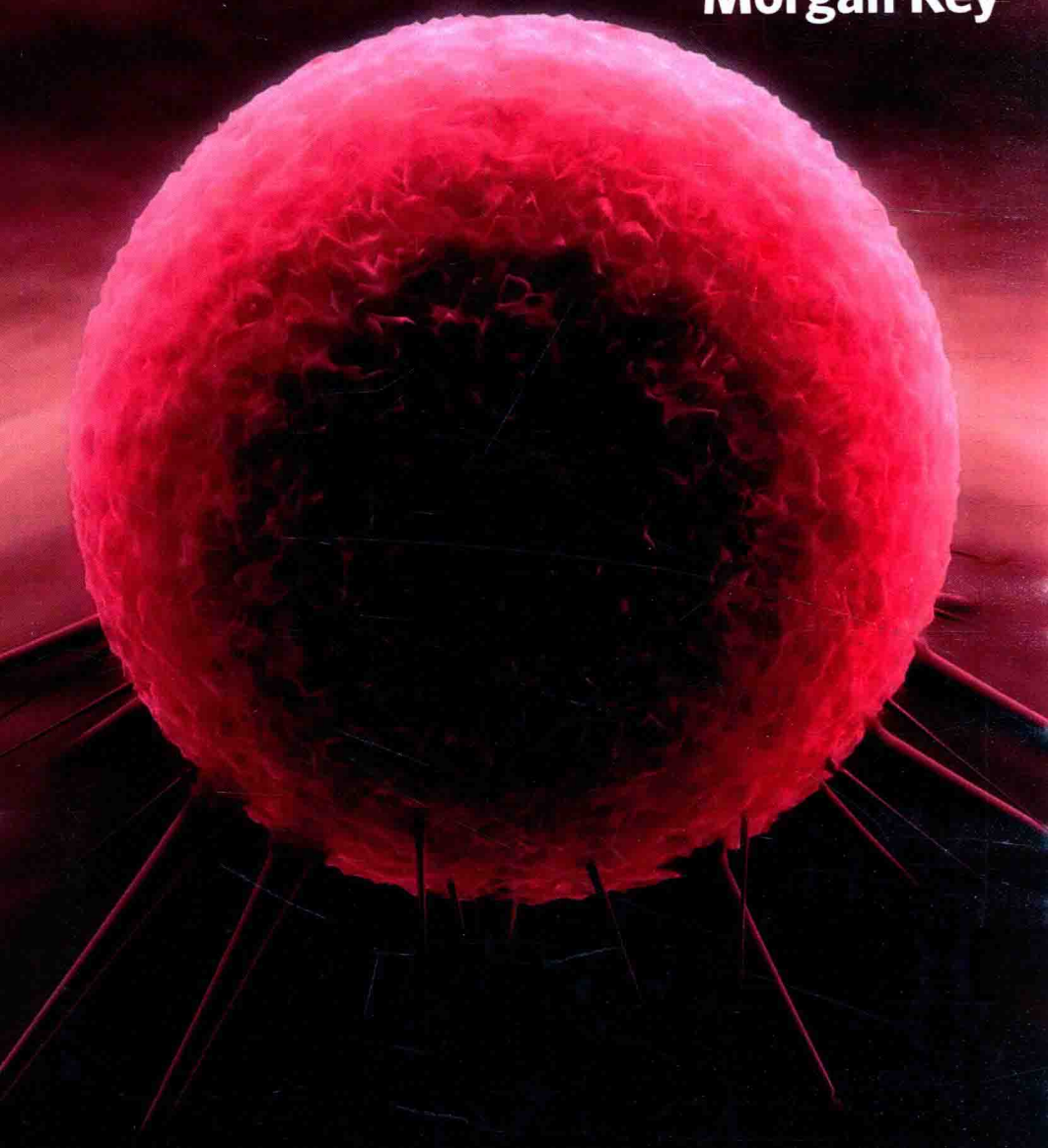
Cytogenetics

Techniques and Applications

Morgan Key



Cytogenetics: Techniques and Applications

Edited by **Morgan Key**

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Preface

This book discusses the current trends in the field of cytogenetics. It involves the specifics and particulars of the methodologies that can be accepted and used in clinical laboratories. The book discusses the basic methods of cell lines, primary cultures and their usage; array CGH for diagnosis of fetal conditions; micro technologies and automations; use of chromosomes as tools to find biodiversity and usage of digital image technology. It also includes methods to deal with acute lymphoblastic and myeloblastic leukemia in patients and survivors of atomic bomb exposure. While focusing on the advanced practices used in this field and its applications, this book demonstrates the urgency to establish cytogenetic labs with modern and latest equipment. This book will aid in accurate and effective diagnosis which will further benefit patients.

Various studies have approached the subject by analyzing it with a single perspective, but the present book provides diverse methodologies and techniques to address this field. This book contains theories and applications needed for understanding the subject from different perspectives. The aim is to keep the readers informed about the progresses in the field; therefore, the contributions were carefully examined to compile novel researches by specialists from across the globe.

Indeed, the job of the editor is the most crucial and challenging in compiling all chapters into a single book. In the end, I would extend my sincere thanks to the chapter authors for their profound work. I am also thankful for the support provided by my family and colleagues during the compilation of this book.

Editor

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Cytogenetic Analysis of Primary Cultures and Cell Lines: Generalities, Applications and Protocols

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1. Introduction

Cytogenetics constitutes an important diagnostic tool to determine and/or confirm specific syndromes nowadays; its use is directed towards the selection of treatments and monitoring of patients using different procedures. These latter are carried out in order to obtain a karyotype from peripheral blood or several tissue biopsies (e.g. biopsies from patients with melanoma, breast cancer, skin biopsies, foreskin samples, abortion products, among others). However, the study of chromosomal abnormalities in culture cells has been limited by complex processes such as achieving cell growth and a good number of metaphases, which in turn hampers the chance to obtain a useful number of metaphase spreads in order to carry out a proper cytogenetic analysis, that should be able to display a good morphology, an adequate dispersion and a correct banding. Cell lines are widely used in different research fields, particularly in invitro models for cancer research. (Burdall et al., 2003)

