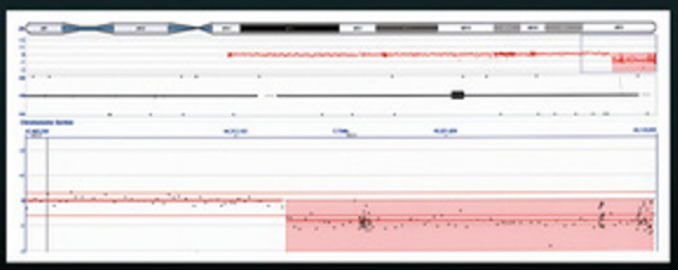
FOURTH EDITION

THE AGT CYTOGENETICS LABORATORY MANUAL

EDITORS: MARILYN S. ARSHAM, MARGARET J. BARCH, AND HELEN J. LAWCE



AGT

THE ORGANIZATION FOR CYTOGENETIC & MOLECULAR PROFESSIONALS

WILEY Blackwell

The AGT Cytogenetics Laboratory Manual

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Fourth Edition

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We dedicate this work to the memories of three extraordinary cytogeneticists who have recently departed, and will be truly missed.

Janet Rowley
April 5, 1925, to December 17, 2013

Dr. Rowley went against the prevailing theories of the causes of cancer to argue that they were chromosomal or genetic in nature. She discovered the first cancer causing translocation, the t(15;17) of APL, and also found that the Philadelphia chromosome of CML was caused by a translocation of chromosomes 9 and 22.

R. Ellen Magenis
September 24, 1925, to February 4, 2014

Dr. Magenis was also unafraid to go against prevailing ideas in science, and was on the cutting edge of research revealing that the seemingly same deletion on the long arm of chromosome 15 was responsible for two different syndromes, Prader–Willi and Angelman. This led to the discovery that imprinting was important in humans, as well as other animals. She contributed the imprinting chapter for this book. There is a genetic disease that even bears her name because of the critical role she played in discovering the genetic cause—Smith-Magenis syndrome, which is caused by a small deletion within the short arm of chromosome 17.

Margaret Barch
July 28, 1945, to February 8, 2015

Margaret was editor of the 2nd edition of the *Association of Cytogenetic Technologists Laboratory Manual*, and co-editor with Turid Knutsen and Jack Spurbeck of the *Association of Genetics Technologists Laboratory Manual*, 3rd edition, as well as an author of several chapters. She was in the process of finishing this 4th edition, along with co-editors Helen Lawce and Marilyn Arsham, and had re-written both the Cell and Cell Division chapter and the Microscopy chapter, when she died unexpectedly. Her spirit is evident throughout this book.

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Preface

The *ACT Cytogenetics Laboratory Manual*, also known as the ACT Technical Manual, was first published by the Association of Cytogenetic Technologists in 1980. Its two editors, technologists Marilyn S. Arsham and Helen J. Lawce, were the driving force behind this work. They did much of the writing themselves and acquired the input of many practicing technologists who shared protocols, advice and troubleshooting strategies. Two additional chapters were published in 1984 and 1985 under the editorship of Gitta Wahrenburg.

In 1991 a second comprehensive edition, edited by Margaret J. Barch, was published with the goal of maintaining the philosophy and quality of content of the original while updating material, including an index, adding chapters on solid tumors, fragile sites and molecular cytogenetics, and producing a professionally published volume. The third edition, edited by Margaret J. Barch, Turid Knutsen, and Jack Spurbeck, was entitled the *AGT Cytogenetics Laboratory Manual* reflecting the change of ACT to AGT (the Association of Genetic Technologists) in 1996. The name change was officiated in order to incorporate the genetic technologies that complement the cytogenetic ones. The third edition also included an expanded treatment of molecular cytogenetics, including comparative genomic hybridization (CGH).

This fourth edition is completely updated and includes chapters on peripheral blood culture, continuous cell lines, prenatal diagnosis and culture, hematological and solid tumor malignancies, fragile sites, an expanded fluorescence in situ hybridization (FISH) chapter, molecular cytogenetics, a treatment of ISCN (International System for Human Cytogenomic Nomenclature), and a new approach to human chromosome identification. Other new topics to the fourth edition include safety, equipment maintenance, compliance, quality control, managerial tools, laboratory information software, laboratory mathematics, and even animal cytogenetics. Together, this constellation of chapters provides a comprehensive resource of both knowledge and skills that are essential to the daily operation of a cytogenetics laboratory.

Our goal has been to collect together in one volume a book which provides an in-depth treatment of the theoretical basis of the field, combined with a wealth of complementary protocols. This manual satisfies that goal by including an exhaustive survey of over 100 techniques for visualization and analysis of chromosome patterns in diagnosis and research. In most cases, protocols, along with their tips, hints, and processing detail, have been submitted by individuals who perform the test. Some procedures may have been edited in order to conform to the manual's general style. Important: Any new procedure, or changes to an existing one, must be properly validated by the performing laboratory before it can be used for clinical purposes.

We have designed this volume to be useful to students as well as to novice and experienced technologists. It is our hope that this new edition captures the vitality and fascination that the field of cytogenetics still holds as it continues to change.

Marilyn S. Arsham
Margaret J. Barch
Helen J. Lawce

Acknowledgments

An African proverb says, “It takes a whole village to raise a child.” This manual is the collective efforts of a village of exceptional people - professionals at all levels who were willing to donate their time, expertise, and insight to create chapters that are rich with information; reviewers who were willing to honestly and carefully critique each manuscript; and editors who helped structure the flow of information. As you read each chapter, you too will appreciate these incredible authors who voluntarily shared their expertise with us. We thank them for their generous contributions and time.

What you won’t see listed in the chapters are the names of the many experts on the review board who worked behind the scenes. We sincerely thank everyone on our review board for their suggestions and evaluations; we couldn’t have done this without you. Please forgive any omissions: Charles Dana Bangs, Jane Bayani, Sue Ann Berend, Adam Coovadia, Pat Dowling, Betty Dunn, Viola Freeman, Atieh Hajianpour, Bing Huang, Bethy Jackle, Wahab Khan, Turid Knutsen, Peining Li, James L. Marks, Susan Olson, Katy Phelan, Richard Van Rheeden, Thomas Wan, Su Yang.

Three individuals deserve a special mention for their extra help. Turid Knutsen, you were the first to complete your chapter, which gave us an example to query publishers, and you stepped in to help edit chapters and format protocols without once asking for accolades. Charles Dana Bangs, your critical eye, encouraging words, and countless donations of time and material brought this manual to a higher level, again without expecting accolades. And Helen Jenks, your willingness and eagerness to step in, with your special way with words, helped us get through a very stressful period. We can never thank you three enough for your support and expertise.

On a personal note, we want to thank all those around us, both personal and professional, family and friends, who helped us along the way. There are too many to individually name, but we want you to know how much we appreciate your willingness to share your time, tips, protocols, pictures, advice, generators, and patience, while we plugged away at this manual, even in the face of nor’easters and hurricanes. Thank you, Bryan Arsham, for your assistance and patience in setting up this new computer so that uploading to AGT and Wiley’s clouds did not take hours per file. We give special thanks to AGT Past President Mervat Ayad for your support in helping build the interface with the publisher and to the editors at Wiley for making the process painless. In particular, we thank Purvi Patel, Metilda Shummy, and the invisible team behind them, for making this project come alive. Most of all, we thank our spouses, Ron, Gary, and John, for your unwavering support. These past 10 years have been an especially challenging time for us, and you have never once demanded anything of us but to finish this manual. You are our true heroes in each of our personal villages.

Finally, and most difficult of all, we thank you, Margaret. You are no longer here as we write these final words for you. You left us too early, and we miss you terribly. Thank you for keeping this manual alive, for giving so much love to each of us, and for being the gracious Southern Belle that you will always be in our eyes. Gary, we know her loss can never be mended, but we want you to know how much we love you for all the love you gave and still give “our” Margaret.

Our “village” is AGT, which reaches across all continents, a vision that far exceeds the expectations of the original 40 California technologists who started it all. This manual is the “child” of AGT’s dedicated technologists. It was conceived four decades ago, when Marilyn nervously stood up at the first annual AGT business meeting in Boston and proposed the concept. Helen joined her a year later, and Margaret kept it going for three more decades. Its need was evident in 1975 and is still evident today. It is therefore our turn to thank you all collectively for sharing your discoveries with us over these last 40 years.

CHAPTER 1

The cell and cell division

Margaret J. Barch[1] and Helen J. Lawce[2]

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1.1 The cell [1,2]

The cell is the basic unit of life – the simplest structure capable of independent existence. The simplest organisms consist of only one cell. Higher organisms are composed of complex colonies of interdependent cells, each colony with a specialized function necessary for the survival of the organism. Cells that have the same general function are often grouped together to form tissues, such as muscle, bone, and connective tissue. Tissues may be combined in larger functional units called organs, such as kidneys, skin, and heart. Organs can in turn be grouped by function into organ systems, such as the respiratory and circulatory systems.

Cells vary greatly in size, but they all must be able to survive and reproduce to be successful organisms. The cell membrane that envelops its contents must be able to control the movement of nutrients into the cell and of ions, molecules, and proteins out of the cell. Energy is converted from food and/or light and is used to synthesize internal components. The information for reproducing cell structures is encoded within its genetic makeup, thus providing the cell with its own self-sufficient capability to reproduce life-supporting needs and to repair genetic damage as needed. When functioning properly, the cell contains all the necessary tools to survive.

1.1.1 Cell membrane

Composition

The cell generally consists of cytoplasm, bounded by a cell membrane, and a nucleus, also enclosed in a membrane. There are exceptions to this model, such as red blood cells that have lost their nuclei during differentiation. The plasma membrane, or cell membrane, defines the boundary of the cell (Figure 1.1) and consists primarily of phospholipids and proteins. The phospholipids form a bimolecular layer, with their hydrophilic ends at the outer surfaces of the membrane and their hydrophobic chains extending into the middle of the membrane. The protein components of the membrane are globular particles distributed through the lipid bilayer; their polar amino acids may be exposed on an outer surface, but nonpolar portions remain in the interior.

Physical barrier

The cell membrane serves as a physical barrier for the cell contents, but it is rather fragile. If one were to tear a hole in this membrane by micromanipulation, the contents would spill out into the surrounding medium. An intact cell can rapidly repair minor membrane damage, but more extensive damage leads to cell death.

* Editors' note: We lost Margaret in the final stages of producing this book. May her spirit shine through and the reader be touched by her love of science, and her passion for passing it on.

The AGT Cytogenetics Laboratory Manual, Fourth Edition. Edited by Marilyn S. Arsham, Margaret J. Barch and Helen J. Lawce.

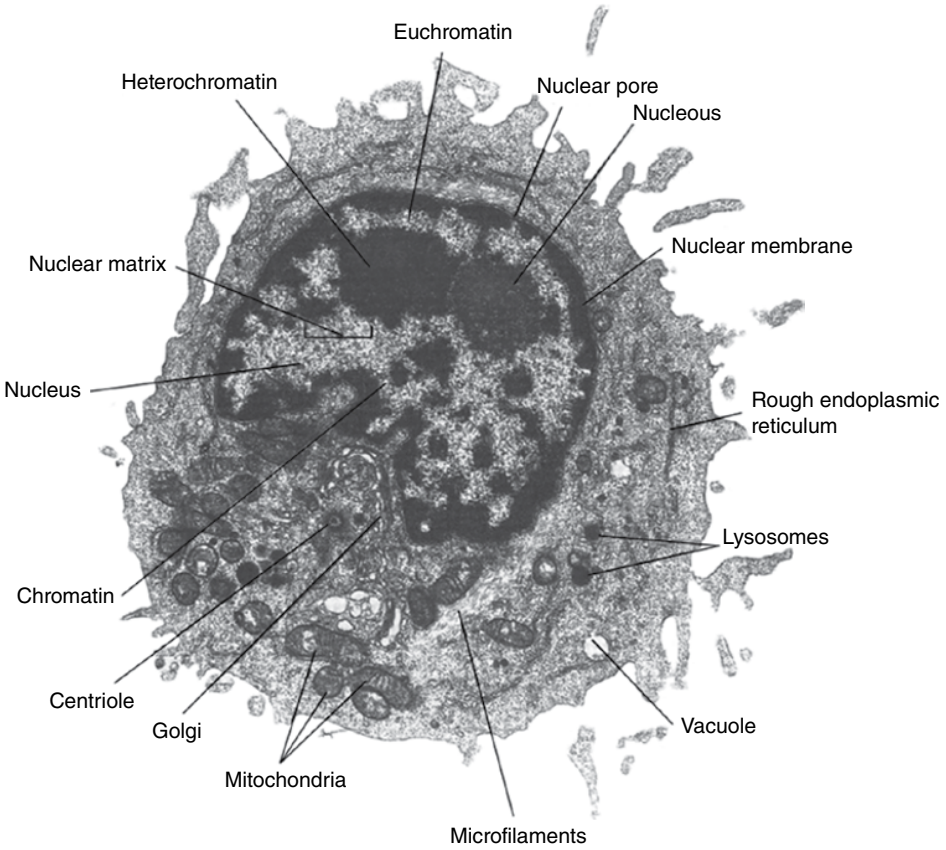


Figure 1.1 An electron micrograph showing the various components of a eukaryotic (human) cell.

Regulatory barrier

The membrane also acts as a regulatory barrier for the entry and exit of molecules and particles. This ability to regulate the passing of substances is called *selective permeability*. Substances can cross the cell membrane by three mechanisms: by free diffusion along a gradient, meaning that substances travel from regions of high concentration to regions of lower concentration; by active transport, which requires energy and moves substances against a concentration gradient; and by enclosure in vesicles that move substances into the cell (endocytosis) or out of the cell (exocytosis). Water can move freely across cell membranes in both directions; it is this property that allows hypotonic solutions (those less concentrated than the inside of the cell) to swell the mitotic cell, thus facilitating chromosome spreading for cytogenetic study.

Glycoprotein functionality

Molecules of glycoprotein (proteins with sugar molecules attached at points along the amino acid chain) exist on the surface of the protein–lipid membrane and sometimes project through it, into the cell. These glycoproteins function in cell adhesion, both to other cells and to culture flask surfaces. Trypsin, a protease (an enzyme that digests proteins), removes these molecules, thereby freeing cells for subculture or harvest. Glycoproteins can be antigenic (e.g., in red cells they determine blood type), and can serve as receptors for viruses, plant agglutinins (e.g., phytohemagglutinin), and hormones. They are further implicated in contact inhibition, a process in which normal cells stop dividing as cultures become confluent. Tumor cells often lose this property and tend to keep growing unchecked in a disorganized fashion when the growth surface is limited. Glycoproteins on the cell surface are also important in cell–cell recognition. If lymphocytes are stripped of their glycoproteins, they no longer accumulate in the lymph nodes.

1.1.2 Cytoplasm

Cytoplasm is the part of the cell within the cell membrane, excluding the nucleus, that consists of water, inorganic ions or molecules, and a variety of organic compounds. In many ways it resembles a colloid, with particles suspended in a continuous gel-like substance called the cytosol. The cytosol, in turn, contains a cytoskeleton of tubules and filaments, dissolved molecules, and water. Among the inorganic molecules are potassium, sodium, magnesium, and calcium. Trace amounts of many heavy metals are also present, as are bicarbonate and phosphate. Tiny granules can also be seen with a light microscope. These granules have been shown to be a series of vacuolar structures, bound by lipoprotein membranes similar to the cell membrane, with some even further differentiated into a complex system of internal membranes.

The large organic molecules (called macromolecules), which give the cytoplasm its colloidal properties, can be grouped into three main classes: proteins, nucleic acids, and polysaccharides. Each class is a polymer built from different subunits (monomers): proteins are made up of amino acid subunits; nucleic acids are polymers of nucleotides; and polysaccharides are built from sugar monomers. Together, the organelles described below and the cytosol make up the cytoplasm.

Proteins

Proteins carry out several important functions within the cell, including structural support, catalysis of metabolic reactions, and regulation of complex cellular processes. Examples of structural proteins are actin and myosin in muscle, and keratin in hair, nails, and hooves. Regulatory proteins include hormones, growth factors, and receptors.

Polysaccharides

Polysaccharides function as food storage molecules and as structural molecules. The two most important polysaccharide food reserves in higher organisms are starch and glycogen, both of which are polymers of glucose sugar. Structural polysaccharides include cellulose and chitin: cellulose is the major constituent of cell walls in plants, and chitin is found in the exoskeletons of insects and crustaceans.

Lipids

Another important organic molecule, although it is not classified as a macromolecule, is the lipid. Lipids encompass a diverse group of compounds that are all soluble in nonpolar, organic solvents. Included in this class are fats, which are used primarily for energy storage; phospholipids, which are found in cell membranes; sphingolipids, which are especially prominent in the cell membranes of brain and nervous tissue; glycolipids, which are important in the myelin sheath of nervous tissue; steroids, which include male and female sex hormones, bile acids, adrenocortical hormones, and cholesterol; and fatty acids, which are components of energy storage molecules.

Endoplasmic reticulum

Endoplasmic reticulum (ER) is contiguous with the outer membrane of the nucleus. It is the site for folding proteins and assembling large molecules in an oxidizing environment. The ER consists of membranous channels lacking ribosomes (smooth ER) or containing ribosomes (rough ER). In the rough ER, ribosomes actively synthesize protein that accumulates in the lumen of the ER. These proteins include secretory proteins that make their way to the cell surface via a complex route, e.g., the rough ER, Golgi complex (see later), and secretory vesicles. Smooth ER is the site of synthesis of lipids and steroids and also for the inactivation and detoxification of drugs and other compounds harmful to cells.

Golgi complex

The Golgi complex (or Golgi apparatus) is a region of flattened vesicles closely related to the smooth ER both in proximity and function. It processes and packages secretory proteins and synthesizes complex polysaccharides. The Golgi also accepts vesicles that "bud off" the ER. These vesicles and their protein contents are processed further and then passed on, via vesicle budding of the Golgi complex, to other components of the cell. Therefore, the Golgi complex is a processing station for both receiving vesicles that fuse with it and also producing vesicles from it in a repackaged form, usually ready for export from the cell.

Lysosome and peroxisome

Two structurally similar organelles are the lysosome and peroxisome, each contained by a single membrane. Lysosomes are storage structures for hydrolases, i.e., enzymes that digest food and cell components that are no longer needed. Peroxisomes generate and degrade hydrogen peroxide. Animal peroxisomes also detoxify other harmful compounds, such as, ethanol, methanol, formate, and formaldehyde, and generate some unusual substances, such as D-amino acids.

Mitochondria

The mitochondrion (plural: mitochondria) is quite large, relative to other organelles, i.e., several micrometers (microns) in length and 1 micrometer in width, about the size of a bacterial cell. All mitochondria in the cells of an individual are maternally derived from those that were present in the egg at the time of fertilization. Therefore, unlike nuclear DNA in which the paternal contribution of genes is 50%, all mitochondrial DNA comes from the mother.

Depending on the organism and cell type, a cell may have only one mitochondrion or it may have several thousand. A typical human cell contains hundreds of mitochondria, each with 2–10 copies of mitochondrial DNA (mtDNA), resulting in thousands of copies of mtDNA per cell. Again in contrast with nuclear DNA inheritance, where each cell receives exactly half the genetic material at cell division, mitochondria are not always evenly partitioned into daughter cells – one cell may receive more (or fewer) copies of mitochondria. Therefore, the number of mitochondria and constituent mtDNAs can be heterogeneous between tissues and even within a given tissue; this is termed *heteroplasmy*.

Within the mitochondria, oxidation of nutrients (oxidative phosphorylation) takes place, providing energy to synthesize adenosine triphosphate (ATP). ATP conserves the energy from the oxidative reaction that would otherwise have been lost as heat and makes it available to the cell for work. Thus, the mitochondria have been called the powerhouses of the cell. They have a double membrane, an outer membrane plus an inner membrane, which are infolded into numerous projections called *cristae*, where oxidation of nutrients takes place.

Mitochondria also command special interest because they contain their own DNA (mtDNA) and ribosomes, although the ribosomes in mitochondria are more similar to those in prokaryotes in size and nucleotide sequence than to ribosomes elsewhere in the eukaryotic cell. mtDNA is usually circular, like a bacterial genome, with no histones attached. Human mtDNA contains 37 genes, including those that specify transfer RNAs, ribosomal RNA, and polypeptides important in ATP synthesis. The mitochondrion even encodes some of its own RNA and polypeptides, about 5% of those it needs. Mutations of mitochondrial genes, even when only a fraction contain mutant mtDNAs, can cause disease if they are located in tissues where mitochondrial function is important; for example, mitochondrial mutations have been implicated in several metabolic diseases, heart disease, and aging.

Ribosomes

In addition to the membranous organelles mentioned above, cells contain other important structures. The ribosome, made up of 50–80 different proteins and three or four different kinds of RNA molecules, is a tiny spherical body on which the synthesis of proteins takes place. They are found either free in the cytoplasm or attached to mitochondria, ER, or the outer surface of the nuclear membrane. Proteins needed for use in the cytosol are usually synthesized on single ribosomes.

Polypeptide chains are made on groups of ribosomes called polyribosomes, or polysomes. The polysome contains a variable number of ribosomes held together by a messenger RNA (mRNA) strand. This mRNA strand determines the sequence of amino acids in the synthesized protein. Signals residing in the mRNA also determine initiation, elongation, and termination of the polypeptide. Antibiotics, such as streptomycin, chloramphenicol, and puromycin, block protein synthesis at one of these three stages.

Centrioles

Tubules and filaments are other versatile cell components. Electron microscopy has shown that centrioles (or basal bodies), which are important in cell division, are found near the nucleus. The centriole contains nine microtubule triplets around its periphery. These bodies occur in pairs, called a diplosome or centrosome, which are perpendicular to each other and are attached to the outside of the nucleus. During the G1/S cell cycle transition the centrioles self-duplicate and migrate to opposite ends of the cell, where they form spindle fibers (also made of microtubules). Spindle fibers help separate chromosomes to their respective daughter cells in cell division.

Many proteins interact with and regulate the centrosomes. Alterations in these centrosome-associated proteins can have pathological consequences. For example, mutations in the TP53 gene can lead to extra copies of the centrosome, predisposing the cell to misshapen spindle apparatus formation, aneuploidy, and tumor formation [3].

In the laboratory, colchicine inhibits cells from completing mitosis by binding to the monomer tubulin, thereby blocking its assembly into polymeric spindle fibers. Colchicine also indirectly disassembles already-formed spindle fibers. Without spindle fibers, chromosomes are unable to move away from the metaphase plate and complete cell division.

Cilia and flagella

Cilia and flagella, the external hair-like projections that function in cell motility, are also made of microtubules. Nine doublet tubules are arranged around the periphery and a tenth doublet forms the core. Like their close relatives, the microfilaments, microtubules are involved in cell movement, cytoplasmic streaming, cell cleavage, and membrane invaginations. Microtubule-initiated motion almost always requires ATP as an energy source.

1.1.3 Nucleus

The nucleus is the information headquarters for the cell. Unlike prokaryotes, such as bacteria and blue green algae that carry their genetic material in the cytoplasm, other more complex organisms confine their genetic information, DNA, within a nucleus. These more evolved plant and animal organisms, including humans, are termed eukaryotes (eu = true; karyon = nucleus). Every eukaryotic cell has a nucleus at some stage of its existence. Some cells have more than one nucleus, and some, such as red cells and platelets, lose their nuclei when they mature. Cells lacking nuclei, however, are severely limited in their metabolic activities.

The nucleus contains a nuclear membrane, chromatin, and nucleoli (see Figure 1.1). It is also the site of ribosome precursor assembly. The term *nuclear matrix* refers to the fibrous material that remains if the chromatin and nucleoli are extracted. DNA within the nucleus determines the cell's morphological, biochemical, and metabolic characteristics.

The appearance of the nucleus is markedly different in interphase (nondividing) and mitotic (dividing) cells. First noted by Brown in plant cells in 1831 [4], the interphase nucleus is a conspicuous spherical body in the cell interior. By light microscopy, it appears as an amorphous network of variably condensed fibers, called chromatin, which are not distinguishable as individual entities. Highly condensed chromatin stains darkly with nuclear stains and is known as *heterochromatin*; the more dispersed chromatin, which stains lightly or not at all, is called *euchromatin*. In cell division, the chromatin condenses into deeply staining, threadlike or rod-like structures called chromosomes (chromo = color; soma = body), which are present in specific numbers in each cell of a given species. This process of chromatin condensation to form chromosomes during division is necessary for the equal parceling of genetic information to daughter cells.

The nucleus is spatially organized with each chromosome in a specific region. This serves to prevent one chromosome from getting tangled with another. Telomeres are attached to the nuclear membrane. Between chromosomal subcompartments are chromatin-free interchromosomal domains. Here reside RNA molecules being processed for export to the cytoplasm. Highly transcribed portions of the chromosomes are positioned next to the interchromosomal domains and since different genes are transcribed in different cell types; the arrangement can vary from cell to cell.

Under the electron microscope, chromatin and chromosomes appear as fibrous structures. This is understandable since they comprise DNA molecules that are themselves filamentous. Fibers of DNA with associated proteins are about 30 nanometers (nm) in diameter, but protein-depleted strands are only about 10 nm in diameter. Chromatin fibers with diameters greater than 30 nm are occasionally observed and are believed to represent coiling or folding of these main fibers.

Nuclear envelope

The nuclear envelope, as the membrane surrounding the nucleus is called, is a porous double membrane with ribosomes attached to the outside. Numerous pores serve as channels for water-soluble molecules to travel between the nucleus and the cytoplasm. Ribosomes, mRNA, chromosomal proteins, and enzymes needed for nuclear activities are also thought to travel through these nuclear pores. The outer membrane is contiguous with the ER at many sites. Inside the nucleus are two obvious structural elements – the nucleolus and the chromatin. During cell division, this nuclear envelope disappears.

Nucleolus

One to four nucleoli appear as darkly staining bodies eccentrically placed within the normal nucleus. They comprise primarily RNA and protein but contain some DNA. Their size will vary, based on the cell type and the metabolic state, i.e., larger nucleoli are seen in rapidly dividing cells and in cells active in protein synthesis. Each nucleolus is formed along the nucleolar-organizing region (NOR) of one or more specific chromosomes; these regions are recognizable during cell division. The nucleolus is the site of ribosome precursor assembly; therefore, all ribosomes in the cytoplasm originate in the nucleolus.

Nucleic acids

The nucleus contains the nucleic acids DNA and RNA along with structural and regulatory proteins. Nucleic acids are involved with protein synthesis and the storage of genetic information. There are two kinds of nucleic acids: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), each a polymer of *nucleotides*. Nucleotides consist of one purine or pyrimidine, a five-carbon sugar, and a phosphate group (see DNA). The sugar in DNA is deoxyribose; in RNA (see RNA) it is ribose.

DNA is the genetic material, and RNA is responsible for carrying out the instructions coded by the DNA. The primary functions of nucleic acids are *gene replication*, i.e., the process of copying sequences of DNA (genes) for distribution to daughter cells, and *gene transcription*, i.e., the process of copying sequences of DNA into complementary strands of RNA. These transcript RNAs may then be translated into corresponding sequences of amino acids during the synthesis of polypeptides (proteins). As previously discussed (see Ribosomes), protein synthesis occurs on cytoplasmic ribosomes.

DNA

The story of how scientists searched for the hereditary material and eventually established that DNA is the genetic material in almost all organisms is a fascinating one. Gregor Mendel's "hereditary factors," Walther Flemming's chromosomal threads, and Walter Sutton's chromosome theory of heredity led the way. Johan Miescher discovered DNA in 1869, calling it nuclein. The beauty of its structure and the logic of the coding process still inspire those who study them today.

Studies with sister chromatid exchange, electron microscopy, and other techniques demonstrate that a chromatid, one of a pair of metaphase chromosome strands, contains a single, uninterrupted, highly folded molecule of DNA. DNA itself is a double helix made up of two strands. Each strand is comprised of nucleotides, each consisting of a sugar molecule, a phosphate group, and one of four bases: adenine (A), guanine (G), thymine (T), or cytosine (C). The nucleotides are arranged side by side, with two bases forming one rung of a twisted ladder, and the phosphate and sugar form the outer structure (Figure 1.2). The sugar in

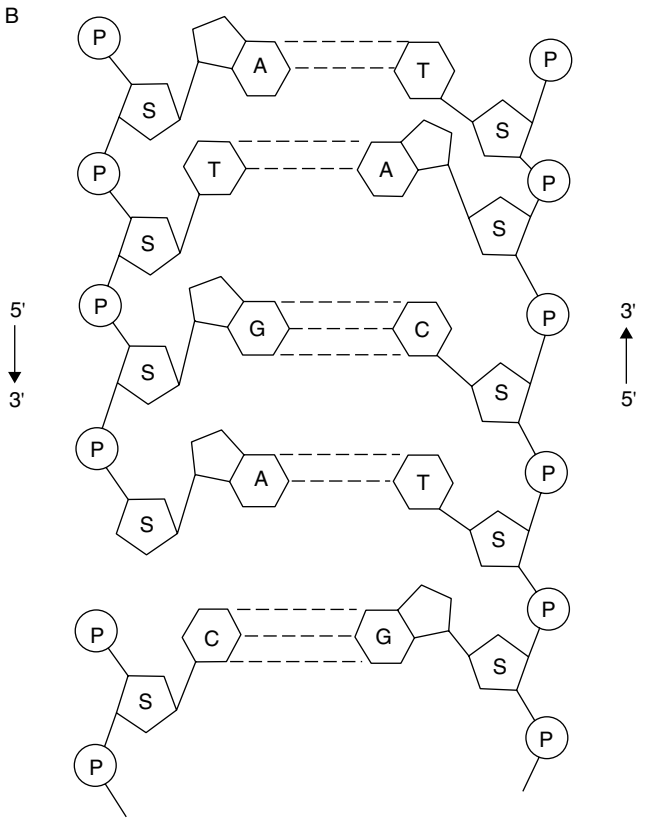


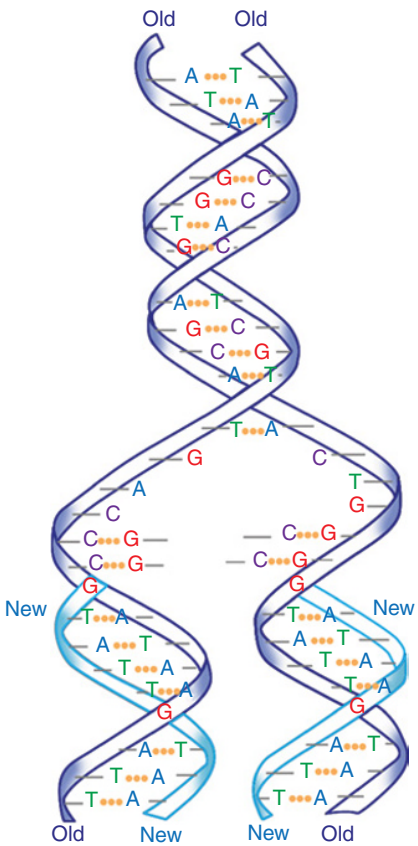
Figure 1.2 Chemical structure where A is adenine, T is thymidine, G is guanine, C is cytosine, S is sugar, and P is phosphate. The left strand polarity is from the 5′ base to the 3′ base, and the right strand has a 3′ to 5′ opposite polarity.

DNA, deoxyribose, has five carbon atoms, the third and fifth of which are bonded together by a phosphate (phosphodiester) linkage. Thus, a single strand of DNA is a polymer of deoxyribonucleotides held together by a 3′–5′ phosphate linkage between their sugars. This is called the sugar–phosphate backbone of the DNA molecule, and it lies on the outside of the DNA fiber; the bases extend inward from the backbone. The free 3′ and 5′ ends give the molecule a polarity, or direction.

Watson and Crick [5,6] determined the double helical structure of DNA in the 1960s using models and X-ray diffraction images. The two strands of polynucleotides have opposite polarity. The bases hold the two strands together by hydrogen bonds (see Figure 1.2). Both strands are coiled in the same direction, so they cannot be separated without unwinding. Minor bases present in mammalian DNA include 6-methyl adenine and 5-methyl cytosine; the latter is found throughout the human genome but is often concentrated in areas of heterochromatin, such as in chromosomes 1, 9, 15, 16, and Y.

The bases in DNA are flat molecules that can stack on top of one another. The double-helical nature of DNA is maintained by these stacking forces and by the hydrogen bonds between the bases. The regularity of the double helix along its axis is possible because an AT pair is the same size and shape as a GC pair.

Prior to cell division, new DNA must be synthesized with great fidelity. This is accomplished by separation of the two strands so that each acts as a template for the assembly of a complementary strand (see Figure 1.3). Thus, two identical copies of the original DNA are produced, each composed of one original strand and one newly synthesized strand (semiconservative replication). This mechanism for producing a faithful copy of the genetic information for each daughter cell is fundamental to understanding techniques such as sister chromatid exchange (see Chapter 6, Chromosome stains). Of the four bases, two are purines (A and G), and two are pyrimidines (T and C). The precise replication of DNA is possible because the pairing of bases is specific: A pairs with T, and G pairs with C. Thus, the sequence of bases in one strand specifies the bases and their order in the complementary strand.



DNA replication illustrating two new helices being replicated semiconservatively.

Figure 1.3 This diagram illustrates two new helices being replicated semiconservatively (Adapted from Lince-Faria et al. 2009 [26]. See insert for color representation of this figure.).

Table 1.1 Genetic code

DNA triplet	RNA triplet	Amino acid
AAA	UUU	Phenylalanine
AAT	UUA	Leucine
TAA	AUU	Isoleucine
TAC	AUG	Methionine (start)
AGA	UCU	Serine
GGA	CCU	Proline
TGA	ACU	Threonine
CGA	GCU	Alanine
ATA	UAU	Tyrosine
ATT	UAA	(stop)
GTA	CAU	Histidine
GTT	CAA	Glutamine
TTA	AAU	Asparagine
TTT	AAA	Lysine
CTA	GAU	Aspartic acid
CTT	GAA	Glutamic acid
ACA	UGU	Cysteine
ACT	UGA	(stop)
ACC	UGG	Tryptophan
GCA	CGU	Arginine
CCA	GGU	Glycine

The nucleotide triplet in DNA specifies a triplet in RNA, which specifies an amino acid (or a start or stop signal). The code is "degenerate" in that each codon is not unique; for instance, UUA, UUG, CUU, CUC, CUA, and CUG all specify the amino acid leucine. A, adenine; C, cytosine; G, guanine; T, thymine; U, uracil.

The way DNA stores information was elucidated in the 1960s [5,6]. It was learned that the genetic code consists of three bases per code word; one triplet, or codon, codes for one amino acid (Table 1.1). A gene, then, can be understood as a linear arrangement of codons giving the instructions for the building of a protein with specific amino acids in a particular order.

It was later discovered that in higher eukaryotes, the coding instructions in a gene are often interrupted by DNA sequences that are not present in the mRNA and are not translated into amino acids in that gene's protein. These interrupting sequences are called introns (for intervening sequence), and the DNA sequences translated into the mRNA that usually code for protein are called exons [7]. The introns are spliced out of the mRNA before it codes for a protein. It is now known that a single gene can make more than one protein. Alternative splicing of introns can lead to multiple transcripts.

High temperatures or high pH conditions break the hydrogen bonds, and the double-stranded helix unwinds, or denatures, into two single-stranded helices. Because G–C pairs have three hydrogen bonds and A–T pairs have only two, the A–T pairs tend to be more unstable, denaturing before the G–C pairs. Therefore, the temperature at which a given DNA will be half denatured, or melted, is used as an index of the amount of G and C in that DNA. The curve of the rate at which this denatured DNA renatures (becomes double-helical once more) is called its Cot curve (Co = concentration of single stranded DNA, t = time). This curve yields other information about the DNA, such as how many sequences are present in multiple copies (repetitive DNA) versus how many are unique. Denaturation of DNA is an important step in fluorescence in situ hybridization (FISH) procedures discussed in a subsequent chapter.

Another measure of the G–C content is the buoyant density of the DNA. This is measured by forming gradients of concentration (and therefore of density) in cesium chloride during centrifugation. The DNA will collect at the

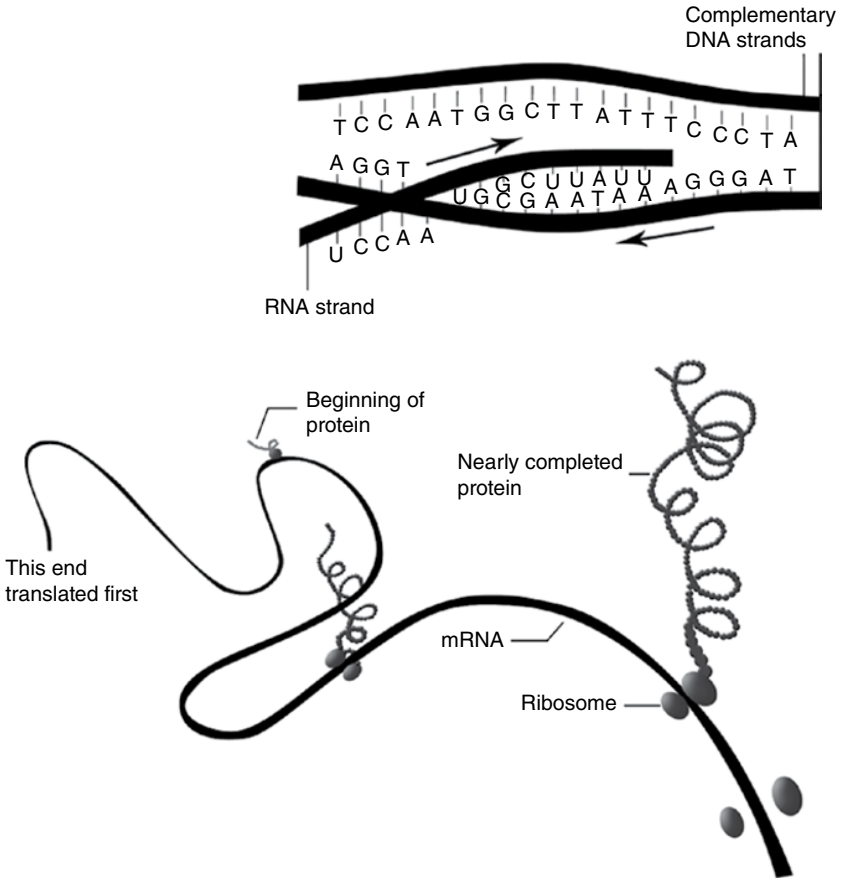


Figure 1.4 DNA transcription involves synthesis of a RNA molecule complementary to one strand of the DNA (top). RNA translation refers to the synthesis of protein molecules specified by the RNA sequence (bottom). Watson 1983 [28]. Reproduced with permission of John Wiley and Sons.

band where the gradient density is equal to the DNA density. This buoyant density depends upon DNA strandedness (single or double) and base composition. (See also 1.1.6, Satellite DNA.)

RNA

Like DNA, RNA is a polymer of ribonucleotides linked by 3′–5′ phosphodiester bonds. RNA differs from DNA in three respects: its ribose sugar has a 2′-H group instead of 2′-OH group; it is single-stranded, rather than double-stranded; and it substitutes the base uracil for thymine to pair with adenine.

DNA does not specify a protein directly; rather, the gene for forming the protein is expressed through an intermediary molecule, the mRNA. Transcription, or mRNA synthesis, uses one strand of the DNA as a template for a complementary strand of RNA (Figure 1.4). After transcription, introns are spliced out and the mRNA molecule moves out of the nucleus to the cytoplasm, where it directs the synthesis of protein in the presence of ribosomes. Transfer RNA (tRNA) binds the appropriate amino acid to its anticodon, a base triplet complementary to a codon in mRNA. Ribosomal RNA assists in actual protein synthesis, by binding the anticodons of the tRNA molecules with the matching codons of the mRNA molecule so that the attached amino acids are covalently linked in the proper linear order.

