.

Sudarshan K. Malhotra
Editor

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FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) AS AN ADJUNCT TO CONVENTIONAL CYTOGENETICS

ANALYSIS OF METAPHASE AND INTERPHASE CELLS

Hon Fong L. Mark

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I. CONVENTIONAL CYTOGENETICS

A. Conventional Cytogenetics Based on Banding

Cytogenetics is a subspecialty of medical genetics. Other medical genetics subspecialties include clinical genetics, biochemical genetics, and molecular genetics. Conventional cytogenetic analysis via banding is a powerful, established technique that can provide a picture of the human genome at a glance. What the clinical cytogeneticist refers to as "banding" is the staining method by which dark and light differential staining is induced along the lengths of the chromosomes. According to the Paris Conference (1972), a chromosome band is the part of a chromosome that can be distinguished from adjacent segments by appearing darker or lighter by one or more techniques. By this definition, chromosomes con-

sist of a continuous series of dark and light bands, with no interbands. Three levels of resolution have been standardized, namely, 400, 550, and 850 bands per haploid karyotype. Further descriptions of the universally adopted cytogenetic nomenclature can be found in An International System for Human Cytogenetic Nomenclature (Mitelman, 1995).

Prior to the advent of banding in the late 1960s and early 1970s, chromosomes were grouped and classified based on size, shape, the position of their centromeres, and the gross chromosome morphology. Unequivocal identification of each unbanded chromosome of the human genome was not possible. The development of Q-banding in 1970 (Caspersson et al., 1968, 1970; Paris Conference, 1972) ushered in an era where many protocols for banding flourished, leading to an unprecedented number of discoveries of numerical and structural chromosomal abnormalities. More descriptive terms were coined, such as the QFQ-technique for Q-banding and the GTG-technique for G-banding. These three-letter codes were described in the Paris Conference Supplement (1975). The first letter describes the kind of banding. The second letter stands for the technique or agent used to induce banding. The third letter signifies the kind of stain employed. For example, GTG stands for G-banding by trypsin using Giemsa as a stain (Harnden and Klinger, 1985).

Most laboratories in the United States use GTG-banding for routine karyotyping. In a routine cytogenetic analysis, a short-term 72-hour-culture of peripheral blood lymphocytes is established by adding the mitogen phytohemagglutinin (PHA), referred to as a stimulated culture. Briefer culture without such an agent is referred to as an unstimulated culture. The former is used most frequently to rule out constitutional abnormalities, whereas unstimulated cultures of peripheral blood or more typically, bone marrow aspirate, is used for the study of neoplastic cells. Long-term tissue cultures of almost any solid tissues are established for a variety of reasons, such as mosaicism detection.

Harvesting of chromosomes for conventional cytogenetics (Moorhead et al., 1960) is a rather lengthy and tedious process. Colcemid, a derivative of colchicine, is usually used to arrest the chromosomes in metaphase. This is followed by a hypotonic treatment to cause the cells to take up water and swell so that the chromosomes can spread well when dropped onto glass slides at a later step. After the hypotonic treatment step, the cell pellet is fixed in several changes of fixative consisting of three parts of methanol to one part of glacial acetic acid. After repeated rinsings, the cells are then dropped onto glass slides to facilitate chromosome spreading and air-dried. Slides are aged for a variable amount of time, then banded and stained by one of the banding protocols. G-banding using trypsin and Giemsa stain is the routine method in the United States (Seabright, 1971; Sumner et al., 1971) because of its simplicity. Prior to the advent of modern-day imaging systems, photographs were taken of the best metaphase spreads, enlarged and hand-cut to separate the images of the chromosomes for identification. Most cytogenetics laboratories now own computer-assisted karyotyping systems. Karyotyp-

ing consists of arranging the 46 chromosomes in the human genome according to shape, size and banding patterns. Thus, conventional cytogenetics is a labor-intensive process requiring highly trained personnel. In addition, conventional cytogenetics depends entirely on the availability of high-quality metaphase cells, thus excluding from analysis the vast majority of cells that are in interphase (Anastasi, 1991). Details of conventional cytogenetics have been described elsewhere (Mark et al., 1993, 1994b, 1995a, 1995b, 1995c; 1996a, 1996b, 1996c; 1997a).

B. Solid Staining

Solidly stained or unbanded chromosome preparations are largely obsolete since the introduction of banding methods in the late 1960s and early 1970s except for selected applications. The scoring of chromosome and chromatid breaks and gaps, for example, can be facilitated by solid staining (see, e.g., Mark et al., 1994c; 1995d, 1995e). Satellites, secondary constrictions, dicentrics, ring chromosomes, double minutes, and fragile sites can be better visualized by solid staining. Chromosome morphometry can also be better performed with nonbanded chromosomes (Priest, 1994). The stains employed are usually one of the Romanovsky-type dyes such as Giemsa, Leishman's, or Wright's stains.