Given the importance of the model used to examine and manipulate potentially relevant molecular and cellular processes underlying malignant diseases, it is necessary to achieve an accurate and comprehensive karyotyping for cultures of different cell lines. In turn, karyotyping provides an insight into the molecular mechanisms leading to cellular transformation and could allow clarifying possible cytogenetic aberrations associated to drugs exposure and the development and progression of different types of cancer. The fast increase observed in cancer incidence is forcing us to carry out more identification studies of cytogenetic biomarkers associated to development of this disease, which could contribute for a better understanding of the carcinogenic process and could also have enormous implications for the development of effective anticancer therapies.

Obtaining metaphase cells for chromosome analysis requires the use of a series of reagents, protocols, and environmental conditions, among others, that will allow us to collect the chromosomes. Metaphase cells must be cultured under certain conditions in order to obtain a proper number of dividing cells, which need to grow and divide fast in this medium as well. Taking into account all the issues mentioned above, an accurate knowledge from the culture medium and its conditions, as well as the techniques and protocols required in

general, will allow a good cell growth in the culture and the collection of chromosome spreads to carry out the cytogenetic characterization and the identification of chromosomal abnormalities present in the cells in study.

This chapter describes the practical aspects of performing cytogenetic studies in primary cultures and human cancer cell lines that have been previously standardized, in order to be applied not only in research but in diagnosis and possible treatment of several diseases.

2. Generalities

The application of cytogenetic studies on tissue and cancer cell lines has become important in recent years, because the presence of some chromosomal abnormalities indicate the prognosis of the disease and the corresponding response to therapy. The most common clinical applications of cytogenetic studies on tissue and cancer cell lines are:

- Establishing the type of chromosomal abnormalities and its frequency
- Identifying the genes located in the affected chromosomal regions in order to establish those possibly implied in neoplasia
- Studying tumorigenic and metastatic behaviors, apoptosis and functionality
- Identifying the mechanisms of action used by hormones
- Establishing models for drug resistance studies
- Establishing the therapeutic potential of different treatments
- Supporting further research.