Approximately 1.2% of the genome encodes for protein via mRNA; yet about 93% produces RNA transcripts. For example, micro RNA (miRNA) performs its regulatory function by binding to a matching region on a strand of mRNA; this will block

the ribosome from reading the strand and will thus disrupt protein synthesis. Likewise, small interfering RNA (siRNA) binds to mRNA and cuts it, thereby preventing translation. The functions of these and other RNAs are under study.

1.1.4 Chromosomes and their proteins

The two main categories of chromosome proteins are histones and nonhistones. Interphase chromatin contains mostly histone proteins, characterized by their basic pH, which is due to large numbers of the amino acids arginine and lysine. Their isoelectric points (pH at which the average charge of the molecule is zero) are always more than 10. Proteins with an isoelectric point less than 10 are classified as nonhistone proteins (NHPs). NHPs tend to be acidic, although their isoelectric points vary from 4 to 9, and are a mixture of proteins with different structural, enzymatic, and regulatory functions.

Histones

Histones, which can be found in a 1:1 ratio by weight to DNA, are classified into five major classes: lysine-rich H1, slightly lysine-rich H2A and H2B, and arginine-rich H3 and H4. More specialized forms can also be found in specific structures, such as, H5, the histone that replaces H1 in nucleated erythrocytes, and protamines, a group of highly basic proteins with a low molecular weight, which replace the histones in mature sperm. H3 and H4 have been highly conserved in evolution, and may actually express the same functionality in all eukaryotes [8]. Histones are also highly conserved in organisms from one tissue to another and between species; therefore, cows and peas have virtually the same histones. In the laboratory, histones can be extracted from chromatin by dilute acids or by high-molarity salt solutions. Acetic acid and methanol, commonly used to "fix" chromosomes, dissolve out some, if not most, of the histones.

Nucleosome

Histones H2A, H2B, H3, and H4 form octomers containing two molecules of each histone, giving rise to a 10-nm sphere or disk (visible with the electron microscope) called a nu-body, or nucleosome. The nucleosome appears to be the basic unit of eukaryotic chromatin. The fifth histone, H1, is implicated in the linking and compaction of these nucleosomes.

The nucleosome is present in dispersed or condensed chromatin, in repetitive areas or unique sequences, and in interphase and metaphase nuclei. It is associated with roughly 140 base pairs (bp) of DNA, which is wound twice around the spherical nucleosome. For reference, an average structural gene is approximately 1200 bp, which would span about six nucleosomes. When chromatin is extended by the removal of H1 histones, a linker region of about 60 base pairs of DNA can be seen between nucleosomes. When this region is uncoiled, the nucleosomes are seen located along the naked DNA like beads on a string. The H1 protein is responsible for condensing these beads into a 10-nm fiber. This is coiled again into the 25-nm strands, which look lumpy or kinky under the electron microscope (see Figure 1.5).

Nonhistone proteins

There are several hundred nonhistone proteins [9], which include all proteins of chromatin other than histones. Even though these proteins are thought to be more numerous and more variable than the histones, they actually make up much less of the chromatin mass [10]. Nonhistone proteins are involved in chromosomal metabolism [1,2], gene expression, and higher order structure.

Euchromatin and heterochromatin

Positive and negative heteropyknosis is used when referring to staining intensity that reflects the degree of coiling or condensation of the chromatin filaments. During the cell cycle, chromosomes condense and decondense, with maximum condensation at metaphase. Chromosomes and segments of chromosomes that are more heavily stained than the rest exhibit positive heteropyknosis. Others that are more lightly stained exhibit negative heteropyknosis. The chromatin in these variable regions, showing condensation unlike the remainder of the chromatin, is termed heterochromatin, while other regions are known as euchromatin. Under the electron microscope there is no difference in the basic structure of euchromatic and heterochromatic chromatin; therefore, differential staining has been attributed to fiber packaging within the heterochromatic regions. In the literature, the term heterochromatin usually refers to positive-heteropyknotic areas, but a given area may be negatively heteropyknotic in one banding technique and positive in another; therefore, use of the term should specify the stain with which it is being evaluated.

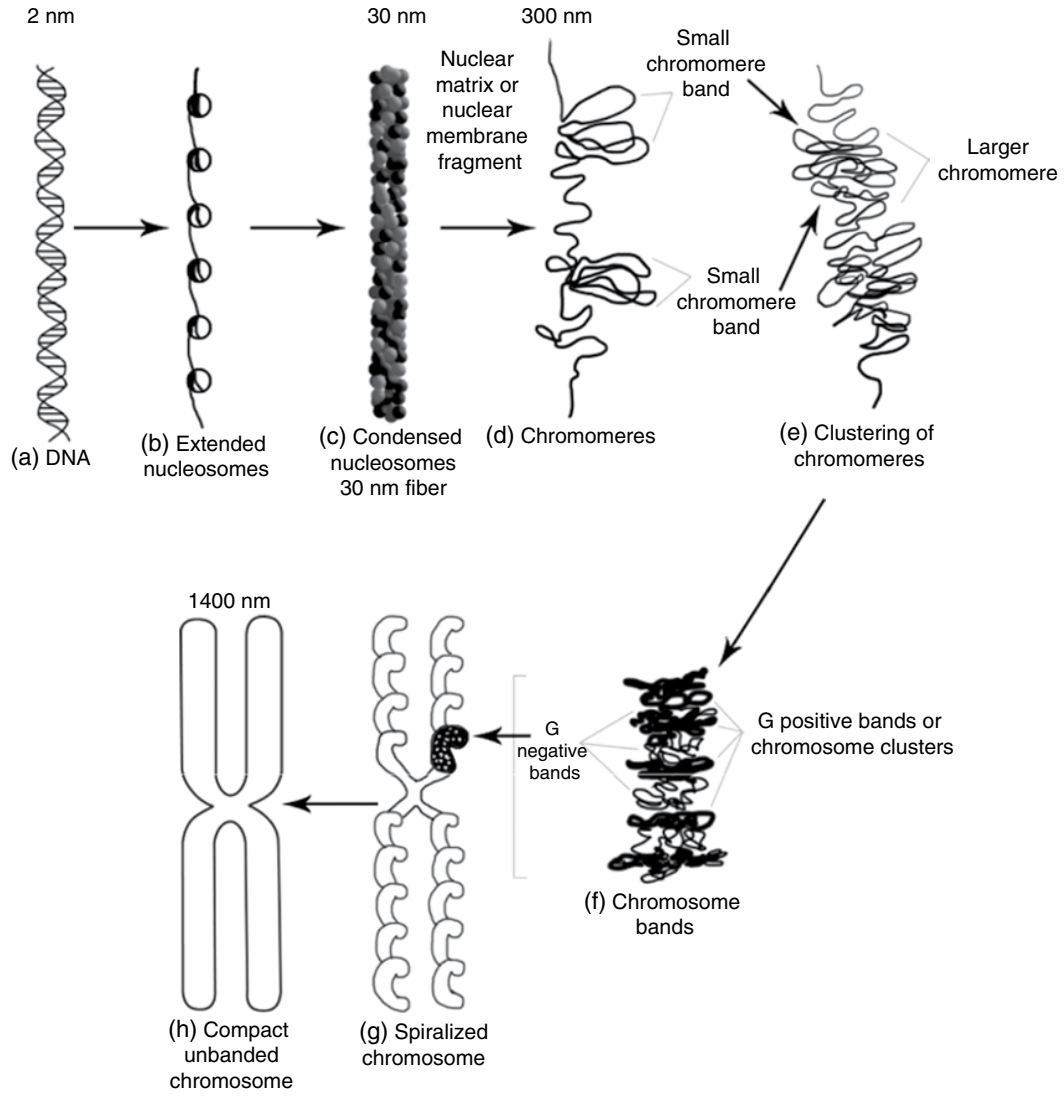


Figure 1.5 Hypothetical relationship between DNA, nucleosomes, chromomeres, G-bands, and spiralized chromosomes (Adapted from Comings, DE. Mammalian Chromosome. In: de la Chapelle A, Sorsa M, eds. *Chromosomes Today*: Amsterdam: Elsevier/ North Holland, 1979, [19–36]).

There are two distinct types of heterochromatin: facultative and constitutive. In humans, facultative heterochromatin is the name given to the condensed, inactive chromatin of X chromosomes in excess of one. It may represent one X in a given cell and its homolog in another, owing to the randomness of X inactivation (see 1.1.5, X inactivation). Constitutive heterochromatin is the name for the differentially staining areas of chromatin and chromosomes, which are evident with different stains and banding techniques and are constant from cell to cell.

Facultative heterochromatin and constitutive heterochromatin resemble each other in several ways: neither codes for protein (in most cases being genetically inactive), and both replicate late in the synthesis phase of the cell cycle. They differ in that constitutive heterochromatin is often rich in repetitive DNA, stains differently from euchromatin with banding techniques, and never elongates or decondenses. Facultative heterochromatin has sequences similar to active DNA, does not stain differently with standard banding techniques, and in some cases can become decondensed and active, as the X chromosome does during meiosis and early embryogenesis.

1.1.5 X inactivation

The manifestation of facultative heterochromatin in most female mammals, including humans, is a visible sex chromatin body that is derived from the second X chromosome. It is commonly referred to as a Barr body. Various staining techniques, as well as phase contrast, can be used to visualize the Barr body. It can be found in almost all tissue cells.

Barr body

Although earlier investigators noticed a "basophilic nucleolus" or "paranucleus," this triangular staining body on the periphery of the nuclear membrane was not related to sex until 1949, when Murray L. Barr and Ewart G. Bertram published their observation of a "paranucleus" in cells from the hypoglossal nerve of female cats, but not of the males, called the Barr body [11]. Expanding on the work by Liane Russell, Mary Lyon in 1961 put forth what we now known as the *Russell–Lyon hypothesis* [12], which stated the following:

1. One of the two X chromosomes is inactivated in human females.
2. The inactivated X may have either maternal or paternal origin in a given cell of an individual, and the choice is random.
3. The inactivation occurs in early embryogenesis.
4. Inactivation is stable, and descendants of a cell with an inactive X inherit that same X in an inactive state except in the germ cells (see later).

X inactivation is often called dosage compensation, that is, a mechanism for producing equal amounts of gene products in females having two X chromosomes and males having only one. We know that this compensation is effected by some mechanism, because females homozygous for hemophilia A have clotting times similar to those of affected hemizygous males. Also, glucose-6-phosphate dehydrogenase (G6PD) levels are similar in normal females (most of whom are homozygous) and in normal hemizygous males [13].

This mechanism, however, is by no means universal. In Drosophila, females are the homogametic sex (XX) and males are the heterogametic sex (XY), just as in mammals. The difference is that in Drosophila both Xs are active (transcribed), but the single X in males produces comparable amounts of gene products. Birds, reptiles, and butterflies have no dosage compensation of Z-linked genes, and the homogametic sex (ZZ), which is male, produces twice the level of gene products as the heterogametic sex (ZW).

Because of the random inactivation of X chromosomes, female mammals are mosaics for the genes on the X chromosome [14]. This is demonstrated by coat color in tortoise-shell and calico cats, which are heterozygous for black and orange X-linked alleles. Another example is the expression of G6PD in women who are heterozygous for the trait. Clones from a single cell produce either the mutant or the wild-type enzyme, never both, but in a random sampling of cells, about 50% produce the wild type and 50% the mutant clones.

Inactivation appears irreversible, even in human/rodent hybrid cells in which the inactive human X is present alone against a rodent background. It also remains stable in cells maintained in culture for many generations [15,16]. Reactivation of the entire X chromosome takes place only in the oocytes at some time before meiosis, and both X chromosomes are transcribed. This has been supported by experiments in which the presence of the X-linked enzyme HPRT (hypoxanthine phosphoribosyl transferase) was found to be at levels twice as great in females as in males in the morula stage of mouse embryogenesis. X inactivation occurs in waves from day 3.5 to day 13 of development. Germ cell progenitor cells in females are inactivated by day 12 [15].

In mammalian cells, an inactivation center is believed to reside on the long arm of the X chromosome at band Xq13 (XIC). There is no known case in which this proximal Xq area, called the *c region*, is deleted in the X chromosome, leading to the presumption that two active X chromosomes would not be viable [15]. In metaphase cells, the inactive X often appears shorter than its active homologue, and is frequently bent in the proximal long arm [17]. In X chromosomes with two c regions, bipartite Barr bodies are seen in some cells.

DNA methylation is believed to play a role in maintaining this repression of the inactive X. The gene *XIST* (inactive X specific transcript) has been found to be active only on the inactive X chromosome [18,19]. This gene, located in the region of the X inactivation center (XIC), does not code for a protein but rather produces an RNA product that "coats" the X chromosome on which it is expressed (i.e., acts in *cis*), and recruits other factors, including those that result in histone deacetylation and methylation, with the overall result of transcriptional repression of genes on the same chromosome. Experimental treatment of cells with 5-azacytidine produces hypomethylation of DNA and partial reactivation of selected loci, but not of the entire X [15,16].

X inactivation and conversion to the heterochromatic state involves interaction between noncoding transcripts such as Xist, chromatin modifiers, and factors involved in nuclear organization. These produce changes in chromatin structure and in the spatial reorganization of the X chromosome [20].

In individuals having one normal and one abnormal X, the abnormal X is usually the inactive one. There are indications, however, that inactivation is initially random even in these individuals but that the cells with an active normal X survive [15,16]. In X;autosome translocations, the normal X is usually inactive, but minority cell lines have been demonstrated. In unbalanced X;autosome translocations (usually offspring of balanced translocation carriers), the translocation chromosome is also usually inactive, but what is interesting is that the inactivation may or may not extend into the autosome.

Some genes on the X chromosome escape inactivation [21]. It is known that DNA synthesis is not synchronous in the late-replicating X but that it starts around the centromere and is followed by the short arm and the proximal part of the long arm. Several of the genes escaping inactivation are found in the early-replicating regions of the otherwise late-replicating X and are thought to cause the abnormalities associated with extra X chromosomes [16].

In the laboratory, the inactive X can be identified by growing cells in bromodeoxyuridine (BrdU) for 40–44 hours, and adding thymidine 6–7 hours before fixation. After staining with Hoechst 33258, the late-labeling X will be bright when examined by fluorescent microscopy. Alternatively, cultures can be grown in the presence of thymidine and pulsed with BrdU, resulting in a pale-staining, late-labeling X.

1.1.6 Satellite DNA

Repetitive DNA found in constitutive heterochromatin is often called satellite DNA because much of it separates from main band DNA by density gradient centrifugation. Satellite DNA has come to mean any highly repeated sequence, whether separable by ultracentrifugation or not. The satellite bands originally described are called classical satellites I, II, III, and so forth (Table 1.2)]. A substantial portion of each fraction is made up of a single family of simple repeats designated by the Arabic numeral corresponding to the Roman numeral [14]. Other pure sequences may be designated by lower case Greek letters, which also relate to the fraction from which they were derived. Polymorphisms that have arisen from mutations can be detected by restriction endonuclease digestion and electrophoretic separation. *Consensus sequences* are ones that are substantially the same, differing by only a few bases.

Alpha (α) and beta (β) satellite DNA, as well as classical satellite DNA, are found at the centromeres of all human chromosomes. Alpha satellite DNA has a consensus sequence so that probes made from a mix of the α satellite probes can be visualized at the centromeres of all the human chromosomes [16] (Table 1.2). More specific α satellite probes can also identify the centromeric regions of specific chromosomes (see Chapter 16, Fluorescence in situ hybridization).

In contrast to the undispersed repetitive sequences found in heterochromatin, dispersed repetitive sequences are found throughout the genome. They are the short (<500 bp) interspersed elements (SINES) and long interspersed sequences (LINES) [16]. SINES contain cleavage sites recognized by the restriction endonuclease Alu1 and are located in the quinacrine pale bands. LINES have cleavage sites for L1 and are located in the quinacrine bright bands. Together the Alu1 and L1 families make up about a third of the total repetitive DNA [14].

Other satellites of interest are microsatellites, SSR (simple sequence repeats), and minisatellites, all of which are interspersed throughout the genome. Microsatellites are di- or trinucleotide tandem repeats and are highly polymorphic. SSR are 3- to 6-bp repeats found in coding and noncoding DNAs and are also highly polymorphic. Minisatellites have longer repeats, usually more that 10 bp, and are usually located at the distal ends of chromosomes. These satellite DNAs are useful for DNA fingerprinting because of their highly polymorphic nature and are also useful as in situ probes [22].

Table 1.2 Characteristics of satellite DNA

Type of satellite DNA	Location	Length	Repeat sequences
Alpha satellite DNA	All human chromosome centromeres; Yq	0.17/0.34 kb	Varies between chromosomes
Beta satellite DNA	Chromosome 1,9, 13, 14, 15, 21, 22, Yq	50–300 kb	68-bp monomers
Satellite I Unit A	Most human chromosomes	0.04 kb	17 bp
Satellite I Unit B	Most human chromosomes	25–48 bp in arrays	$(A\text{-}B\text{-}A\text{-}B\text{-}A)^n$
Satellite II	Most chromosomes	0.05 kb	$(GGAAT)^n$
Satellite III	Chromosomes 9 and 15, also most others	0.05 kb	$CAACCCGA^A/_G T(GGAAT)^n$

1.2 The cell cycle

1.2.1 Interphase

The transition from interphase to cell division (mitosis) and back to interphase is called the cell cycle; therefore, when the cell is not dividing, it is said to be in interphase. This is the time when the nucleus is metabolically active. The nucleus is spatially organized with each chromosome in a specific region. This separation serves to prevent one chromosome from getting tangled with another. Telomeres are attached to the nuclear membrane. Between chromosomal subcompartments are chromatin-free interchromosomal domains, where RNA molecules are being processed for export to the cytoplasm. Highly transcribed portions of the chromosomes are positioned next to the interchromosomal domains and since different genes are transcribed in different cell types, the arrangement can vary from cell to cell.

Originally, it was assumed that the cell synthesized new DNA just previous to cell division. With the use of autoradiography and Feulgen staining techniques in the early 1950s; however, DNA synthesis was found to take place hours before any sign of mitosis, during interphase [23]. Furthermore, it is now known that synthesis of new DNA does not take place all at once. During the synthesis (S) period of 6–8 hours, some DNA will replicate early, and other DNA will replicate late. A given part of the chromosome, however, will almost always replicate at a certain time in the S period; for example, the inactivated X will always replicate late, as well as most of the dark-staining G-bands, and the light-staining G-bands will replicate early. The late-replicating portions of the chromosomes are usually considered genetically less active than the early-replicating areas. The discrepancy in replication timing between regions (and even whole chromosomes in the case of the X chromosome homologues) provides the basis for the banding technique called replication banding (see Chapter 6, section 6.3.2, Replication banding), which is most useful in discriminating the active from the inactive X chromosome.

There are four distinct stages in the cell cycle. Each phase is driven by cyclin-dependent kinases (CDKs) in conjunction with regulatory subunits called the cyclins. Cyclins are negatively regulated by cyclin-dependent kinase inhibitors (CKIs) such as p16, p21, and p27. CDKs phosphorylate target proteins at different stages of the cell cycle. For example, the retinoblastoma protein, pRb, associates with a transcription factor called E2F when pRb is hypophosphorylated. This association physically prevents E2F from dimerizing with its partner protein DP and thus inhibits the cell's passage into the synthesis phase. When pRb is phosphorylated by CDKs, it no longer binds E2F, and the cell cycle is allowed to progress through the synthesis phase. Mutations in the *RB1* gene are responsible for the eye cancer called retinoblastoma. Another cell cycle regulatory protein, TP53, can likewise stall the cell cycle at the G1/S transition to allow time for DNA repair or to initiate programmed cell death (apoptosis) when the damage is severe. Indeed, cell cycle regulation is intimately tied to DNA damage response: it is crucial that the cell have a series of checkpoints to prevent damaged DNA from being replicated, but checkpoints also exist at other points in the cell cycle. Alterations in any of these elements (e.g., cyclins, CDKs, pRb, *ATM*, *TP53*, and many others) have been observed in many human cancers, and several have their own cancer syndrome associated with alterations (e.g., TP53 mutations and Li–Fraumeni syndrome, and *RB1* and retinoblastoma) [3].

The time intervals for the four stages are consistent within a given cell type but vary between cell types. The four stages of the cell cycle are G1 (gap one), S (synthesis of DNA), G2 (gap two), and M (mitosis). The histone proteins of the chromosomes are, like DNA, synthesized during S. When the cell is in a resting stage and is not cycling, as when nutrients are scarce, it is said to be in G0.

The average mammalian cell cycle spans 18 hours, with a range of 12–24 hours, and a typical schedule for the portions of the cycle is nine hours for G1, 5 hours for S, 3 hours for G2, and 1 hour for M (see Figure 1.6). However, early embryonic cells can complete the cycle in 30 minutes, and an adult liver cell may take 1 year. These differences are due to shorter or longer G1 and G2 stages. If the cell is arrested at some point in S or G2, it cannot undergo cell division. During G2, the final preparations for mitosis are completed, and unless protein synthesis is inhibited, the cell will divide.

Different drugs inhibit the cell cycle at various stages. S inhibitors include amethopterin (methotrexate), hydroxyurea, and cytosine arabinoside. Naturally occurring mitotic arrestants include Vinca alkaloids, colchicine (also an alkaloid), and podophyllin.

1.2.2 Cell division

It has been firmly established since the time of Louis Pasteur that cells arise from pre-existing cells [24,25]. In order to donate genes to progeny, the parent, whether it is a single cell or multicellular organism, duplicates its genetic material (e.g., chromosomes) and transfers the copies to the offspring. Diploid organisms possess chromosomes in pairs called homologues, one inherited maternally and one inherited paternally. These pairs of chromosomes are normally similar or identical in shape, with a given gene found in a specific position (locus) on each chromosome of a pair. Even though these homologous genes determine similar functions or characteristics, they often are not identical. There are commonly alternate forms (alleles) of a gene on each homologous chromosome that code for two different expressions of the same gene, such as body color in fruit

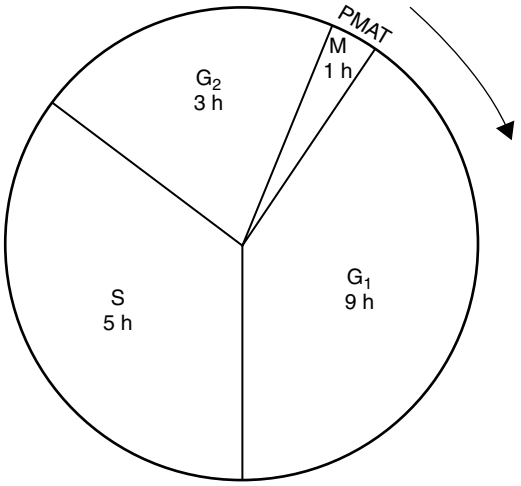


Figure 1.6 This pie graph demonstrates the four major stages of the human cell cycle and each stage's relative timeframe, e.g., Gap 1 (9 hours), Synthesis (5 hours), Gap 2 (3 hours), and Mitosis (Prophase, Metaphase, Anaphase, Telophase) (1 hour).

flies. If a fly inherits the allele for yellow from both parents, it is said to be homozygous for that trait. If, instead, it receives one allele for yellow and one for gray, it is said to be heterozygous for the trait. The color it expresses (gray or yellow) is called its phenotype, and the description of the actual genes it is carrying is its genotype.

In the case of the sex chromosomes in humans, males have only one X chromosome and the genes on that X are present unpaired, in a single dose. This condition is neither homozygous nor heterozygous, but hemizygous. Dosage compensation (see 1.1.5, X inactivation) provides a mechanism for the production of comparable gene products between homozygous and hemizygous individuals.

The genetic information present in each cell is transmitted during cell division, both in mitosis and meiosis. Mitosis is the division of somatic (nonreproductive) cells, and meiosis is the specialized division that occurs only in the formation of gametes (ova and sperm). One reason for the existence of two types of cell division is the fact that somatic cells need a full (diploid) complement of chromosomes; whereas, gametes need only one-half a complement (haploid), because they will ultimately fuse with another gamete during fertilization to become diploid in the resulting zygote.

1.2.3 Mitosis

In humans, the diploid ($2n$) number of chromosomes is 46. During the S stage of the cell cycle, each chromatid replicates itself. The cell at the end of S phase is still diploid, but the DNA content doubles as the chromatids are replicated to produce a chromosome comprised of two chromatids. Thus, at the end of the S phase, the cell is $2n$, but $4c$, where c = DNA content. At mitosis, the chromosomes line up at the metaphase plate and one chromatid from each chromosome goes to each daughter cell. The cycle continues and the chromatids are then replicated at S phase. Mitosis can be divided into four stages: prophase, metaphase, anaphase, and telophase (see Figure 1.7).

Prophase

Prophase is the stage of progressive coiling of the already doubled chromosomes, which appear long and threadlike. In middle to late prophase the chromosomes can be seen as discrete units, each containing two chromatids and a centromere. During prophase, the nucleolus becomes undetectable under the light microscope. With the electron microscope it is apparent that the nucleolus becomes dispersed throughout the nucleus. In the cytoplasm, the centrioles (one pair of which has budded off of the other) start to migrate to opposite poles of the cell and to form the microtubules that make up the mitotic spindle.

Between prophase and metaphase (in a stage often called prometaphase), the nuclear envelope breaks down in most organisms, releasing the chromosomes into the cytoplasm, which is contained by the cytoplasmic membrane. The chromosomes move rather erratically toward the equatorial plane (metaphase plate) of the cell. At this time the spindle fibers are not yet attached to the spot in the centromere (called the kinetochore) to which they later anchor for chromatid separation.

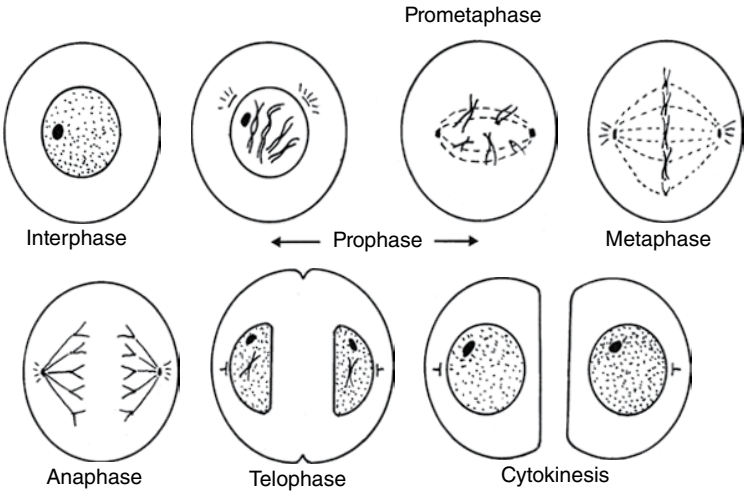


Figure 1.7 During the 1 hour that mitosis typically takes, replicated chromatin condenses in prophase to form identifiable chromosomes, and the nuclear envelope breaks down. Chromosomes that line up at the metaphase plate are attached at their kinetochores with spindle fibers that are connected at the other end to the centrioles at each of the poles. At anaphase, the spindle fibers pull the duplicated chromosome arms to opposite poles to form the two daughter cells in telophase, when the nuclear envelope is re-formed. Cytokinesis is the formation of new cell membranes between the daughter cells, each with a complete, identical set of genetic information.

Metaphase

At metaphase, the mitotic spindle is complete: the centrioles are in place at opposite poles, the chromosomes are lined up at the metaphase plate with the spindle fiber attached to each kinetochore, and the kinetochores are facing opposite poles of the cell. The mitotic spindle consists not only of these chromosome-to-pole fibers but also of continuous pole-to-pole fibers, which bypass the chromosomes altogether. At metaphase, chromosomes are at their most contracted state. This is also their least metabolically active state.

Anaphase

Anaphase begins with the division of the centromeres and, therefore, the separation of the chromatids. Once separate, each chromatid is called a daughter chromosome. The chromosomes move to opposite ends of the cell along the spindle fibers. Increased understanding of the mechanism of chromosome movement is being gained and some think there is potential to exploit this knowledge in the development of new cancer therapies [26,27].

Telophase

The final stage of mitosis is telophase. In telophase, the chromosomes uncoil, the nucleolus reappears, the nuclear envelope reappears, the spindle fibers disappear, and the nucleus takes on the morphology of the interphase cell. During or directly after telophase, the cytoplasm is divided by the formation of new cell membranes in a process called cytokinesis, and cell division is complete. The result of mitosis is two daughter cells, each with a complete, identical set of genetic information. For a video of mitosis in a live cell, search on "mitosis" or "cell division" on YouTube (www.youtube.com). A good example is found at: http://www.youtube.com/watch?v=aDAw2Zg4IgE.

1.2.4 Meiosis

Meiosis is often called the reduction division, because it reduces the number of chromosomes in each daughter cell to the haploid (n) number, which is 23 in humans. Meiosis takes place in the reproductive organs – the ovaries in females and the testes in males. The process of meiosis transforms cells called primary spermatocytes in the male testis and primary oocytes in the female ovary into haploid spermatids and ova, respectively. When fertilization occurs, the ovum and sperm fuse to form a

diploid zygote. Meiosis differs from mitosis in its reduction to four separate n (haploid) nuclei and in the creation of new gene combinations by crossing over so that the daughter chromosomes are composites of the parent chromosomes. The parent cell, for example, contains a pair of chromosomes number 1, one from each of its parents. During meiosis, these homologs exchange genetic material between them (crossing over – see later) so that the spermatid or ovum receives a single chromosome 1 that is derived from, but is not identical to, either of the two chromosomes 1 that the parent possessed. The same sequence of events has simultaneously occurred for chromosomes 2, 3, and so on. The consequence of this feature of meiosis is an increase in the phenotypic variation of sexually reproducing organisms, which provides selective advantage of great importance. The mechanism of this genetic exchange will become apparent in the description of meiosis.

Meiosis has two nuclear divisions, meiosis I and meiosis II. In meiosis I the homologous chromosomes (homologues) separate, and in meiosis II the chromatids separate (as in mitosis). This results in four cells, each with one haploid set of chromosomes. In the female, only one of these becomes a viable ovum, and the rest become polar bodies. In the male, all four spermatids can mature into spermatozoa.

Compared with mitosis, meiosis is a complicated and lengthy process. Like mitosis, each stage of meiosis has a prophase, metaphase, anaphase, and telophase. Prophase I is especially complex and is divided into five consecutive substages: leptotene (also called leptonema), zygotene (zygonema), pachytene (pachynema), diplotene (diplonema), and diakinesis (see Figure 1.8).

Prophase I

Leptotene

At this substage the nuclear chromatin begins to condense for division, but the chromosomes are not yet evident and the double nature of the strands cannot be discerned. The electron microscope shows that both ends (telomeres) of the chromosomes are attached to the nuclear envelope. By light microscopy, the leptotene cell has an enlarged nucleus and finely dispersed chromatin. Once the cell has entered leptonema, it is committed to meiosis.

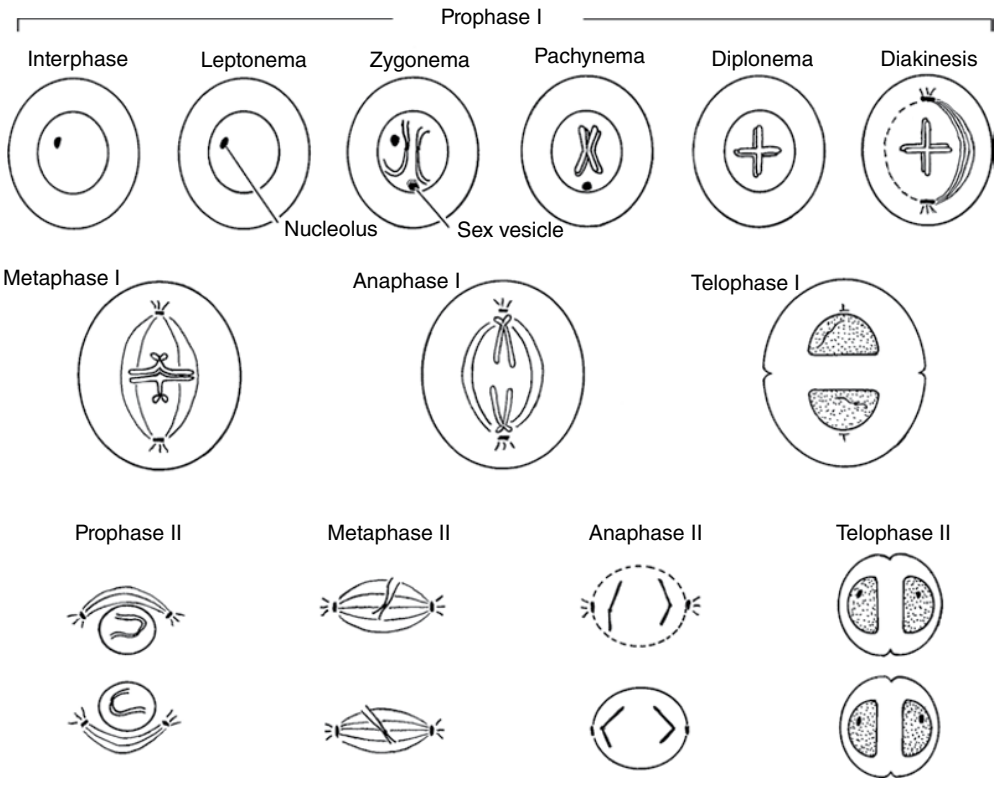


Figure 1.8 Meiosis, which takes place in the reproductive organs, starts with diploid cells and ends with haploid germ cells, i.e., spermatids and ova. Genetic crossing over, critical for genetic variation, occurs in prophase I. A second cell division, meiosis II, is responsible for the reduction of the diploid count to two haploid sets of chromosomes.

Zygotene

Homologous chromosomes, which still resemble long threads, align themselves side by side and attach to each other, allowing homologous loci to lie next to each other. This alignment is called synapsis (the Greek word for joining together). Electron microscopy reveals a structure holding the chromosomes close to each other; this structure is called the synaptonemal complex. The exchange of genetic material probably takes place in these complexes. This exchange is called crossing over, or recombination. In the female, the two X chromosomes are homologous and behave exactly like the other chromosomes. In males, however, the X and Y chromosomes are not homologous and condense to form a small, dark-staining body called the sex vesicle. Evidence shows that the X and Y are aligned end to end in this vesicle. Each set of homologues in synapse is called a bivalent, because the two chromosomes do not yet appear differentiated into chromatids. During zygotene the nucleolus is still visible and is associated closely with some of the bivalents. Zygonema ends when all homologues have been paired.

Pachytene

During this stage of prophase I, the bivalents shorten and become very thick, and crossing over occurs. Two nonsister chromatids cross over, but the other two remain unchanged. In good preparations, the bivalents can be seen as two parallel strands (chromosomes) on which there are a number of dark-staining, beadlike structures called chromomeres. These chromomeres are constant from preparation to preparation, so their number and position can identify some of the bivalents. Chromomeres are thought by some workers to be analogous to the G-band patterns of mitotic prometaphase chromosomes (see Figure 1.5). In this stage, the bivalent, owing to its four closely opposed chromatids, is known as a tetrad.

Diplotene and diakinesis

At the diplotene stage the nucleolus detaches from its associated bivalents, and the bivalent chromosomes begin to separate as their centromeres pull them apart. They are still attached, however, at points called chiasmata (a single point is a chiasma), which are the sites of genetic crossing over. Chiasma means cross, and chromosomes having only one chiasma typically assume a cross-like appearance. Each chiasma acts as a sort of knot that holds the paired chromosomes together so that the chromatids do not separate. In normal human meiosis, there is usually at least one chiasma per bivalent. If no chiasmata are present, nondisjunction can occur, leading to aneuploidy. In males, diplonema marks the disappearance of the sex vesicle, and the continued end to end association of X and Y can be observed.

Diakinesis occurs at this point in males: the chiasmata appear to move toward the ends of the bivalents (terminalize), the nucleolus dissipates, and the nuclear envelope disappears. In human females, however, meiosis is halted before diakinesis in the ovaries of the unborn female fetus. The oocytes remain in this special diplonema stage, called dictyotene, as long as 50 years or until each oocyte is singly ovulated after puberty before reaching the diakinesis stage. Meiosis is never completed unless fertilization occurs. When diakinesis is finished, the cell is ready to move into metaphase I.

Metaphase I

Here the spindle is formed and the bivalents line up at the equatorial plane. In females, the spindle is off-center in the cell and determines, by its position, which of the daughter cells will inherit most of the cytoplasm at anaphase. The bivalent chromosomes are lined up so that maternally and paternally derived chromosomes randomly face one pole or the other. This will allow them to sort independently to the daughter cells; each daughter cell will contain a mix of both paternal and maternal chromosomes. Excluding crossing over, this shuffling of 23 pairs of chromosomes can produce about 8 million genetically different gametes.

The form of the individual bivalents in metaphase I depends upon the number of chiasmata present. Bivalents with one chiasma form a cross-shaped structure at diakinesis, which proceeds to form a rod-shaped structure at metaphase when the chiasma terminalizes. Two terminalized chiasmata form a ring-shaped structure at metaphase; three chiasmata create a figure eight; and four or more will appear with additional loops. The X and Y chromosomes sometimes appear separated in metaphase I and are then called univalents.

Anaphase I and telophase I

Whole chromosomes, centromeres intact, move to opposite ends of the cell during these stages. In the oocyte of the female, one of the two daughter cells receives most of the cytoplasm and becomes the secondary oocyte. The other cell, mostly nucleus, becomes the first polar body. In humans, the cycle proceeds directly to meiosis II without an intervening interphase stage.

Metaphase II

In human meiosis there is no true prophase II; the cells pass directly into the second meiotic metaphase. The 23 chromosomes, each composed of two chromatids, move to the equatorial plate (metaphase II). At this stage, the chromosomes appear somewhat spiralized and fluffy. Although only a haploid set of chromosomes is present, i.e., one of each human homologue, there is still a diploid amount of DNA because the replicated strands have not yet separated. In anaphase and telophase, the two chromatids finally separate and go to two daughter cells so that the end product of meiosis is four haploid cells, each with one complete but different set of genetic material. In females, the spindle is again off-center, giving rise to a very large cell called an ovum and a second polar body. The first polar body may also undergo meiosis II, creating two additional polar bodies. Of the resulting four haploid cells, the ovum is theoretically the only viable gamete. For a video demonstration of the processes of meiosis, search 'meiosis' on YouTube (www.youtube.com). For example, this URL may be helpful to grasp the basic concepts: http://www.youtube.com/watch?v=R_LUJSqeSrI.

1.3 Recombinant DNA techniques

A breakthrough in the study of DNA occurred when researchers discovered bacterial enzymes that cut DNA at specific sequences [28]. These restriction enzymes often make staggered cuts in the two DNA strands, leaving short, single-stranded tails on the ends of both fragments. These single-stranded ends easily bind to complementary fragments by base pairing. Two fragments that have attached in this way can be permanently joined by adding DNA ligase, a repair enzyme that produces a recombinant molecule.

1.3.1 Bacterial-plasmid cloning

Further advances in this field took advantage of the fact that many bacteria contain plasmids, tiny circular DNA molecules, and that these plasmids can replicate autonomously in bacteria. Plasmids from the bacterium *Escherichia coli* (*E. coli*), which had only one recognition site for the restriction enzyme EcoRI, were cut by the enzyme; foreign DNA, also cut with EcoRI, was spliced in; and the plasmids were sealed with ligase. The hybrid plasmids were then transferred back into E. coli, where they carried out the instructions of the inserted DNA and reproduced with the bacteria's own DNA [29]. Using these techniques, researchers were able to isolate bacteria that had acquired a gene of interest and then make an enormous number of copies (cloning), owing to the rapid reproductive rate of bacteria [8]. This technique found practical uses; for instance, large amounts of insulin could be made for use by patients with diabetes.