C. G-Banding

Giemsa banding (G-banding) is the most widely used technique for the routine analysis of mammalian chromosomes in cytogenetics laboratories. With this method a series of dark and light stained regions along the length of the chromosome are produced and are grossly similar to the Q-bands discussed below. A number of protocols are used to induce G-bands. Most common are the treatment of slides with a protease such as trypsin or the incubation of the slides in hot saline-citrate (Seabright, 1971; Sumner et al., 1971) after appropriate slide aging. The chromosome banding patterns resulting from these treatments are distinct and quite consistent from experiment to experiment, leading some to postulate that G-bands reflect the inherent genetic composition and underlying structure of the chromosome. Dark G-bands have been found to correlate with pachytene chromomeres in meiosis. Generally, they replicate their DNA late in the S-phase, contain DNA rich in adenine and thymine (AT) base pairs, appear to contain relatively few active genes, and may differ from light bands in terms of protein composition. It has been hypothesized that the differential extraction of protein during fixation and banding pretreatments from different regions of the chromosome are important in the mechanism by which G-bands are obtained (see discussion by Rooney and Czepulkowski, 1992).

Although it has been approximately 30 years since chromosome banding was first discovered, the optimal banding conditions for each preparation are still often

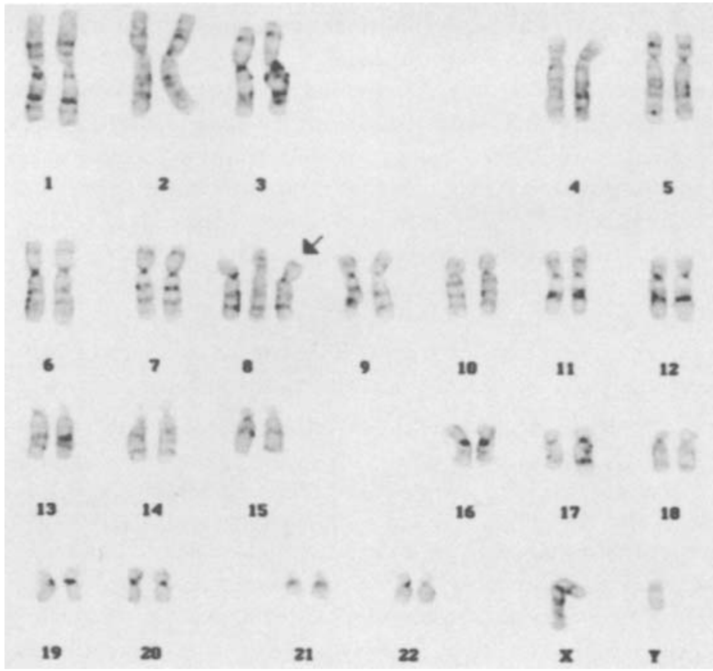

Figure 1. Computer-generated GTG-banded karyotype for illustrating chromosome 8 trisomy. Arrow points to an extra chromosome 8. Reprinted with permission of Medicine and Health/Rhode Island.

determined by trial and error. Cytogenetic technology is still more of an art than a science. An example of a GTG-banded karyotype is shown in Figure 1.

D. High-Resolution Banding

Although early G-banding was instrumental in delineating many structural chromosomal abnormalities, the level of resolution resulting from this technique is relatively low, with 400 to 450 bands per haploid genome, depending on the nature of the specimen. To obtain a higher level of resolution, at a band level of 550 to 850 or even greater, longer chromosomes at an earlier stage of mitosis, such as prophase or prometaphase, are needed. Protocols utilized to obtain such high-resolution G-banding were first described by Yunis and Sanchez (1975) and Yunis (1976). High-resolution banding protocols involve cell synchronization with agents such as methotrexate followed by a subsequent removal and the use of short exposure times to Colcemid or Velban. High-resolution banding, which is a mod-

ified G-banding technique, is most useful for the delineation of structural chromosomal rearrangements, such as microdeletions and microduplications. Examples of these include deletions and cryptic translocations involving 17p13.3 in Miller–Dieker syndrome, deletions of chromosome 15q11.2 in Prader-Willi, and Angelman syndromes, 22q11.2 deletions in DiGeorge syndrome and velocardiofacial syndrome, deletions of 17p11.2 in the Smith–Magenis syndrome, deletions of 7q11.23 in Williams syndrome, deletions of 4p16.3 in Wolf-Hirschhorn syndrome, deletions of 5p15.2 in Cri-du-Chat syndrome, deletions of Xp22.3 in Steroid Sulfatase Deficiency/Kallman syndrome, and microduplication at 17p11.2, which leads to Charcot–Marie–Tooth disease. However, even with high-resolution analysis, many of these deletions and duplications are not visible. For the detection of subtle structural rearrangements, FISH with molecular probes is a more sensitive technique than G-banding and high-resolution banding analysis (Mark, Jenkins, and Miller, 1997a).