The knowledge of novel chromosome rearrangements and breakpoints identified could be useful for further molecular, genetic and epigenetic studies on human cancer that could lead us to understand the mechanisms involved in the development and progression of this disease.

2.1 Characteristics of cell cultures

Cell cultures can be divided into two groups, depending on the substrate used for cell growth:

- Suspension cultures: Cells are cultured by constant agitation in a liquid medium. Cell cultures are prepared by diluting cell suspensions.
- Monolayer cultures: Cells adhere to a solid (glass or plastic) or semisolid (agar, blood clot) surface, forming a cell surface, which can be observed by light microscopy or phase contrast. Cultures are maintained by releasing cells from the substrate using mechanical or enzymatic procedures, continuing their life cycle in new cell subcultures.

2.1.1 Requirement of cell growth

There are several variables that determine whether a cell will multiply *in vitro* or not, some of these depend directly on the conditions of the growth medium and some do not.

- The growth medium must possess all the essential, quantitatively balanced nutrients. It must include all the necessary raw material to promote the synthesis of cellular macromolecules; it must also provide the substrate for metabolism (energy), vitamins and trace minerals (their primary function is catabolic) and a number of inorganic ions implied in the metabolic function.

- Physiological parameters: temperature, pH, osmolarity, redox potential, which must be kept within acceptable limits.
- Cell density and subculture mode
- Serum is added to the basal medium to stimulate cell multiplication and interaction with the other variables contained in the system. It serves as a source of macromolecular growth factors. Serum is a very effective supplement that promotes cell division because it contains different growth-promoting factors. Complete serum contains most of low molecular weight nutrients required for cell proliferation. Serum may neutralize trypsin and other proteases, provide a protein "carrier" to solubilize water-insoluble substances (such as lipids) and has the ability to provide hormones and growth factors to cells.

2.1.2 Contamination of cell cultures

Cell cultures can be contaminated by fungi, bacteria, mycoplasma, viruses, parasites or cells from other tissues. It is mistakenly thought that tissues obtained using aseptic techniques from apparently healthy animals are sterile; however, it is common to find bacteria, mycoplasma, viruses or other microorganisms in these tissues. Fungi and bacteria are universally distributed in nature and are relatively resistant to environmental factors such as temperature, radiation, and desiccation, among others.

These organisms can appear in cultures due to several factors:

- Through dust particles carried by air currents.
- Aerosols produced by the operator during handling.
- Through non-sterile equipment.

Viruses and mycoplasmas are found in nature mainly in cells and body fluids, and these are more sensitive than fungi and bacteria to environmental factors. The most important sources of contamination with mycoplasma are aerosols and sera used in culture mediums. Other routes of entry for the virus are other infected cell cultures, serum or spray. Three factors are determining the effectiveness of a sterility test:

- Sensitivity and spectrum of the medium used.
- Incubation terms and time.
- Sample Size

The medium used for these tests should be sensitive and have a broad spectrum to detect anaerobic bacteria, fungi and mycoplasmas in routine testing. Cultures for bacteria and mycoplasma should be incubated aerobically and anaerobically, in order to avoid the loss of detection of some microorganisms. It is recommended to test for sterility at different times in the initiation and harvesting of the cell culture (beginning, middle and end).

2.1.3 Contamination of cell cultures by other cells

A very common contamination, generally not considered by researchers working in cell culture, is cross-contamination between cell cultures, both at the intra and interspecific level (MacLeod et al., 1999; Marcovic & Marcovic 1998; Masters et al., 2001; van Bokhoven et al., 2001; Masters 2002). Several cases of cross-contamination between cell cultures have been

documented in the last years, this has been possible by some sources that are able to provide certified cell lines, which can be used when contamination in the cell culture is suspected. Approximately, a 20 to 30% of cell cultures are contaminated at the intra or interspecific level, and it is believed that this value is higher due to the large number of contaminated cultures, on which there is no suspicion. The most convenient way to avoid contamination is to use rigid sterilization and aseptic techniques, the culture medium must be proven for contamination before use, working with cell cultures in laminar flow chambers, decontaminate the work area on a daily basis and furthermore, when there is manipulation of different cell lines.