Other advances in the study of DNA and genetics were made when Sanger, Maxam, and others devised methods for determining the base sequence of a given DNA molecule [30,31]. This capability led to the sequencing of mitochondrial DNA and bacteriophage λ. Sanger's dideoxy method was eventually used to sequence the entire human genome. Because restriction enzymes cut DNA at specific nucleotide sequences or recognition sites, the length of each fragment produced depends on the distance from one recognition site to the next. Harmless natural variations exist, such as the one at a point about 7000 nucleotides away from the beta-globin gene on chromosome 11. A recognition site for the restriction enzyme HpaI is present at that point in the DNA of some people, but not others. If the site is present, a short fragment containing the beta-globin gene, 7600 bp long, is produced. If absent, the beta-globin-negative fragment is 13,000 bp long. These normal variations have been named restriction fragment length polymorphisms (RFLPs).

1.3.2 Electrophoresis

Fragments formed by restriction enzymes can be further separated by electrophoresis, a process in which the DNA fragments move through porous agarose gels (for large fragments) or polyacrylamide gels (for small fragments) under an electric field. Smaller fragments migrate more quickly, providing a visual way for determining fragment sizes by their positions in the gel. This separation comparison has proven useful in the study of heritable diseases like sickle cell disease [32].

Direct detection of the sickle cell globin gene has been demonstrated using the restriction enzyme MstII, which cuts within the beta-globin gene, as well as many other places. In the normal beta-globin gene, MstII cuts at the sequence CCTG**A**GG, producing two fragments of 1150 and 200 bp. The sickle cell mutation changes the sequence to CCTG**T**GG, thereby eliminating the recognition site; therefore, MstII produces only one 1350 bp fragment [28].

1.3.3 Southern blotting

Detecting whether a person has the two smaller fragments (normal) or the single 1350 bp fragment in sickle cell disease testing is complicated by the fact that there are so many fragments of similar sizes. This is overcome by using radioactively labeled probes in a technique called Southern blotting (see Figure 1.9).

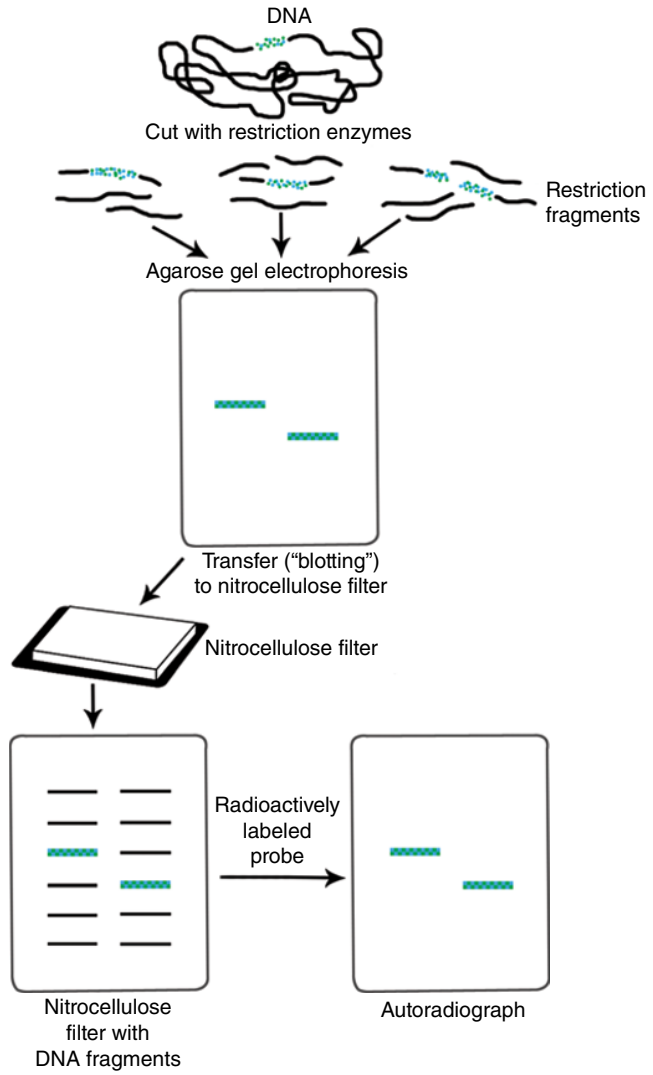


Figure 1.9 DNA fragments are separated according to size by agarose gel electrophoresis. They are then transferred to a nitrocellulose filter where they are exposed to radioactive probes which hybridize with complementary sequences. The radioactive signals are detected by autoradiography using X-ray film. Watson 1983 [28]. Reproduced with permission of John Wiley and Sons.

A probe is a short sequence of purified DNA that is complementary to the DNA of interest. In order for the probe to attach, the double-stranded DNA must be denatured by heat or alkali. The probe used to detect the sickle cell gene is a fragment of the cloned beta-globin gene, made radioactive so that it can be detected in the presence of large amounts of nontarget DNA.

DNA to be tested for the sickle cell mutation is cut with MstII. The resulting fragments are then separated by agarose gel electrophoresis and treated chemically to denature them. Next, the fragments are transferred (blotted) onto a nitrocellulose filter, to which they become bound. The transfer retains the pattern of fragments that was produced in the agarose gel. Next, the filter is exposed to the radioactively labeled probe. Once hybridized to its complementary DNA, the radioactivity can be detected by placing the filter next to X-ray film, which exposes the film to produce an autoradiogram [28,32].

If a band corresponding to a DNA fragment of 1350 bp shows up on the autoradiogram, it represents the sickle cell gene. If two bands of 1150 and 200 bp appear, they represent the normal gene. If bands representing both the longer and the two shorter fragments are present, the individual has inherited the sickle cell gene from one parent and the normal beta-globin gene from the other parent and is a carrier for the sickle cell trait [28].

1.3.4 Synthetic oligonucleotides

Because it is unusual for a genetic mutation to exist in a restriction site, other means of detection must be used. One option is to use synthetic oligonucleotides (oligo = few). These short molecules are engineered to match portions of a normal gene exactly. If there is a change in just one base, the hybrid molecule will be unstable and will denature easily. These oligonucleotide probes can therefore be used to detect genetic defects that involve a point mutation or change in a single base [28,32].

1.3.5 Polymerase chain reaction

Another useful tool available to molecular geneticists is the polymerase chain reaction (PCR), which allows small amounts of DNA or RNA to be amplified, producing millions, even billions, of copies [33]. This makes it possible to make, from tiny samples of DNA, amounts great enough to be analyzed using restriction enzymes, oligonucleotides, or direct sequencing. The low cost of this method, in addition to its flexibility and precision for amplifying a short stretch of DNA, has resulted in PCR testing largely supplanting the bacterial plasmid cloning method for many clinical and research applications.

1.4 The human genome

1.4.1 Genomic DNA variations

Genomic DNA variation can provide useful methods by which to identify individuals. For instance, RFLP analysis is sometimes referred to as fingerprinting. However, these variations may also play a role in human phenotypic variation and disease. The single nucleotide is the smallest unit of variation. Single nucleotide polymorphisms (SNPs), by definition, are present in at least 1% of the population and may account for a large fraction of human genetic variation. Recently, surprising observations regarding human genetic variation on a larger scale were made. First, alterations in DNA copy number of large segments of DNA were reported in normal individuals. These segments (copy number variations, or CNVs) ranged from kilobases to megabases of DNA. The size of these alterations was below the resolution of karyotypic analysis and above the resolution of most molecular techniques, thus their discovery relied on the technique called array comparative genomic hybridization (aCGH), which is a sensitive method to detect genomic DNA imbalances at a lower limit of statistical resolution of several kilobases. Fifty-six percent of the clones overlapped with known coding regions and some included one or more genes [34].

Further research has demonstrated that individuals carry their own unique CNV signatures, and that CNV databases from populations or ethnic groups carry variations and similarities. Similarity between groups with unusual phenotypic characteristics or disease, has led to some CNVs being recategorized as pathogenic rather than benign population polymorphisms. The DNA architecture lying immediately adjacent to a region is thought to convey susceptibility to rearrangements observed as CNVs. Certain DNA regions contain low copy repeats (LCRs) that may be in direct head-to-tail orientation or in inverted tail-to-tail orientation; these repeats may facilitate nonallelic homologous recombination (NAHR) during meiosis [35] resulting in duplications or deletions. Many of the chromosomal deletion/duplication syndromes share this etiology. For example, velocardiofacial–DiGeorge syndrome (VCF/DGS) is usually caused by a deletion that results from unequal crossover events between two 240 kb LCRs termed LCR22-2 and LCR22-4 on chromosome 22 long arm in band q11.2. These LCRs may also cause duplications in the same region. Similarly, LCRs flank the Charcot–Marie–Tooth/HNPP (hereditary neuropathy with liability to pressure palsies) region on chromosome 17p12. Duplications lead to Charcot–Marie–Tooth syndrome (MIM: 118220) and deletions cause HNPP (MIM: 162500). Depending on the size, CNVs may be observed using a number of different methods, including chromosome analysis, FISH, and chromosome microarray analysis. Although many larger CNVs have been described using chromosome and FISH analysis, most of the recent additions to the CNV databases, and much of what we know of CNV formation, have been uncovered using array technologies [36].

The sequencing of the human genome, first published in 2001, has opened up a new era in understanding genetics from a functional viewpoint [37,38]. One early surprise in this process was the relatively low number of genes resulting in a peptide or protein. The number is believed to be 21 ~ 30,000 rather than the expected 100,000. Only about 1% of the genome is comprised of exons. Introns account for another 25%.

A consortium of international scientists is seeking to identify all biologically functional elements in the genome (ENCODE project). The results of their study of the first 1% have revealed more surprises [39,40]. The majority of DNA is transcribed into RNA, and the transcripts overlap one another extensively, but about half are not constrained evolutionarily. A number or previously unknown start sites for DNA transcription have been identified. Regulatory sequences are symmetrically distributed around transcription start sites with no bias toward upstream regions. Chromatin accessibility and histone modification patterns are highly predictive of both the presence and activity of transcription start sites.

Although alternative splicing of transcripts has been known for some time, the ENCODE project has verified that splicing occurs for virtually all genes. Also, a given gene can code for more than one protein. The estimate is that the average protein-coding region produces 5.7 different transcripts. Exons may be spliced into transcripts from other genes, even from different chromosomes. So, while the absolute number of protein coding genes identified by the Human Genome Project was surprisingly low, through alternative splicing and post-translational modifications, the actual protein repertoire of the human genome is quite large. When this is superimposed on the different gene expression and protein interaction networks in particular cells and tissues, the 'protein function' repertoire becomes larger still.

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CHAPTER 2

Cytogenetics: an overview

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> Chromosomes have attracted many microscopists not only because these sausage-like bodies represent vehicles of genetic material (and hence, are biologically important) but also because they are hypnotically beautiful objects.
>
> T. C. Hsu, *Human and Mammalian Cytogenetics: An Historical Perspective*, 1979, Springer-Verlag

2.1 Introduction

Cytogenetics is the study of chromosome morphology, structure, pathology, function, and behavior. Chromosomes are best studied at mitotic or meiotic metaphase, although some studies, such as fluorescence in situ hybridization (FISH) methods, may utilize interphase cells. The metaphase chromosomes may be studied in spontaneously dividing tissues or in cells that have been stimulated to divide in culture. Spontaneously dividing cells may be found in bone marrow, lymph node, testis, chorionic villi of placenta, some leukemic blood, solid tumor, some pleural or ascitic fluid, fetal ascites or cystic hygroma fluid, and often in fetal or newborn blood. Cells that are cultured before cytogenetic study include all of the above (for study of other components that may not be spontaneously dividing), plus blood lymphocytes, amniotic fluid, skin, and other tissues containing fibroblasts.

Once dividing cells are obtained, mitotic arresting agents (such as Colcemid®, colchicine, or Velban) are used to collect the metaphase cells. Then the cells are processed (harvested). This is usually performed with suspensions of cells, although some cells are harvested "in situ" directly on the cultured cells in a Petri dish. During the harvest procedure, hypotonic solutions are used to increase cell volume, which spreads the chromosomes apart, and methanol–acetic acid is used to fix (preserve) them for study. Slides are prepared from fixed cells, either in suspension (dropped onto slides), or in situ, on the growth surface (fixed cells are allowed to dry on the flask, coverslip, or the slide on which they were cultured). Either way, cells are then stained with appropriate stains, observed, and analyzed. Computer-generated images are now used to arrange the chromosomes in pairs on karyograms (Figure 2.1). The chapters that follow discuss these methods in detail.

2.2 History of human cytogenetics

Cytogenetic methodologies have been developed gradually [1,2]. The earliest known observation of chromosomes was by Eduard Strasburger in 1875, using plant material, and by Walther Flemming in 1879–1889, using animal material. Flemming coined the terms *mitosis, chromatin, prophase, metaphase, anaphase, and telophase.* W. Waldeyer coined the term *chromosome* in 1888.

Although these workers in Germany first observed human chromosomes about 100 years ago, technical problems made counting the chromosomes quite difficult. Painter [3] published the human diploid number in 1923 as 48, and this was believed to be the true number for three decades. This was because chromosomes were difficult to spread out in the cell, and because the differences between primary constrictions (centromeres) and secondary constrictions (heterochromatic

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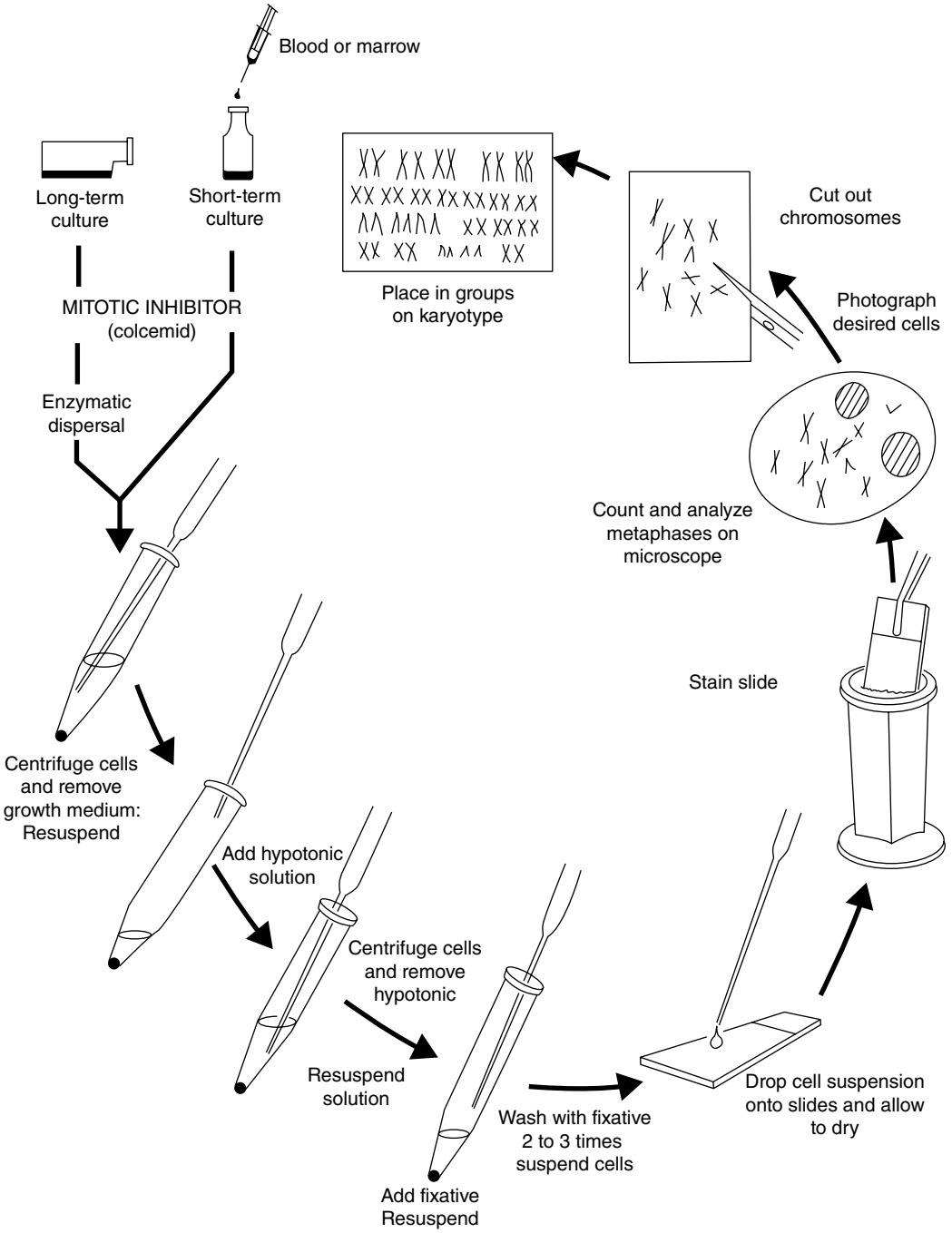


Figure 2.1 Schematic of chromosome preparation methods. Cutting chromosomes from prints has largely been replaced by computerized karyotyping systems. At the "Stain slide" step, the slide may be used for FISH instead of banded chromosome preparations. FISH studies can be performed on interphase cells at almost any stage, including on fresh, unharvested tissues (e.g., formalin-fixed, paraffin-embedded tissue sections or cerebrospinal fluid dropped onto slides and fixed in situ), or on fixed tissues and/or cells, whether harvested or not. Metaphase FISH can be performed on unstained cells from harvested cultures, or sequentially after staining chromosomes with banding methods or solid stain.

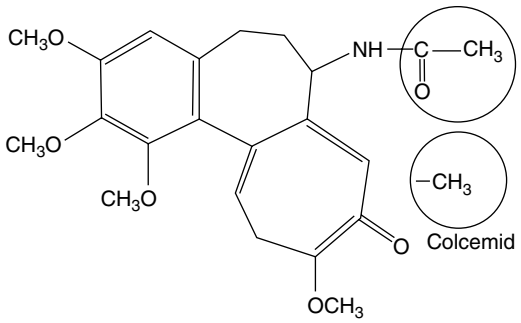


Figure 2.2 Colchicine and Colcemid®. Colcemid® is deacetylmethylcolchicine: the acetyl group of colchicine (upper circle) is replaced by a methyl group (lower circle).

regions of chromosomes 1, 9, 16, and the stalk regions of chromosomes 13, 14, 15, 21, and 22) were obscure. It was easy to interpret two chromosomes in association with each other as one chromosome, and, conversely, it was difficult to be sure that what appeared to be a large chromosome was not, instead, two smaller chromosomes in association. Even today, with much more sophisticated methods, it can still be a challenge, without some experience, to differentiate between a translocation and closely aligned telomeres, or between a centromere and a twisted or broken chromosome region, or a stretched heterochromatic region.

The other great difficulty was in obtaining dividing cells to study. It was not until tissue and cell culturing methods were sufficiently developed to grow fetal lung tissue that Tjio and Levan [4] were able to use this material in 1956 to get a clear look at the chromosome number for the first time. They used colchicine (Figure 2.2) to arrest cells in metaphase and then used hypotonic treatment [5,6] to swell the cells to a much larger volume, so that the chromosomes were less likely to be crowded together. However, the method used to spread the chromosomes onto slides was still the squash preparation, in which tissue was pressed between a coverslip and the slide. This method required harsh aqueous acetic acid fixatives in order to soften the tissues enough to squash flat, and created preparational artifacts, losing some chromosomes from many cells. Using these techniques for clinical purposes was also highly impractical, because of invasive tissue collection procedures. It was not yet possible to culture peripheral blood, and so the only tissues available for chromosome studies were bone marrow, testicular biopsies, and skin. When Nowell [7] discovered a way to stimulate blood lymphocytes to divide, and Moorehead et al. [8] described a method to spread alcohol–acetic acid–fixed lymphocyte chromosomes by air drying it became possible to examine human chromosomes obtained by noninvasive methods, with much less technical artifact than ever before. This, together with the discoveries of the chromosomal causes of Down syndrome, Turner syndrome, Klinefelter syndrome, and the Philadelphia chromosome in CML, led to the development of the science of clinical cytogenetics in the 1960s.

Metaphase chromosomes were originally stained with Giemsa or aceto-orcein, producing unbanded ("solid stained") chromosomes, which could only be grouped by size and shape, but not identified individually, aside from chromosomes 1, 3, 9, 16, and Y, if they were normal. Research methods to identify individual chromosomes, including autoradiography, had met with limited success. Thus, many abnormalities either went undetected or could not be completely characterized.

In 1968, Caspersson et al. [9] published a fluorescence method (Q-banding) using quinacrine mustard stain that differentiated the pairs of chromosomes in lower animal and plants; this paper was largely ignored, however, until Caspersson's group [10] showed that each human chromosome had a distinct pattern of bands using his method. Finally, many human chromosome abnormalities could be characterized by the patterns of the light and dark regions (bands), which allowed identification not only of each pair, but also of small chromosomal segments. Methods subsequently described included the much more popular G-bands, using trypsin and Giemsa, which did not require fluorescence, as well as reverse (R-bands) and constitutive heterochromatin (C-bands) stains (Figure 2.3). Other methods followed, as discussed in Chapter 6. These stains expanded the detection and characterization of many more cytogenetic abnormalities, especially for the field of oncology, where the discovery that specific chromosomal rearrangements were associated with certain neoplastic conditions led to a dramatic improvement in both diagnostic and prognostic capabilities for clinicians (see Chapter 11, Cytogenetic analysis of hematologic malignant diseases and Chapter 12, Cytogenetic methods and findings in human solid tumors) than what could be seen with solid staining. Chapter 6, Chromosome stains, discusses these and other staining procedures.

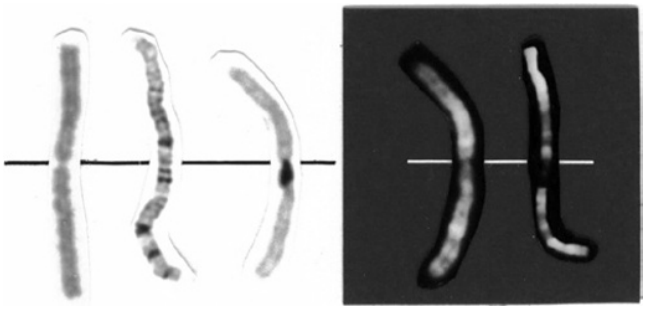


Figure 2.3 Comparing banding methods. A human chromosome 1, stained with (left to right) solid Giemsa, trypsin G-bands, C-bands, Q-bands, and R-bands.

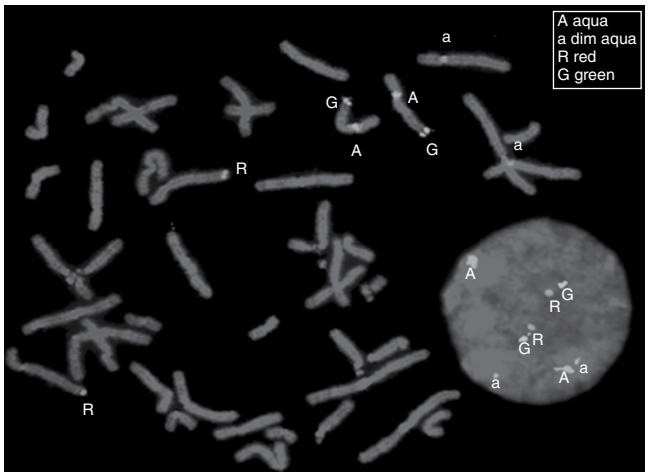


Figure 2.4 Fluorescence in situ hybridization on metaphase and interphase cells. FISH may be used on metaphase or interphase cells. This metaphase has been hybridized to three probes with three different colored fluorophores. The chromosome 4 long arm telomere is in red, chromosome 9 centromeric, alpha-satellite DNA is in aqua, and the chromosome 9 long arm is in green. Note that the signals can be enumerated in an interphase cell (right), as well as in the metaphase chromosomes. The small aqua on the B-group chromosome is due to similarities between the alpha satellite DNA between chromosomes 4 and 9. See insert for color representation of this figure.

In 1976 Jorge Yunis [11] revolutionized the field by demonstrating that short exposures to low concentrations of Colcemid®, along with cell synchrony methods, would yield elongated chromosomes with nearly twice the number of bands, termed "high-resolution" banding, that could then be used to detect smaller and more subtle chromosome abnormalities. Microdeletions, such as those responsible for Prader–Willi [12] and Angelman [13] syndromes on chromosome 15 long arm, DiGeorge/velocardiofacial syndromes on chromosome 22 long arm [14,15], and Miller–Dieker syndrome [16] on chromosome 17 short arm, were eventually discovered by using these methods.

In the 1980s, prenatal diagnosis by chorionic villus biopsy (CVS) became available to couples desiring earlier diagnosis than could be provided by amniocentesis. CVS could be successful at 10–12 gestational weeks, whereas amniocentesis becomes feasible at 14+ weeks since there is inadequate fluid volume present at earlier gestational ages. Knowledge of the cytogenetics of various neoplasms and correlation with diagnosis and prognosis of disease also became much better defined.

Also in the 1980s, the development of molecular cytogenetics sent the rapidly changing field into another revolution, uncovering for the first time the nature of cryptic rearrangements or changes, often visible only at the molecular level. The new FISH methods included whole chromosome probes (paints), single-copy probes, and centromere-specific probes, allowing many different kinds of questions to be answered, sometimes in the absence of metaphase cells (see Figure 2.4).

During the 1990s and early 2000s, FISH probes became commercially available for many different constitutional and acquired diseases. Specialized methods were developed to stain each chromosome a different color, such as SKY, M-FISH, and color-banded FISH probes, as well as comparative genomic hybridization (CGH) and panels for all subtelomere regions or all centromeres. Probes that could detect specific deletions and translocations in interphase cancer cells became commercially available in many forms for many different cancers, allowing for precise determination of disease diagnosis (e.g., *BCR/ABL1* translocation for diagnosing CML in certain situations; prognosis (e.g., *ATM, TP53,* 12 centromere aneuploidy, etc., for evaluating chronic lymphocytic leukemia (or CLL); response to treatment (e.g., *ERBB2,* also called *HER2/neu* gene amplification testing) for determining the therapeutic course for certain invasive breast carcinomas; residual disease (RD) status (e.g., *PML*/RARA rearrangement recurrence in acute promyelocytic leukemia (APL); and transplantation engraftment success (e.g., using X and Y centromere probes for detecting the presence and percentage of opposite-sex donor marrow cells).

Finally, comparative genomic hybridization was refined into chromosome microarray analytical tests, including mCGH or aCGH for array, allowing a high-throughput FISH analysis for hundreds or thousands of genes or chromosomal DNA sequences in a single assay (see Chapter 18, Genomic microarray technology for the cytogenetics laboratory). Table 2.1 shows a timetable of selected landmarks in the history of cytogenetics. No doubt the revolutions in this rapidly evolving field will continue to surprise us.

2.3 Cytogenetics methods

In this overview chapter we will discuss the general flow of activities within the cytogenetics laboratory, as well as the theories behind their processes, i.e., harvesting, slide-making, staining, chromosome analysis (Figure 2.1), and case reporting, as it applies to all specimens. The chapters that follow will discuss in more depth the methods that are used to process specific tissue types received by cytogenetics laboratories, such as, blood, bone marrow, tumors, amniotic fluid, chorionic villi, skin, and products of conception, as well as molecular (FISH) methods that can further interrogate the cells obtained from these tissues.

The methods used to study a tissue depend upon what the tissue is and what cytogenetic questions need to be addressed. Constitutional chromosome aberrations are typically studied in blood lymphocytes, but other tissues from different germ layers, such as skin and gonad, or tissue from placenta, may also be studied when looking for tissue mosaicism (see 2.6.2, Mosaicism). In addition, there may be occasions in which blood is not available (e.g., the patient is deceased), but skin or other tissue is still viable.

Acquired conditions associated with neoplasms must study the site of the malignancy, e.g., tumor, lymph node, metastatic site, bone marrow aspirate, bone core in the event of a dry tap, or neoplastic blood, if appropriate to the diagnosis. Situations may arise, however, when a second, non-neoplastic source may be required in order to determine whether a karyotypic abnormality is neoplasia-related or constitutional. For example, trisomy 21 in bone marrow from a leukemic child may be acquired or constitutional.

Tissues that are in suspension, such as amniotic fluid, bone marrow, and peripheral or cord blood, are easiest to culture, because they do not require dispersal before being set up in culture. Solid tissues, such as, chorionic villi, skin, solid tumors, and products of conception, however, will require some enzymatic dispersal before culturing for the best results.

2.3.1 Work flow

Specimen procurement

The first step in cytogenetic analysis is to obtain the correct tissue in the correct collection medium/anticoagulant in appropriate containers, at the correct temperature and in a timely manner. Some tissues must be received immediately, such as many brain tumors; others may be delayed for 1–3 days, such as the average blood sample. The tissue of choice depends on whether the referral is for constitutional or acquired disorders. All laboratories should create and provide standard specimen request forms for submitting physicians and laboratories so that the client will know what information is needed by the cytogenetics laboratory.

Collection containers must be sterile, and in most cases, are either standard stock (e.g., syringes, vacutainers, glass test tubes, urine collection cups) or are provided by the cytogenetics laboratory (sterile Petri dishes, plastic centrifuge tubes with or without transport medium). The technologist, however, should pay close attention to any suboptimal growth pattern that affects multiple patients drawn under similar conditions, as there could be unexpected toxicity in a lot or type of container being used by the physician or laboratory. Many types of specimens (e.g., solid tissues, such as chorionic villi, products of conception, solid tumors) must be kept moist during transport by using isotonic saline or various collection/transport media. Small samples of blood, bone marrow, or other fluids will survive much better in medium as well, since fewer cells dry onto the collection tube surface, and some of the nutritional and buffering requirements are met by the tissue culture medium.

Table 2.1 Major discoveries and landmarks in cytogenetics, 1865–2001

Year	Investigator	Discovery
1865	Gregor Mendel	Discovered principles of heredity
1867	Gustav Giemsa	Invented Giemsa stain mixture
1882	Walther Flemming	Described mitosis, used fixation, staining
1888	Heinrich Wilhelm Waldeyer	Named chromosomes
1893	Oscar Hertwig	Published *Cell and Tissue*, the first cytology textbook
1902	Theodor Boveri, Walter Sutton	Demonstrated presence of pairs (homologues)
1905	Nettie Stevens	Reported presence of Y chromosome in males
1914	Theodor Boveri	Theorized that chromosome changes could cause cancer
1921	John Belling	Introduced squash method for chromosome spreading
1922	Theophilus Painter	Published that humans have 48 chromosomes based on testicular sections
1937	Albert Blakeslee and A. G. Avery	Used colchicine for arresting metaphases
1941	Frits Zernike	Phase contrast microscope developed
1951	George Gey	Established HeLa cell line
1952	Arthur Hughes; Kyoko Makino and Kazuo Nishimura; T. C. Hsu	Described the use of hypotonic solution in cytogenetics
1953	James Watson and Francis Crick	Described double helix structure of DNA
1956	Jo Hin Tjio and Albert Levan	Established chromosome number of man as 46 using colchicine, 0.04–0.004 μg/mL for 20 hours, and hypotonic on embryonic lung cultures from four fetuses
1958	Charles E. Ford	First report of chromosome abnormalities in leukemia
1958	David Hungerford	Reported 0.075 M KCl improves chromosome morphology over sodium citrate hypotonic solution
1959	Jerome Lejeune	Discovered an extra chromosome 21 in Down syndrome patients
1959	Patricia Jacobs and John Strong	Described XXY in Klinefelter syndrome
1959	Charles E. Ford	Reported 45,X Turner syndrome
1960	J. A. Böök and Berta Santesson	Described triploidy in humans
1960	Klaus Patau	Described trisomy 13
1960	John Edwards	Described trisomy 18
1960	Peter Nowell	Described phytohemagglutinin A (PHA) effect in lymphocyte culture
1960	Paul S. Moorhead, Peter Nowell, W. J. Mellman, D. M. Battips, and David Hungerford	Described blood culture and harvest protocol using PHA, colchicine, hypotonic, 3:1 methanol–acetic acid fixative, Giemsa stain
1960	ISCN	First conference on chromosome nomenclature (Denver, CO)
1961	Mary Lyon	Described X inactivation
1963	Jerome Lejeune	Described deleted 5p in Cri-du-chat syndrome
1964	Patricia Farnes and Barbara Barker	Described pokeweed mitogens use
1968	Torbjörn Caspersson, L. Zech, E. Modest, G. Foley, and U. Wagh	Described Q-bands in *V. Faba*, *Trillium*, and Chinese Hamster
1969	Herbert Lubs	First described fragile X in families

Table 2.1 (Continued)

Year	Investigator	Discovery
1969	Joseph Gall and Mary Lou Pardue	First described in situ hybridization using radioactive probes
1969–1970		Amniocentesis becomes clinically used for cytogenetic diagnosis
1970	Torbjörn Caspersson, Lore Zech, C. Johansson	First Q-bands in humans described
1971	ISCN	Third meeting for human chromosome nomenclature (Paris, France). First meeting to describe banding for ISCN
1971	Adrian Sumner; S. R. Patil; Maximo Drets and Margery Shaw; Marina Seabright	Described various G-band methods
1971	Bernard Dutrillaux, Jerome Lejeune	Published R-bands in the USA
1973	Janet Rowley	Described Ph chromosome as a t(9;22), not a deletion of 22q using Q-bands
1974	P. Perry and Sheldon Wolff	Described sister chromatid exchange staining with BrdU and Giemsa
1974	David Cox, Virginia Niewczas-Late, Margaret Riffell, and John Hamerton	Described in situ harvesting method
1975	Samuel A. Latt	Described replication banding methods
1975	C. Goodpasture and S. E. Bloom	Described NOR silver staining of chromosomes
1977	David Peakman, Marilyn Moreton, Barbara Corn, Arthur Robinson	Described coverslip in situ harvesting method
1979	Jorge Yunis	Described high-resolution G-banding methods
1980	J. G. Bauman, J. Wiegant, P. Borst, P. van Duijn	First in situ hybridization with fluorescent fluorophores
1981	P.R. Langer, A. A. Waldrop, D. C. Ward	Described biotin-labeled probes for in situ hybridization
1981–1984	N. Wake	Improved methods for solid tissue culture using collagenase dissociation
1982–1983	Z. Kazy; R. H. T. Ward; B. Brambati and A. Oldrini; L. G. Jackson and J. M. Hahnemann	First reports of CVS use for prenatal diagnosis
1986–1988	Daniel Pinkel and Joe Gray	Developed interphase and metaphase FISH
Mid 1980s		Automated (computerized) capture/karyotyping systems developed
1987	Jack Spurbeck	First described robotic harvesting method
1989	Hermann-Josef Lüdecke	First described microdissection of chromosomes
1991	A. Kallioniemi, O-P Kallioniemi	Described comparative genomic hybridization (CGH)
1996	Evelyn Schröck, Thomas Ried	Described multicolor spectral karyotyping
1996	Affymetrix company	Introduced the first gene chips, also called microchips/microarrays
1999	ICGSE	Rough draft of human genome established
2000–2001	Various companies	Commercially available microarrays for high-resolution cytogenetic analysis

Specimens to be cultured must never be frozen or exposed to excessive heat, because live cells are required for cytogenetic preparations cell culture. The exception is cells cryopreserved in special freezing media to keep them viable. Refrigeration is helpful for some specimens, especially those, like products of conception, which may have a risk for contamination, but because the specimen could freeze by accident at only a few degrees colder than standard refrigerator temperatures, and some refrigerators are not well controlled, room temperature for specimen storage and shipping is often recommended.

Communication between the laboratory and referral personnel is very important each time a clinical sample is sent, in order to confirm that these specimen variables and requirements are met, and to obtain a complete patient name, diagnosis, reason for referral, birth date, billing information, and details for delivery. Many culture, harvest and analysis decisions depend on the reason for referral, and although this may sometimes be difficult to obtain, it is nevertheless crucial.

Unless otherwise required by state or federal regulatory agencies, all specimens received by the laboratory must have at least two identifiers. One is usually the patient name, and the other may be patient birth date, medical record number, and/or other identifying data. These identifiers match with the order requisition. If a specimen arrives without a patient name or unique identifier, many laboratories refuse it, and request another sample. If no other sample can be obtained, the laboratory should keep careful records of the circumstances, and note the deficiency on the final report. The sender should be alerted regarding samples with insufficient patient identification, and the referring MD, hospital unit or laboratory should provide a signed release form verifying and accepting responsibility for specimen identification before the specimen is fully processed. Under certain situations, however, a rejection or delay in processing may not be in the best interest of the patient (e.g., brain tumor), or when the specimen has been obtained via an invasive or irreplaceable procedure (e.g., amniotic fluid, bone marrow, or solid tumor tissue). Unless otherwise directed by your facility, the laboratory may choose to accept the specimen for processing if the person collecting the specimen can irrefutably confirm the specimen's identification, and the referring office or laboratory sends a release form to the cytogenetics laboratory. Careful records should be kept of the circumstances, and the deficiency should be noted on the final report.

Specimens received for genetic testing are ordered by the physician; therefore, in most cases the cytogenetics laboratory performing the test does not see the patient. This becomes a problem when informed consent forms are required, especially for prenatal genetic testing. Informed consent confirms that the patient was made aware of not just the reason for and what is expected from the proposed procedure, but also the risks involved, benefits projected and alternatives available. The physician is generally the one who explains the test to the patient, and the patient must sign the informed consent form before the procedure is preformed; however, a copy of this signed consent is not always forwarded to the cytogenetics laboratory. If the form is prepared by the physician, it may also not include information pertinent to the specific test being performed by cytogenetics; therefore, it is important that laboratory directors, in conjunction with the institution's legal staff, review the content of all informed consent forms for their specific tests in order to ensure that appropriate information has been covered, and that a copy be maintained for reference in the laboratory. If a copy of the signed form is not being sent to the laboratory with the specimen or faxed upon test ordering, laboratory personnel should devise some method by which the physician can indicate that an informed consent was signed for that particular procedure, and that, in the event that the laboratory will require a copy of the form for verification (e.g., laboratory inspection), the physician will provide the copy or scanned image of the document within a specified time. If the result of a test sent for constitutional chromosome analysis is abnormal or questionable, whether prenatal or postnatal genetic counseling must be recommended on the final report, in order to help the patient(s) understand their options and to answer any questions they may have. Neoplastic situations are generally handled by the oncologist, but they also may refer patients for genetic counseling if it would be helpful to the patient.

Specimen logging

Once the specimen has been properly received, it should be recorded into the laboratory log, whether this is computerized or in book form, and be given a unique, generally consecutive accession number. The accession number format is devised by laboratory management to meet all regulatory and state guidelines. Some institutions incorporate letters indicating specimen type, and a numeric prefix to indicate the year, for example, BM-15-001531 could indicate the 1531st bone marrow specimen received in 2015. The log or system worksheet should have at least the laboratory accession number and two patient identifiers, for example, patient's name, physician's name, birth date, sample type, and date of receipt in the laboratory. All paperwork and sample containers should be double-checked to ensure that each has been correctly recorded and labeled. The receipt date should be compared to the date of service, or the date the specimen was procured. The time of day the sample was received should be recorded as it may explain how specimens were subsequently cultured and harvested, and may help differentiate between multiple specimens on a patient, etc. Any unusual delay in specimen receipt and other variables, such as specimen condition, or any other observation that could compromise the viability of the specimen should be noted.

After the specimen is logged in, a culture and harvest schedule must be assigned according to specimen type and urgency of the results, as well as the day of the week and time of day that it is received in the laboratory. Spontaneously dividing samples, such as bone marrow or solid tumors, may be set up for a direct (uncultured) harvest, and may also be cultured for one or more days. Other samples may require culturing for several days to a week or two to stimulate growth, some with mitogens (e.g., blood lymphocytes), some following mechanical dispersal (mincing with scissors or scalpels or forcing tissue through a mesh screen, as for solid tumors and lymph nodes), or enzymatic disaggregation with collagenase and/or trypsin (e.g., solid tissues, tumors, chorionic villi) (see individual chapters for details). Some less urgent samples may be refrigerated at 4 °C until they can be fit into the laboratory workweek schedule.

Once the sample has been cultured and is ready to process for cytogenetic analysis, a harvest protocol specific to the sample type and culture method (e.g., in situ versus suspension harvests) is performed.