E. Q-Banding

Quinacrine banding (Q-banding) using either quinacrine mustard or quinacrine dihydrochloride was the first banding method reported (Caspersson et al., 1968). Prior to Q-banding, the only chromosomes in the human genome that could be unequivocally identified by size and morphology alone were chromosomes 1, 2, 3, and 16 and the Y chromosome.

Quinacrine dihydrochloride (quinacrine, atebrin) is an acridine dye that binds to DNA by intercalation or by external ionic binding. Upon treatment, the chromosomes exhibit a series of bright and dull fluorescent bands. The distal long arm of the Y chromosome is particularly bright with Q-banding. The Q-banding pattern resembles that of G-banding, with Q-bright bands corresponding to G-dark bands. Notable exceptions are centromeric regions of chromosomes 1, 9, and 16 and the acrocentric satellite regions.

As in G-banding, it has been postulated that the pattern of banding reflects the underlying chromosome structure and genetic composition of the chromatin. It has been hypothesized that regions that fluoresce brightly are rich in AT nucleotides (Ellison and Barr, 1972), whereas the guanosine–cytidine (GC)-rich areas of the chromosomes tend to exhibit duller fluorescence (Comings et al., 1975; Comings, 1978).

One of the most useful features of this method of banding is the identification of the human Y chromosome. With Q-banding, the distal long arm (Yq12) of a normal Y chromosome appears very bright and is easily distinguishable. Thus, Q-banding is especially useful for identifying sex chromosome variants, such as XYY. Prior to FISH and the advent of interphase cytogenetics using FISH, the fluorescent Y body (or Y-chromatin) was often used to examine Y chromosome copy number in interphase cells (Pearson et al., 1970).

Although simple, quick, and reliable, Q-banding has two serious drawbacks. One is the requirements of special optics, filters, and light source for fluorescent microscopy, which are expensive. The other is the rapid quenching of the fluorescence, making Q-banding less than ideal for microscopic analysis, especially compared with methods such as G-banding which produce permanent preparations. For these reasons, most cytogeneticists today no longer perform Q-banding on a routine basis.

F. C-Banding

Constitutive heterochromatin, first described by Heitz (1928), constitutes approximately 20% of the human genome. It is the structural chromosomal material seen as dark staining material in interphase as well as during mitosis (Rooney and Czepulkowski, 1992). Constitutive heterochromatin is distinguished from facultative heterochromatin which is represented by the inactivated X chromosome in mammalian females (Lyon, 1962) and which manifests itself as the Barr body or sex chromatin (Barr and Bertram, 1949). Whereas constitutive heterochromatin can be visualized with the C-banding technique, facultative heterochromatin cannot.

Constitutive heterochromatin banding (C-banding) stains constitutive heterochromatin located in pericentromeric regions of all human chromosomes, but is most prominent in the secondary constrictions of chromosomes 1, 9, and 16 and the distal long arm of the Y chromosome. C-banding therefore can be used sometimes to characterize karyotypic abnormalities involving these chromosomes. C-dark bands are postulated to contain satellite DNA and are ideal for studying heteromorphic variants in the human genome, such as variations in the lengths and positions of the secondary constriction regions of chromosomes 1, 9, and 16 and distal Yq. C-banding protocols involve treatment with acid, alkali, and hot saline and subsequent staining with Giemsa. A popular protocol uses a saturated solution of barium hydroxide (Sumner, 1972), whereas the original method (Arrighi and Hsu, 1971) used sodium hydroxide as the denaturing agent. The formation of C-bands is postulated to be due to preferential destruction of DNA in non-C-banded regions of the chromosomes (Comings et al., 1973; Pathak and Arrighi, 1973), but the mechanism of C-banding is not at all clear.

G. R-Banding

Reverse or R-banding (Dutrillaux and Lejeune, 1971) is so called because bands that are pale by G-banding often appear dark by R-banding, and vice versa. In general, R-banding produces a reverse pattern to that obtained with either Q- or G-banding. R-bands replicate their DNA early and are said to contain active genes.