2.2 Human cancer cell lines

Many human carcinoma cell lines have been developed and are widely used for laboratory research, mainly in studies of tumorigenic and metastatic behaviors, apoptosis, functionality, and therapeutic potential, and particularly as in vitro models for cancer research. Among these cell lines are the following: MCF-7, SKBR3, TD47 and BT474.

2.2.1 Characteristics

MCF-7 is a cultured cell line from human breast cancer, which is widely used for studies on breast cancer biology and hormones' mechanism of action research. The cell line was originally derived at the Michigan Cancer Foundation from a malignant pleural effusion found in a postmenopausal woman with metastatic breast cancer. The cells express receptors as biological responses to a variety of hormones including estrogen, androgen, progesterone, glucocorticoids, insulin, epidermal growth factor, insulin-like growth factor, prolactin, and thyroid hormone with non-amplified HER2 status (Osborne et al., 1987)

The cell line SKBR3 is a highly rearranged, near triploid cell line, derived by Fogh and Trempe (1975) from a pleural effusion and overexpresses the HER2/c-erb-2 gene product. This cell line shows only a weak ESR2 (ERß) expression and no ESR1 (absence of functional ERα) and PGR expression, indicating that this cell line represents models of estrogen- and progesterone-independent cancers, with capability for local E2 formation and possible action via non-ER mediated pathways. ERß expression level in tumor cell lines is characterized by a significantly slowed proliferation (Hevir et al., 2011). ERß may negatively regulate cellular proliferation, promote apoptosis and thus may have not only a protective role in hormone-dependent tissues, such as breast and prostate, but also a tumor-suppressor function in hormone-dependent tissues (Lattrich et al., 2008).

Human breast ductal carcinoma BT474 cell line was isolated by Lasfargues et al (1978). It was obtained from a solid, invasive ductal breast carcinoma from a 60-year-old woman; cells were reported as tumorigenic in athymic mice and were found to be susceptible for mouse mammary tumor virus, confirmed as human with IEF from AST, GPDH, LDH and NP (Lasfargues et al., 1979).

T47D is a cell line derived from human ductal breast epithelial tumor, it was isolated from a pleural effusion obtained from a 54 year old female patient with an infiltrating ductal breast carcinoma (Keydar et al., 1979). These cells contain receptors for a variety of steroids and calcitonin. They express mutant tumor suppressor protein p53 protein. Under normal culturing conditions, these cells express progesterone receptor constitutively and are

responsive to estrogen. They are able to lose the estrogen receptor (ER) during long-term estrogen deprivation *in vitro*. Culture conditions, receptor status, patient age and source and tumor type for each cell line are shown in Table 1 and Figure 1.

Cell line	Source code	Passage no	Receptor status	HER-2 status	Tissue source	Tumor type	Patient age	Culture conditions
T47D	ATCC: HTB-133	P20	ER+ PR+	Negative	PE	IDC	54	RPMI 1640 + 10% FBS + 2 mM L-glutamine +antibiotic-antimycotic solution (1X)
MCF-7	ATCC: HTB-22	P16	ER+ PR+	Negative	PE	AC	69	
SKBR3	ATTC: HTB-30	P15	ER− PR−	Positive	PE	AC	43	
BT474	ATTC: HTB-20	P12	ER+ PR+	Positive	IDC	IDC	60	DMEM + 10% FBS + 2 mM glutamine + antibiotic-antimycotic solution (1X)

Table 1. Characteristics of the breast cancer cell lines AC, adenocarcinoma; IDC, invasive ductal carcinoma; PE, pleural effusion; P, passage number. Media conditions: FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle's Medium. Cell lines were maintained at 37°C and 5% CO2 in the indicated media

2.2.2 Cytogenetic abnormalities found in human cancer cell lines

MCF-7 cell line has a modal number from 82 to 86 with 56 types of aberrations: 28 numerical and 28 structural aberrations. The most common aberrations in MCF-7 cells are der (19) t (12;19)(q13;q13.3) and add(19)(p13) (Figure 2A).