2.3.2 Culture methods

With the exception of some sample types, such as STAT interphase FISH samples on directly harvested cells, some period of culturing will be necessary before the specimen can be harvested. This period varies from one day to several weeks, depending on sample type. For example, bone marrow cells would be cultured 0–5 days, whereas skin fibroblasts would require 1–3 weeks to grow enough to harvest. Other conditions, such as sample size, viability, and urgency of the result can also affect the culture duration. The proper culture duration may be critical to obtaining the results, because abnormal tumor cell lines may be lost over time or, as with some bone marrows, may appear only after a few days in culture. Culturing is performed using strict aseptic techniques, because microbes will cause culture failure.

There are two general categories of tissue culture:

1. Suspension cultures, such as bone marrow and blood, and some types of tumors, such as some neuroblastomas and most lymphomas, which are suspended in growth medium and do not attach to the culture vessel; and
2. Attached monolayer cultures, or anchorage-dependent cultures, such as amniocytes, skin fibroblasts, etc., which require adherence to a culture surface to grow.

These two growth patterns are often called short-term and long-term cultures, but these categories are less distinct now that "long-term" cultures are often harvested in 2–3 days (solid tumor attached cultures) to 6–7 days (in situ amniocytes). Lymph node and solid tumors may be set up in both suspension and attached cultures for specific conditions, such as with a differential diagnosis of lymphoma versus sarcoma, or when the tumor type requires both culture types (e.g., neuroblastoma or other round blue cell tumors). These cultures may be incubated in an open or closed system. Open cultures are able to exchange gases with the atmosphere surrounding the culture. Closed systems are sealed tightly and do not exchange gases with the atmosphere. Closed systems have less chance of microbial contamination entering the culture, but open systems allow the culture to release gas phase byproducts of metabolism, which may be toxic to the cells. Open cultures are advantageous for in situ culturing and harvesting methods, in which cultures are grown and harvested on coverslips or slides that are inserted into Petri dishes or commercial slide flasks (Figure 2.5).

The pH of the cultures, as indicated via a bicarbonate buffer in the media, can be maintained by

1. allowing the CO_2 and other metabolites from the cultured cells to adjust the culture pH (closed system);
2. gassing the culture flask or tube from a canister with the desired gas mixture and closing it tightly to prevent gas exchanges (closed system); or
3. providing the incubator with a constant, controlled flow of the desired gases from external gas tanks (open system).

Closed systems are often put into incubators with a controlled flow of gas as a backup against leaks in the flask. The usual gas mixtures are 5% CO_2 in ambient air, which is composed of about 15–18% oxygen, or 5% CO_2 in 2–5% O_2 with the balance (93–95%) inert N_2. The 5% CO_2 adjusts the culture medium to pH 7.25–7.40 via the buffer in the medium, and the low-oxygen formula of the 2–5% O_2 mixture has been shown to increase the growth rate of many cell types.

Some cell cultures are grown with no oxygen, and may be gassed only with nitrogen. An example is transformed cell lines from patients with Fanconi anemia, which are sensitive to the presence of oxygen, and may not grow in its presence. Media with buffering systems other than the CO_2–bicarbonate equilibrium are also available, for example, HEPES buffer. This type of buffer is useful for transporting cells or tissues to the laboratory when the CO_2 component is not available, and standard media would lose the pH. Human cell cultures derived from human tissue are best grown at the physiologic body temperature of 37–37.5 °C, as growth curves fall off sharply over 38 °C, often resulting in cell death. Incubators should have alarms to alert personnel to temperature extremes and minimum-maximum temperature recordings for monitoring daily fluctuations (see Chapter 20, Selected topics on safety, equipment maintenance, and compliance for the cytogenetics laboratory).

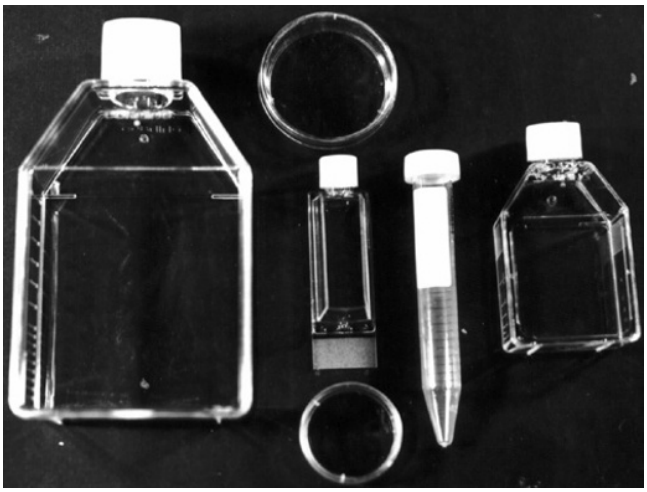


Figure 2.5 Tissue culture plasticware. Common tissue culture plasticware used by cytogenetic culture methods include the T-75 flask (a) and T-25 flask (f), which are appropriate for growing attached or suspension cultures, as are the chamber slide (c) and Petri dishes (b,d). The smaller Petri dish (d) also comes with a coverslip inside for in situ culture and harvesting. The 15-mL conical centrifuge tube (e) is used for growing (in culture) and harvesting blood, bone marrow, and other suspension cultures, and for harvesting attached cells that have been brought into suspension for a harvest.

Table 2.2 Tissue culture media commonly used for cytogenetics

Medium	Type of culture	Comments
RPMI 1640	Blood, bone marrow, solid tumor culture	Developed for lymphocytes but has been used for every application
RPMI 1603	Blood, bone marrow	Yunis recommended this medium for prophase; difficult to find
MEM or Eagle's MEM	All	Minimum essential medium
MEM Alpha	All	Enriched; available with ribonucleosides, deoxyribonucleosides
DME	All	Dulbecco's modified Eagle's
Ham's F10	All	Enriched medium with low phenol red dye
Ham's F12	All	Enriched medium with low phenol red dye
Medium 199 or TC199	Fragile X (historically), prophase	Low folic acid
Chang	Originally a prenatal medium with growth factors; now available specifically for amnio, marrow, and blood cultures	Can be used for POCs, tumor cell cultures as well
AmnioMAX	Amnio, CVS	Can be used for POCs, tumor cell cultures as well
McCoy's 5A	All	Highly enriched; good for transport medium
MarrowMAX	Bone marrows, Hem/onc bloods	Ready to use, fully supplemented with serum, a human stromal conditioned medium, antibiotics, and L-glutamine

There are scores of tissue culture media available. The most commonly used media are listed in Table 2.2. Basal medium is supplemented with serum for protein and growth factors. Most laboratories prefer fetal bovine serum. Newborn calf serum is cheaper, but may be toxic; colostrum-free newborn calf serum is less expensive and may be as effective as fetal bovine serum. Serum concentration in complete medium varies from 2% to 20%, with more serum yielding faster growth in some cases.

Some cells, however, are sensitive to the presence of fetal bovine serum and may not divide as well with high concentrations (e.g., neuroblastoma cultures, lymphocytes), and others may arrive with serum in them (e.g., blood samples) and may not require as high a concentration of serum in the culture medium as other specimen types do. Other media supplements include antibiotics, L-glutamine, and optional additions, such as selenium, insulin, and giant cell tumor conditioned medium (see specific chapters for culture methods). Once supplemented, the medium is called complete medium.

Cultures that are valuable or may be needed for future use may be frozen in medium with 5–10% dimethyl sulfoxide (DMSO) or glycerol to prevent ice crystal formation, and kept in liquid nitrogen until such time as they are needed.

2.3.3 Harvesting

Once cells are growing either spontaneously or in culture, they must be processed (harvested) to obtain metaphases for study. Harvesting cells for cytogenetic analysis is a stepwise procedure that may be and often is accomplished by automated instrumentation. However, the success of the final preparation will depend on the resourcefulness and skill of the technologist. Controlling and working around inevitable problems and variables, such as cell density (too high or too low), low mitotic index, disease-related poor chromosome quality, unusual cell types, and environmental changes often requires thought, imagination, and inventiveness.

The three constant features of the metaphase harvest protocol are mitotic arrest, usually with Colcemid®; hypotonic treatment, usually with solutions of KCl, sodium citrate, or combinations of these salts; and fixation, usually with 3:1 methanol–acetic acid solution. There are, however, several other variable options, which would depend upon the type of culture being harvested or the type of results most desired.

2.3.4 Removal of attached cells and centrifugation steps

After mitotic arrest and just before the use of the hypotonic and fixative steps, an optional step for attached cells intended for a suspension harvest is the removal of the mitotic cells by mechanical methods (rapping flasks sharply on bench top or scraping with a rubber policeman) or by enzymatic methods. The latter is commonly accomplished with trypsin and/or EDTA solutions. This mechanical/enzymatic removal would only be needed for cells that are growing attached to the surface of the flask or dish. An advantage to the mechanical removal is that mitotic cells come off before the nondividing cells, and such methods enrich the harvest for metaphase cells by leaving the interphase cells behind. Either way, the culture may be refed and reincubated for further study, providing the mitotic arrestant used is reversible. Colchicine, for example, binds irreversibly, and cannot be washed off like its synthetic analog, Colcemid®.

Another variable step is centrifugation, which is employed during suspension culture harvests to change from one solution to the next. Each solution, such as culture medium, hypotonic solution, and fixative, is removed following centrifugation. In contrast, in situ harvesting is accomplished by adding the harvesting solutions (hypotonic and fixative) to cells growing on coverslips or slides inside of Petri dishes or in chamber slides, and since cells are held in place by anchorage to the growth surface no centrifugation between steps is necessary. Some laboratories have modified the in situ harvest method to work with bone marrow suspension cultures in Petri dishes, because the cells usually settle to the bottom of the dish; if pipetting is done carefully, the cells are not disturbed.

Centrifugation is never complete, and a number of cells remain in the supernatant or are lost to damage from shear forces between cells or with the centrifuge tube walls. This is why the number of centrifugation steps should be kept to a minimum. It is also why the in situ harvesting method may yield more metaphases than do similar cultures from suspension harvests. The speed, or revolutions per minute (RPM), of centrifugation required for most cells to be recovered depends on the radius of the arm of the centrifuge. A centrifuge with a 10-in. radius yields higher gravities at 1000 RPM than a centrifuge with a 6-in. arm would at the same number of revolutions, because the larger radius travels farther (faster) per revolution. The equation for calculating the gravities for a given centrifuge at a given speed in revolutions per minute is

$$g = \left(1.118 \times 10^{-5}\right) \times R \times S$$

where g = relative centrifugal force (gravities), R = rotating radius in centimeters (distance from center of rotation to bucket center), and S = rotating speed (RPM).

Therefore, the gravities that a cell will experience at 1000 RPM in a centrifuge with a 4-in. (10 cm) radius is about 110, and the gravities that the same cell would experience in a centrifuge with a 6-in. (15 cm) radius at 1000 RPM is about 170. This may be important when one uses a new centrifuge with a different design, as the difference in gravities (centrifugal force) can make a difference in the yield, either by incomplete migration to the pellet (too slow) or by breakage due to shear forces (too fast).

Of the harvesting steps, the one usually considered the most important is the hypotonic solution because it affects spreading, chromatid width and separation, and, in bloods and bone marrows, the elimination of red blood cells. However, chromosome spreading and morphology are also controlled by Colcemid® concentration to a high degree [17]. Many so-called hypotonic problems in cell spreading are actually Colcemid® problems (Figure 2.6). Cell culture density and slide-making variables, including glass slide quality, are also equally important to spreading and may be responsible for spreading problems. Permutations of all the steps affect the quality of the final product.

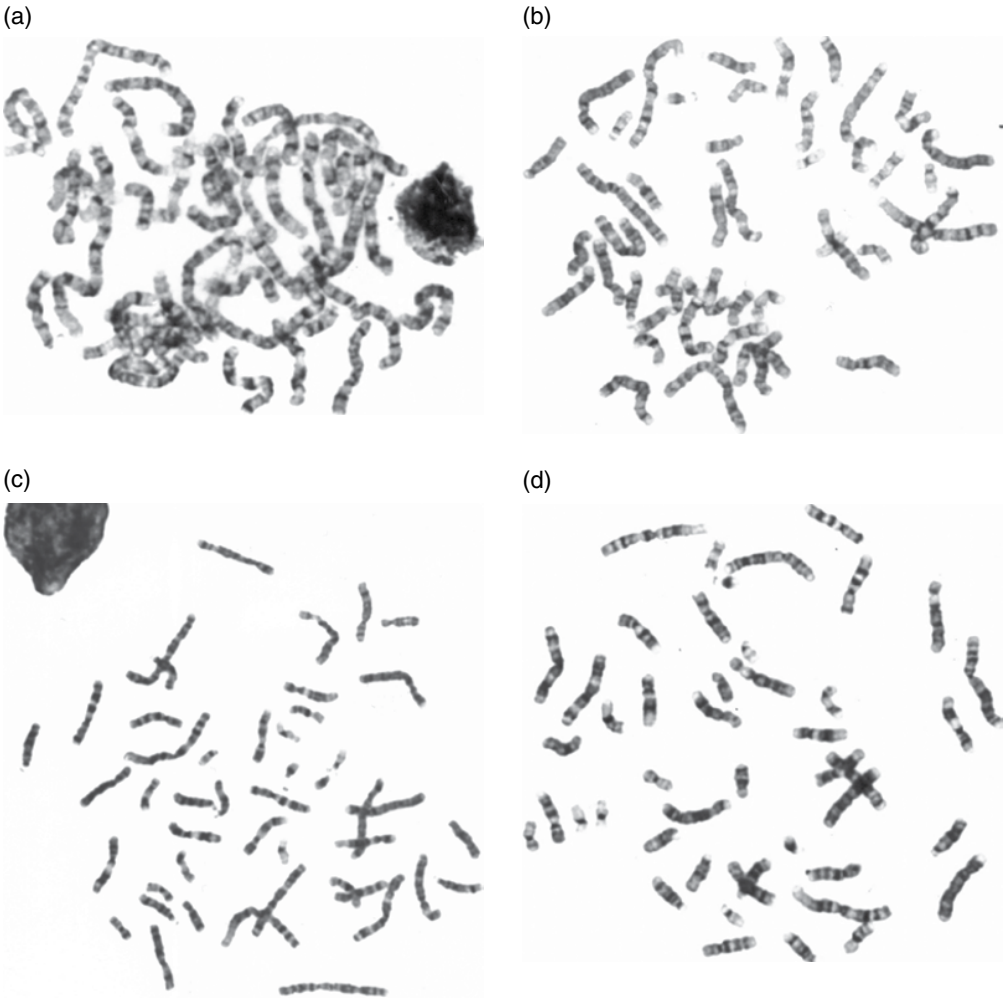


Figure 2.6 Colcemid® concentration effect on blood chromosome quality. The following examples show representative blood cells from a Colcemid® experiment using various concentrations of Colcemid® for 30 minutes. (a) No Colcemid® added. Mitotic index was very low, because mitoses were not accumulated. Metaphase chromosomes are bent, with chromosomes unable to spread out due to the effect of the spindle; (b) 0.02 μg/mL Colcemid®. Mitotic index on the slide was better, less chromosome bending is evident, and spreading is improved due to spindle poisoning; (c) 0.05 μg/mL Colcemid®. Chromosomes show better spreading and are straight, but are beginning to shorten and show fewer bands; (d) 0.1 μg/mL Colcemid®. Mitotic index was very high (not shown) because there are enough Colcemid® molecules to poison all of the microtubules of the spindles of all cells in the culture. Also, chromosomes are well spread and have few crossed chromosomes. However, chromosomes are highly contracted, and many small abnormalities, such as the Prader–Willi and DiGeorge deletions, would not be detectable at this band level.

2.3.5 Mitotic arrest: Colcemid®

The first step in most harvest procedures is to arrest cells in the mitotic stage required for standard cytogenetic analysis: metaphase. The effect of the most commonly used mitotic arrestant Colcemid® is to prevent formation of the spindle fiber apparatus, which would normally pull the sister chromatids to opposite poles for incorporation into the two daughter cells. Colcemid® also causes chromosome condensation, a process that becomes accentuated when increasing the time of exposure and the concentration. Modern cytogenetics laboratories strive for medium-length to very long chromosomes in order to increase the chance of detecting small rearrangements, so Colcemid® dosage and exposure are often reduced to a minimum. However, an understanding of the effects of Colcemid® can allow the technologist to vary the dosage and exposure time and still maximize the quality and quantity of mitotic cells in the harvest, depending on the culture and harvest type, the desired type of study, and other variables. The condensation effects which occur with longer time exposures seem to be greater with certain cell types, such as blood, bone marrow, and CVS, than with others (amniocytes, solid tumors), and may be at least partly related to cell cycle time and disease state. Also, some cell types are more sensitive to the concentration of Colcemid® than others. For many attached cultures, the slower growing cells seem to tolerate longer Colcemid® times. This condensation effect of Colcemid® is mitigated with the use of anticontraction agents, such as ethidium bromide (EB), 5-bromo-2′-deoxyuridine (BrdU), etc. (Figure 2.7; see Chromosome anticontraction methods).

The dose-dependent contraction effect of Colcemid® is well known [17–19], but increased dosage of Colcemid® also dramatically increases mitotic index, straightens chromosomes, sharpens chromatid edges, and increases chromosome spreading as it releases them from the mitotic apparatus [17–21] (see Figure 2.6). Experiments in our laboratory suggest that as Colcemid® concentration increases from 0.01 μg/mL to 0.1 μg/mL, there is a G-band coalescing effect, so that chromosomes with a long appearance do not have as many sub-bands as seen at the same physical length with lower dosages. See Table 2.3 for Colcemid® concentrations at various dosages.

The optimal concentration for each tissue type should be tested for every laboratory, and Colcemid® should be added with a micropipette to known volumes of medium in the culture in order to strictly control concentration. The concentration can be increased in the presence of EB or other anticontraction agents (see 2.3.9, Chromosome anticontraction methods later). In fact, EB causes chromosomes to spread poorly [22], and experiments in our laboratory led to the conclusion that spreading dramatically improves using 0.05 μg/mL of Colcemid® with EB over that seen with 0.02 μg/mL Colcemid® with EB (see Figure 2.7) in tumor cultures.

Colcemid® is a synthetic analog of colchicine, an alkaloid derived from autumn crocus (see Figure 2.2). Some laboratories still prefer colchicine, which is more toxic, and may therefore retard the cell cycle speed and effectively yield longer chromosomes. Some cultures may stop dividing and yield no metaphases due to the toxic effect of colchicine, although this is not common. If it is suspected that a culture is not as mitotic as it should be when harvested with colchicine, it is possible that Colcemid® would be a better choice. Velban (vinblastine sulfate) is also used for some or all tissues in some cytogenetics laboratories, and is a good substitute in tumor cultures and other difficult specimens when Colcemid® seems ineffective.

Often the mitotic arrest is blamed for contracted chromosomes, but other factors may be involved. In blood and bone marrow cultures, overinoculation may result in depleted medium and increased metabolic byproducts that cause irreversible chromosome contraction. Slide-making variables, such as cell suspensions that are too concentrated or contain excessive debris, result in crowded metaphases that appear more contracted than cells from slides made from more dilute suspensions of the same pellet. In our experience, specimens from compromised patients, such as leukemics, drug-treated patients, and neonatal intensive care patients, may always appear contracted in spite of our best efforts. The experienced technologist learns to use all of the tools available to ensure the best possible results, and Colcemid® is one of the most important.

2.3.6 Hypotonic treatment

The second major step in harvesting cells is treatment with a hypotonic saline solution to increase cell volume (Figure 2.8) so that the chromosomes have adequate space to spread out during slide preparation. Since the cell membrane is semi-permeable, water moves slowly into or out of the cell by osmosis, which equalizes the concentration on both sides of the membrane. Thus, if the concentration of the solution outside the cell is higher than inside (hypertonic), the cell will lose water and shrink. Alternatively, if the concentration outside the cell is lower than inside the cell (hypotonic), it will absorb water and swell. Hypotonic solutions work in this way, by creating a concentration gradient across the cytoplasmic membrane, so that water moves in by osmosis, though active transport may also be involved [19]. If the potassium pump is poisoned, the cells do not swell [19]. Various hypotonic solutions and their uses are listed in Table 2.4.

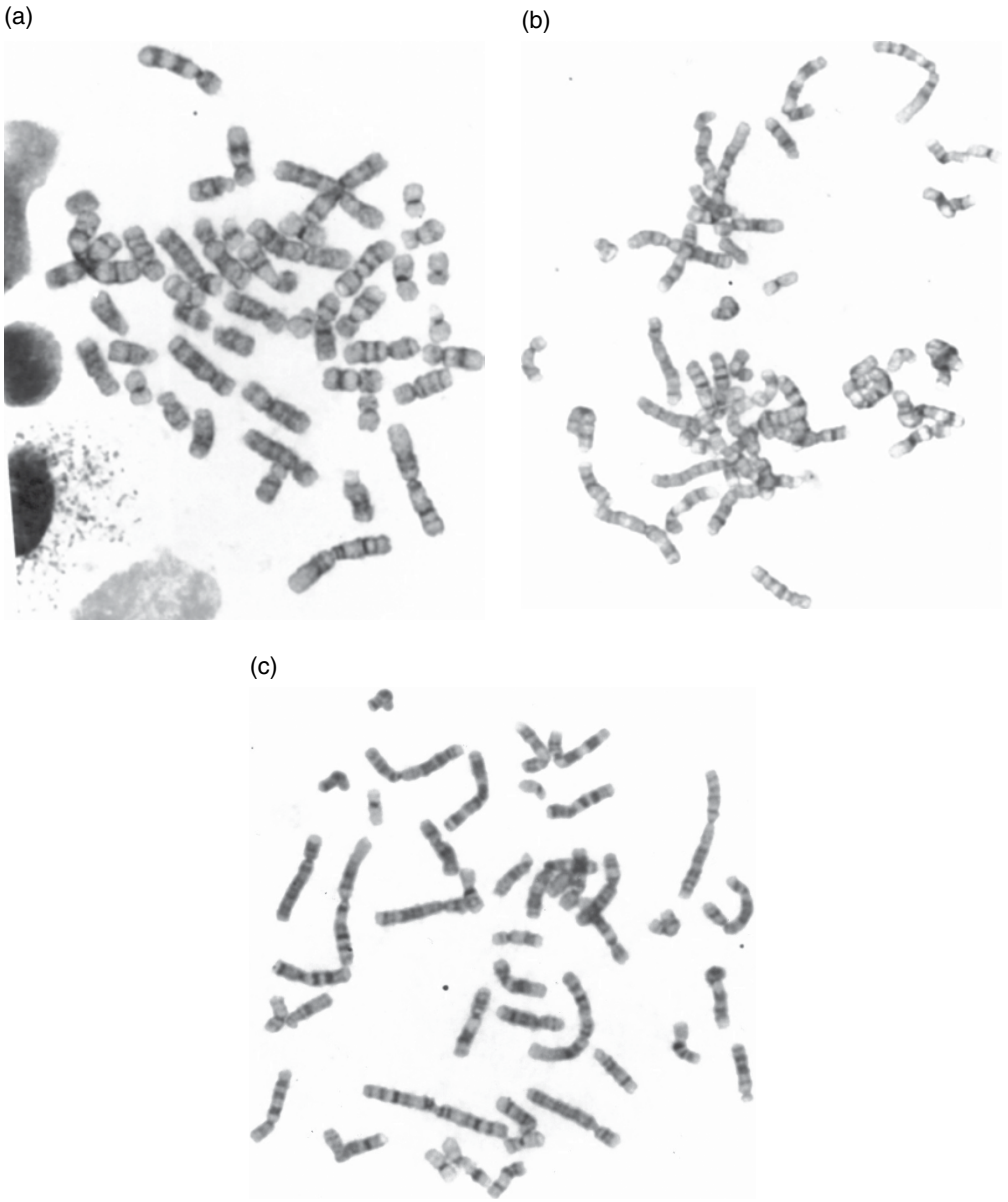


Figure 2.7 Colcemid® and ethidium bromide (EB) concentration effect on melanoma cells. Representative cells from an experiment on melanoma cells. (a) Harvested with 0.02 μg/mL Colcemid® and no EB. (b) Harvested with 0.02 μg/mL of Colcemid® and 5 μg/mL of EB. Chromosomes are longer than without EB, but tend to clump together and cross each other. (c) Harvested with 0.05 μg/mL of Colcemid® and 5 μg/mL of EB. This concentration of Colcemid® mitigates the clumping effect of the EB and reveals better band resolution for abnormal chromosomes than without EB.

Prewarming the hypotonic solution to 37 °C may increase effectiveness by speeding up water transport across the cell membrane and possibly by softening the cytoplasmic membrane, which has a lipid component, giving it more stretching capability. The type of salts used in the hypotonic can affect the width and sometimes the length of the chromatids, Sodium citrate, for example, often yields wider chromatids than KCl, and Ohnuki's hypotonic usually yields longer chromatids than KCl.

Many cell types are sensitive to the hypotonic treatment, for example, solid tumors or acute lymphocytic leukemia (ALL bone marrows), with some being easily damaged by over treatment and some being resistant to swelling. In our hands, the best results are obtained when the hypotonic volume and treatment time are kept at the lowest effective level. We feel that most cells seem to respond well in the first 10–20 minutes of exposure. Problems due to large volumes of hypotonic solution (excluding in situ

Table 2.3 Colcemid® concentrations

µg/mL	Microliters to add (per 5 mL culture)	Syringe equivalents (25G needle)
0.05	5	½ drop
0.02	10	1 drop
0.05	25	2 ½ drops
0.1	50	5 drops
0.2	100	10 drops

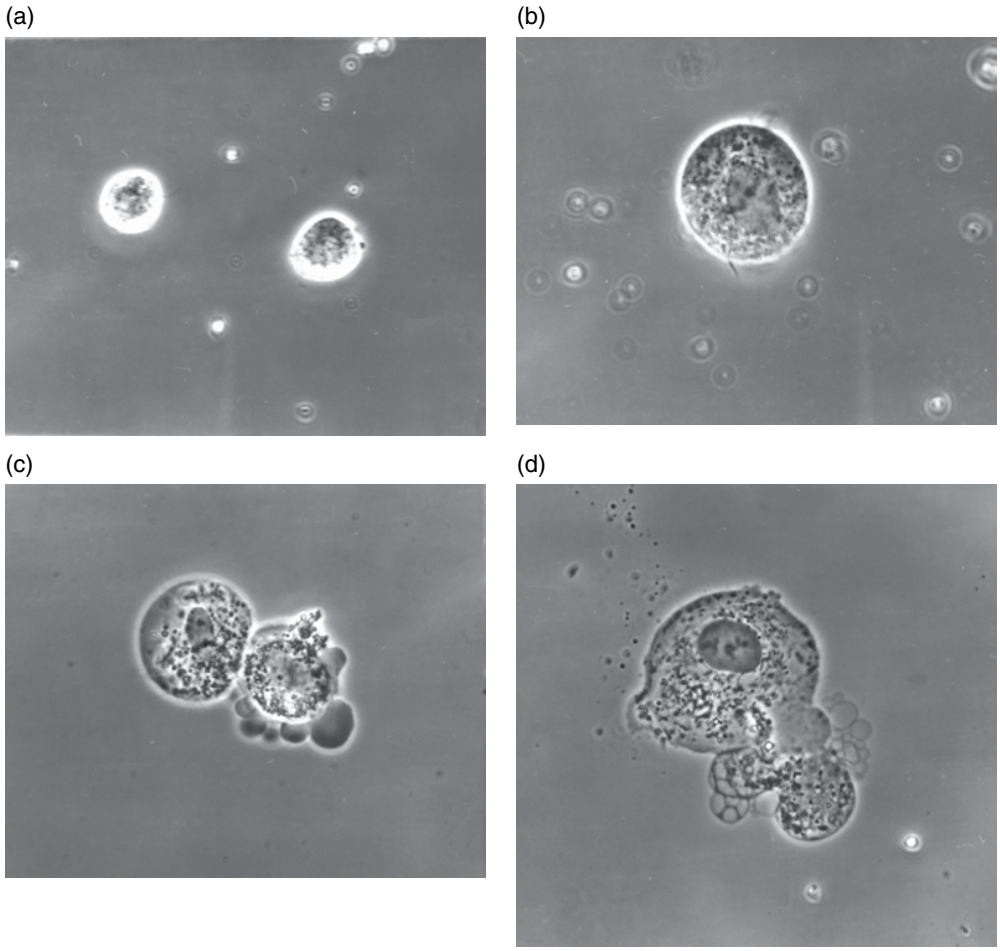


Figure 2.8 Hypotonic action on tumor cells in 0.075 M KCl. (a) Within 3 minutes of addition of hypotonic solution, cells have not begun to swell. (b) At 10 minutes of hypotonic duration, cells are double in volume, and membrane is stretched but still strong. (c,d) After 30 minutes and 45 minutes of hypotonic, respectively, cells have begun to lose integrity and collapse under the weight of the coverslip. A hole has opened in this cell (d) and cytoplasmic contents are streaming out. If this were a metaphase cell, with the nuclear envelope disassembled, some of the chromosomes could leak from the hole, leading to hypodiploidy or lowered mitotic index.

harvests) may include loss of mitotic cells due to increased fragility during centrifugation, as well as incomplete migration to the pellet. Long exposures to hypotonic may cause weak areas in the cytoplasmic membrane (see Figure 2.8), which, if they burst at any point, will allow some or all of the chromosomes to escape. This can lead to a (false) low mitotic index, scattered chromosomes, partial metaphases, or tight "imploded" cells. Holmquist and Motara [19] report that after a long hypotonic incubation period, the cells return to their original size.

Table 2.4 Formulas for hypotonic solutions

Solution	Formula	Usual harvest type
0.075 M KCl (0.56%)[a]	5.59 g/L	All
0.4% KCl	4 g/L	Neoplastic cells for increased spreading
CHS (0.4% KCl with EGTA and HEPES)	3 g KCl + 4.8 g HEPES + 0.2 g EGTA/L, pH 7.4	Cancer hypotonic solution
THC[b]	9 parts 0.075 M KCl, 1 part 0.25% Trypsin-EDTA, 0.08 μg/mL Colcemid®	Neoplastic cells: trypsin/hypotonic/ Colcemid® to increase spreading, morphology
Sodium citrate[c]		
0.7%	7 g/L	All attached cell types, especially amniocytes
0.8%	8 g/L	
1.0%	10 g/L	
Sodium citrate and KCl mixtures	Many combinations of various concentrations of KCl, sodium citrate, dilute serum or medium	Amniocytes, fibroblasts
Dilute Hanks' BSS	1 part Hanks': 3–6 parts distilled water	Attached cell types
Dilute serum (calf, fetal bovine)	1 part serum: 3–6 parts distilled water	Attached cell types
Ohnuki's hypotonic	4.1 g KCl in 1 L water+ 2.33 g $NaNO_3$ in 500 mL water+ 0.9 g CH_3COONa[d] ($NaC_2H_3O_2$) in 200 mL water, or 5 mL 55 mM sodium nitrate, 2 mL 55 mM sodium acetate, 10 mL 55 mM potassium chloride	

Many methods call for addition of a few drops to several milliliters of fixative to the hypotonic at the end of the incubation period. The in situ harvest method requires this "prefixative" step to help prevent mitotic cells from becoming dislodged during addition of the first fixation. This prefixation begins the process of hardening the cells and preserving the chromosomes, and makes cells more resistant to damage from centrifugation and the shock of the pure fixative. It also encourages the lysing of any red cells present, and since the lysed cells do not migrate to the pellet, the result is a cleaner cell preparation after centrifugation. However, nucleated red cells, as found in some blood samples (e.g., newborn blood), as well as blood from lower vertebrates (birds, reptiles, and fish), do not lyse in hypotonic or fixative.

2.3.7 Fixation

The third constant feature of chromosome harvesting is fixation of the cells. This process removes water from the cells, killing and preserving them, hardening membranes and chromatin and preparing the chromosomes for the banding procedure. Banding patterns are not possible in formalin-fixed chromosomes, but the gentler 3:1 methanol–acetic acid changes the chromatin structure less, or in a different way, allowing subsequent banding. The first fixative may create turbulence at first when added to the remnant hypotonic solution. During this period of turbulence, the fixative is usually added slowly, or metaphase cells can be lost to breakage. Then the fixative is added more quickly. It is important to gently but thoroughly mix the cell pellet into suspension to prevent irreversible cell clumping. Once in the first fixative, the cells become stronger (are hardened), and subsequent fixations may be added much more quickly.

After first fixation, cells may be left to stand for some period of time to allow them to harden before additional manipulations. Cold fixative may improve chromosome morphology, and many protocols call for 1–24 hours for first fixative and/or final fixation in the refrigerator or freezer. Once cells are in the first fixative, they may be stored for days or weeks or more before slides are made. The composition of methanol–acetic acid fixative will change with time, at first becoming more acidic and eventually becoming contaminated with acetates as a reaction takes place between the acid and the methanol; this is the reason that so many protocols require freshly made fixative. Fixative also absorbs water from the air, which diminishes its fixative properties. This deterioration of fixative also occurs in stored cell pellets. Storing fixed cell pellets at low temperatures

decreases the fixative deterioration, and the cells are preserved better for longer periods. The Mayo Clinic Cytogenetics Laboratory is able to use very old cell pellets (up to 8 years) from bone marrow and phytohemagglutinin (PHA)-stimulated blood cultures which were kept in a –70 °C freezer in a 1.8-mL cryotube.

Certain types of plastic tubes are broken down by fixative, and the byproducts can ruin the cells. The experience of our laboratory and of other laboratories is that polystyrene tubes start to break down within a few days of exposure to fixative, whereas polypropylene tubes seem to resist fixative well.

For suspension-type harvests, the first fixation may be the final step in the harvest before cells are centrifuged out and slides are made, if the pellets are small, clean, and free of red cells (e.g., amniocytes, solid tumors). Additional fixation steps are required for large or red blood cell (RBC)-contaminated pellets to get rid of debris that would interfere with slide-making and banding. Cells with red cell components are usually washed with two additional changes of fixative before slides are prepared.

When slides are made, if the cells do not spread well, additional fixation steps may also be helpful, especially if there is still a brownish tinge to the supernatant from remaining, incompletely lysed red cells.

2.3.8 In situ harvesting

Cox et al. [23] described the in situ harvest method in 1974 using colonies of amniotic fluid that were grown and harvested in Petri dishes. The sides of the dish were removed and the cell growth surface was taped to a slide for microscopy. Peakman et al. [24] modified this method to growing cells on coverslips inside Petri dishes. This way the coverslips are harvested in situ ("in place") in the dishes, and removed and attached to slides for microscopy. They developed the method to deal with maternal cell contamination, but the method became very popular due to the improved turnaround time and the advantages of clonal analysis.

The advantages to harvesting cells on the growth surface (usually on a coverslip in a Petri dish or a special slide flask with removable top) include the following:

1. Harvests may be performed days earlier than with suspension harvests, improving turnaround times.
2. Cultures may grow better on the glass surface than on plastic ware. This is sometimes true for prenatal and tumor specimens; however, some specimens (e.g., very bloody amniotic fluid specimens) may prefer a flask environment because it dilutes out the RBCs and improves amniocyte contact with the culture ware.
3. Clonal analysis may be performed to rule out pseudo-mosaicism and culture artifact. There are one to three viable amniocytes per milliliter of amniotic fluid, with generally less viable cells available as the gestation advances beyond 25 weeks. Each amniocyte tends to grow out a single colony, so chromosome aberrations that are confined to part of the colony must have occurred after colony formation. Such cells are therefore spurious artifacts of culture, and may be discounted, in most cases. Aberrations that occur in the entire colony may, on the other hand, represent true mosaicism, especially if multiple colonies from multiple, independent cultures display the same whole-colony abnormality. Clonal analysis applies these ideas to form rules for studying in situ harvested cultures, examining a certain number of colonies rather than a certain number of metaphases, ignoring partial colony mosaicism.
4. Morphology of the parent colony is available for correlation with karyotypes. This may be especially useful in solid tumor studies, to distinguish fibroblastic stromal cell colonies (normal cells) from tumor colonies with unusual morphology.
5. Since they are usually inside culture ware with removable lids, in situ harvests may be automated [25] (see 2.3.13, Automatic harvesting devices and slide-making chambers/drying chambers).

Typically, in situ harvests are performed on attached cultures, since the mechanism that aspirates the harvest fluids would remove suspended cells with the discarded materials. However, the Mayo Clinic laboratory has success with an automated harvest method even with bone marrow suspension cultures, as the desired cells are settled to the bottom. About 10–20% of the bone marrow cells are removed during the harvest, but the metaphases are not selectively lost. Experiments in that laboratory suggest that significantly more bone marrow metaphases are recovered with the in situ than suspension harvests because the fragile metaphases are lost in the centrifugation steps.

Drawbacks to the in situ system include the following:

1. It is sometimes difficult to get attached cultures at optimal densities at a convenient time. Metaphase cells in the center of the colonies may not spread well so it is important to have rounded up mitotic cells around the periphery of the colonies. There is often a 2-day window in the culture duration where conditions are optimal. It is important, however, not to harvest all cultures on the same day for quality control.
2. The coverslip is delicate and can be broken or dropped face-down, scratching the cells.

3. There may be a higher chance of mixing up cultures because the coverslips themselves are usually not labeled. Many protocols require labeling the bottom of the Petri dish because the top can be switched. Some laboratories have been able to label the coverslip using a diamond-tip or other marking pen. Slide flasks (flaskettes) are more easily labeled than coverslips, and can be labeled both on the plastic flaskette top and on the label of the bottom, which will become the microscope slide when the harvest is complete and the top is removed.
4. Metaphase quality is limited by the procedure: only one attempt per culture can be made to spread cells and optimize chromosome morphology. Often, no backup culture for a specific coverslip is available if the harvest or spreading maneuvers are unsuccessful. Most laboratories have a mechanism for a backup suspension culture in case of need for FISH, extra counts, etc., using either cells from the Petri dish surface that remain after the coverslip has been removed, or a separate flask culture set up at the same time as the dishes.
5. Small Petri dishes can dry out faster than flasks in low humidity situations.

If growth is too dense when checking for harvest, some culture coverslips can be trypsinized or scraped so that there is sufficient surface for new growth, and then fed with fresh media. If time permits, the culture can rest before being inoculated with an overnight mitotic inhibitor for harvest the next morning, or left in culture until the next morning, when it would then be evaluated for harvest. Because colonies have been disrupted, and lifted cells could have replanted at a secondary location, the resulting culture can be used for cell counts, as in the flask method, but not for colony counts.

For poor-growing or at-risk cultures, it is important to check for colony growth around the inner periphery of the dish, off the coverslip surface, before adding Colcemid®. If there is any colony growth off the coverslip that is not an extension from a colony on the edge of the coverslip, the coverslip can carefully be removed to a new Petri dish for harvesting. The colonies growing off the coverslip in the original vessel can be trypsinized and replanted onto a new coverslip or flask, and be used as a backup for counts, if needed. Even though these cells can no longer add to the colony count, the number of colonies that were present before trypsinization should still be noted in the specimen worksheet.

Routine setup of a backup flask culture (in situ or flask) is a good policy, both as an insurance policy against harvest problems and for other uses, such as sending for DNA analysis, freezing cells in liquid nitrogen, etc.

In situ harvest steps include:

1. Addition and incubation of Colcemid® or other mitotic arrest solution.
2. (a) (Optional) Removal of half of the culture medium and addition of an equal volume of hypotonic solution, and/or (b) Removal of all culture medium. Addition of hypotonic solution for a period of time.
3. Usually, addition of an amount of fixative (cold or room temperature) equal to the hypotonic for a brief incubation to help adhere the mitotic cells to the slide or coverslip.
4. Removal of hypotonic solution and (usually slow) addition of first fixative, which is allowed to stand for a few minutes or more to harden and adhere metaphases.
5. One to three additional changes of fixative to get rid of all water possible. Then the cells may be left in fixative until it is convenient to dry them for chromosome preparations.
6. After harvest, coverslips are mounted (specimen side up or down) and the slide is labeled. The coverslip may be mounted with the cells down (between slide and coverslip), or with the cells up (so that the coverslip need not be removed for sequential staining of cells).