R-banding can be induced by incubation in a saline solution at a high temperature followed by Giemsa staining, in which case metaphases are analyzed by conventional brightfield microscopy. This is the so-called RHG technique, or R-banding by heat using Giemsa. It can also be obtained by staining with acridine orange after hot phosphate buffer treatment that results in bands being green and red in color, in which case fluorescent microscopy is required. The latter is called the RFA technique (Bobrow and Madan, 1973), or R-banding by fluorescence using acridine orange. Other variations of the protocols used to induce R-banding are also possible.

Although the exact mechanisms of chromosome banding remain unclear, the fact that R-bands fluoresce yellow-green and G- and Q-bands fluoresce orange-red is consistent with the hypothesis that R-bands are GC-rich and are more resistant to denaturation than AT-rich regions, as acridine orange fluoresce yellow-green when bound to double-stranded DNA and orange-red when bound to single-stranded DNA. The main advantage of using R-banding over G-banding is that the telomeric regions of certain chromosomes that stain faintly using Q- and G-banding can be better visualized using R-banding (Wyandt et al., 1973, 1974). This method of banding is useful in cases where a structural chromosomal rearrangement near the terminal region of a chromosome is suspected.

H. Additional Comments on Conventional Cytogenetics

Other less commonly used staining and banding techniques include nucleolar organizer regions (NOR) staining, in which the genes that are thought to be actively transcribed can be selectively stained using silver nitrate (Goodpasture and Bloom, 1975; Howell et al., 1975; Bloom and Goodpasture, 1976). With silver staining, NOR bands appear as one or more dotlike structures of varying size located on the stalks (not the satellites) of the acrocentric chromosomes. Studies have also shown that only the active NORs are impregnated by silver. Another technique that is only used rarely is differential replication staining. Other conventional cytogenetic techniques include 4',6-diamidino-2-phenylindole (DAPI)/distamycin A (DA) staining (Schweizer et al., 1978), telomere staining (Bobrow et al. 1972), CT banding (Chamla and Ruffie, 1976; Scheres, 1976) and kinetochore staining, all of which are nonroutine techniques.

A multitude of other banding and staining techniques induced by a variety of agents, including antibiotics, antibodies and restriction enzymes, have been described by Verma and Babu (1995). For example, in restriction endonuclease banding, the treatment of slides with restriction enzymes prior to staining with Giemsa generally produces a modified C-banding pattern depending on the choice of enzymes. A banding pattern similar to G-banding can be obtained with *Hae*III (Rooney and Czepulkowski, 1992; Verma and Babu, 1995). This particu-

lar technique is useful in occasional instances where one of the more common techniques is not informative and newer molecular technologies such as FISH are not available.

Most of the above staining and banding techniques depend on brightfield microscopy, discussed thoroughly in many other texts (e.g., Rooney and Czepulkowski, 1992; Kaplan and Dale, 1994; Barch et al. 1997), the details of which are beyond the scope of this chapter. Q-banding and fluorescent R-banding, on the other hand, need instrumentation for fluorescent microscopy, also discussed extensively elsewhere (see above).

Summarizing, G-banding is the method of choice for many cytogenetics laboratories involved in diagnostic testing. C-banding is used occasionally to study centromere position, dicentric chromosomes, and Y chromosome variants. Q-banding is rarely used nowadays, although in the past it had been used more often for routine analysis as well as for the study of heteromorphisms of the centromeres, shortarms of the acrocentric chromosomes, and the distal long arm of the Y chromosome. The application of solid staining has been restricted as described above. NOR (silver) staining was used to analyze short arm variants of the acrocentric chromosomes and for the identification of small markers. With the advent of FISH and other FISH-based techniques to be discussed in the remaining sections of this chapter, the importance of some of the older staining and banding techniques has been somewhat diminished. However, conventional cytogenetics using G-banding remains a highly informative test and will most likely continue to serve as the preferred standard of reference.

II. *IN SITU* HYBRIDIZATION

In situ hybridization with radionuclide-labeled probes has been reported since the late 1960s (Pardue and Gall, 1969). Autoradiography, however, requires long periods of exposure that are not practical for most applications. Biotinylated DNA probes and probes modified with other reporter molecules were introduced in the 1980s (Burns et al., 1985; Manuelidis, 1985a, 1985b; Nederlof et al., 1989; Guttenbach and Schmid, 1990; Lichter and Ward, 1990; Guttenbach et al., 1995). These early colorimetric *in situ* hybridization assays were performed with immunocytochemical stains, such as horseradish peroxidase, and were detected with a light microscope. Some laboratories today still adhere to these systems because of preference, while others have little choice because of financial constraints. Methods of nonisotopic *in situ* hybridization possess several advantages over the older radioactive methods, such as high sensitivity and specificity, rapid results, probe stability, and direct mapping without fastidious statistical analysis (Mark et al., 1997a; Viegas-Pequignot, 1992).