SKBR3 cell line has a modal number from 71 to 83, with 48 types of rearrangements: 27 numerical and 21 structural rearrangements. The most common aberrations in this cell line are del(1)(1p13) and add(17)(17q25) (Figure 2B).

BT474 cell line demonstrated to have a modal number from 65 to 106, with 67 different rearrangements: 35 numerical and 32 structural aberrations. The most common aberrations in this cell line are: Additional material of unknown origin on chromosome 14: add(14)(q31), derivatives from chromosomes 6: der(6)t(6;7)(q25;q31) and 11: der(11)t(8;11;?)(q21.1;p15;?), losses in chromosomes 15, 22 and X chromosome and a gain on chromosome 7 (Figure 2C).

T47D cell line have a modal number of 57 to 66, with 52 types of rearrangements: 26 numerical and 26 structural. The most common aberrations in this cell line are: der(X)t(6;X)(q12;p11); der(8;14)(q10;q10); del(10)(p11.2); der(16)t(1;16)(q12;q12) dup(1)(q21q43) and der(20)t(10;20(q21;q13.3) Figure 2D. The cell lines SKBR3 and BT474 exhibited amplification of HER-2 gen by FISH and the cell lines MCF-7 and T47D not have amplification for this gene.

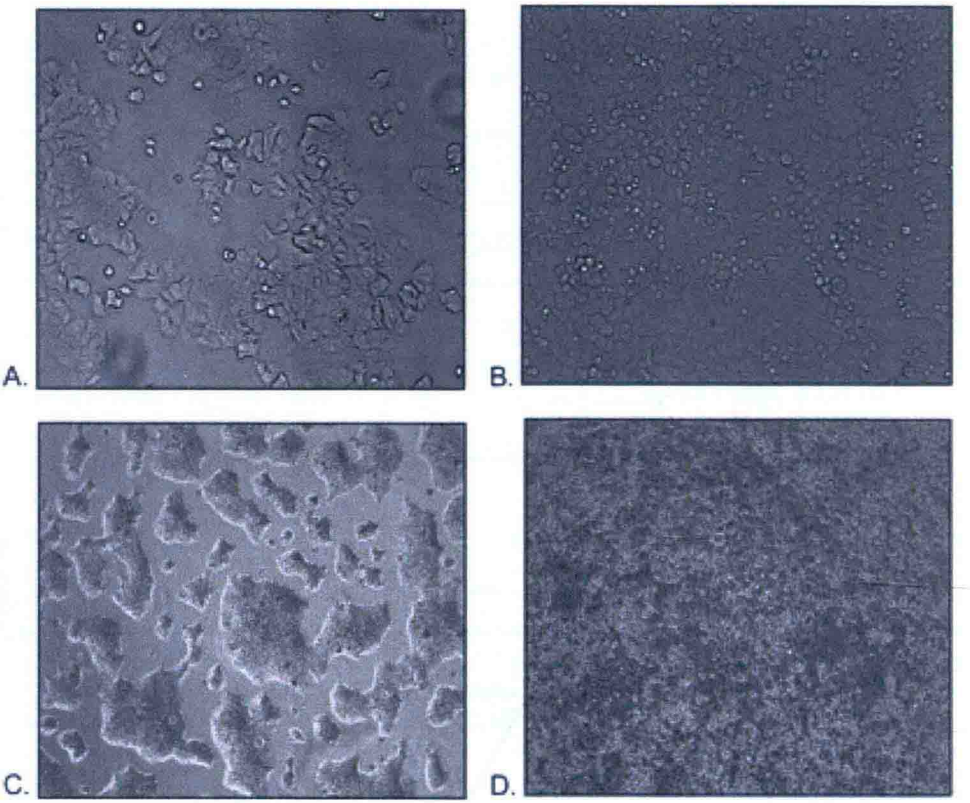

Fig. 1. Inverted microscopic pictures of representative breast cancer cell lines in a monolayer culture. A) MCF-7; B) SKBR3; C) BT474; D) T47D