The critical steps for the in situ harvesting methods are:

1. Gentle treatment of cultures and often gentle addition of hypotonic and first fixative to retain loosely attached mitotic cells, and to prevent false low mitotic index from dislodged, lost cells, or cell movement due to dislodging and reattachment elsewhere, away from the parent colony.
2. Consistency in the amount of fluid left on the culture before the next solution is added. If much culture medium is left behind, it makes the hypotonic less effective. Large amounts of residual hypotonic make the first fixation less effective at removing water. In the final drying step (equivalent to slide-making), it is essential to remove all of the fixative to prevent waves of drying fixative from dislodging cells or breaking them open.

Cell drying will be discussed later in the chapter.

A modified in situ harvesting method may be used for harvesting very small numbers of cells from uncultured specimens for FISH studies. For example, bladder washings to be used for interphase FISH may be concentrated by centrifugation, placed on a glass slide in a small drop of fluid such as medium or saline, and harvested on the slide by draining the drop of medium off, replacing it with a drop of hypotonic, incubating for a few minutes, adding fixative, draining off the excess fluid, and fixing cells again. Many of the cells remain on the slide if the fluid is drained carefully. Certain types of cells (e.g., cerebrospinal fluid) may also be prepared for interphase FISH in a similar fashion, omitting the hypotonic step, and pre-treating the slide preparations with protease such as pepsin in order to remove the protein from the cytoplasm.

2.3.9 Chromosome anticontraction methods

As chromosomes progress through the stages of interphase and mitotic prophase toward metaphase, they condense from long, string-like structures into shorter and shorter bodies. As they condense, the banding patterns coalesce, with sub-bands merging into bands and major bands merging (see Figure 2.9). This serves to package the chromatin into neat, small units for distribution to the two daughter cells, where they return to the decondensed state as soon as possible. Most metabolic functions take place in interphase.

In the early 1970s, cytogenetic analysis was usually performed on mid-metaphase chromosomes that contained 300 or 400 bands per haploid set. In 1976, the first of several techniques to obtain elongated chromosomes with 500–2000 bands per haploid set was reported by Yunis [11]. The technique involved increasing the proportion of late prophase and early metaphase cells by synchronizing the cells with an amethopterin (methotrexate) block during synthesis, followed by a thymidine release and much lower Colcemid® concentrations than had been traditional until that time. Released cells in synchrony can then proceed from the S stage of the cell cycle into early metaphase, when they are harvested.

This technique led to the discovery of several very small chromosome deletion syndromes [12–16,26–29] and was useful in defining the exact nature and breakpoints of other chromosome aberrations. The subsequently developed techniques with varying synchrony chemicals and DNA-intercalating anticontractants were also used to improve mitotic index, chromosome morphology, and banding quality in all stages of metaphase for blood, bone marrow, solid tumor culture, amniotic fluid, and CVS cultures. Yunis and colleagues [30,31] and some others have used the techniques to find new chromosome abnormalities in leukemic bone marrow specimens and other human neoplasms [32,33]. Variations of the synchrony method were introduced by several authors [31,34–37], and chemical additives to prevent chromosome contraction, used alone or in combination with synchrony, were developed [34,37–44]. Techniques for all types of specimens were consequently developed using shorter exposures to lower concentrations of mitotic-arresting solutions. Most laboratories now offer prometaphase, prophase, or both types of chromosome analysis for blood cultures. This produced familiarity with and appreciation for the long chromosomes with more intricately detailed banding patterns and a trend toward using longer chromosomes in all cytogenetic studies resulted.

There are two main ways to utilize chromosome anticontraction methodologies: to obtain a high-quality regular study (e.g., bone marrow, amniotic fluid, and tumor cultures) or to obtain prophase and prometaphase chromosomes (Prader–Willi, Angelman, DiGeorge, Miller–Dieker syndromes) (Figure 2.10a,b). Our laboratory has also used a synchrony method to enrich solid tumor cultures for abnormal cells.

For focused prophase and prometaphase studies, where specific chromosome regions are being questioned by the clinician, the time required to complete the study is not much longer than for a regular study, if the preparation quality is good. Often, however, the study is not focused, and each chromosome pair must be examined on uncrossed, unobscured homologues. It is best to analyze a few relatively shorter cells because some inversions and deletions may actually present better on the shorter chromosomes (Figure 2.11).

The quality of very long chromosomes is dependent on good slide-making and staining methods and optimal photography or imaging. The most common technical problems are slide-making issues, overtrypsinization, overstaining, unsharp image, poor Köhler illumination (see Chapter 14, Köhler illumination), use of the wrong contrast photographic paper or film, or computer imaging problems.

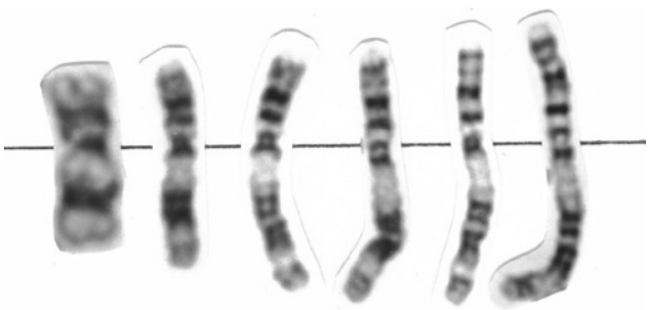
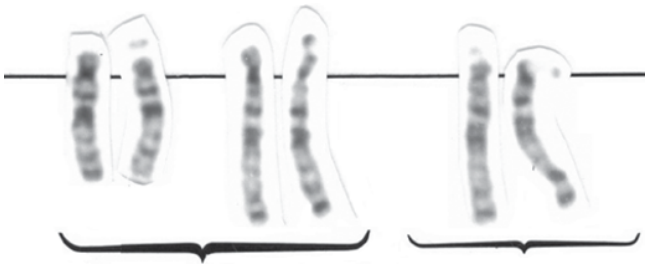
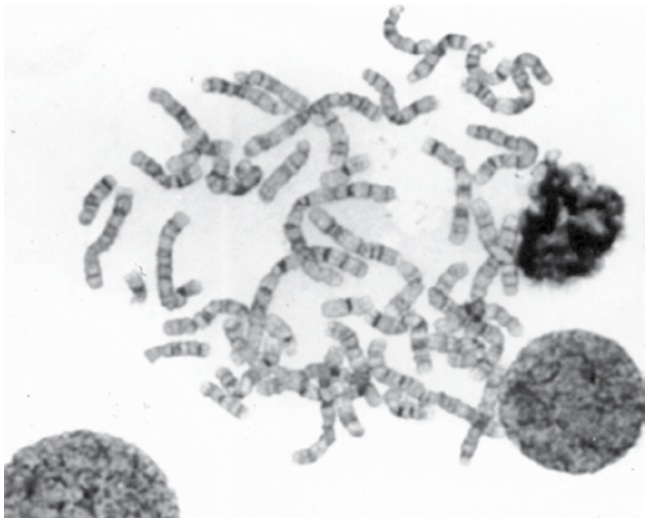


Figure 2.9 Forming metaphase bands. G-banded chromosome 11 at various band levels to illustrate how prophase and prometaphase bands (right) coalesce to form metaphase bands (left).



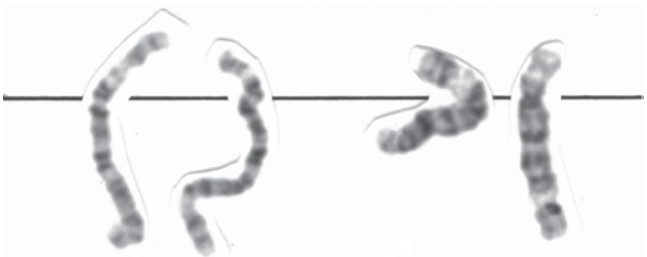
(a) Patient 1 Patient 2



(b)

Figure 2.10 Two uses of anticontraction chemicals. (a) To detect microdeletions and other small aberrations: two patients with Prader–Willi (deletion of chromosome 15) were used. *Patient 1 (left-hand pair):* Band-level resolution is not high enough to determine the presence or extent of the deletion for chromosome 15. *Patient 1 (right-hand pair):* Chromosomes 15 from the same patient are at a high enough band level to determine the presence of a deletion at band 15q11.2. *Patient 2:* This deletion is from band 15q11.2 through the proximal q13 band (called q13.1). Note: Deleted chromosomes for both patients are placed on the right. Both deletions were confirmed with FISH. (b) A second use of anticontraction chemicals is used to obtain a higher quality preparation in samples that are normally poor, such as bone marrow and tumor specimens. This abnormal bone marrow was harvest with ethidium bromide and shows improved quality.

(a)



(b)

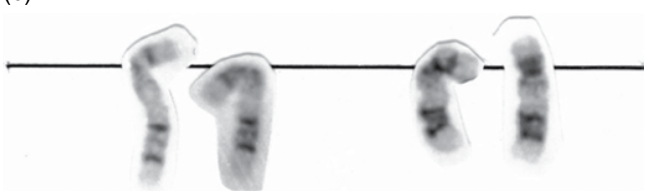


Figure 2.11 Advantages of short chromosomes. Relatively short chromosomes are also helpful in certain situations. (a) A pericentric inversion of this chromosome 6 (left) at the 700 band level is difficult to visualize because the centromere is not strongly constricted; whereas at the 550 band level (right), it is much easier to visualize. (b) Smith Magenis syndrome is caused by a small deletion in the proximal short arm of chromosome 17. At higher band levels (left), the abnormality seems to be more subtle than at shorter levels (right). This deletion was confirmed with FISH.

There are several methods of determining how many bands per haploid set (BPHS) a cell contains [45–50]. Band counting is still quite subjective, however, and does not give information on how crisp the patterns are, how many overlapped and bent chromosomes are present, or whether the cell displays the abnormalities in question. Most laboratories report the band level of the karyotypes in the final report, which is very important because it gives some idea of the degree to which the study answered the questions of the referring physician. Late metaphase chromosomes are at <400 BPHS level, mid-metaphase is 400–549 BPHS, early metaphase is 550–699 BPHS, late prometaphase is about 700–849 BPHS, early prometaphase is about 850–999 BPHS, and prophase is at or more than 1000 BPHS [47] (Figure 2.12). Usually, in our laboratory, neoplastic

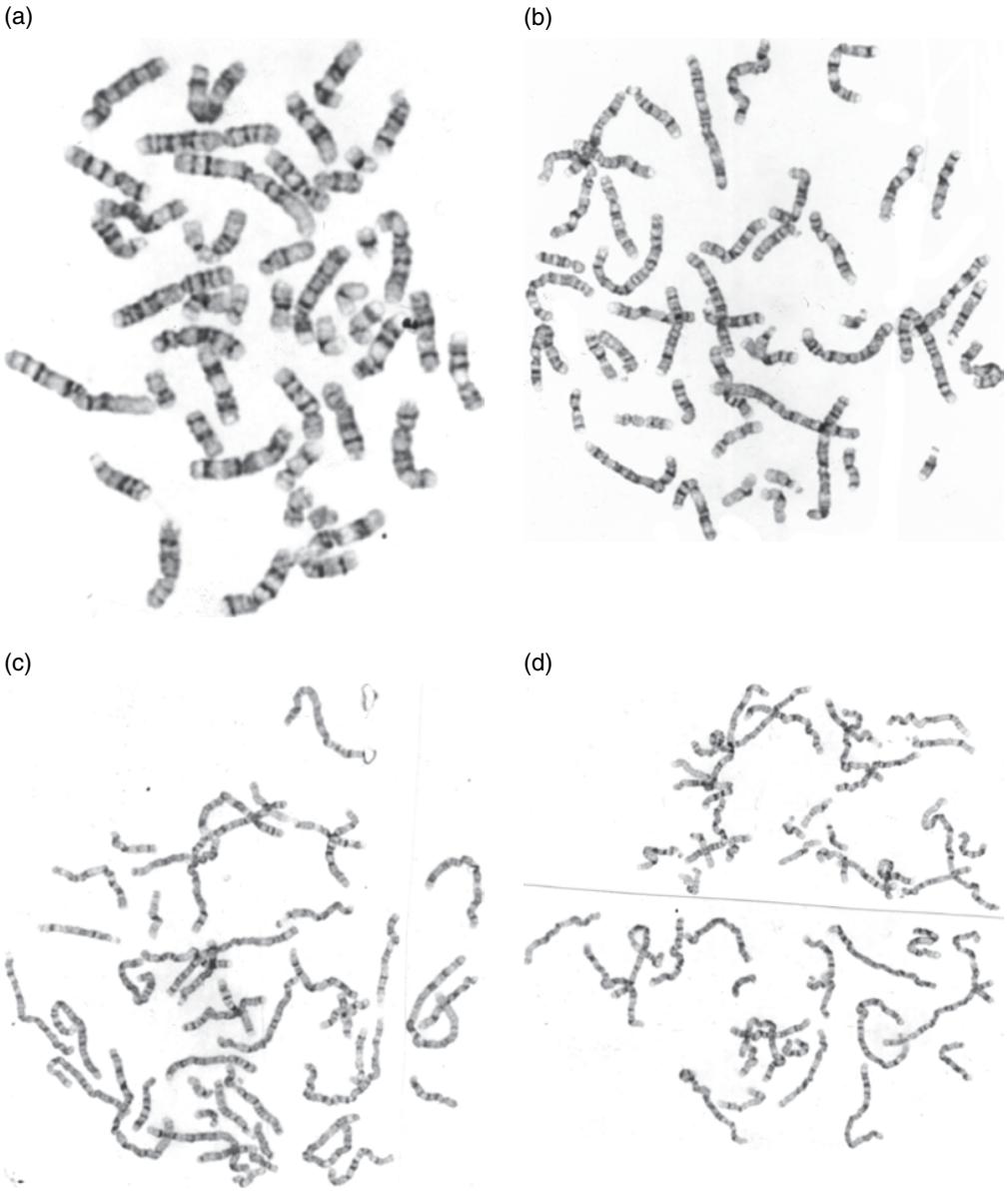


Figure 2.12 Metaphase cells at various band levels. (a) About 400–550 bands per haploid set (BPHS). This length is acceptable for certain types of studies, such as malignancies. (b) About 550–700 BPHS. This is a level most people feel comfortable with for analyzing cells at the microscope, and most abnormalities will be evident at this stage. (c,d) 700–850 BPHS is generally suitable for microdeletions greater than 3~5 Mb, and other small aberrations.

Table 2.5 Chemical agents used to produce cell synchrony

S-block agent	Release agent
Amethopterin (methotrexate), 10^{-7} M, 16–18 hours[a] (MW = 454.4)	Thymidine, 10^{-5} M, 5–6 hours[a] (MW = 242.2), OR BrdU, 12 μg/mL, 5.5 hours[b]
5-fluorodeoxyuridine (FdUrD or FUdR) 0.1 μM (bone marrows)[c] 3.3×10^{-7} M, 16–18 hours[d] (MW = 246.2)	Thymidine 3.3×10^{-3} M[c] 3.3×10^{-5} M, 5 hours[d] (MW = 242.2), OR BrdU as for amethopterin
Thymidine excess 300 μg/mL 16–18 hours[e]	2-deoxicytidine, 10 μM, 4 hours 15 min[f] (MW = 227.2) OR BrdU 50 μg/mL, 5–8 hours[e], OR just rinse and feed OR no rinse or release[i]
BrdU excess 10 μg/mL, 15–17 hours[g] 200 μg/mL, 15–17 hours[h]	Thymidine, 0.3 g/mL, 6–7 hours OR just rinse and feed 6 hours[c]

[a] From Reference 11; [b] Reference 40; [c] Reference 52; [d] Reference 52; [e] Reference 53; [f] Reference 37; [g] Reference 41; [h] Reference 35; [i] Reference 56.

specimens are in the late to mid metaphase range, amniotic fluid, and fibroblast cultures are in the early metaphase to prometaphase range, and bloods vary from early metaphase to early prometaphase. This variability reflects the quality that each type of tissue is capable of attaining as a result of intrinsic chromosome characteristics in various cell types [17]. Tumors and CVS cultures, for example, often seem to have short chromosomes under the most ideal conditions. If the resolution is unsatisfactory, it may be necessary to repeat culturing and analysis.

There are two basic methods of obtaining prometaphase and prophase chromosomes: (i) cell synchrony and (ii) additives to prevent contraction. They may be combined into still other permutations of synchrony and additive methods that may be synergistic. In theory, the synchrony method works by stopping cells in synthesis, collecting a large population of cells ready to begin division together. When the cells arrive at an early stage of metaphase together, they are harvested and put on slides. By timing the release period properly, one can obtain a large number of early metaphase and prophase cells.

The chemicals used to block synthesis include amethopterin (methotrexate), 5-fluorodeoxyuridine (abbreviated FdU, FUdR, or FdUrd), 5-bromo-2′-deoxyuridine excess (abbreviated as BrdU, BUdR, or BrdUrd), or thymidine excess. These chemicals are added to exponentially growing cells (e.g., 2–3-day stimulated blood cultures, bone marrows the first or second day of culture, or young primary or early subcultured monolayer cultures.) The block is released with thymidine (for methotrexate or BrdU excess type blocks), BrdU (for methotrexate), or 2-deoxycytidine (for thymidine excess) (Table 2.5). Drouin et al. [51] report that release time is about 30 minutes longer for BrdU than for thymidine.

2.3.10 Mechanism of action of synchrony chemicals

Amethopterin (methotrexate) interrupts the purine pathway by inhibiting dihydrofolate reductase, which interrupts the purine pathway. Tetrahydrofolate, the active form of folic acid, is a coenzyme that carries one-carbon groups for transfer reactions, such as synthesis of amino acids, purines, and thymidine. This includes the conversion of dUMP to dTMP via thymidylate synthetase. The removal of amethopterin and the addition of thymidine allow synthesis of dTMP via a thymidine kinase in the salvage pathway. 5-Fluorodeoxyuridine is also used to synchronize cells and has an effect similar to that of amethopterin, acting as an antagonist to thymidylate synthetase. Thymidine releases FdU cell blocks in the same way that it releases amethopterin blocks. Webber and Garson [52] report using FdU to synchronize bone marrow cultures, and Gibas et al. [53] developed a 24-hour CVS technique using FdU synchrony to improve chromosome morphology.

BrdU, an analog of thymidine, can also be used to release cells from amethopterin-type blocks. It will produce a greater number of bands, because it also inhibits chromosome condensation [43], and it can be used to produce RBA reverse chromosome bands in the preparation [35,54]. However, Yunis [33] reports that using BrdU as a releasing agent can select against some cancer cells and in high concentrations (>10 μg/mL) can produce differential stretching of chromosomes, which leads to artifactual homologue discrepancies. Numerous other methods produce synchrony [51] (see Table 2.4).

Excess thymidine inhibits DNA synthesis by feedback effects on the synthesis of other nucleotide precursors.

Excess thymidine inhibits DNA synthesis by feedback effects on the synthesis of other nucleotide precursors. These blocks can be released by washing cells and reculturing with ordinary medium, by adding the nucleoside 2-deoxycytidine [52], or by washing and incubating in BrdU. The advantage to this last method is that it requires no toxic chemicals. The method Sutherland reported for inducing fragile X with excess thymidine and no release [55] has also been adapted for obtaining longer chromosomes [56]. Excess BrdU probably works like an excess of its analog, thymidine. Because the block is not complete, some laboratories use the high thymidine method with no release step, and subsequent harvests may have reduced mitotic indices but long chromosomes. Also, releasing the block by washing off excess BrdU and incubating in normal medium can produce G-bands and R-bands in the same preparation with acridine orange staining or FPG (fluorescence plus Giemsa) methods [35]. Eichenbaum and Krumins [41] use this technique for amniotic fluid cultures. Cold synchrony has also been used for blood and bone marrow preparations [57]. Yu et al. [58] use a cold synchrony for 24-hour CVS preparations. Sha et al. [36] use a one-step S-block method for prenatal diagnosis in amniotic fluid cultures using a 3-hour pre-harvest exposure to high concentrations of BrdU, thymidine, and Colcemid® (200 μg, 0.3 μg, and 0.25 μg/mL, respectively).

The timing of the block is usually 16–20 hours. Less time may not yield enough blocked cells, and more time may cause cell death owing to the prolonged absence of thymine [59]. At the end of the S block, the old procedure was to centrifuge and wash cells in medium, and then reculture the cells in medium supplemented with agents that would facilitate release, such as thymidine. We do not find the washing steps critical, and Barnes and Maltby [60] report a similar observation. Lew [61] reports a release method for amethopterin-blocked cells in which thymidine is simply added to the blocked cultures. This is the thymidine release method our laboratory uses. Webber and Garson [52] report that washing after FdU synchrony is unnecessary. Wheater and Roberts [37] report that a thymidine block may be released by simple addition of 2-deoxycytidine, without washing. They believe that the washing steps can adversely affect mitotic index and chromosome length. It is important that medium used to reculture blood cells not have phytohemagglutinin because PHA is unnecessary at this point in the culture and can cause red cell agglutination.

Note that cells are arrested at whatever point they were stopped in the 6- to 9-hour synthesis period. Holmquist and Motara [19] report that the block seems to occur at the boundary between the early- and late-replicating chromosome synthesis periods, whereas Camargo and Cervenka [62] and Richardson et al. [63] report that arrest occurs at the late G/G2 stage. Drouin et al. [51] report that methotrexate, high thymidine concentration, and FdU may all cause accumulation of cells both at the G1/S interphase, and at the R/G transition (point at which R-positive bands stop replication and G-positive bands begin replication, about one half to two thirds of the way through the S phase). When released, they must go through the remainder of that S period and then continue through the G2 period, which takes another 2–5 hours. This variation in the arrest point explains why cells are not more precisely synchronized.

High thymidine concentrations do not prevent entry into S phase, but they inhibit DNA synthesis and prolong the S-phase period. Different cell types have completely different S and G2 times, so that solid tumor cells will require different (usually longer) release times than blood cultures. For blood cultures in our laboratory, we use 4.5 hours release time, which is shorter than what we used several years ago with a cooler incubator (37 °C versus 37.5 °C). Blood culture release time varies from 4.5 hours to 5 hours and 10 minutes [64]. In our laboratory, solid tumor cultures are released for 6–7 hours, with Colcemid® added for the final 2–4 hours. Morris and Fitzgerald [65] report that leukemic marrows have different cell cycles than normal hematologic specimens and that release times for these marrows may be disease- and patient-specific. Webber and Garson [52] recommend 7–8 hours release time for leukemic marrow cultures, and Gibas et al. [53] use a 7-hour release time for FdU-synchronized CVS samples. Barnes and Maltby [60] suggest that culture variables, such as serum type, may affect cell cycle time.

There are reports of synchrony of solid tumor [33,65] and CVS 24-hour cultures [53], but synchrony is underutilized for many monolayer-type cultures. We have seen in our laboratory two testicular stromal tumors with clonal abnormalities found only in synchronized cultures. The finding of abnormal clones only in synchronized cultures has been true in our laboratory for other tumors as well; the synchrony method reveals abnormal clones that are not as common or are not present in other harvests. There may be some abnormal periodicity in the abnormal cells, or the synchrony may simply enrich the culture for abnormal cells by collecting them all night during the block.

One disadvantage of synchrony techniques is the fact that cultures to be harvested on Monday must be synchronized on Sunday afternoon or evening, when many clinical laboratories are not open. By varying bone marrow and blood culture setup times and blood culturing periods (72–96 hours), most technologists can accommodate synchronized samples in the work schedule. Blood cultures may be stored at refrigerator or room temperature for a day or more before culturing. Bone marrow or suspension tumor specimens arc best stored in culture medium in the refrigerator until the day before harvest and then cultured overnight with amethopterin or another synchrony agent. Our laboratory makes use of an on-call technologist for the weekend, and one of the duties of the technologist is to add amethopterin on Sunday to cultures that require synchronization.

Yunis [33] and Webber and Garson [52] suggest that for best results, bone marrow cells should be incubated for 3–8 hours before the addition of amethopterin so that the cells tolerate the blocking agent better. This is not always practical, however,

and we do not always follow this practice for bone marrow samples received late in the day; they are simply put in culture and immediately synchronized. We suggest performing an unsynchronized culture for all samples. This ensures results and controls against unforeseen technical problems, and it is especially important in bone marrow cultures because they tend to vary widely in cell cycle and in their response to cytotoxic agents.

The shelf life of stock solutions of amethopterin and thymidine, in our experience, seems to be almost indefinite at refrigerator temperatures. Gloves should be worn with all anticontraction chemicals, because they all interact with DNA and may be dangerous.

2.3.11 Additives to prevent chromosome contraction

Numerous chemical agents bind to or intercalate into DNA or chromatin and, when added for periods before fixation, are capable of preventing normal chromosome contraction during metaphase [35,36,38,40,42,66–72,74,75]. Some of these agents show a preference for certain areas of the chromosome. BrdU and 5-azacytidine preferentially bind to G–C-rich areas, and Hoechst 33258, DAPI, and distamycin A show a preference for A–T-rich regions. These chemicals inhibit contraction differentially rather than lengthening the chromosome homogeneously [42].

Actinomycin D (dactomycin), acridine orange, and ethidium bromide seem to bind uniformly and prevent contraction of chromosomes homogeneously, although Schollmayer et al. [54] found differential condensation using acridine orange. Because long-term cultures are considered more difficult to synchronize, ethidium bromide and actinomycin D techniques have been developed to elongate chromosomes of fibroblasts [39], pleural effusions [73], and amniotic fluid cultures [76]. Some of these agents decrease mitotic index, but this disadvantage may be offset by increased metaphase quality. Agents may be added to the culture before or at the time of addition of the mitotic inhibitor or during the hypotonic period (Table 2.6). 9-Aminoacridine has also been shown to intercalate chromatin and yield longer chromosomes than treatment with ethidium bromide [75].

Table 2.6 Chemical additives used to produce elongated chromosomes

Ref. no.	Author	Tissue type	Chemicals used	Concentration	Exposure time	Mitotic arrest
74	Dewald and Dines	Pleural effusions	AMD[a]	5 μg/mL	1 h	Last hour
43	Yunis J.[b]	Lymphocytes	AMD	5 μg/mL	1 h or more	Colcemid®, last 10 min
42	Yu et al.	Human fibroblasts	AMD	2 μg/mL	1 h	Velban 0.01 2 g/mL, last h
40	Ikeuchi T.	Lymphocytes	EB[c]	10 μg/mL	2.5 h	Colcemid®, last hour
76	Hoo et al.	Amniocytes	EB	5 μg/mL	4.5 h	Colcemid®, 0.6 μg/mL, last 1.5 hour
68	Latos-Bielenska and Hameister[b]	Lymphocytes	EB AMD AMD + EB Hoechst 33258 Hoechst 33258 + AMD Hoechst 33258 + EB Hoechst 33258 + EB + AMD	2.5×10^{-5} M (MW = 394.3) 2 μg/mL 2 μg/mL; 2.5×10^{-5} M 60 μg/mL 60 μg/mL; 2 μg/mL 60 μg/mL; 2.5×10^{-5} M 60 μg/mL; 2.5×10^{-5} M; 2 μg/mL	2 h 2 h 2 h 2 h 2 h 2 h 2 h	Colcemid®, 0.7 μg/mL, 7 min Colcemid®, 0.7 μg/mL, 7 min Colcemid®, 0.7 μg/mL, 7 min Colcemid®, 0.7 μg/mL, 7 min Colcemid®, 0.7 μg/mL, 7 min Colcemid®, 0.7 μg/mL, 7 min Colcemid®, 0.7 μg/mL, 7 min

[a] Actinomycin D.
[b] Used in combination with an amethopterin/thymidine or BrdU cell synchrony technique.
[c] Ethidium bromide.

2.3.12 Combination of synchrony and additives for longer chromosomes

Several successful methods have been developed for chromosome elongation using a combination of synchrony and anti-contraction chemical additives. Yunis [33,40] reported use of amethopterin blocks followed by release with thymidine or BrdU and adds prefixation agents, such as, acridine orange or actinomycin D, to peripheral blood and tumor cell cultures. BrdU release with 1 hour or more of actinomycin D (AMD) inhibition yielded up to 2000 BPHS [43], and Yunis hypothesizes a synergistic effect between BrdU and AMD on elongation. Little or no decrease in mitotic index, no differential contraction, and few chromosome breaks are reported with this technique. Yunis prefers a high concentration of BrdU [40] in order to achieve more bands, and recommends a slightly longer release (5.5 hours). Perhaps with the use of combination techniques, the release times are less critical owing to the anti-condensation action of the added agents. Latos-Bielenska and Hameister [74] use various combination techniques (synchrony plus BrdU, Hoechst 33258, ethidium bromide, and AMD) successfully in a clinical laboratory. They recommend using several techniques for each patient to allow for patient response variables. Our laboratory uses a combination of amethopterin synchrony and ethidium bromide on peripheral blood cultures routinely with great success.

Other methods to enhance chromosome elongation and quality are harvesting manipulations, such as special hypotonic solutions (e.g., Ohnuki hypotonic and cancer hypotonic solution; Table 2.4), reduced time and concentration of Colcemid® to reduce band coalescence, and cold or higher alcohol content fixatives.

2.3.13 Automatic harvesting devices and slide-making chambers/drying chambers

The high workloads of modern cytogenetics laboratories made the automation of the harvesting and slide-making [25] steps important. Besides saving time, they also contribute to the consistency of cytogenetic preparations. There are several automated harvesting machines available, and a number of slide drying cabinets that control humidity and air temperature for optimizing the conditions for each sample type.

2.4 Slide-making

Once cells have been well-fixed in 3 : 1 methanol–acetic acid, they are dropped onto glass slides and dried using specific conditions for optimal chromosome spreading and morphology.

Although most of the variables for good slide-making are well understood, there is still a craft-like aspect to the procedure, making it impossible to verbalize all the necessary skills. One must simply practice until the intuitive part of the skill is mastered, and there will always be some technologists with a better feel for it. Laboratories may find slide-drying chambers both useful and cost-effective, because they allow environmental conditions to be set to specified requirements. Meanwhile, manual slide-making with adjustments for ambient conditions, whatever they may be, is still a necessary skill in many laboratories, and an understanding of manual slide-making is the basis for making slides under any circumstance.

2.4.1 History of slide-making

In the early days of slide-making, before banding methods, we were much less constrained in our methods to get chromosomes to spread out, because nonbanded chromosomes always stained well. In the 1950s and early 1960s, chromosomes were spread by squashing the cells (Belling, 1921, using plant material) [77] between slide and coverslip. If done with a stain such as aceto-orcein, a temporary wet mount preparation was obtained. Alternatively, the cells could be squashed in 50% or 60% aqueous acetic acid, and then the coverslip removed. The resulting slide could be stained with Giemsa. Some of the material, however, stuck to the cover glass, and even the elaborate methods devised to cope with this problem, such as, freezing slides on a block of dry ice or plunging the squashed, coverslipped slide in dry ice-cold methanol before popping the coverslip off with a razor blade [78], were not always helpful. Partial metaphases were the rule. The squash method was still being used for intact tissues (e.g., solid tumor direct preparations) in the late 1960s. It lost favor because the aqueous acetic acid fixatives that had to be used to soften cell membranes precluded banding patterns.

The air-drying slide-making methods we have today evolved in two steps. The first was when Rothfels and Siminovich accidentally allowed some in situ-harvested slides of monkey cultures to dry out by evaporation; they found that the chromosomes were spread out in a single plane without mechanical force [79]. Nowell [7], who described the use of PHA for lymphocyte culture, still used the squash method of slide preparation. It was Moorhead et al. [8], who modified Rothfels and Siminovich's air-dry techniques for lymphocyte cultures to give the slide-making method we still use four and a half decades later.

The fixative used by early workers was 3 : 1 : 1 methanol–acetic acid–chloroform and was known as Carnoy's fixative. The chloroform was found to be dispensable for cytogenetic purposes and was no longer used in fixatives after the 1960s. Three to one methanol–acetic acid is sometimes called modified Carnoy's fixative since it has no chloroform.

Early slide drying methods involved manipulations to improve spreading such as flaming cell preparations. One method was to dip slides in 95% ethanol, drop the fixed cell suspension onto the alcohol, and pass the slide through Bunsen burner or alcohol flame. Another was to drop slides in dry-ice-cooled water, remove the slide and then drop cells on the slide, ignite the fixative in a flame, and dry the slide on a hotplate. The idea of flaming is to effectively boil the cell open to spread the chromosomes. Some of these methods denature chromosomes and preclude banding, but if done very gently, flaming is still a method that can be used for certain extreme problems, such as spreading tumor cells with large ploidy numbers and refractory cytoplasm. However, in the early 1970s, to accommodate the new banding methods, most laboratorians learned to use air-drying methods instead of flaming. Slides were made by dropping the fixed cells onto wet slides and allowing them to dry by evaporation.

At this point in the development of air-drying techniques an inexplicable variability in chromosome spreading became apparent: something occasionally went wrong with the slides, with the chromosomes perhaps spreading well one day but not the next day. John Melnyk at the City of Hope in Duarte, California, was one of the first to uncover the reason. He was attempting to design an automatic slide-making machine, and he used compressed air to blow air on the cells to cause evaporation of the fixative. He found that some tanks of air worked better than others and traced the poor result to low water content in the compressed air in the tanks that had been filled with air on a dry day. When the cells dried too fast in the presence of the dry airflow, there was insufficient relaxation of the cytoplasmic membrane for the chromosomes to flatten out and move out from the center of the cell. The result was a pile of chromosomes and a crinkled, film-like membrane over them. City of Hope is located in a southern California desert environment, and the worst days for slide-making were when a dry desert wind blew.

Technologists in the early to mid-1970s were also discovering this correlation in slide-making with humidity, often from the other perspective. In the eastern, southern, and midwestern regions of the country, the humidity became so high at times that the cells dried too slowly, causing poor banding, tight cells, cell loss due to scattering chromosomes, and other difficulties. Consequently, various methods were developed to improve air-drying methods for slide-making, and most of these deal with relative humidity, which is linked to air temperature. (Warmer air is capable of holding more moisture than cold air. Thus, relative humidity is not a straight percentage but rather is the percentage of water present in the air compared to what air at that temperature is capable of holding.)

These correlations have been explored in controlled experiments by Spurbeck et al. [80], who measured cell spreading (cell volume) in relation to increasing humidity and temperatures, and found a positive correlation between increased humidity and spreading at a set temperature, up to a certain threshold. Beyond a certain optimal humidity, cells begin to lyse, spilling chromosomes out, and only the resistant, small (tightly spread) cells remain intact. This fits well with the observations made by technologists on a more intuitive level. The authors also reported that warmer air yields more spreading due to the ability of warm air to hold more moisture. However, warm air created in winter by building heating systems will drive moisture out of the air, and therefore, these warm air conditions will usually yield less spreading.

2.4.2 Theory of slide-making

Air drying of cells that are fixed in 3:1 methanol–acetic acid is based on the theory that chromosomes, which are contained in cells that are much enlarged and have much thinner cell membranes than before the harvest, will be supported by the layer of fixative on the slide in the first few seconds after application to the slide. Then, as the fixative evaporates, the layer of fixative becomes thinner and the meniscus pushes down on the top of the cell, enlarging the area of the cell and pressing the metaphase chromosomes between the upper and lower membranes, spreading them out (see Figures 2.13 and 2.14). This physical relaxation and collapse of the cell membrane takes some time, but this slow process stretches the chromosomes, thus determining to some extent what the banding level will be [81]. If the fixative dries before the cytoplasm has relaxed and cells have spread out, there will be a visible cytoplasmic background and the cells and chromosomes will be thicker (darker on the phase contrast microscope and after staining), shorter, and often poorly spread out [80]. The thick cytoplasmic covering will usually prevent good staining or in situ hybridization, and the stained chromosome morphology will be unsatisfactory in many cases (see Figure 2.15). Well-spread metaphases also have a cytoplasmic membrane intact over the cells, but it is very thin and requires special stains to visualize, such as 0.2% fast green [17]. If drying takes a very long time, the weakened cytoplasmic membrane may develop holes or may rip open, spilling some or all of the chromosomes out and losing the metaphase to analysis or causing pseudohypodiploidy (see Figure 2.13). On such preparations, it is not uncommon to see cells that are poorly spread, as well. This may be due to the rolling of the cells in the layer of fixative, which has currents and other forces causing cell motion. A cell that is rolling at the time of final drying has not relaxed and will appear tightly spread [80] (see Figure 2.13). This theory has been systematically developed, explored, and supported by the experiments of Spurbeck et al. [80]. The cell rolling can explain occasions in which the opposite occurs: very high humidity may sometimes yield a cytoplasmic background that can be alleviated by decreasing drying time (our technologists call this a "backward day"). Low humidity has been reported to cause scattered chromosomes [82], an exception to the paradigm. We conclude that slide-making is an empirical

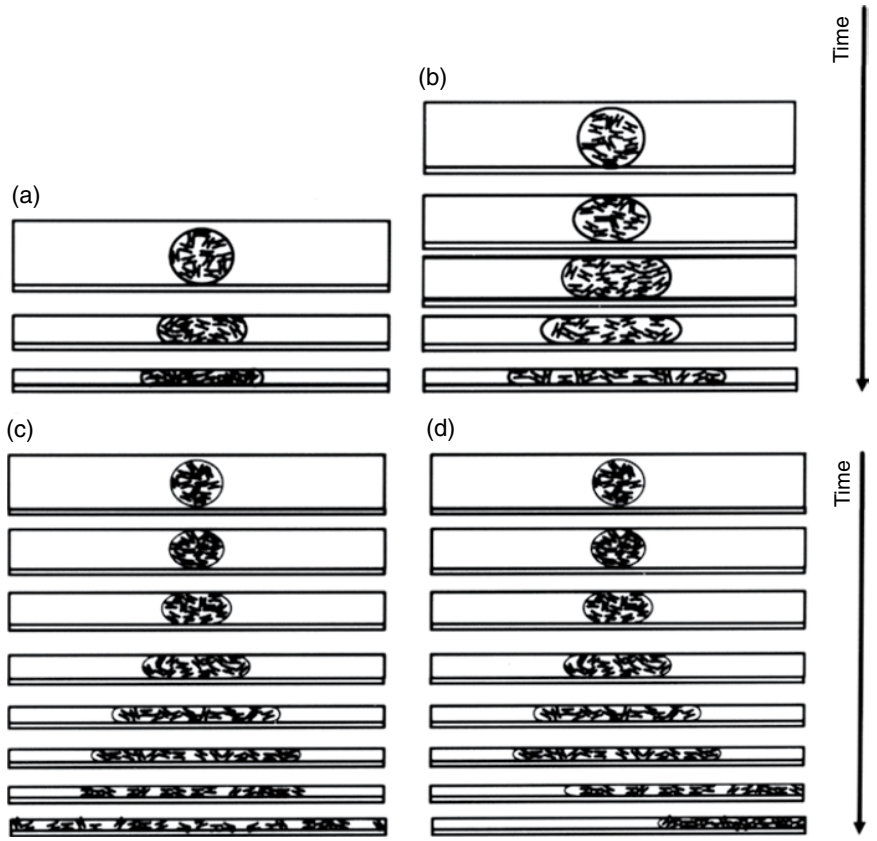


Figure 2.13 Chromosome spreading and drying time. Metaphases spread as a function of the duration of drying time. This figure illustrates a side view where the top of the fixative solution is represented by a single line, and the slide surface is represented by a double line. Time is represented from top to bottom. (a) Metaphases that dry too fast are often tight with many overlapping chromosomes. (b) Metaphases that dry at the optimum rate (for a top view, see Figure 2.14) have few overlaps and are not broken. (c) Metaphases that dry too slowly are characterized by both broken metaphases and (d) by tight, "rolled" metaphases. Courtesy of Jack Spurbeck. Reprinted from Spurbeck J. Dynamics of chromosome spreading. In: Spurbeck JL, Zinmeister AR, Meyer KJ, Jalal SM. *Am J Hum Genet* 1996; 387–393, © 1996, John Wiley and Sons, with permission.