3. Cytogenetic techniques from tumoral tissue samples and cancer cell lines

Obtaining metaphase cells for chromosome analysis requires the use of a series of reagents that will allow us to collect the chromosomes. Metaphase cells must be grown *in vitro* under certain conditions in order to obtain a proper number of dividing cells. Cells used for chromosome collection must be able to grow and divide fast in the culture medium. Different types of cells may require specific growth factors and medium supplements; once the basic requirements for each cell type are known, the appropriate culture medium is selected, checking sterility appropriately. After the culture has reached the 80% of confluence, it must be harvested and fixed to make a cytogenetic suspension. Cultures are growth arrested and accumulated in metaphase or prometaphase by inhibiting tubulin polymerization and thus preventing the formation of the mitotic spindle (e.g., using colcemid or velbe). Following exposure to colcemid or velbe, cells are treated with a hypotonic solution to enhance the dispersion of chromosomes and fixed with carnoy fixative (Methanol: Acetic Acid). Once fixed, the cytogenetic preparation can be stored in cell pellets, under fixative conditions and 20°C for several months. Fixed cells are spread on slides and air-dried, to be finally banded for the correct identification of chromosomes. Obtaining an

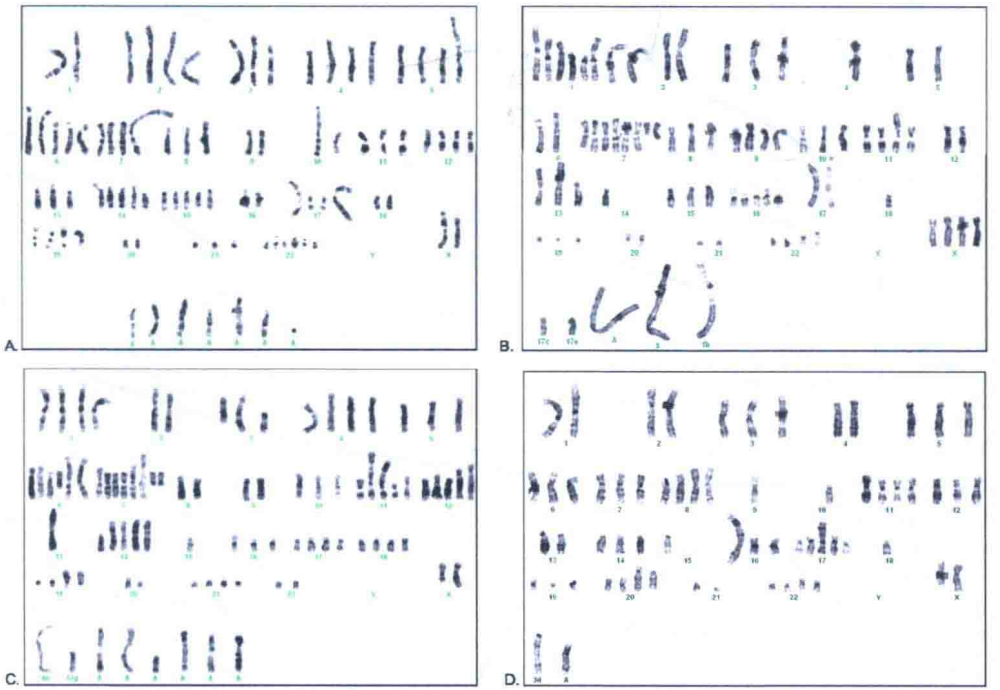

Fig. 2. Karyotypes from breast cancer cell lines. A) MCF-7; B) SKBR3; C) BT474; D) T47D15-ml conical centrifuge tube

adequate quality on chromosome spreads is multifactorial; this will be discussed in detail further on. The amount of metaphases obtained is sometimes inadequate for chromosome analysis, thus it is always necessary to keep growing the cell line.

3.1 Materials, reagents and equipment

3.1.1 Equipment

- Laminar flow chamber
- Incubator
- CO2 Incubator
- Serological bath
- Centrifuge
- Refrigerador
- Microscope with camera
- Inverted microscope
- Magnetic Stirrer
- Micropipettes
- Analytical balance
- Vacuum Pump

3.1.2 Materials

- 75-cm^2 tissue culture flasks
- 25-cm^2 tissue culture flasks
- Sterile disposable plastic transfer pipettes
- Glass slides
- Coverslip sheets
- Petri dishes

3.1.3 Reagents

Solutions should be kept in the dark at -20°C or 4°C, according to the manufacturer's instructions. According to frequency of use, reagents should be aliquoted and frozen. Reagents should be thawed before use and stored at 4°C. Frequent freezing and unfreezing may cause alteration of the culture medium by inactivating the components.

Medium: The most commonly used medium for cell cultures are Dulbecco's modified Eagle's medium-DMEM, RPMI 1640 and DMEM-F12, among others. If the medium does not contain Glutamine, L-glutamine should be added (final concentration 2mM); this is an essential amino acid that is unstable and has a short life at room temperature. To each 500 ml bottle of medium, add 50 ml of Fetal Bovine Serum, 5 ml of L-Glutamine (200 mM) and 5 ml of antibiotic-antimycotic solution (100x). Store the medium up for a month at 4°C. In order to establish primary cultures it is recommended to add also hydrocortisone, estradiol and insulin to the culture medium, providing enough nutrients to induce cell growth.

Serum: Fetal bovine serum; the proportion commonly added is 50ml of serum per each 450 ml of medium. Usually, the presentation of fetal bovine serum is 500 ml, so this amount should be aliquoted in 50 ml aliquots which must be stored at -20°C and thawed at 4°C or room temperature prior to use. It is advisable not to thaw the medium at high temperatures (37°C or more), as this could alter its composition.

Collagenase stock solution: Type 2 collagenase. To make the stock solution, dissolve 215 U/mg collagenase in distilled water to obtain a final concentration of 2000 U/ml, filter the solution through a 0.2-µm filter and prepare 1 ml aliquots, these can be kept stored for 2–3 months at –20°C. The working solution of 200 U/ml is prepared immediately before use, adding 1 ml collagenase each 9 ml of complete medium. This solution should be kept at 4°C.

Arresting agents:

Colcemid: Colchicine inhibits microtubule assembly by binding to a high affinity site on β-tubulin. Colchicine binding occurs in a nearly irreversible manner and exerts a conformational change in tubulin, as well as in colchicine itself. (Daly, et al. 2009). Colcemid is used on cell lines displaying a high-speed replication and is applied to a final concentration of 0,01 µg/ml for 2.5 hours.

Velbe: Described as a vinca alkaloid, also called vinblastine, this agent is derived from the periwinkle plant, *Catharanthus roseus*, and is noted as the most successful anticancer agent within the past few years. Binding of the vinca alkaloids to β-tubulin occurs fast and reversibly at an intermolecular contact point (Daly, 2009). It is recommended to use Velbe if the rate of cell replication is low at a final concentration of 0,01 µg/ml in a maximum of 16 hours.

The application of these reagents can arrest cells in metaphase and helps chromosomes contraction, allowing an easy recognition of these cells in pro-metaphase or metaphase. The use, exposure time and Colcemid or Velbe concentration varies and depends on several factors, including cell type and overall growth characteristics.

Hypotonic solution: Saline solution that allows chromosomes dispersion within the cell membrane, facilitating its observation and recognition. In order to obtain chromosome preparations from cell lines, the following hypotonic solutions can be used; the selection of this solution will depend on the degree of chromosome condensation obtained.

0.075 M potassium chloride (KCl): Use 5.59 g KCl and make up to 1 liter of aqueous solution. Use the solution at 37°C.

20 mM potassium chloride (KCl) and 10 mM sodium citrate ($Na_3C_6H_5O_7$): Use 1 g KCl and 1g sodium citrate and make up to 500 ml of aqueous solution. Use the solution at 37°C. Its use is recommended with longer chromosomes, that may be twisting or overlapping.