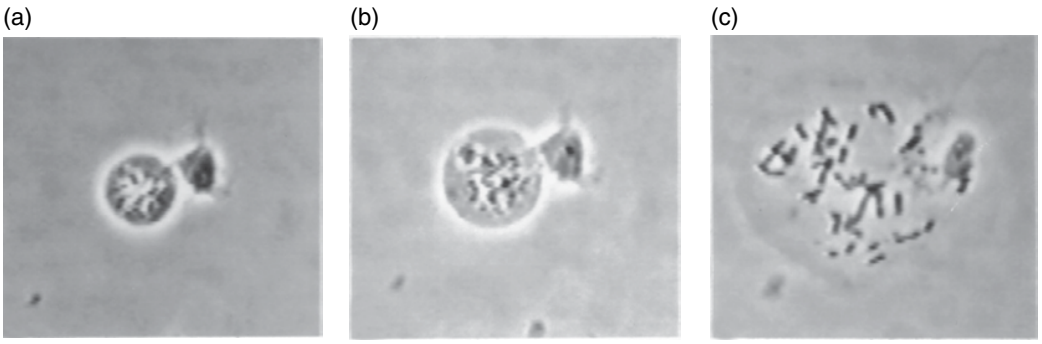


Figure 2.14 An amniocyte cell during the drying process. Videotape images of an amniocyte during the drying process at early (a), intermediate (b), and near-optimum (c) stages. Courtesy of Jack Spurbeck. Reprinted from Spurbeck J. Dynamics of chromosome spreading. In: Spurbeck JL, Zinmeister AR, Meyer KJ, Jalal SM. *Am J Hum Genet* 1996; 387–393, © 1996, John Wiley and Sons, with permission.

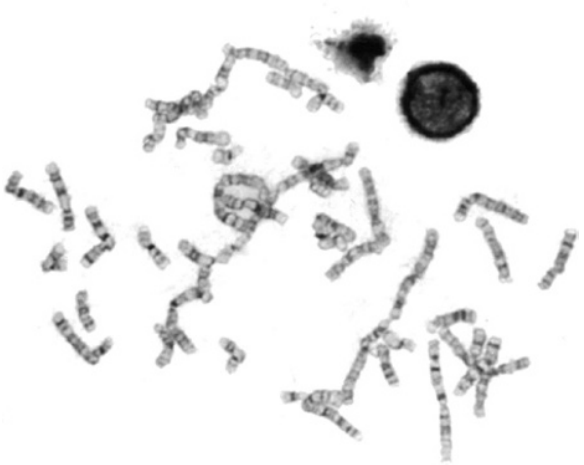


Figure 2.15 G-banded metaphase from poor slide-making. This picture demonstrates a G-banded metaphase that dried too fast and is encapsulated in the cytoplasmic membrane. Chromosomes did not have time to spread out completely, and trypsin digestion is uneven due to interference by proteins in the thick membrane over the chromosomes.

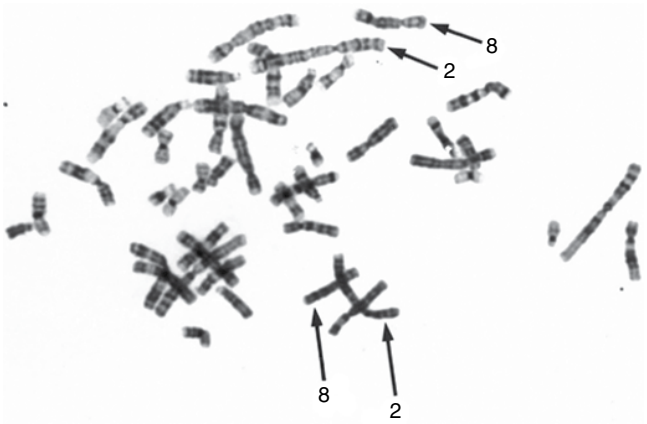


Figure 2.16 Differential drying. One side of the cell has dried at an optimal rate, and the other side has dried too fast, creating more contraction of chromosomes on that side. Note, for example, the two chromosomes 2 and the two 8s (arrows).

and qualitative skill, at least by today's level of understanding. There may be effects by other environmental factors yet unknown. Barometric pressure is probably not one of these factors, because Spurbeck et al. did test for barometric pressure effects, and did not find it to be an important variable (in the range of 29.74–30.03 inches of mercury). Claussen et al. [83] did some work on fixed cells drying on slides under different atmospheric conditions, and found that spreading involves significant water-induced swelling of mitotic cells during evaporation of the fixative from the slide.

Once the methanol–acetic acid-fixed chromosomes are dried onto a glass slide, they stick fast until physically scraped off, and staining may be accomplished without losing cells from the slides. Further changes in spreading are not possible after cells dry, although Claussen et al. [84] has discovered that chromosomes may be stretched with a micromanipulator to exhibit more bands. Cells fixed in other fixatives, such as formalin-fixed paraffin-embedded cells, do not stick to glass upon drying unless the glass is treated by coating (e.g., silanizing) or the glass is positively charged. Such preparations are often used for interphase FISH studies.

The length of chromosomes is somewhat affected by slide drying, as evidenced by cells that have dried differentially, with various band levels on different sides of the cell (see Figure 2.16). Differential staining is usually due to differential drying

speeds on opposite sides of the cell, and can be caused by debris nearby, uneven heating, water remaining in the vicinity of the cell, oil on the slide, or draw lines on the microscope slide created in the manufacture of the glass, among other variables. Drying can also differ from the inside to the outside of the cell for the same reasons. Unstained chromosomes can be viewed using a phase contrast microscope, and most slide-making protocols call for an assessment of spreading and chromosome spreading, morphology, and contrast using such a microscope, or using an ordinary light microscope with the condenser adjusted to view shadows of the unstained cells. Chromosomes may appear gray to black, depending on drying conditions, and the resulting banding quality will reflect the contrast seen in the unstained preparations [85] (Figure 2.17). We feel that this phase contrast microscopic assessment is one of the most important parts of the harvesting process.

The goal of the slide maker is to obtain chromosome slide preparations that have the following characteristics on the phase microscope:

1. Mostly medium gray to dark gray chromosomes. Light gray chromosomes yield trypsin-sensitive cells with poor contrast between bands, and very black phase contrast may have increased cytoplasmic background and be resistant to trypsin, although these banding guidelines may be different in some laboratories.
2. Little chromosome scattering (overspread, broken metaphases) because this can cause diagnostic problems when mosaicism is suspected or can mask true mosaicism at a low percentage.
3. Minimal number of chromosome overlaps, for accurate counting and band analysis.
4. Very thin or absent cytoplasmic background, if possible, so that trypsin G-banding will be optimal (see Figures 2.12 and 2.14).
5. Proper cell density on the slide: too few cells on slides can result in preparations that are time consuming to scan for metaphases. Slides that are too dense may interfere with spreading and G-banding, and FISH preparations from these slides may be difficult to interpret due to crowded signals and confusion as to which cells have been scored in a given field.
6. Chromatids that are together, not split apart. This may also be a function of the Colcemid® and hypotonic steps.
7. Absence of debris, such as glass chips from pipettes or slides, glove powder, diatoms from pipette bulbs, or bits of tissue from explants, which will interfere with proper slide drying.

2.4.3 Slide-making variables

Normally, on a good day, one should be able to drop fixed cells onto a clean, wet slide at a 20–30° angle lengthwise on a paper towel, drain the excess water off, maybe flood the slide with a little fixative to improve water removal and produce a consistent drying surface, and dry the slide flat or at an angle to get good preparations. If this does not do the trick, manipulations to improve spreading and morphology are numerous, and include the following variables. (Note: if an entire harvest yields consistently poor quality preparations, regardless of specimen type or environmental conditions, there may be a problem with the culturing or harvesting conditions. This could include too large an inoculum of cells in the culture, or hypotonic or fixative that was made incorrectly or contaminated with some other chemicals.)

2.4.4 Wet versus dry slides

Some laboratories prefer to drop fixed cells on wet slides, which facilitates spreading due to the immediate retraction of the water meniscus as soon as the acid alcohol hits it. Holmquist and Motara [19] report that by dropping cells onto wet slides, the energy of dehydration from fixation is returned as a change in the free energy of mixing between fixative and water, which spreads the cells. Others prefer to use dry slides, and under good environmental conditions, this method should work [80]. If wet slides are used, the retreating water should be eliminated by immediate draining onto paper towels, blotting with KimWipes, and flooding with fixative. Residual water spots cause spreading and staining irregularities due to localized drying time variations. For coverslips in dishes, it is crucial to completely dry the edges of the coverslip by diligent withdrawal of residual fixative. This ensures a consistent drying milieu across the coverslip and reduces cell breakage from waves of drying fixative.

Wet slides may further facilitate spreading control by the use of different temperatures of water coating to speed up or slow down drying time. Cold, wet slides will slow drying, increasing spreading, whereas higher room temperature or warm wet slides will accelerate drying time. The thickness of the water film may be varied by draining the slide on a paper towel more or less thoroughly, and this can help control spreading by using a thicker film of water on very dry days. Also, extremely fragile metaphases, such as acute lymphocytic leukemia bone marrow specimens, may yield more metaphases with very thin water layers, or even just by coating slides with moisture from the breath.

A method to ensure complete removal of water from the slide and uniform drying across the slide is to flood the slide with a few drops of fixative after the cells have been applied to the wet surface. This also may aid spreading by exerting pressure on the upper cell membrane to encourage the flattening or relaxation of the membrane to cause more spreading. Some laboratorians use slides that have been coated with fixative instead of water.

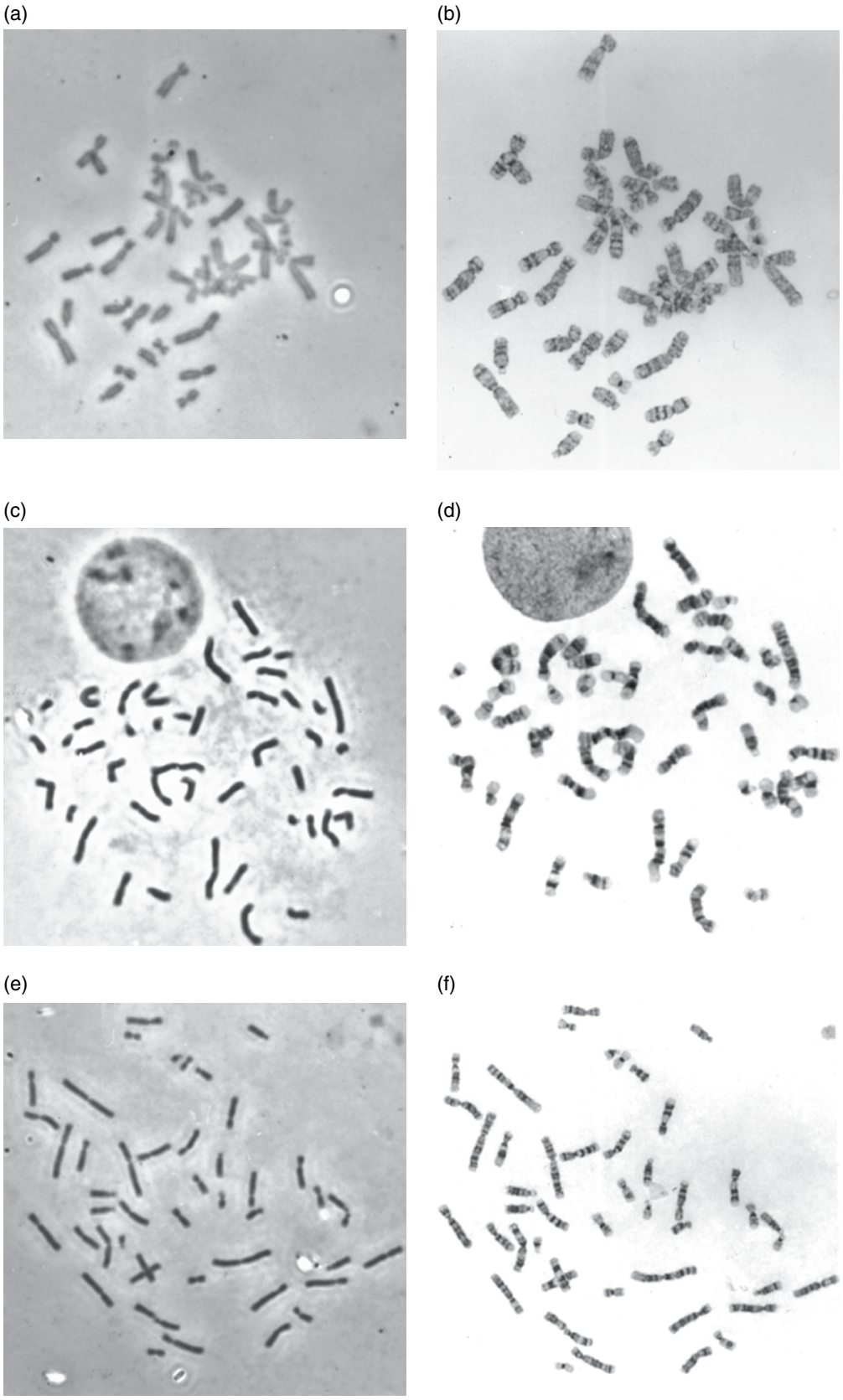


Figure 2.17 G-band quality comparison. Three cells are shown with different phase contrast images and their subsequent G-banding quality. (a) Too gray on phase contrast image. (b) Subsequent G-bands also lack contrast. (c) Too dark on phase contrast. (d) Subsequent G-bands are harsh, with high contrast. (e) Nice, medium dark cell on phase contrast. (f) Subsequent cell shows good contrast and chromosome morphology.

2.4.5 Angle of the slide

Some technologists drop cells onto horizontal slides, but they are in the minority. Most technologists allow the cell suspension to travel after it hits the slide, by angling either along the long axis (e.g., label end up or down) or along the short axis (e.g., one long edge down). Spreading may improve when cells travel and roll as the fixative level first becomes thin, although this is undocumented and does not apply to in situ harvest slide drying. The effect of dropping cells on slides angled along the long axis is that the water and fixative drain from the upper end of the slide to the lower end and pool there; this may cause faster evaporation at the upper end and drying may be uneven, both between cells at each end and within cells, giving different banding responses to trypsin from cell to cell and even within a metaphase (see Figure 2.15). Slides made with one long edge down tend to be more uniform, especially if the angle is kept at 20–30°, and cells are placed in the upper one-third of the slide and allowed to move downward. Cell suspension drops should not pool at one edge of the slide but should be in uniform circles of spreading that are about 20 mm in diameter and well centered on the short axis. Greater tilt angles may speed up the drying process at the upper end of the slide compared to the lower end, giving a somewhat uneven, inconsistent slide. Uniform drying can be achieved by tilting slides at one angle for part of the drying time and another for the final drying. Changing the angle of the slide and rapping the slide edge on the bench top are said to improve cell spreading for some technologists.

2.4.6 Ambient humidity and temperature

It is important to remember that relative humidity is the percentage of moisture actually in the air compared to its maximum potential saturation at that temperature; therefore, since warm air is able to hold more moisture than cold air, a relative humidity value of 50% means a much lower absolute moisture content for cold air than for warm air. Thus, slides spread differently according to air temperature, given the same relative humidity. Higher ambient temperature usually (but not always) translates into better spreading. For drying cells from in situ harvests, it is humidity that affects results over all other variables, with best results obtained at 5–10% higher humidity than that which would be ideal for suspension harvests. In dry air, fixative evaporates fast, whereas in moist air, evaporation occurs slowly. Neither fast nor slow evaporation is ideal, because either can result in poor phase contrast and spreading, scattered chromosomes, or visible cytoplasmic background around the metaphase. Drying time should be roughly 30–45 seconds, based on our experience. In dry climates, it is necessary to slow drying times, and in humid climates, to speed them up. Some ways to provide humidity are to dry slides in front of a humidifier, on top of wet paper towels, or over steam in a sink. In very humid, damp climates, a technique that encourages drying is needed, such as the use of 6:1 methanol-acetic acid fixative, as reported by Yunis et al. [64]. Note that his work was performed in Minnesota, where humid summers are not uncommon. Some areas of the country experience drastic climate changes during the year, and the technologist must become adept at handling all types of conditions. The most difficult situation is accommodating for rapidly changing weather conditions when making slides. Consistency is very difficult in such circumstances, and it may be best to leave slide-making until conditions stabilize.

The ideal relative humidity for slide-making can vary owing to differences in temperature, hygrometer accuracy, technique, and specimen type. In our experience, harvests from attached monolayer cultures tend to have more fastidious moisture requirements for good drying than the blood and bone marrow specimens, and the in situ-harvested samples seem to spread better at high humidities (e.g., 50–55%). Lundsten and Lind [86] suggest 20 °C and 45% humidity, with the optimum range between 40% and 50%. Spurbeck et al. [80] find 50% humidity and 25 °C to be the optimum setpoints, and they lower the humidity to 35% if chromosome scattering is a problem. Yu et al. [58] recommend 25 seconds of drying time for high-resolution preparations. The experienced technologist can usually tell if the humidity is not optimal by simply observing the drying time of the slide and the phase contrast image that results. Laboratory technologists have found ways to deal with environmental conditions that affect slide-making. For example, laboratories in the East and Midwest often use dehumidifiers in their slide-making area to compensate for high humidity. Humidifiers are also useful in many parts of the country, especially in winter, when the air is dried by heating systems. These may range from one or two small baby room humidifiers available in department stores, to the large, commercial ultrasonic room humidifiers with humidity controls (humidistats) that automatically control humidity in entire rooms. Lundsteen and Lind [86] report the use of a specially designed "climate room" for slide-making. Another way of compensating is to have a slide-making cabinet, as it is easier to control the temperature and humidity in a small area than it is in a large room. There are commercially available slide-making cabinets that are very popular. In regions such as the American northwest, it is rare that the air is saturated with moisture as it may often be in other parts of the country, and simply warming slides and/or increasing airflow is usually sufficient to get good preparations.

Other sources of humidity are breathing on slides as if to fog the glass, which gives a different effect than blowing to create air flow; wetting a pad of paper towels on which to place the drying slides (a microclimate just above the toweling is more moist than the ambient air), or if that does not work, placing a slide box lid over the top of the drying slide on the moist towel; using wet paper towels on a hot plate; placing wet cheesecloth over air flow vents in a hood to control humidity

inside the hood; and placing wet paper towels with an inverted Petri dish over in situ-harvested coverslips. Several laboratories recommend leaving the back of the slide wet rather than drying off excess water droplets. It is sometimes useful to flood the drops of cells with 6:1 methanol–acetic acid instead of the usual 3:1 ratio, and this accelerates evaporation. Sometimes, on very rainy, wet days, slides can look good on phase contrast microscopy but do not G-band well, lacking contrast. Sometimes this can be cured by putting the unstained slides in the hot oven for another round of drying (aging). The temperatures which affect slide-making results are that of the ambient air, that of the slides and water with which they are coated, and that of the surface on which they are dried (hot plate versus forearm versus benchtop). By varying one or more of these temperatures, we control the speed with which evaporation takes place. We have, for example, switched from room temperature fixative and slide rinse water to cold fixative and water in very dry conditions in order to counteract the rapid evaporation. Drying in situ coverslips on wet paper towels with ice around them has been a common technique in many laboratories. Drying slides in a very cold or very warm room can disrupt proper cell spreading and chromosome morphology, and make banding suboptimal. It is important to have control over the temperature of the laboratory. Hansen [87] recommends changing the temperature of the water that the slides are coated with before dropping cells on the slides to help control spreading. She uses water from 20 °C to 48 °C and prefers 41 °C to 47 °C for most slides.

2.4.7 Fixative ratio

The alcohol in the fixative is a cell-hardening agent, and the acetic acid is a softening agent. By changing the ratio of these two components, the cell membrane can be strengthened or softened to get less or more spreading, respectively. Thus, some laboratories might choose to use 2 : 1 methanol–acetic acid instead of the usual 3 : 1 ratio for bone marrow cultures that are difficult to spread, or for in situ harvested amniocytes. Alternatively, they might put amniocytes into 6:1 methanol–acetic acid on days when scattering is a problem. We recommend a conservative approach of keeping with the standard 3:1 fixative unless all else fails, because this yields the optimum banding quality and preserves cells best. We may vary the ratio of the fixative used to flood the slide after the cells are dropped on in order to accelerate drying, on occasion. Multiple fixations can strengthen cell membranes, improve chromosome morphology, and rid the sample of debris such as lysed red cells. Overnight fixation in the refrigerator or freezer can also strengthen cells and make them tolerate slide-making better. Tumors and difficult bone marrow specimens such as ALL marrows often yield better spreading and sharper chromosomes after incubation in the freezer.

2.4.8 Quality and freshness of fixative

Methanol–acetic acid does two things to the cell: as the pH of the cell is lowered, most acidic groups like -COO- become -COOH, permanently denaturing them; then the fixative dehydrates the cell and replaces the water with methanol, altering proteins and DNA [18]. Very dry cells are required for good spreading. Since both methanol and acetic acid are hygroscopic (absorb water from the air), the two components can be contaminated by water in the reagent bottles. The mixture also absorbs water upon standing.

Water contamination in fixative will decrease the spreading and quality of the slide [17]. Also, bad lots of alcohol and acetic acid do occur occasionally. Some years ago, the manufacturer of the acetic acid we had used for years changed the plastic liner material on the bottle. The acid became contaminated with this material, becoming useless for cytogenetic purposes. It is good practice to test new lots on a small harvest before risking a large one.

2.4.9 Height from which cells are dropped

Although a study by Lambson et al. [88] showed no correlation between chromosome spreading and the height from which cells are dropped (between 0.1 and 0.31 meters), increased height is sometimes used to increase spreading. Gibas and Jackson [89] specify the use of increased heights for dropping leukemic bone marrow cells. Our laboratory uses this as a last resort on large-ploidy, tightly compacted cells from solid tumors that will not spread any other way.

2.4.10 Wicking effects

When the slide is held up in the air during cell dropping, the coating of water and fixative are accumulated at the edges of the glass slide. Water can move back onto the cell spreading area and affect drying. Placing the slide on a paper towel with the long edge parallel to the bench, and quickly draining the excess fluid can solve this problem. The slide can be brought back to horizontal and/or the opposite long edge can similarly be drained on the towel. This also seems to encourage cell spreading due to the wicking effect of the slide draining. The goal is a consistent drying surface that leads to a constant rate of drying that in turn yields the most consistent quality spreading across the slide.

2.4.11 Air flow

Cell drying speed can be increased or decreased by changing the airflow over the slide. This can be accomplished by blowing on slides, by waving them in the air, or by using a fan or, as is sometimes used in the in situ method, an aquarium air pump. The air pump has the added advantage of a humidifying capability, when the air is passed through a bottle of water before releasing it over the slides. Most effective slide-drying chambers make use of a small fan that can be adjusted to produce various movement rates. Be aware of air movement in the slide-making area: people whisking past or air conditioning vents may be a source of drying problems. One caveat when blowing on slides to dry them: cells from the mouth can be deposited on the slide, and cause interpretation problems with interphase FISH studies.

2.4.12 Dilution factor

A common mistake in slide-making is applying too many cells on the slide so that there are lots of metaphases from which to choose. Crowded cells do not spread and band as well as optimally diluted cells because of physical crowding and an altered drying kinetic, as well as other factors, such as extracellular RNA and protein. Cell crowding also slows drying time. If spreading is a problem, consider further diluting the specimen.

2.4.13 Slide cleaning and labeling

Slides may be used with success from the manufacturer's package, depending on how clean they are [90]; however, cleaning slides before use facilitates uniformity of spreading and increases metaphase quality and banding quality (any oil on the slide will ruin spreading). Our laboratory cleans slides by placing them in 95% ethanol just before use, wiping them with a tissue three or four times in one direction, and then dipping them back in the alcohol before coating them with water for slide dropping. Some other laboratories prefer to use detergents or combinations of detergents and alcohol cleaning. If labeling manually, be sure to use a number 3 pencil or harder. It is unproductive to clean slides and then allow pencil lead debris to dislodge from the label during staining and stick to the metaphases. Indelible marking pens are also used successfully and avoid this problem. Many laboratories have pre-made labels from the LIS for labeling slides; however, caution must be taken as with any pre-labeling system not to use them on the wrong patient. When converting to pre-printed labels, check them to make sure both the glue and printer ink are resistant to aging techniques, staining and de-oiling procedures.

2.4.14 Slide type

It is remarkable how difficult slide-making can become when changing the type or brand of slides, or when the manufacturer changes something in slide production. Find a brand that holds a uniform layer of water, without streaks or beading up, with no pits or manufacturer's "draw lines" in the glass, and it should produce decent slides. As glass slides age, they oxidize, starting around the edges. Spreading that differs from edge to center is a sign of a slide that is oxidized. Manufacturers are starting to make available to the cytogenetics community slides that are stored under nitrogen inside of foil packages to circumvent the oxidation problem.

2.4.15 Cell and culture type

In our hands, there are certain cell types that lend themselves to slide-making better than others. All uncultured, "direct" harvests (from bone marrow, CVS, or solid tumors) present more difficulty in the making of good slides than their cultured counterparts. Blood specimens tend to be the easiest from which to make good slides, and bone marrows tend to be more difficult, with amniotic fluid specimens in between. Epithelial cultures are more difficult than fibroblast cultures (e.g., certain amniotic fluid or tumor cultures) because they do not seem to swell as large in the hypotonic solutions.

2.4.16 Culturing and harvesting techniques

Culturing variables (e.g., micro versus macro blood cultures, number of bone marrow cells used for inoculum, primary versus secondary or subcultured fibroblast cultures) and harvesting techniques (e.g., type, amount, duration, and temperature of hypotonic solution and use of ethidium bromide) can affect the quality and spreading of the cells and may require some slide-making adjustments. Primary amniotic fluid cultures contain more dead epithelial cells than subcultures do, and these cells may interfere with drying and contribute to scattering and other drying problems. Blood or bone marrow cultures inoculated with too many cells per milliliter of medium will never look as good as they would have had the culture not been crowded.

2.4.17 In situ cell drying

Once residual fixative is thoroughly removed from around the coverslips or off of the chamberslide, the manipulations are performed to assist chromosome spreading according to the ambient conditions, if commercial or other slide-drying chambers are unavailable. If conditions are perfect, few manipulations are necessary. In dry weather, humidity may be supplied as for suspension slide-making (wet paper towels under the dishes, ice to slow the drying, breathing over slides/coverslips, steaming over hot water or humidifier) as well as using moist air created by bubbling air through a flask of water and then gently onto the coverslip/slide.

If no metaphases are seen, the most likely explanation, given a healthy culture, is metaphase breakage, and the next coverslip should be dried faster. If cells are present but chromosomes are encapsulated in cytoplasmic membranes, the next coverslip should be given more humidity and a longer drying time. Coverslips with heavy growth will never give as good spreading in the center of the colonies, as the interphase cells will physically prevent them from relaxing; picking coverslips at the correct cell density is almost as important as manipulating cells during drying.

2.4.18 Slide-making for FISH studies

Several references state that slides for in situ hybridization will hybridize best if they appear gray rather than black on phase microscopy. While this is true, the darker or more cytoplasmic cells may be worked with. First, we flood the slide with fixative and allow it to dry; this step will lighten most cells and make them flatter and more accessible to the probe. The other pre-hybridization steps to deal with cytoplasmic or three-dimensional cells are to soak them longer in 2× SSC before performing the hybridization, and/or to pretreat them with a pepsin step (see Chapter 16, section 16.9.4, Protease Pretreatment for Standard Slide Preparations). Three-dimensional cells may yield a large signal depth of field, requiring a lot of focusing up and down during analysis to detect all signals. During hybridization, difficult specimens may be denatured at higher temperatures and for longer times to be sure that the DNA is fully single stranded. Otherwise, almost all slide-making conditions have yielded good FISH results, in our hands.

2.4.19 Slide aging

When G-bands were first integrated into the clinical laboratory scheme, one troubling aspect of the new method was that only slides that had been allowed to sit for 3 or 4 days were suitable for banding. Until they were aged, chromosomes resisted banding procedures, so turnaround time was lengthened. After experimenting, it was discovered by many laboratorians that heating the chromosome preparations on the slides in an oven or on a hot plate for 12–24 hours at 40–60 °C or 20 minutes at 90–95 °C would obviate the problem, and slides could be G-banded immediately. This process has never been entirely understood, but it seems likely that driving off water is involved. The major change in chromosomes caused by aging may be the oxidation of the protein sulfhydryl groups [91]. Aging of slides is reported to degrade the chromosomal DNA [92], and this may explain why some banding methods and FISH methodologies are so affected by artificial aging by slide heating. All G-band methods, and many other methods as well, are preceded by artificial aging via heating or sometimes microwaving slides. The aging process is very important, as it gives better contrast and crispness even to Q-banded chromosomes, which do not really require pre-aging. Because Q-bands do not require aging, sequential Q-banding to FISH has been useful in our laboratory. It is also important to be consistent in aging (or not aging) slides for other methods, such as C-banding and G-11, to control treatment times. In our experience, slides for FISH can be aged or not. Signals tend to be much sharper and more discrete for aged preparations; however, overaging can make signals dim or may cause failure of the hybridization. Slides that are to be saved for several weeks or months for FISH or other applications may be kept in a desiccated, oxygen-deficient environment (e.g., under nitrogen gas in a Ziploc bag or in liquid nitrogen) at –20°C until needed, in order to keep them from aging naturally. Slides that have been baked for more than 10 minutes in a 90–95°C must be re-hydrated before use for FISH (see Chapter 16, section 16.9.4, Aging and baking slides). It is possible to ruin slides for G-banding by leaving them in a hot oven for too long, (e.g., 90 °C for 2 hours). Because Q-bands do not require aging, sequential Q-bands to FISH are useful in our laboratory.

2.5 Chromosome staining

There are three stains capable of differentiating all chromosomes: G-, R-, and Q-banding (Figure 2.2). The analytical stain of choice in the United States is G-banding, because it is simple and gives a lot of detailed information. Some parts of Europe use R-banding for a primary stain, and Q-banding has been preferred by some cytogeneticists for certain types of study (e.g., hematologic malignancies, and CVS direct preparations, on which it is difficult to obtain good G-bands) because of its independence from pretreatment variables and the usefulness of the bright Y chromosome for determining sex. However,

Q-banding is becoming less important for these applications as laboratories improve bone marrow methods using G-bands, and as CVS direct preparations are being replaced in some laboratories with interphase FISH for aneuploidy on uncultured chorionic villi cells. The Q-band variants are also very useful for ascertaining chromosome origin (see Chapter 6, section 6.2.1, QFQ Clinical significance, and Chapter 10, section 10.3, Germ cell tumors – UPD and imprinting). This was once especially helpful in determining whether engraftment had taken place in post-bone marrow transplant specimens (now interphase FISH is used instead, since much larger cell numbers can be scored), in looking for uniparental disomy of chromosomes 14 or 15, and to demonstrate complete isodisomy in teratomas, among other things.

Other stains that may be used in certain cases include AgNOR (silver) for staining acrocentric stalks with active nucleolar organizing genes; C-banding to stain the genetically inert heterochromatin; G-11 to stain a subgroup of heterochromatin and differentiate between, for example, chromosomes 19 and 20 or X and Y; C-dot stain to distinguish centromeric areas; endonuclease stain (Alu-banding, etc.) to allow specific patterns of heterochromatin; and FISH with various probes to discover the origin, makeup, and order of chromosomal and subchromosomal units (see Chapter 6, Chromosome stains; Chapter 16, Fluorescence in situ hybridization). After staining has been accomplished, slides are examined and metaphase cells are photographed or electronically imaged (Chapter 15, Computer imaging), chromosomes are analyzed (Chapter 7, Human chromosomes: Identification and variations; Chapter 9, Constitutional chromosome abnormalities; Chapter 11, section 11.7.2, Cytogenetic methodology; Chapter 12, section 12.2.16, Chromosome analysis), and reported (Chapter 8, ISCN: The universal language of cytogenetics; Chapter 23, Laboratory information system).

2.6 Chromosome microscopy/analysis

Once chromosome preparations have been banded, usually with G-banding in most laboratories, chromosomes are analyzed under a microscope for both numerical and structural aberrations. The slide is scanned in a methodical, field-by-field manner, either by traveling up and down or right to left on the slide using a low power lens (10–16×), searching for metaphase cells. Once found, each cell is examined under high power (60× or 100× lens, usually oil immersion type). After determining whether the cell is of reasonable quality, it is counted, and at least partially analyzed, looking for sex chromosomes and checking other chromosomes of interest, depending upon the reason for referral. For example, for a referral of CML, the 9s and 22s would be checked routinely for the translocation between 9 and 22 long arms that characterize the disease (see Chapter 11, section 11.4.1, Chronic myelogenous leukemia). For a referral of Down syndrome, the focus would be on detecting an extra copy of chromosome 21 (see Chapter 9, section 9.1, Numerical abnormalities). The stage coordinates for each cell are recorded on the analysis sheet along with the chromosome count and any abnormalities seen, as well as the sex chromosome makeup and any other pertinent findings. Often, a notation of the position of certain chromosomes is recorded for each cell, so that it can be reidentified at another time or by another technologist. For example, the chromosome closest to the 6 and 12 o'clock positions or 3 and 9 o'clock positions may be recorded; this is usually unique for all the cells of one study. Some cells are completely analyzed under the microscope to ensure that each chromosome matches its pair (homologue), and also to determine if all the bands are present and in the correct order. Experienced technologists can tell all chromosomes apart by size, shape, and banding patterns. Chromosomal banding patterns are best learned by repeated karyotyping, using a good example of chromosomes at various lengths (see Chapter 7, Human chromosomes: identification and variations).

A suitable cell is usually reasonably well spread and well banded, although quality may be compromised in various disease states; however, in cancer analyses, it may be the poor cells that will demonstrate clonal abnormalities. Once a suitable cell is found, it is counted by one of several methods:

1. Locating the cell under the high-power oil lens of a microscope and counting in a systematic fashion, by mentally dividing the cell into halves, quadrants, or other subdivisions, and counting one subdivision at a time, continuing until all subdivisions are completed. Counting may be done in ones or twos. Usually, cells counted by this method are recounted to confirm the original chromosome number.
2. Printing a photograph or captured image of the cell from an imaging system, and then marking the print with a pen or marking crayon to facilitate accuracy.
3. Electronically counting the image using an imaging system software feature; this marks all chromosomes by mouse-click and keeps a tally.
4. Preparing a karyogram from a captured image, either by manually cutting out each chromosome from a print or by using imaging software electronically "cut" the karyogram or to mark each chromosome on a spread as it is analyzed on the screen. Usually, cells counted by methods 1–3 are recounted to confirm the original chromosome number.

5. To determine the cytogenetic makeup of each patient's sample, multiple cells, usually twenty per case, are counted (e.g., most routine cytogenetic referrals involving a constitutional abnormality) and/or completely analyzed (e.g., all neoplastic cases). Often cells from more than one culture are analyzed for reasons of quality control, because sometimes the abnormal cells are confined to a certain culture or cultures. If a cell has more or less than 46 chromosomes, the cell should be analyzed to determine what is extra or missing, and this information, along with other data, such as the microscope stage coordinates, is recorded on the analysis sheet or into the LIS/imaging system, if the laboratory is paperless.

The best representative cells, normal or abnormal, are photographed or imaged (see Chapter 14, section 14.5, Capturing the microscopic image and Chapter 15, Computer imaging) and some or all are completely analyzed (e.g., comparing band presence, position and size on every homologous pair). This may be accomplished by making a hard copy of some or all of the cells, and analyzing the rest by eye under the microscope or from a computer image of a spread (a cell which has not been cut out or karyotyped) or on a print. To analyze by eye, chromosomes are all identified by first finding the homologues of each pair in some methodical sequence and then comparing their bands for differences. Some technologists prefer to go through the cell in numerical order from chromosome 1–22 and X and Y, and some find the smallest chromosomes first or the acrocentric chromosomes first, and then go on to the others. Within these five photographed images collectively, coverage must be represented at least once for every chromosome pair, for example, no overlapped or unclear regions. Some laboratory protocols call for two different technologists to check each cell or to perform different parts of the study as a quality check. It is recommended that any question of abnormalities be either flagged for director review when prepared on an analytical software system, or recorded on the analysis sheet and initialed by the director or designee after verification.

Most guidelines require documentation of two karyograms from each abnormal stemline clone, and one for abnormal sidelines and normal cells; for normal cases, at minimum, a total of two or three per case are prepared.

Once analysis is complete, a karyotype will be devised which will explain the normal or abnormal findings in specific nomenclature format (see Chapter 8, ISCN: The universal language of cytogenetics). Most guidelines also require a minimum of two karyograms (pictorial representation via the systematic arrangement of chromosome pairs in numerical order) per nonmosaic case. Specimens found either constitutionally mosaic or showing neoplastic clonal evolution may require one additional karyogram to represent each additional cell line.

2.6.1 Chromosome abnormalities

Each chromosome is composed of two chromatids joined together at the primary constriction, which is usually called the centromere. The sections of the chromosome on either side of the centromere are called arms. Human chromosomes are classified morphologically based on the relative position of the centromere. In any metaphase spread of human chromosomes, some chromosomes have their centromere in the middle, some have it at one end, and some have it somewhere between these two points. The terminology of chromosomes reflects these differences. Chromosomes are described as metacentric (centromere in the middle), submetacentric (centromere a little away from the middle so that one arm is definitely longer than the other), acrocentric (centromere close to one end), or telocentric (centromere right at the end) (see Figure 2.18 and Chapter 7, section 7.2, Description of human chromosome shapes). There are no truly telocentric chromosomes in the normal human karyotype, although some tumor rearrangements may appear telocentric. In the international system for human chromosome nomenclature, short arms are designated as p arms (for petit), and long arms are called q arms [32].

Every metaphase chromosome must have a centromere to attach to the spindle fiber apparatus during cell division. Chromosomes that do not have centromeres tend to be lost from the daughter cells because they lag behind in anaphase and are excluded from the reformed nucleus in telophase. The exception to the rule is the formation of small "double minute" (dmin) chromosomes found in some tumors. These dmins are commonly the site of gene amplification, and may have a mechanism for inclusion in daughter cells. Some chromosomes also have a secondary constriction, called a stalk (stk), which is a variable, thin portion that does not stain very darkly with Giemsa stain. Stalks frequently appear on the short arms of acrocentric chromosomes, connecting the satellites (small knobs of chromatin usually found at the ends of the short arms of the acrocentrics) to the centromere. These secondary constrictions on the acrocentric ("D" and "G", see Figure 2.18) chromosomes are sites of ribosomal RNA synthesis in the interphase nucleus and are termed the nucleolus organizing regions (NORs). Secondary constrictions are sometimes seen on other chromosomes, such as chromosome 9, just below the centromere. On photographs, secondary constrictions may be confused with gaps because they are so thin and pale; they can be seen through the microscope, however, and may thus be differentiated from true gaps. Gaps in chromosomes may be due to chromosome breakage, as in Fanconi anemia, or fragile sites, such as fragile X syndrome [93]. The normal human chromosome number is 46. This is termed the diploid or 2*n* complement, which results from the fertilization of a haploid egg, with 23 chromosomes (the human '*n*'

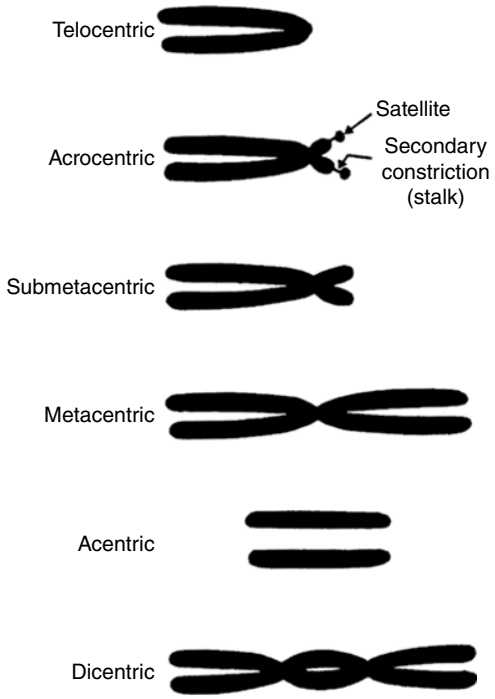


Figure 2.18 Chromosome shapes. The size and shape of a chromosome is an integral part of identifying a chromosome, along with its banding pattern (not shown).

number), or by a haploid sperm, both of which are termed gametes (see Chapter 1, section 1.2.4, Meiosis). This number can vary due to a number of errors in cell division, that is, during meiosis, fertilization, or mitosis. If the error happens meiotically, it can result in aneuploidy (cells other than diploid), or if the error is due to a mistake of fertilization (e.g., extra sets of chromosomes from either a second sperm or the polar body of the egg), it can lead to ploidy errors, such as triploidy – 69 chromosomes – or tetraploidy – 92 chromosomes. If the error arises post zygotically during mitotic division, it may lead to mosaicism, or the presence of more than one clone in the zygote (see later). Such findings would be called constitutional, meaning present in the subject's basic hereditary makeup. Alternatively, one cell in the bone marrow of an adult could undergo nondisjunction of chromosome 8, leading to a clone with trisomy 8 and hematologic disease (see Chapter 11). This finding would be called an acquired abnormality, since it is not innate, and represents a new change in the genetic makeup of that clone of cells. Acquired abnormalities are present only in the neoplastic cells, but not in the normal, nondiseased cells, so often these studies require extreme attention to find the abnormal cells.