Fixative: Reagent used to stop the action of hypotonic solutions and which in turn, has several functions throughout the procedure related to hemolysis, dehydration, chromosomes fixation and removal of debris membrane that may interfere with the chromosome extended. This reagent is prepared with three parts of absolute methanol and one part of glacial acetic acid. This should be freshly prepared just before its use and should be kept always cold (-20°C).

10x Trypsin-EDTA: Stored frozen in 1 ml aliquots. Diluted 1:10 in PBS when required to obtain a 1x working solution. Store indefinitely at 4°C. Place at room temperature or 37°C before use.

Phosphate-buffered saline (PBS): pH 7, used for diluting solutions.

Stains:

Wright's stain: This stain is usually obtained as a powder. Cover a flask with aluminum foil and insert a magnetic stirrer. Add 0.5 g stain and 200 ml methanol. Stir for 30 min. Filter using a filter paper into a foil-coated bottle. Close the lid tightly and store the bottle in a dark cupboard for at least a week before its use. The stain should be diluted immediately before use at 1:4 with pH 6.8 buffer.

Giemsa: This stain is usually obtained as a liquid. Before use, the following mixture must be prepared: 0.2 ml Giemsa, 0.2 ml Sorensen Tampon and 4.6 ml water (the amount used to dye a slide).

Saline-sodium citrate (SSC) buffer: This is a widely used weak buffer, which is used to carry out several washes and to control stringency during *in situ* hybridization. The 20x stock solution consists in mixing 3M sodium chloride and 300mM trisodium citrate. To make the stock, dissolve 38,825 g sodium chloride (NaCl) and 22.05 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in 200 ml of water. Adjust to pH 7 with NaOH or HCl if necessary, make up to 250 ml and sterilize by autoclaving procedures.

Sorensen Buffer: This buffer is used for G-Banding. The working solution consists in two solutions: KH_2PO_4 and Na_2HPO_4. Prepare the buffer as follows:

- Sln A: KH_2PO_4. Dissolve 4559 grs in 500 ml of sterile distilled water

- Sln B: Na_2HPO_4. Dissolve 4755 grs in 500 ml of sterile distilled water.
- Take 500 ml of solution A and mix it with 496.8 ml of solution B, keep it at 4°C.

HCl 0,2 N: Used for G-Banding. To prepare 1000 ml of solution, add 8,25 ml HCl 37% and 500 ml H_2O into a glass container. Store at room temperature.

3.2 Cell culture methods from tumoral tissue

To ensure cell growth and obtain cells in metaphase, it is important to take into account all the sampling conditions. Sterile, non-necrotic tumor samples must be collected in a transport container using optimal conditions of sterility; for example, a sterile tube containing sterile culture medium, an antimycotic and a double concentration of antibiotics, which should be transported to laboratory facilities under controlled temperature.

The tissue sample must be representative, sterile, and viable. To ensure fast cellular growth and prevent contamination with other cell types, the cultures must be incubated in a small culture flask (25cm²) or directly on microscopic slides mounted in multi-well chambers. Cell attachment, proliferation, and mitotic rate should be monitored by daily x examination through an inverted microscope.

The steps to obtain metaphases are:

3.2.1 Dissociation of solid specimen: Enzymatic and mechanical procedures
3.2.2 Culture initiation
3.2.3 Culture harvesting and metaphases
3.2.4 Banding techniques
3.2.5 Freezing of viable cells

The way of determining the time of harvest, colchicine use and exposure to hypotonic solution will depend on the cell type and its growth rate.

3.2.1 Dissociation of solid specimen

Materials

- Collagenase (2000 U/ml)
- Appropriate culture medium (RPMI 1640, DMEM-F12) containing 10% fetal bovine serum (FBS), antibiotic-antimycotic solution (1X), L-glutamine (2 mM), Hydrocortisone, 17β-estradiol and insulin.
- PBS (1X)
- 25-cm² tissue culture flasks
- 15-ml conical centrifuge tube
- 5-ml and 10-ml plastic pipettes
- Petri dishes
- Microscopic slides mounted in multi-well chambers x 6 wells
- Tissue dissection equipment: tweezers, scissors

Procedure

Mechanical Disaggregation (Figure 3)