During the cell count, an experienced technologist can also detect many structural chromosome rearrangements. A chromosome that is missing material is said to be deleted for that portion. If a segment is repeated, it is termed a duplication. A chromosome may also exhibit an exchange of material with another chromosome (called a translocation), which can either be balanced or unbalanced. In a balanced translocation no chromosomal segments are lost or gained. An unbalanced translocation involves loss or gain of one or more chromosomal segments resulting in partial monosomies or trisomies, respectively. These may imply, in a non-neoplastic chromosome study, a parental balanced translocation, inversion, or other rearrangement from which the unbalanced chromosome is derived by meiotic mis-segregation. An acquired unbalanced translocation in a malignancy is, on the other hand, usually caused by a mitotic event in the malignant cells. Sometimes a karyotype must be performed on a stimulated blood culture from a cancer patient in order to determine the origin of an abnormality seen in the cancer, if all the cells examined in the study show the same abnormal karyotype.

Sometimes a balanced translocation in an individual may form unusual meiotic configurations in preparing for disjunction in the gametes. A phenomenon called 3:1 meiotic disjunction can occur, with unequal sharing of the translocation chromosomes as the cell divides. This can result in an offspring with aneuploidy and unbalanced translocations, and can also lead to a state called uniparental disomy, in which both homologues of a pair of chromosomes come from the same parent, and there is no contribution from the other parent. For example, a translocation involving chromosomes 1 and 15 in one parent could

nondisjoin so that the subsequent daughter cell acquires both normal and translocated chromosomes 15. When the two gametes merge, one of the now three chromosomes 15 will become lost as a result of anaphase lag (loss of a chromatid at anaphase because it is not attached to the spindle and so does not get drawn to the pole), a phenomena known as trisomy rescue (see Chapter 10, section 10.2.2, Whole chromosome uniparental disomies). If the lost chromosome was from the second partner, the embryo will acquire two chromosomes 15 from the same parent, and thus have uniparental disomy for chromosome 15. Depending upon which parent the two chromosomes 15 came from, the offspring will have either Prader–Willi or Angelman syndrome (see Chapter 10, section 10.2.1, Chromosomal syndromes).

Another type of translocation commonly seen in constitutional studies is the fusion of two acrocentric chromosomes leading to a bi-armed chromosome termed a Robertsonian translocation, as a result of the discovery of such translocations in grasshoppers by Dr. W.R.B. Robertson in 1916. More than one per thousand liveborn children carry such a translocation, and it can be either balanced or unbalanced. Some Down syndrome children have Robertsonian rearrangements of the extra chromosome 21 with involvement of one of the other acrocentric chromosomes (note: the chromosome number would be 46, even though the genetic material is unbalanced). These may be inherited from one of the parents who can carry the balanced form of the Robertsonian translocation. Genetic counselors would give a much higher recurrence rate for parents of a translocation Down patient than for parents of a Down syndrome child with 47 chromosomes and an extra 21 because half of the gametes will contain the translocation and of these, due to improper disjunction, some may carry two normal chromosomes 21 as well as the translocation copy.

Some translocations occur at clusters within specific sequences, as with the familial translocation between chromosome 11 short arm and chromosome 22 long arm at bands 11q23.3 and 22q11.2. This is the most commonly occurring translocation in constitutional abnormalities, and the breakpoints occur at long AT-rich palindromic sequences (sequences with mirror image bases that hairpin back on themselves). It is not known why palindromic sequences are prone to translocation.

Other types of imbalance include duplications and deletions caused by unusual alignment of chromosomes when there is an inversion present. An inversion may involve the centromere of the chromosome (pericentric inversion) or not (paracentric). During meiosis, in order for the chromosome to pair its entire length gene for gene, the inverted chromosome has to make a loop configuration inside an inchworm shape taken by the homologue (Chapter 9, Figures 9.17 and 9.18). This may result in unbalanced gametes (see Chapter 9, section 9.2.4, Inversions), with particular duplications and deletions (or deficiencies). Carriers of inversions have an increased risk for miscarriages and unbalanced offspring because of this meiotic error.

Cytogenetic imbalance can also arise from extra chromosomes not identifiable by any definitive banding patterns. These are called marker chromosomes. Sometimes a marker can be characterized with FISH and special stain methods to be composed of multiple chromosomes mixed together, or they may be a small part of one chromosome with insufficient banding to be recognizable. Chromosomes may form ring structures, and these may be formed from a single chromosome or from multiple chromosomes. They may be constitutional or acquired in cancer. They may represent a single homologue in a euploid karyotype or they may be supernumerary. Rings often appear in different forms in different cells from the patient due to sister chromatid exchange (see Chapter 6, section 6.3, Sister Chromatid exchanges, and Chapter 13, Chromosome Instability Syndromes), becoming double in size, a figure 8, or may even open up and appear as a nonringed chromosome during this process.

Aneuploidy may also arise from technical artifact from the preparation (often termed "random chromosome loss"). The small chromosomes are commonly lost because of a small tear in the cytoplasmic membrane through which they may escape, either during harvest or slide-making. One of these escaped chromosomes may also drift into a neighboring metaphase during slide drying. Rules for distinguishing between technical artifact and true mosaicism, as defined by the International Standing Committee for Chromosome Nomenclature in the ISCN book [94], is to take these random gains and losses into account, with true mosaicism requiring the presence of two or more cells with an extra specific chromosome in order to be called significant ("clonal"), and three or more cells with a missing chromosome to be considered clonal.

2.6.2 Mosaicism

Mosaicism usually arises after fertilization, through inaccurate segregation of chromosomes at mitosis. When this occurs, different cell lineages (hence, karyotypes) that have originated from a single zygote will be present in different cells or tissues within that individual, such as the finding of some cells in a specimen with 45 chromosomes and only one X chromosome, and the remaining cells with 46 chromosomes and two X chromosomes, as seen in mosaic Turner syndrome individuals. Other examples of documented mosaic syndromes include Pallister–Killian syndrome, trisomy 20 mosaicism, and confined placental mosaicism (see Chapter 5, section 5.6.2, Confined placental mosaicism and Uniparental disomy). A much less common type of mosaicism, called chimerism, is the result of two embryos (zygotes) fusing into one, so that there is a mixture of two cell lineages that are completely unrelated (e.g., half male and half female, or half trisomic for chromosome 13 and half for chromosome 18).

If two or more chromosomally distinct cell lines are found, it becomes reportable if at least two chromosomes are observed with the same structural abnormality or additional chromosome, or at least three cells are observed with the loss of the same chromosome [94]. In vitro culturing can lead to a mitotic error (cultural artifact); therefore, finding the identical abnormality in an independent culture(s) becomes critical to interpreting mosaicism (see Chapter 4 for a discussion of primary cultures). When mosaicism is suspected but is either not found, or is found but does not meet reportable standards, another specimen source, for example, skin, buccal mucosa or another tissue, or the use of FISH, may help confirm or rule out its presence.

Two mechanisms have been postulated to account for errors of segregation, as shown in Figure 2.19. Nondisjunction is the failure of the two chromatids to separate at the centromere at metaphase, causing both chromatids to go to the same daughter cell, leading to one hyperdiploid (more than the correct diploid number) cell and one hypodiploid (less than the correct diploid number) cell. Anaphase lag, the second mechanism for errors of segregation, is the loss of a chromatid at anaphase due to the lack of its attachment to the spindle, and so it does not get drawn to the pole. This causes one hypodiploid and one normal daughter cell.

The level of mosaicism among the various tissues of an organism depends on the stage of development at which the error in division occurred (Figure 2.20). If misdivision occurs at the first cell division after fertilization, it is possible for all tissues in the body to be affected, with mosaic lines of 50% each, assuming equal survival and mitotic activity. If the misdivision occurs after the three cell types (ectoderm, mesoderm, and endoderm) have been established, the abnormal cells could be localized to one cell type (e.g., skin fibroblasts only); if it occurs later still, abnormalities could appear in only one organ of the body (e.g., bladder). The phenotypic effect could be dependent on the percentage and location of abnormal cells present in the body. Thus, it is important to be aware that analysis of a different tissue source in prenatal studies may be necessary (e.g., peri-umbilical blood, cultured urine) in order to find certain mosaic states, or that sometimes extra-fetal tissues, such as placenta or fetal membranes, will show an abnormal line (confined placental mosaicism, which can lead to growth retardation), while the fetus is karyotypically normal (see Chapter 5, section 5.6.2, Confined placental mosaicism and uniparental disomy). See Figure 2.21.

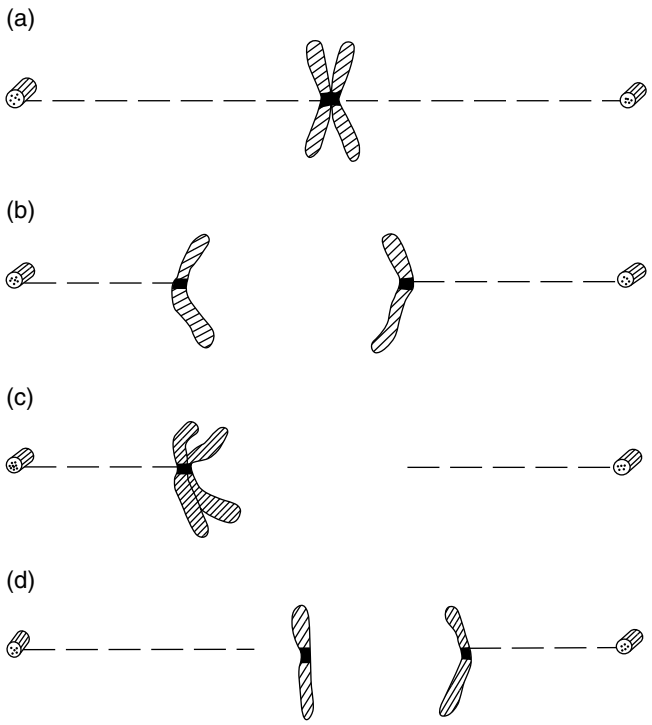


Figure 2.19 Normal and abnormal chromatid separation at mitosis. (a) Chromosomes at metaphase plate between centrioles. (b) Normal disjunction at anaphase – one chromosome at each centriole. (c) Nondisjunction at anaphase – two chromatids travel to one centriole, resulting in a cell with an extra chromosome and a cell with a missing chromosome. (d) Anaphase lag – one chromatid fails to attach to the spindle and is usually excluded from the nuclei and lost from both daughter cells.

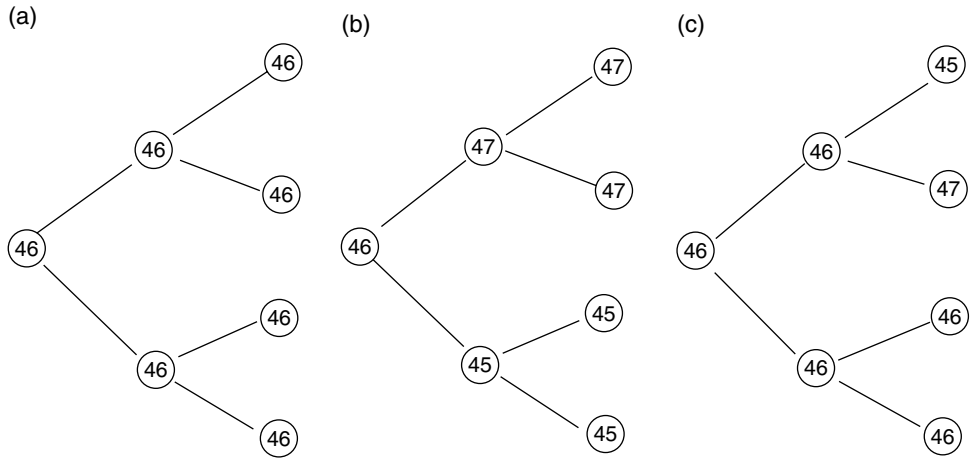


Figure 2.20 The development of mosaicism in the early zygote. (a) Normal cell division. (b) Unbalanced segregation at the first mitotic event caused by nondisjunction. This results in cell lines with 45, 46, and 47 chromosomes, and is often present in all tissue types examined. (c) Unbalanced segregation at the second mitotic event caused by nondisjunction. This also results in cell lines with 45, 46, and 47 chromosomes; however, if tissues have begun to form into separate germ layers, one or more of the abnormal cell lines could be confined to a single tissue type, such as skin, which is derived from the particular germ cell layer involved.

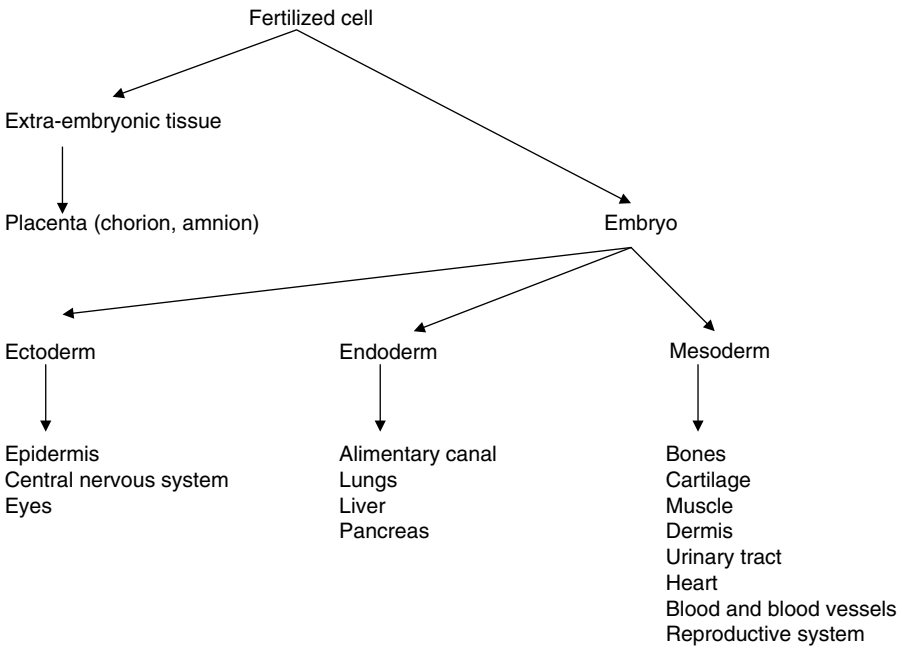


Figure 2.21 Genealogy of the organs of the body. In checking for mosaicism, tissue from different groups should be examined. Note, for example, that blood is mesodermal in origin, and skin is ectodermal.

Guidelines from the Association of Clinical Cytogenetics in the United Kingdom [95] describe a three-level definition for interpreting mosaicism, by Hsu and Benn [96], when finding at least one abnormal cell in prenatal studies:

Level I, when a single aberrant metaphase is found within only one, otherwise normal colony, or in only one suspension culture harvest.

Level II, when an abnormality is confined to only one culture vessel, whether it includes one entire colony or aberrant chromosomes are noted among normal cells from one monolayer flask.
Level III, or true mosaicism, when the abnormality has been documented in two or more independent culture vessels.

See Chapter 5, section 5.4.8, Mosaicism, for the actions to be taken and factors to consider when resolving a potential prenatal mosaic situation.

There is no way to totally eliminate the possibility of mosaicism in the patient under study, because not all cell types are present in the sample being studied. The sample is representative of the cells of the body from which it came, and not representative of other tissues from the individual. However, it is possible to count enough cells to eliminate certain levels of mosaicism within that tissue source. Hook's tables [97] apply to lymphocyte studies, and the studies of Clausen et al. [98] and Cheung et al. [99] are calculated for amniotic fluids. In each study, the statistical analysis is based on the assumption that the sample obtained is truly random (though it probably is not) and that all cells grow at the same rate and behave the same in the in vitro culture flask (which is probably not true either). These tables are very useful as a guideline for assessing the confidence levels for mosaicism, because they show the percentage of mosaicism excluded and the confidence levels that may be accepted if a specific number of cells have identical counts (note that "counts" may denote analysis as well, to look for structural mosaicism). The recommended count of 20 identical cells on a patient sample means that mosaicism of greater than 14% may be excluded with a confidence level of 95%. Ruling out mosaicism of greater than 10% for the same confidence level requires counting 30 identical cells on a sample. Increasing counts to 50 identical cells will only improve the detection rate to 6%, with the same confidence level; thus the small improvement may not justify the additional 20 cells counted. On the other hand, if there is enough clinical evidence to warrant the investigation for low-level mosaicism, it may be necessary to examine a greater number of cells or to obtain a sample from a different tissue source, especially when the clinical diagnosis strongly suggests a syndrome that is not found by studying 20 metaphases from a lymphocyte culture. Additional cells may also be warranted for a cancer workup or for the investigation of fragile sites or breakage syndromes; however, even a single, abnormal neoplastic cell may be reported if that aberration was previously reported in a prior specimen from that patient [94].

Because long-term incubation can increase the risk for cultural artifact, finding an abnormal cell in prenatal material requires its own criteria for differentiating true mosaicism from sporadic artifactual changes (pseudomosaicism). For example, each cell lineage should be observed in more than one independent primary culture; therefore, every colony in all primary cultures, except the one that had the abnormality, would be checked until a second colony can validate the finding. If it was only observed in that one original culture, but the finding correlates with clinical findings, it may be necessary to perform alternative testing (e.g., FISH, array, or follow-up amniocentesis). Abnormal findings in prenatal tissue could also stem from extra-fetal tissues, such as placenta or membranes (confined placental mosaicism), while the fetus is normal. In particular, an extra copy of chromosome 2 or chromosome 7 has been reported as an artifact of prenatal cultures and usually have no clinical significance (see Chapter 5, section 5.6.2, Confined placental mosaicism and uniparental disomy). See Figure 2.21. These situations have been discussed extensively in the literature [100–112]. (See also Chapter 5, Prenatal chromosome diagnosis). If no further cells (or colonies, in the case of in situ culture analysis) from an independent culture are found identical to the one abnormal cell (or colony) already noted, then the aberrant cell can usually be assumed to be artifactual, and mosaicism of greater than 10% can be excluded at a 95% confidence level. If other cells with the same aberration are found in a second, independent culture source, low-level mosaicism may be present and should be reported, particularly if the karyotype of the minor line represents a clinically described condition and clinical findings are consistent with that cytogenetic finding. It is important to stay current with new guidelines for interpreting mosaicism that are published from time to time.

Another method for detecting mosaicism is to utilize interphase fluorescence in situ hybridization (FISH), if probes are available for the interphase detection of the abnormality. FISH can be used to extend the number of cells scored for a given abnormality and to investigate alternative tissues with different germ cell layer origins, such as epithelial tissue in skin or buccal smears. It is important to first know how the patient's metaphase cells behave with the probe used, since alpha satellite DNA probes may have variant patterns in some individuals, with extra signals or even missing signals in the normal cells (see Chapter 16). Microarray CGH analysis also provides analysis for delineation of structural abnormalities, comparison of inherited abnormalities between parent and child, or investigation of possible cryptic unbalances to complete the report (see Chapter 18).

2.6.3 Chromosome breakage

Cultured chromosomes may naturally exhibit gaps and breaks in chromatids as a result of cultural artifact. The lesions vary from a staining gap where the break took place to complete displacement of the chromosome fragment far removed from the chromosome. These gaps and breaks can lead to chromosome rearrangements, including translocations, inversions, and ring chromosome formations. There are autosomal recessive conditions that predispose individuals to chromosome breaks and

radial formations (Chapter 13, Figure 13.2) and consequently to cancer. Examples include Bloom syndrome, Fanconi anemia, and Ataxia telangiectasia (see Chapter 13, Chromosome instability syndromes).

Some regions of the genome that have repetitive DNA sequences may exhibit chromosome gaps and breaks in specific breakpoints in the karyotype known as fragile sites. An example of a fragile site is the fragile Xq27.3 that causes the phenotype for Martin Bell (fragile X) syndrome. The test for this condition was a cytogenetic breakage study until the advent of a DNA test for the repeat DNA that is now the standard of care for diagnosing the fragile X both in carriers and in affected individuals. It is important to document breaks and gaps in chromosome studies because there may be a clinical cause.

2.6.4 Karyotyping a cell

The noun karyotype originally meant just an arranged layout of the chromosomes of a single cell, but with time the meaning expanded to include the written description of the cut-out cell because the two were both considered descriptions of the same thing. However, in the 2005 *International System for Cytogenetic Nomenclature* (ISCN), in order to distinguish between the two ideas, the chromosome layout became called a karyogram, and the written description as well as the chromosomal makeup of the individual became known as the karyotype. The verb for making the karyogram is not described by the ISCN, and since "karyogramming" does not resonate, we will continue to use the verb "to karyotype" as the act of arranging chromosomes for analytical comparison. Here we will discuss the principles used in the making of a pictorial karyogram. See Chapter 8 for the use of ISCN nomenclature to describe the karyotype in writing.

The karyogram is an orderly arrangement of chromosomes (46 in human, 44 autosomes arranged in pairs, one from each parent, and two sex chromosomes) according to international conventions [112]. The chromosomes may be cut from a photographic print and arranged on a preprinted form with areas marked for each chromosome and its group or they can be digitally arranged via cytogenetic analytical software. If manually cut, chromosomes may be fastened to the form by gluing, taping, or using photographic dry mounting tissue and heat. In most clinical laboratories, however, manual methods have been replaced with digital imaging software systems that can automatically arrange chromosome images onto a karyogram format, with guidance from the operator using mouse and keyboard commands. Computer-assisted karyotyping has helped to enhance the quality and reduce the time required to prepare a karyogram.

Chromosomes were originally categorized by size and morphology into seven groups, A-G, by the 1961 Denver Conference [113] (Figure 2.18). With the advent of banding, the 22 pairs of autosomes (nonsex chromosomes), numbered 1–22, became individually identifiable. The sex chromosomes were named X and Y. Although they were not given numbers, they were assigned to groups based on their size; the X chromosome to the C group, and the Y chromosome to the G group. When chromosomes are not banded (see Chapter 6, section 6.1.5, Conventional (solid) staining methods), they can be assigned to groups but cannot all be identified individually. When they are banded by the G-, Q-, or R-banding methods, each chromosome can be positively identified by number as well as by group. The distinguishing characteristics of each chromosome when G-banded are given in Chapter 7, Human chromosomes: identification and variations.

Chromosomes are always arranged on the karyogram with the short arms uppermost. Groups are arranged in alphabetical order. The sex chromosomes may be placed together at the end or separated to their groups; either way is acceptable.

The largest chromosomes are in the A group (two each of numbers 1, 2, and 3). Chromosomes 1 and 3 are metacentric chromosomes, 1 being larger than 3, and the number 2 chromosomes are slightly submetacentric.

The B group consists of two pairs of chromosomes that are large and strikingly submetacentric; the short arms about one-fourth the length of the long arms. Without banding treatment they cannot be identified individually. They are numbered 4 and 5.

The C group contains the most chromosomes and is the most difficult group to karyotype in both banded and nonbanded spreads. The chromosomes are medium-sized and are all submetacentric; they are numbered from 6 to 12. The X chromosome also belongs to this group. In the nonbanded state, these chromosomes cannot be individually identified, so they are arranged roughly in order of decreasing size: the 6s are the largest, and the 7s and the X are the next largest. Chromosomes X and 7 are about the same size, but the X is more metacentric than the 7. The 11s are the most metacentric, and the 9s are variable size depending on the length of the heterochromatic region just below the centromere. The 8s, 10s, and 12s have the shorter short arms, and the 12s are the most submetacentric.

The D group consists of three pairs of medium-sized acrocentric chromosomes, numbered 13, 14, and 15. In the nonbanded spread, all Ds look alike. Tiny short arms with satellites are often visible and vary greatly in size and intensity of staining.

The E group consists of three pairs of chromosomes that are about the same length as the D group chromosomes but have clearly defined short arms. The 16s are metacentric, and the 17s and 18s are submetacentric. The 18s have the shortest short arms in this group.

The F group contains two pairs of small metacentric chromosomes, 19 and 20, which cannot be individually distinguished without banding treatment.

The G group contains two pairs of autosomes, numbers and 22, and the Y sex chromosome. The 21s and 22s are small acrocentrics that frequently have short arms with satellites, similar to the Ds. The Y chromosome can be very similar to the other Gs, although it does not have satellites, and may have more defined short arms. It can also vary in size and is sometimes larger than the 18, although its short arms are always small.

The normal female has two X chromosomes and no Y chromosome, so that the nonbanded spread has 16 C group chromosomes and only four G group chromosomes. The normal male, with only one X chromosome, has only 15 C group chromosomes but has five G group chromosomes because of the presence of the Y.

A note about satellites: other chromosomes (autosomes, the Y chromosome) can acquire satellites as a normal variant (see Chapter 9). In addition, the short arms of the acrocentric chromosomes can acquire the Yqh region without an effect on the phenotype, and can be seen in females as well as males.

2.6.5 Banded karyograms

Karyotyping banded chromosomes follows the same principles described for the nonbanded chromosomes but uses the additional information provided by the banding pattern to identify each chromosome by number. Because the band number depends upon the elongation of the chromosome, band descriptions vary for a given chromosome and are difficult to verbalize. Chapter 7, Human Chromosomes: Identification and Variations, shows G-banded chromosomes at various lengths.

2.6.6 Haploid band number and band levels

Band level is a term representing the total number of bands countable in a haploid set of autosomes plus the sex chromosomes. The band level that can be obtained by the various banding procedures depends on the degree of mitotic chromosome condensation achieved by the cell when it was harvested. It is desirable to aim for the highest level of band resolution as possible. Landmark bands that were the first demonstrated on metaphase chromosomes represented a band level of about 250 bands per haploid set. At this stage in late metaphase, all chromosomes can be identified individually but even quite large abnormalities are invisible. The Paris Conference schematic [114] represents chromosomes at the 400-band level; this is obtained from chromosomes at a slightly earlier stage of metaphase.

A band level of 550 (see Figure 2.12) provides sufficiently high resolution for detection of many small rearrangements without being prohibitively difficult to analyze. This level corresponds to prophase or prometaphase, when many small, distinct bands can be demonstrated that will later condense to form the well-known landmark bands (see Table 2.7). Blood and amniotic fluid samples are capable of obtaining this length, but bone marrows are usually shorter. Band levels of up to 850–900 bands per haploid set are obtainable using stimulated blood cultures, and amniotic fluid cells are capable of occasional high band lengths as well. These very high band levels are used to define very small abnormalities. However, for detecting certain abnormalities such as pericentric inversions, may be more apparent in shorter cells, so cells of various lengths should be examined.

There are a number of methods to determine the band length of a given cell [45–50]. One method, developed at the Cytogenetics Laboratory of Vancouver General Hospital [49], uses the dark G-bands from five different chromosomes, counting all bands except the centromere on the X and 10, 11p, 12q, and 1p31–p32. The total obtained is related to the haploid band levels, from 350 to 850. The number of bands counted represents approximately 7% of the total haploid count at band levels of 500 and above, and slightly less at lower band levels. The sum of the bands on these segments on the ISCN schematics is as follows: 18 bands at the 350-band level, 21 bands at 400, 40 bands at 550, and 60 bands at 850. In counting

Table 2.7 Comparison of bands on four chromosomes at two different band levels

Chromosome	Approximately 400	550–600 Band level
7q	2 dark bands	Each of the two major dark bands splits into 2 dark and 1 light
10q	3 dark bands (upper or proximal is darkest)	Proximal dark band splits to 2 dark and 1 light; also 1 dark band appears in the next distal light band, 10q22
11p	2 dark bands, very close together	The 2 dark bands separate and a thin dark band appears, close to terminal
Xp	1 large dark band	The 1 dark band splits into 2 dark bands with 1 light band between them; distal dark band is the largest

p = short arm; q = long arm.

the actual bands on the chromosomes of a spread, the total obtained might be intermediate to the four guidelines, in which case an extrapolation can be made. Alternatively, certain bands can be used as a reference to assess the entire cell, since they split into additional bands at specific band levels [50]. Many laboratory guidelines require band assessment, and often band level of the best quality cell is reported on the final report. The College of American Pathologists (CAP) requires the reporting of band resolution on the final report for non-neoplastic studies.

Many patient samples will show variations in regions of certain chromosomes, such as increased or decreased heterochromatic regions, variation in sizes of short arms, stalks, and satellites of acrocentric chromosomes, and so on. These variables, or heteromorphisms, which usually carry no phenotypic effects, are covered in detail in Chapter 7, Human chromosomes: identification and variations.

2.6.7 The complete cytogenetic study

There are guidelines for the components of the complete cytogenetic study, which are followed by most laboratories. These include the guidelines of the American College of Medical Genetics (ACMG) [115], College of American Pathologists (CAP) [116], and European Cytogenetic Association (ECA) and other country-directed guidelines [117]; in addition, there may be state or country-specific standards also available. These guidelines are updated regularly and can be printed from the websites of the organizations mentioned above (see Chapter 25, section 25.2.7, Credentialing and guidelines). Guidelines will specify how many metaphase cells are to be examined, photographed or imaged, analyzed or karyotyped, and how many interphase cells for FISH are to be examined and imaged.

For most constitutional studies, counting 20 cells and finding them identical is sufficient to provide the cytogenetic diagnosis. It may, however, be necessary to examine a greater number of cells (see 2.6.2, Mosaicism) or to obtain further cells from a sample of a different tissue source if the clinical diagnosis strongly suggests a syndrome that may not be detected by studying 20 metaphases from a lymphocyte culture (e.g., Hypomelanosis of Ito, Pallister–Killian syndrome). Cancer predisposition studies for the presence of fragile sites and breakage may also require analysis of an even greater number of cells for meaningful statistics.

For many samples, the quality of the spreads chosen for analysis should be the best that can be found on the slide. The major exception to this rule is in cancer cytogenetics, in which the abnormal cells are sometimes of poorer quality than the normal cells, and may be nondiploid (e.g., near-tetraploid or near-haploid). For this reason, some laboratories routinely document the quality of a study during analysis: for example, *excellent* for spreads with sharp, clear bands and no overlaps; *good* for spreads in which all chromosomes can be identified, though some overlaps are present; and *fair* for spreads that can be analyzed but are unsuitable for capturing and karyotyping.

Exceptions to the rule of reporting prenatal mosaicism in multiple colonies include the appearance of a colony with an extra chromosome 2 or chromosome 7, both of which have been reported as an artifact of prenatal cultures and are usually considered to have no clinical significance. However, even a single aberrant cell in a bone marrow sample could represent a neoplastic line and may need to be reported, if relevant to the clinical picture [94]. The laboratory director may choose to report a single aberrant cell if it seems relevant, considering the clinical findings or patient history.

2.6.8 Karyograms, karyotypes, and the final report

After a case has been analyzed microscopically, images are made of representative metaphase cells either with a photographic or digital system. The number of images to be made will be established by the laboratory director, but guidelines generally include minimal recommendations. Prints of chromosome spreads that are not karyotyped enable technologists to check their analyses. Some laboratories, particularly those in cancer work, photograph as many as 30 consecutive spreads for later analysis.

All photographic materials, both negatives and prints, must be clearly identified with the patient's name and the case number, and careful records must be kept of the photographic work, either in a special file or in each patient's file, following the protocols established for each laboratory. An identification system for storing digital images is usually programmed into the computer imaging system, or can be added during analysis.

The final report should be prepared as soon as the study is completed, and copies sent to the relevant referral physicians, hospital records departments, or laboratory/hospital information computer systems. The report should include (per ACMG/CAP guidelines) the patient name and birth date, identification number such as medical record number and test encounter number laboratory accession number, referring physician, test requested and performed, clinical indication for the study and ICD10 code if known, source of the specimen (amniotic fluid, blood, etc.), date of service, date received, culture type and number of vessels examined, number of cells and colonies examined, number of analysis and karyograms prepared, stains used,

quality, band level, karyotype, cytogenetic results, interpretation, recommendations, preliminary and final report dates, and the Director's signature. The results are expressed using ISCN (the current version), as well as in a verbal interpretation, which can correlate the findings with the clinical presentation and can comment on recommendations for genetic counseling or further cytogenetic or other studies of the patient or the family of the patient.

2.6.9 Sources of error in analysis and reporting

It is extremely important to avoid errors in genetic studies, because prognostic, diagnostic, and outcome information, treatment decisions, and often the life and death of a patient may depend upon accuracy. Because almost all stages of a cytogenetic study involve subjectivity and individual expertise on the part of the cytogeneticist, it is important to be aware of some potential sources of errors.

Mistakes can occur at various stages, from specimen collection to the actual interpretation of the karyotype. Cases can be mixed up during culturing, harvesting, slide-making, slide or record sheet selection at the microscope, printing of the film and karyotyping. Each case must be properly labeled throughout the cytogenetic procedure – preferably by the patient's name and an identification number (e.g., accession number, bar code, medical record number, etc.). The CAP guidelines require two separate, unique identifiers on each sample. It is also helpful to work in an atmosphere that is free from distractions and to handle each case separately when setting up cultures, making slides, performing microscopy, printing, adding probes to slides, and karyotyping. Extreme care should be taken to avoid cross-contamination of specimens during harvesting and slide-making. It may sometimes be necessary to repeat work using other cultures or new samples to clarify discrepant results.

Poor growth of cultures and poor banding also present the greatest sources of potential error to the technologist. Cultures that yield few metaphases of poor quality, such as compact spreads and cells with many overlapping chromosomes or background debris, may result in incorrect microscopic analysis because the technologist may fail to detect mosaicism, translocations, ring or marker chromosomes, or other very small chromosome abnormalities. If the technologist is not satisfied, new slides should be prepared, a repeat culture performed, or a repeat specimen requested. It is still as important as ever to obtain the highest quality banding resolution possible, because even though microarray technologies are improving and increasingly available, they do not cover the entire genome, do not detect balanced rearrangements, and will not be offered to all patients. Poor quality banding may also be a source of error by preventing the detection of translocations, duplications, deletions, or other structural abnormalities. If the technologist has any doubt, new slides should be prepared, a harvest repeated on a culture, or a repeat specimen requested.

The banding pattern of each chromosome should be closely examined in each study, and every chromosome pair should be compared band for band at least once to ensure that no differences exist between homologous chromosomes. If an unusual pattern is observed, all means necessary, including supplementary banding or molecular procedures, should be employed to rule out or confirm any abnormality. Preparation of more than one karyotype helps to ensure that all chromosomes can be visualized without crossovers or other clarity obstruction. If there are still regions of some chromosomes that are unclear, examine more cells on the slides on the microscope or on prints. Some unusual findings may turn out to be due to variations in the slide-making and staining, limitations of the image reproduction system, or to the location of the chromosome within the metaphase: chromosomes tend to stretch on the outer edge of the metaphase, overlapping chromosomes can confuse banding patterns, and extraneous stain can be mistaken for chromosome segments.

Some abnormalities, termed "cryptic," are not readily detectable by karyotype analysis. For example, if the light telomere bands from two different nonhomologous chromosomes exchange, neither will appear much different than the normal state. Subtelomere specific probes have been designed to investigate such rearrangements (see Chapter 16, section 16.3.1, Subtelomeric-specific probes). Another example is seen in chronic myelogenous leukemia, where the diagnostic translocation between the long arms of chromosomes 9 and 22 may be obscured by involvement of a third chromosome or may present as a small insertional rearrangement that does not include a translocation of the entire distal segments of the 9 and 22. FISH for the translocation and/or polymerase chain reaction (PCR) DNA testing should clarify these rare cases because the cryptic rearrangement still results in a fusion between the *BCR* and *ABL1* genes.

Finally, obtaining another opinion is valuable. Consultation with other technologists or laboratories may prove invaluable in many cases.

2.7 Laboratory procedure manual

All of the methods used in the cytogenetics laboratory should be updated regularly and be made available to all laboratory personnel working with them. They may be in a loose-leaf binder or on a computer-based system. The computer-based system, if on a common drive of an institutional intranet, is easiest to keep up to date for everyone. If a paper based methods

system is utilized, when newer, updated versions are created, all copies of the old methods must be archived with date that the method was discontinued so that all personnel will be using the same guidelines for every method. Personnel must review all methods annually, or whenever changes are made, to be sure that they are correct and that the correct methods are being used. It is a good practice to have all personnel review all of the methods at least annually to be sure that current and correct methods are being used. The laboratory director and department chair, if appropriate, are also responsible to review the methods semi-annually or when any change is made.

The essential parts of a laboratory protocol are:

Title: The title should have a name that would represent the procedure uniquely, along with a filing identification for easy retrieval. The name of the writer(s) of the procedure, as well as laboratory director and institution, could also be included in this initial section.

Purpose: What the method does, what it is used for, or how it relates to patient care.

Principle: How the method works. This is important for troubleshooting.

Materials/Supplies: What needs to be present in the laboratory in order to undertake the method; ordering information, such as vendor, size or volume of stock reagent along with its catalog #; how to formulate and store the reagent solutions; whether any specific safety conditions are required in their handling; and what the expiration times are after a stock reagent has been defrosted or opened, and for solutions that have been prepared from that reagent.

Method: This should include any safety warnings specific to the use of any reagent, as well as what steps are used to complete the procedure. ***Tips***: Sometimes procedures are not always straightforward and there may be specific variables or limitations, which when they occur, may have troubleshooting tips to handle these occurrences. These can be added either within the procedure or after the Method section. ***References*** and ***Additional readings***: Any literature references would be placed at the end of the Method section.

Approval signoffs: Before any procedure can be put into production, approval signoffs by laboratory director and/or department chair must be documented, which would include the date, their name(s) clearly typed or printed, followed with their signature(s). Signatures must be verifiable. This process is repeated for each change and for routine annual reviews. All laboratory personnel responsible for performing any procedure must also document that they reviewed all procedures and any subsequent changes to a procedure, at initial implementation and annually thereafter, even if no changes have been implemented.

The laboratory should also keep a record of policies. These can include the probes used for various panels, turnaround times, guidelines for an acceptable band length for prometaphase studies, etc. These are also reviewed yearly or semi-annually by the technologists and the laboratory director and/or department chair.

This chapter, while detailed, is a simplified glimpse into the workings of a cytogenetics laboratory as it existed when this book was written. Cytogenetics is a continuously changing field, and it is important to continue learning from the literature and networking with others at meetings and whenever we can. There are few schools teaching this necessary laboratory science, and so it is our duty to pass our experience and knowledge on to others, and their duty to do the same.

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Contributed protocols

IMPORTANT: *No protocol included in this manual should be used for clinical testing unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements for all chemicals used in the laboratory.*

Protocol 2.1 Slide-making

Contributed by Oregon Health & Science Center, Knight Diagnostic Laboratories, Clinical Cytogenetics Laboratory

I. Principle

Good chromosome banding is dependent on appropriate slide preparation. The purpose of this procedure is to obtain an adequate number of metaphases/slide with appropriate morphology. Variables affecting preparation include relative humidity, room temperature, drying time, and number of cells/slide. Chromosomes that are too flat do not produce crisp banding; chromosomes that are too raised appear too dark and have a halo appearance under phase microcopy. These dark cells frequently result in uneven and poor banding. The perfect phase contrast image is one that is crisp and even with no visible cytoplasm.

Safety warning

All tissue specimens should be handled as biohazardous, using Universal Precautions. Use the laminar flow hood for all steps up to harvest spin. Wear a laboratory coat and protective gloves for all steps through slide-making. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with Sanimaster 4 (made fresh weekly) or 70% ethanol. Cover cuts with protective bandages even when gloves are worn. Dispose of Pasteur pipettes in sharps container. Wash hands thoroughly after removing gloves.

II. Materials

1. Chromosome cell suspension
   Dilute pelleted suspension with fresh fixative to achieve a cell dilution that is thin enough to allow proper chromosome spreading but thick enough to find mitotic figures easily (cloudy but not milky-appearing suspension).
2. Float glass slides with painted end for labeling
3. Pasteur pipette and bulbs
4. Phase microscope with 16× lens
5. Blotting material, such as KimWipes
6. Hygrometer and thermometer for determining ambient humidity and temperature
7. Tray with wet paper towels for dry conditions

III. Method

The following is an example of a slide-making method for a clean, average sample on a day with 40% relative humidity and a temperature of 72 °F (22 °C).

1. Remove glass slides from the box and place them in a Coplin jar containing 95% ethanol.
2. Remove one of the slides from the ethanol and clean the upper surface of the slide with a KimWipe, using two or three forceful strokes to clean off manufacturing residues.
3. Dip the slide back in the ethanol and then into a beaker of clean, distilled water. Dip it in the water 8–10 times, or swirl it until the water coating is even and the signs of the mixing of alcohol and water disappear.
4. Hold the slide horizontally and make sure the water coating is even. Then place the long edge of the slide on an absorbent towel to drain excess water.
5. Gently aspirate some of the fixed cell suspension into a Pasteur pipette and, holding the slide at a 20–30° angle on the towel and starting at the nonlabel end, drop 2–4 drops of cell suspension from about 1/4 in. from the slide surface. The drops should not stream down the slide but should make round patterns. Drain the slide on paper towel.

6. Drop 4–6 drops of fixative across the slide, overlapping the drops.
7. Drain the slide on a paper towel or with a KimWipe.
8. Label the slide with patient information, slide number, and date.
9. When the slide has dried until rainbow colors appear on the surface, warm the slide on the back of the hand or the thigh for a few seconds.

IV. Notes

Technique variations for different environments:

1. For conditions in which the humidity is very high (45% or greater), try drying the slide continuously on hand, leg (thigh), or warming plate (temperature 35–40 °C).
2. For conditions in which the humidity is very low (30% or less), place slide on a stack of water-soaked paper towels and leave until completely dry.
3. Slide-making under other conditions may require combinations of drying environments that can only be discovered by experimentation. For example, use a very thin layer of water on very humid days or thicker on very dry ones. Drain slide after each drop of suspension is added on humid days and wipe back of slide several times on very humid days before warming. Do not wipe back of slide before putting on wet paper towels on very dry days.
4. Check on a phase microscope. If cells are gray or chromosomes are scattered, warm longer. If cells are very dark and show visible cytoplasm, reduce or eliminate warming.

Protocol 2.2 Slide-making

Anonymous contributor

I. Principle

Even though slide-making is the final step in the harvesting procedure, a technologist must have the ability to troubleshoot and customize a procedure to yield optimal results.

II. Materials

1. Methanol
   Store in an enclosed and secure flammable cabinet, separate from acids, other reactive substances and human contact. Never lift bottle by cap. Transport in a rubber safety carrier. Label all receptacles containing this reagent. Use proper fume ventilation and PPE when handling reagent. Disposal must be in accordance with federal, state and local environmental control for flammable materials.
2. Glacial acetic acid
   Store in a cool, dry, well-ventilated place away from incompatible materials. Never lift bottle by cap. Label all receptacles containing reagent. Transport in a rubber safety carrier. Label all receptacles containing reagent. Disposal must be in accordance with federal, state and local environmental control regulations. Use proper fume ventilation and PPE when handling reagent.
3. Modified Carnoy's fixative
   Mix 1 part acetic acid to 3 parts methanol. Keep at 20 °C. Prepare fresh daily, as needed. *Warning*: use proper fume ventilation and PPE when handling reagent. If spreading is a problem in dry climates or regions, see Note 1.
4. Wheaton staining tray for 20 slides, Science Products #900200, Fisher Scientific #08-812.
5. Microscope slides
6. Sterile, distilled water

Cleaning slides

Slides are first visually inspected; any slide with even a questionable flaw should be discarded in the sharps container. With the scratchy label side all facing the same direction, slides are placed diagonally in a glass slide container, with both slide front and back freely exposed to the cleansing reagent. Fill the dish with fixative (see Note 2) so that the slides are fully immersed, and allow to sit for 15 minutes. Fixative is carefully decanted, and slides are rinsed twice in sterile distilled water.

Fresh sterile, distilled water is added so that the slides are completely immersed, and the container is stored in the refrigerator. Expiration: 3 months. NOTE: Fixative must be discarded in container collector for Flammable Material pickup.
7. Warming plate at 40 °C and 60 °C
8. Coverslips 24 × 50 mm
9. Sealant (use under fume hood)
10. Tweezers for lifting clean slides out of staining tray
11. Paper Towels (for drainage)

III. Method

Slide-making is performed in a humidified, temperature-controlled environment (see Note 3). Never leave more than one centrifuge tube uncovered at a time. Do not drop more than one case at a time.

1. Cells should have had at least three 10 mL fixative changes and should have had its final centrifugation (1000 RPM for 10 minutes) so that the cells are in a pellet, ready for slide-making.
2. Depending upon size and condition of cell pellet, withdraw fixative up to an equal volume to the cell pellet. Resuspend pellet gently but ensuring that all material has been released in suspension.
3. Test slide surface by removing a slide from the water holder and running fresh fix down its surface. If the fixative does not flow straight and smoothly, for example, it buckles in pockets, discard slide and try another. If the problem persists, try another slide container and/or change fixative reagents. If the problem still persists, try a different slide lot or manufacturer (see Note 4).
4. If FISH is required and a more concentrated slide is desired, make the FISH slide first and then dilute pellet for routine cytogenetic analysis.
5. Labels for slides should have at least two patient identifiers, including patient name and lab accession number, vessel #, slide #, tech initials and date made.

Slide-making

6. Slide-making method 1: Lay wet slide onto paper towel and run a couple drops of fixative on the water surface. Resuspend cell pellet, withdraw 0.5 mL of cell suspension in Pasteur pipette and gently release two drops equidistant onto horizontal slide. Allow slide to remain flat for 1 minute. It should not become dry. Gently roll slide to its horizontal edge, drain excess, dry back, wave once and place onto 40 °C hotplate until dry. Check under phase microscopy to see if cells are spread sufficiently and metaphase concentration is of ideal density. Add more fixative if cells are too dense and try again. If the fixative dries too quickly (before a minute), increase humidity or reduce time it lays on paper towel before transferring to the hotplate. Label and initial.
7. Slide-making method 2: Remove slide from storage container without draining water from surface. Add a couple drops fixative to water and release two drops of cell suspension equidistant on slide while slowly tilting slide to its horizontal edge. Hold horizontal for one minute. Slide should not be dry yet. Dry back, blow gently on surface and place on 40 °C hotplate until dry. Check under phase microscopy for spreading and morphology. Label and initial.
8. Slide-making method 3: (a) place two drops fixative on slide, tilt slide at a 45° angle and gently run 2 or 3 drops down the center of the slide. (b) Release cell suspension from a higher distance. Lift slide to a vertical position; hold again for one minute, allowing the cells to drain slowly down the slide surface. Blow on slide, dry back, and place on 40 °C hotplate until dry. Check under phase microscopy. Label and initial.

Slide quality evaluation

9. If the distance between nuclei is too dilute (you see only sparse nuclei under a microscope field), bring pellet to a 10 mL volume with fixative and centrifuge at 800–1000 RPM for 10 minutes. Remove supernatant, resuspend in less fixative and re-drop.
10. When complete, resuspend pellet in 5 mL fresh fixative, close tightly and store in refrigerator for 6 months (see Note 5).
11. Artificially age slides for G banding on a 60 °C hotplate overnight. For emergency results, bake slides in a 96 °C for 45 minutes.

IV. Notes

1. Getting chromosomes to spread properly in very dry or very humid conditions can be difficult. Replacing the 1 : 3 ratio of acetic acid to methanol with 1 : 2 or 1 : 1 acetic acid–methanol solution, may help. The validation process must ensure that all subsequent steps have not been detrimentally affected by the ratio change.

2. FIXATIVE is a caustic reagent and requires advance planning in its usage – from storage and preparing, to use and discarding, in order to avoid any potential health hazard risks. Its fumes should be controlled via a fume hood. The staining dish filled with fixative should remain closed and undisturbed while slides are immersed. The dish should also be properly labeled of its contents and danger. A bottle containing either fixative or its reagents should never be carried by holding only the top of the jar; large reagent containers should be carried inside a bottle carrier specially designed for high-risk transport [for example, we use Rubber Bottle Carrier (red), 1 gal/5 pts, Lab Safety Supply #3328R]. Once complete, the fixative should be easily discarded with minimal handling and with no risk of spillage. As accidents may occur, precautions must be taken that, if spillage does occur, that it is contained and not within physical harm to the user. Spill directions should be visually displayed and/or easily referenced. In the event of a spill that does not harm the user, follow your institution's guidelines for toxic chemical spills. In the event of spillage onto the user, remove any potentially affected clothing immediately and immerse in the safety shower for 15 minutes. Seek medical attention immediately, whether you feel a burning sensation after the wash or not.
3. Fluctuation in temperature and humidity can affect the spreading of chromosomes during slide or coverslip drying. Hypotonic time may need adjusting if slide-making results are consistently less than optimal. Optimal harvest parameters in our laboratory are:

Humidity	Temperature	Hypotonic time
40–60%	75–88 °F (24–31 °C)	25 min (unstimulated); 10 min (stimulated)

4. Slide-making depends heavily on the ability for metaphase chromosomes, held together within a fragile membrane, to flow unimpeded on the slide surface until it can rest and spread, without bursting too early (chromosome "soup") or holding too tightly (chromosome "fudge"). Any obstruction that increases or decreases friction, or any change in environmental conditions (seasonal changes, humidity, temperature and airflow) that will increase or decrease fixative evaporation time, could affect slide quality. Culture and harvest parameters can also affect metaphase spreading.
5. Fumes from the fixative solution are corrosive and may eventually affect a refrigerator's electrical parts. Covering the tube with parafilm may help, but it is still not a total solution. Fixative is also flammable; therefore, care must be taken with historically held cell pellets. Storing these pellets in the freezer may help retard DNA degradation.

Protocol 2.3 Making wet slides for chromosome analysis

Adapted from a personal communication with Laura Adomaitis.

I. Principle

Making slides depends not just on the quality of the harvest, but it also depends on the atmospheric conditions of the day and on the quality of all reagents and materials used. The cytogenetics technologist must be resourceful enough to be able to recognize certain recurring problems and through an understanding of the process, be able to handle the unforeseen.

Safety precautions

Fixative is caustic and flammable and thus should be used and stored with proper fume ventilation, with full protection from spillage (see Note 1, Handling fixative).

II. Materials

General Supplies

1. Sterile bottled water, 1000 mL
2. Glass Pasteur pipettes, 5¼ in.
3. Sterile plastic Pasteur pipettes, 5¼ in.
4. Microscope slides
5. Microscope coverslips
6. CytoSeal
7. Glass slide tray/holder

8. Humidifier/temperature readout and controlled environment
9. Hot plates set to 40 °C (for drying), 60 °C (for aging overnight), and/or 90–96 °C if same-day aging is needed for STAT cases)
10. Phase microscope
11. Chemical harvest fume hood
12. Access to eye wash and shower
13. Vinyl labcoat and gloves

Reagents

1. 1 : 3 Fixative: Add 1 part glacial acetic acid to 3 parts absolute methanol. This solution should be made fresh periodically throughout the day. CAUTION: FLAMMABLE, CORROSIVE. Use proper PPE and proper ventilation to reduce caustic vapors.

III. Procedure

Preparing clean slides

1. Inspect each slide to ensure there are no scratches, streaks or chips. Discard defective slides in sharps container.
2. Stack slides into glass tray inserts.
3. Inspect glass dish to ensure there are no cracks. Glass dish should be able to hold 100 mL of liquid. Place dish within a protective tray (to control accidental spillage) under the chemical fume hood.
4. Add 25 mL of glacial acetic acid to 75 mL of methanol and carefully pour into glass dish. (Adjust volume if glass dish does not hold 100 mL) (see Note 2, Handling spillage).
5. Slowly immerse one slide tray insert into the glass dish of fixative. Cover and allow tray to sit for 5 minutes. Leave metal holder attached to tray. Meanwhile, fill a second glass dish with 100 mL of sterile, bottled water and place alongside the fixative dish.
6. After 5 minutes, carefully lift the slide tray insert above the fixative, allow fixative to drain into the dish, and place the tray of slides into the second glass dish with sterile bottled water.
7. Take this second dish to the sink, pour off water while running copious water, and add enough fresh, sterile bottled water to immerse the upper edge of the slides. Label dish cover with contents, date of cleaning and initials of preparer, and place in 4 °C until ready to use. Slides can be prepared in advance and stored at 4 °C for 2 weeks.
8. Continue the process with the second slide tray. Slowly immerse the next slide tray insert into the fixative dish under the fume hood and repeat the process until all slide trays have been cleaned and stored.

"Dropping" slides

NOTE: Work with only one patient at a time and keep only one centrifuge tube uncovered at a time. Change pipettes between centrifuge tubes, even on the same patient.

1. Confirm that humidity is 60–80% and temperature is 70–85 °F (21–29 °C) (the lower the temperature, the higher the humidity should be) in the area where slides are being made.
2. Depending on the size of the fixed cell pellet, supernatant is aspirated to 0.5 mL level or in equal volume with the size of the cell pellet. Cell suspension should be slightly opaque (see Note 3, When cell clumps are visible).
3. Confirm that name and accession # on tube matches slide label to be affixed. Number labels with Tube ID and unique slide identifier #. For example, 72T1 and 72T2 would be the first two slides made from culture 72T.
4. Remove one cold, wet (water) slide with forceps and run a few drops fixative along its surface (see Note 4, If fixative does not run down the slide evenly). Wipe back. Drop cell suspension onto wet slides in one of several methods below. **Do not rush the drying step.** Allow the fixative to complete its motion. You will see Newton's rings (rainbow) appear along the fixative surface. A ruffled edge indicates that the fixative "bubble" has imploded and the cells are planting. Place the slide on a 40 °C hotplate until surface is dry.
5. Label slide and check under the phase microscope. If the concentration and spreading is optimal, make one more slide. If, however, the quality or quantity can be improved, change what needs to be changed (see Note 5: Troubleshooting) and re-drop.

Slide dropping positions

Horizontal method

Place slide on countertop. Run fixative over its surface. Drop 2 equidistant cell suspensions onto the fix/water mixture. After 30 seconds, drain the residual fixative onto a paper towel and wipe the back. Wave the slide up and down once and watch for Newton's rings (see Note 6, The vanishing rainbow). As soon as ruffled edges appear, place slide onto the 40 °C hotplate.

Tilted horizontal method

Tilt slide on its long side at a 30° angle. Run fixative gently across surface. Gently place 2 drops at the top edge of slide and tilt the slide as the cells run down the short width of the slide. Blow gently and watch surface. When Newton's rings and edge ruffles appear, place on hotplate to dry. If, however, the cells are tight, wave the slide at a slow pace for 30 seconds. If they are still tight, tap gently on counter twice, wave, repeat tap and wave until slide is dry.

Tilted vertical method

Remove slides with water still retained on surface of slide. Place a couple of fixative drops to the surface and tilt slide lengthwise at a 30° to 60° angle (see Figure 2.22). Add the cell suspension while tilted, allowing cells to run down the surface, from label to lower edge. Tap moderately on counter edge, about 1 second between taps, until slide is dry. Place on 40 °C hotplate until completely dry.

IV. Notes

1. Handling fixative: Acetic acid can cause severe burns. Although methanol may neutralize some of its toxicity, it is still a reagent that should be used with a fume hood and any contact should be immediately treated. Do not carry any bottle containing fixative by the lid, as accidents have been reported because the top was not securely closed. Thermo Fisher Scientific SDS for acetic acid provides the following first aid measures for acetic acid exposure [1]:

   Eye contact: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required.

Figure 2.22 Tilted vertical slide method. Tilt wet, cold slide at a 30–60° angle, rinse with fixative, and allow two drops of cell suspension to run from label to lower edge. Tap moderately on counter edge, about 1 second between taps, until slide is dry.

Skin contact: Wash off immediately with plenty of water for at least 15 minutes. Immediate medical attention is required.
Inhalation: Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required.
Ingestion: Do not induce vomiting. Call a physician or Poison Control Center immediately.

2. Handling spillage: In the event of a spill while under the fume hood and within the protective tray, suction spillage into glass decanter and discard in liquid chemical waste receptacle. If the spill occurs in any other uncontrolled manner, dial 'O' for Operator and call for a "Code 100" to alert the assistance of the Chemical Emergency Cleanup team. See First Aid Measures for immediate attention.
3. When cell clumps are visible: Allow larger clumps to settle (or remove them to a labeled tube), or take the cell-fixative suspension from above the particles, without remixing) in order to have a cleaner slide preparation.
4. An uneven fixative runoff: Many factors can obstruct the metaphase rhythm and expansion during the slide-making process. Air movement caused by the fume hood fan, centrifuge vibration on the countertop, nearby construction rumbling the foundation, can all affect the critical moment when the cell collapses onto the glass surface. Therefore, conditions need to be ideal when making slides. Sometimes, however, the cause may have nothing to do with the air or countertop. In the early 2000s, a trusted slide manufacturer decided to improve their slides with a chemical that made their appearance whiter and for a lab, gave a much cleaner look. But the chemical created submicroscopic nicks into the glass surface that caused havoc to the cytogenetics community. If the fixative does not run smoothly down the slide, the cells may also find difficulty resting flat on its surface. Discard or re-clean the slide, and if the problem persists, find a new lot or different manufacturer, preferably one that the cytogenetics community currently uses with proven success, and compare results.
5. Troubleshooting
   a. Too crowded
      - Dilute and re-drop.
   b. Lots of nuclei but very few metaphases
      - Too much blood set up in culture; not enough buffy coat; too long in hypotonic or wrong concentration; mitogen expired; Colcemid® not added; harvested at improper time range; pregnant women have a low yield; children or patients on medications may also have a low yield.
   c. Too sparse
      - Re-spin and make more concentrated.
   d. Too tight
      - Check humidity; slides are drying too fast; re-fix and try again; use warm slides (e.g., drop cell suspension while slide is on 40 °C hotplate); blow on slide while still wet; shorten wait time before placing on hotplate; try another slide method; dilute; re-fix and aggressively pipet before dropping; layer 1 : 1 or 2 : 1 (methanol : acetic acid) fixative on slide before dropping the cell suspension (this should slow down the drying process).
   e. All I got was chromosome soup
      - Check that humidity isn't too high or temperature too low; fixative is drying too slowly; too long in hypotonic; dry flat; try a different method; do not blow on slide or wave.
   f. Short, dark, and stubby
      - Slides drying too quickly; check humidity/temperature. Could be a harvest problem: take off more supernatant before adding hypotonic; leave more supernatant over pellet before first fixative; slow down the fixation process; reduce time or quantity in Colcemid®.
   g. Chromosomes constantly too skinny
      - Try using blood/bone marrow hypotonic with a ratio of 1 : 2 sodium citrate/KCl.
   h. Intruding cytoplasm
      - Re-fix and mix well. Could be a harvest problem: not enough time in hypotonic; slow down fixation process.
   i. Uneven homologues, especially on the periphery
      - Slow down slide-making; hold back placing slide on hotplate until the edges show ridges. Could be a harvest problem: slow down first fixative procedure.
   j. Mushy chromosomes
      - Check fixative reagents; fix pellet a couple more times; increase slide aging; decrease trypsin time during banding; decrease stain concentration.
   k. Full of holes
      - Insufficient aging; insufficient drying after banding before covering
      - Hot plate temperature too high (should be 60 °C for overnight or 96 °C for the "quick-bake" aging process in 30–45 minutes).

The vanishing rainbow

Although Robert Hooke first described this rainbow phenomenon in his 1664 book *Micrognathia*, Isaac Newton in 1717 was the first who studied its formation. The refraction of light at varying interferences between the convex fixative drop resting on the flat glass surface creates this rainbow effect. As the convex fixative bubble "flattens," the cells become pressed onto the glass slide surface. This provides a visual indication for when the fixative meniscus collapses. http://en.wikipedia.org/wiki/Newton's_rings

Reference

1. Thermo Fisher Scientific Material Safety Data Sheet (MSDS) for Acetic Acid, CAS# 64-19-7. Revised July 30, 2010. 2nd rev.

Protocol 2.4 Slide-making

Adapted from the third edition of the *AGT Cytogenetics Laboratory Manual*, contributed by Brigham and Women's Hospital, Boston, MA, p. 51.

I. Principle

Slide-making is best done after the cells have been in fixative at 4 °C overnight. However, good results can be achieved from material that is freshly harvested.

II. Procedure

1. Aspirate the remaining supernatant from the last centrifuge procedure. (If tubes have been sitting overnight, mix and then centrifuge the tubes for 10 minutes at 1200 RPM.) Add sufficient fresh 3 : 1 fixative (methanol-acetic acid) to make the cell suspension slightly turbid. Note: The amount of fixative added will vary depending on the pellet size.
2. Using a diamond-tipped pencil, follow your institution's requirements for labeling slides, for example, case number and second patient identifier, tube letter, and slide number. Depending on atmospheric conditions and the characteristics of each sample, obtaining the best results may necessitate a variety of methods for preparing the slides before dropping the cells. Slides can be dipped in cold distilled water; steamed over a hot water bath; steamed with breath; dipped in methanol and allowed to dry; or soaked in 6 : 1 fixative.
3. Gently resuspend the cell pellet, using a Pasteur pipette. Drop 3–6 drops of cell suspension at one end of the slide and allow the drops to spread across the slide surface. Place the slide on a slide warmer at 75 °C to dry.
4. Check the slide for the presence/absence of metaphase spreads and for the degree of spreading using a phase contrast objective. Use this information to adjust or modify your slide-making conditions. Record the quality of the preparation in the Harvest Quality Assurance book.
5. Slides can be stained with fluorescent stains immediately. For Giemsa staining, it is best to allow the slides to dry on the slide warmer at 75 °C for approximately 2 hours and 15 minutes or bake for 1 hour at 90 °C.
6. Unstained slides are stored on metal trays by case number in slide files.

Protocol 2.5 Slide preparation

Adapted from a protocol from the third edition of the *AGT Cytogenetics Laboratory Manual*, that was contributed by Stanford University, Palo Alto, CA, pp. 61–63.

I. Principle

Chromosome slide preparations are made by dropping harvested cell suspensions on a wet slide, flooding with fixative, and air-drying. Properly prepared slides will yield mitotic figures that are well spread and of a contrast, when observed with phase microscopy, that will allow adequate G banding and subsequent chromosome analysis to be performed. Many different methods exist for slide preparation, and the following is presented as one protocol which, when consistently applied, will

satisfy the above requirements. It is not intended to constrain individual technicians methodologically, only qualitatively. Any variation that produces results consistent with the following quality control guidelines is acceptable.

Specimen

Harvested cell suspension from any chromosome analysis protocol. Slide preparations are best made on the same day of harvest, mainly as a matter of convenience. Slide preparation can be delayed for one or more days without problem and may in some cases benefit (e.g., difficult bone marrow) harvests. Metaphase spreading on harvests left longer than several days may be problematic.

II. Materials and equipment

1. KimWipes
2. Microscope slides, frosted (VWR #48312-079)
3. Paper towels
4. Absolute methanol, (JT Baker #9070-1)
5. Glacial acetic acid (JT Baker #9507-1)
6. Pasteur pipette, 5-3/4 (VWR #14673-010)
7. Oven, 90 °C
8. Phase contrast microscope

Reagent preparation

9. Methanol-acetic acid fixative: One part glacial acetic acid to three parts absolute methanol. Make fresh.

Quality control

Quality control is an ongoing process every time slides are made. Each slide should be examined under phase microscopy to determine if a sufficient number of metaphases show adequate spreading and contrast. Never make more than two or three slides without monitoring their quality. For specifics of manipulating the process to optimize spreading and contrast, see Troubleshooting.

Unacceptable results: Preparation in which a preponderance of metaphases are of such poor spreading or contrast as to preclude successful G-banding and analysis.

III. Procedure

1. Store slides in a Coplin jar of absolute methanol. Remove slide from methanol and polish with a folded KimWipe. Dip the slide back in methanol and swirl it in a beaker of distilled water until the methanol is dissolved and a uniform, thin film of water covers the slide.
2. Holding the ground glass end between thumb and finger, vertically blot the long side of the slide on a paper towel to remove excess water. Keeping the edge in contact with the paper towel, lower the slide until it forms a 30° angle with the benchtop, with uniform water film up.
3. From a horizontally held Pasteur pipette, 1–2 inches above the slide, place 3 drops of cell suspension evenly spaced onto the slide, moving successively toward the frosted end. The droplets should strike the slide one-third of the width from the top of the horizontally positioned slide and should burst and spread evenly as they strike the slide.
4. Tilt the slide back to vertical and drain it a moment. Lower it again to a 30° angle and, dropwise with a Pasteur pipette, flood the slide with fresh 3:1 methanol–acetic acid fixative, starting at the upper corner and moving toward the ground glass end. This will evenly displace any remaining water and allow the slide to dry uniformly.
5. Again drain the slide for a moment, wipe off the back, and then air-dry the slide in such a manner as to provide good chromosome spreading and morphology (see Troubleshooting).
6. Label slide according to laboratory requirements, which may include laboratory number, second patient identifier, culture letter, unique sequential number, and date.
7. Artificially age the slide in an oven at 90 °C for 30 minutes.

IV. Troubleshooting

Procedural consistency is essential when preparing chromosome slides. This will subsequently allow consistent and systematic G-banding. To achieve consistency, each slide must be monitored with phase contrast microscopy. Slides are examined for good cell concentration, chromosome spreading, morphology and mitotic index.

1. Good chromosome preparations should be spread adequately for easy counting and analysis. Overspreading should be avoided as this will cause artifactual hypomodal chromosome counts and difficulty in photomicroscopy.
2. Chromosomes should appear sharp and dark, without "light halos" surrounding them. Likewise, they should not be fuzzy, pale or "grayed out."
3. The most critical and variable step is air-drying the slides. This must be worked out for each session, then adhered to and monitored for each slide. In general, poorly spread chromosomes with halos are drying too fast and may be slowed by placing them to dry on a wet paper towel or at a vertical or steep angle, breathing lightly on the slide after fix flooding or by chilling the fix. Also in general, pale, gray, or overspread chromosomes are drying too slowly and may be corrected by lowering the drying angle, briefly warming the slide, or waving the slide before setting it down to dry.
4. Drying is very sensitive to humidity and temperature, and so will vary from day to day. A certain amount of imagination and experimentation is helpful. Remember, though, that simplicity of technique is desirable and will lead to more consistent results and subsequently to easier and better trypsin G-banding.
5. Heating of the slide is also important. This is artificial aging and should be done within the limits stated in the procedure. Overheating or underheating, whether by varying temperature or time, will lead to under- or oversensitivity to trypsin pretreatment during G-banding.

References

1. Personal communication with Mike Brown's technique, Clinical Cytogenetics Lab, Oregon Health Sciences University, Portland, Oregon
2. Hack M, Lawce H, eds. *The Association of Cytogenetic Technologists Cytogenetics Laboratory Manual*. San Francisco: Association of Cytogenetic Technologists, 1980.

Protocol 2.6 Slide preparation procedure

Adapted from a protocol from the third edition of the *AGT Cytogenetics Laboratory Manual*, that was contributed by the University Medical Center, Ohio State University, Columbus, Ohio, pp 73–74.

I. Principle

A homogeneous cell suspension is dropped on detergent or acid alcohol-cleaned slides from a height and dilution such that the metaphase spreads as well as the chromosomes themselves will be spread apart from one another without breaking the cell membranes. A ruptured cell membrane will result in chromosomes scattering on the slide surface, producing metaphases that are both difficult and inappropriate to analyze. After dropping the cell suspension, the slides are placed briefly on a hotplate and then into a hot oven to aid the drying process. The slides are banded according to the technique deemed necessary for the type of analysis. Slide preparation is the single most important step in achieving good results. Poorly prepared slides will result in poor banding and tedious analysis.

II. Materials

1. Fixative
   a. Methanol, analytical reagent grade
   b. Glacial acetic acid, analytical reagent grade

Preparation of working solution

1. Measure with a 100 mL graduated cylinder, 75 mL (3 parts methanol) to 25 mL (1 part) glacial acetic acid.
2. Pour into 100 mL bottle and cap lightly. Label as "Fix" with warning: CORROSIVE/FLAMMABLE.
   Prepare fixative immediately before it is needed. Always keep bottle capped when not in use. Discard daily any unused portion into safety canister, according to your institution's Chemical Management Guidelines. Mark date, type and concentration of compound, quantity discarded and initials on tag attached to safety can.

Quality control

1. Microscopically check first slide dropped, assessing quality and quantity of metaphase spreads present.
2. Adjust dropping technique accordingly for successive slides dropped.
3. Check microscopically each successive slide dropped from that culture. Readjust dropping technique accordingly.
4. Check first slide on each remaining culture tube to be dropped, and each successive slide thereafter, for chromosome spreading and background cytoplasm.
5. Tolerance limits: Aim for well spread chromosomes with no cytoplasm.

III. Procedure

1. Using a Pasteur pipette, place three drops of fixed cell suspension evenly over clean slide immersed in 37 °C distilled water.
2. Slides can be shaken and/or blown on gently to facilitate chromosome spreading.
3. Place on wet paper towels for 5–30 seconds if humidity 40%.
4. Place on 65 °C slide warmer for 5–30 seconds, as necessary.
5. Check slide using phase contrast microscope for quality of cell suspension, number of metaphases, quality of metaphases, and spreading. Document on specimen setup sheet the quantity and quality of metaphase spreads present in each culture tube.

IV. Troubleshooting

1. Slide too thick with nuclei and/or mitotic figures
   a. Dilute with more fixative.
2. Slides too thin
   a. Spin and remove some of the fixative.
3. Poorly spread and/or cytoplasmic background
   a. Increase height from which cells are dropped from pipette onto slide.
   b. Use additional washes in fixative.
   c. Increase concentration of glacial acetic acid in fixative.
   d. Substitute 50% glacial acetic acid in fixative.
   e. Use steam and less time on slide warmer.
   f. Use combination of above methods.
4. Scattered chromosomes and/or broken spreads
   a. Decrease height from which cells are dropped from pipette onto slide.
   b. Use more time on slide warmer.
   c. Use colder water and/or frozen slides.
   d. Use combination of above methods.
5. If none of the above rectifies the problem, refrigerate and drop the following day.

CHAPTER 3

Peripheral blood cytogenetic methods

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3.1 Using peripheral blood for cytogenetic analysis

Peripheral blood is the most commonly utilized tissue for determination of constitutional karyotypes because it usually yields the longest and best-banded chromosomes (Figure 3.1) and can be obtained easily. Peripheral blood is obtained in a relatively noninvasive manner; is inexpensive to culture; requires only short culture durations; can be recultured from the original sample; and can be mailed successfully. Peripheral blood lymphocytes are not dividing in normal, healthy adults; they must be stimulated to divide by exposure to mitogens, such as phytohemagglutinin or pokeweed antigen.

Clinical uses of stimulated peripheral blood cultures include determination of the constitutional karyotype of patients requiring genetic diagnosis or genetic counseling; determination of the constitutional karyotype of families of individuals with chromosome abnormalities to determine carrier status of a familial translocation; comparison of lymphocyte karyotypes with those of other tissues (skin, amniotic fluid, or chorionic villus sampling) for confirmation of structural and numerical abnormalities or mosaicism; determination of the constitutional karyotype of patients with hematologic malignancies or solid tumors for comparison with chromosome abnormalities in their neoplastic cells (see Chapter 11, section 11.7.2, Cytogenetic methodology); assessment of chromosome damage in individuals exposed to environmental hazards; aid in the diagnosis of chromosome breakage syndromes such as Bloom syndrome, Fanconi anemia (Chapter 13, section 13.2, Fanconi anemia), and ataxia telangiectasia; and to provide material for interphase and metaphase FISH (fluorescence in situ hybridization) studies. Most acquired abnormalities in hematologic malignancies are studied with bone marrow or unstimulated blood cultures; however, certain hematologic malignancies, such as chronic lymphocytic leukemia, are best studied with stimulated blood cultures, using various mitogens (e.g., pokeweed mitogen or IL-4) for various culture durations (see Chapter 11, Protocols 11.3–4). This chapter will deal mainly with culture of non-neoplastic blood cultures.

Clinical uses of unstimulated blood cultures include determination of the acquired karyotypes of spontaneously dividing cells from hematologic malignancies (see Chapter 11, section 11.7.2, Cytogenetic methodology), and constitutional karyotypes from directly harvested or 24-hour cultures of neonatal blood samples (e.g., umbilical cord blood from newborns or periumbilical blood samples (PUBS) obtained prenatally). This is possible because of the presence of immature cells (blasts) in these samples that are capable of dividing and yielding mitotic cells that can be analyzed for STAT chromosome results.

Although blood cultures have many advantages, they are not the best choice for every situation. Evaluation of cases in which mosaicism could be present could miss some of the different cell lineages when only blood is studied, because some cell lines may be lost over time (especially in rapidly dividing tissues) or may not manifest in blood tissue at all (e.g., Pallister–Killian syndrome and mosaic trisomy 20). There are three germ layers in the embryonic blastoderm: **endoderm**, the innermost layer that gives rise to the epithelium of the digestive tract, the respiratory organs, bladder, vagina, and urethra (from which many amniotic cells may originate); **mesoderm**, the middle layer, which gives rise to all connective tissues, including muscular, skeletal, circulatory, lymphatic, and urogenital, plus body cavity linings; and **ectoderm**, the outer layer, which forms skin, nervous system, and so forth. Blood is from mesodermal germ cell layers and will not exhibit cell lines that are confined to ectoderm or endoderm, nor will it confirm abnormalities seen in some extra-fetal cell lineages such as certain confined placental mosaicism (CPM). In spontaneous abortuses, it is extremely important to study extra-fetal material in addition to blood, skin,

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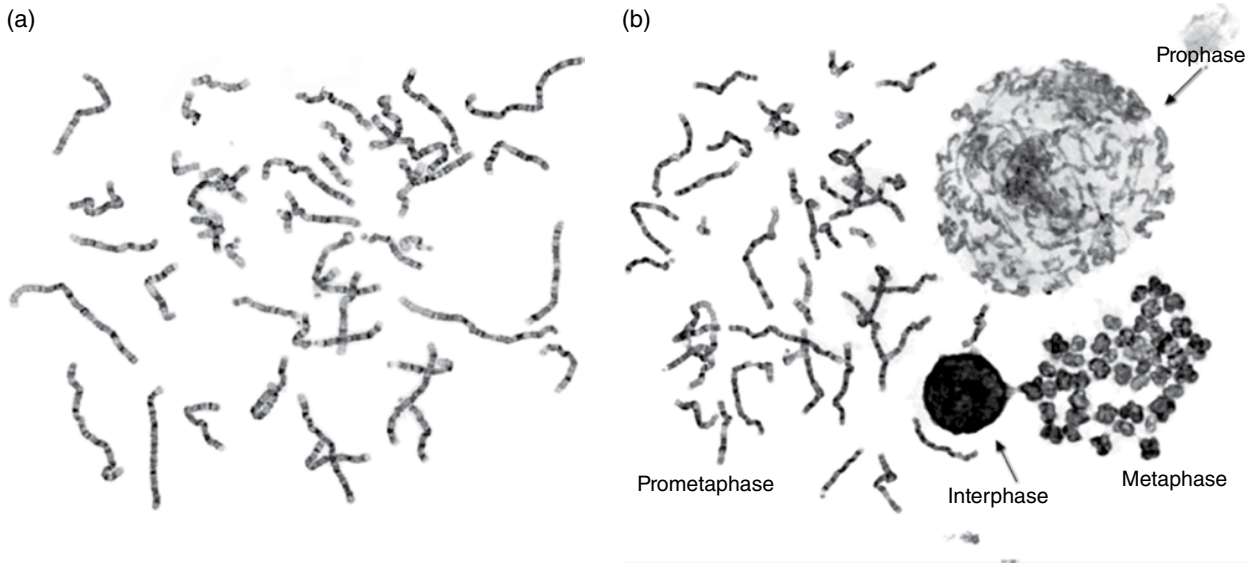

Figure 3.1 (a) A metaphase cell from peripheral blood stimulated with phytohemagglutinin, synchronized with methotrexate, released with thymidine, and arrested with Colcemid®. The quality that can be attained from lymphocyte cultures, combined with the ease of specimen procurement, makes this the tissue type of choice for most constitutional cytogenetic studies. (b) One field showing an interphase, prophase, prometaphase, and metaphase cell.

etc., to look for confined placental mosaicism (see Chapter 5, section 5.6.2, Confined placental mosaicism and uniparental disomy). In suspected mosaics, other tissue (skin, gonad) should be examined cytogenetically, if possible, in addition to blood cultures.

Research uses of stimulated blood cultures include creating lymphoblast cell lines; performing microdissection and PCR (polymerase chain reaction); gene mapping; comparative genomic hybridization (CGH); chromosome organization research; animal karyotyping; and chromosome evolutionary studies.

3.2 Special uses of peripheral blood cultures

3.2.1 Chromosome instability syndromes

Peripheral blood cultures are used to study the genetic syndromes with DNA repair, replication, or recombination defects. Examples of these include Fanconi anemia [1–8], ataxia telangiectasia [9,10], and Bloom syndrome [9]. The phenotypic pattern includes growth restrictions, immune deficiencies, and predisposition to malignancy. Fanconi anemia is an autosomal recessive disorder characterized by progressive pancytopenia, diverse congenital malformations, and a predisposition to malignancies [3–5]. Clinical signs vary and can include some or all of the following: cafe-au-lait spots, absent thumbs, microcephaly, radio-ulnar synostosis or aplasia, hypotelorism, short stature, renal and genital anomalies, heart defects, hearing loss, intellectual disabilities, low birth weight, and aplastic anemia [2]. Owing to the variable expression of the disorder, diagnosis by clinical manifestations alone is unreliable. Fanconi anemia cells exhibit a higher percentage of chromosome breaks and radial formations than cells from normal individuals when cultured with DNA-damaging clastogenic agents, such as diepoxybutane (DEB) and mitomycin C (MMC) [2]. Breakage analysis combined with the clinical picture provides an accurate diagnosis of patients with Fanconi anemia. Treatment for Fanconi anemia patients is different from that for other anemias, so it is very important to obtain the correct diagnosis. For example, treating a Fanconi anemia patient with radiotherapy, chemotherapy, or immune suppression before bone marrow transplant would have devastating results because of their chromosome instability. Current therapy strategies include bone marrow transplant, androgen therapy, and hematopoietic growth factors.

Cytogenetic breakage analysis is most commonly performed on peripheral lymphocytes. Cultures are set up in the usual manner and cultured in the clastogenic agent for at least 48 hours. Metaphases (50–l00) of nonbanded Giemsa slides are scored for breaks and radial formations. Control cultures are performed also to determine effectiveness of the clastogenic agent and to determine reference ranges [2]. Prenatal testing has been performed [6] on periumbilical blood samples and on chorionic villi and amniotic fluid cells for couples with a previously affected child. Normal baseline breakage frequencies are being developed on these latter cell types, as well as normal and affected fibroblasts. Various cell types have different sensitivities to clastogenic agents, so the dosage has to be carefully established.