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CELL CHARACTERIZATION: PRINCIPLES AND TECHNIQUES OF MICROSCOPY, FLOW CYTOMETRY AND IMMUNOFLUORESCENCE

CARACTERIZAÇÃO CELULAR: PRINCÍPIOS E TÉCNICAS DE MICROSCOPIA, CITOMETRIA DE FLUXO E IMUNOFLUORESCÊNCIA

CARACTERIZACIÓN CELULAR: PRINCIPIOS Y TÉCNICAS DE MICROSCOPÍA, CITOMETRÍA DE FLUJO E INMUNOFLUORESCENCIA

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ABSTRACT

This chapter aimed to present the two main techniques for cellular study: optical, immunofluorescence, and confocal microscopy, and flow cytometry. Both have specific techniques for sample preparation, but all are based on immunological or immunocytochemical methods. The chapter explains the operating principles of these two instruments and their variations, as well as the principles of immunological techniques and fluorescence. It also presents concepts and methods for preparing immunocytochemistry samples, along with all the preceding procedures on how different types of antibodies are produced and which should be selected for each purpose—whether for cell surface, intracellular, or organelle and nuclear labeling. At the end of each technique, the reagents to be used and their preparation methods are presented.

Keywords: Optical Microscopy. Immunofluorescence Microscopy. Confocal Microscopy. Flow Cytometry. Immunocytochemistry. Antibodies.

RESUMO

O presente capitulo teve como objetivo apresentar as duas principais técnicas para o estudo celular, a microscopia óptica, de imunofluorescência e confotal e a citometria de fluxo. Ambas têm técnicas específicas para preparação de amostras, mas todas se baseiam em técnicas imunológicas ou imunocitoquímicas. O capitulo traz os princípios de funcionamento desses dois equipamentos e suas variações, bem como o princípio das técnicas imunológicas e o princípio da fluorescência. O capitulo também traz conceitos e técnicas de preparo de amostras de imunocitoquímica, como também todos os procedimentos anteriores, sobre como são produzidos os diversos tipos de anticorpos e quais devem ser escolhidos para cada objetivo, se marcação da superfície celular, intracelular ou dentro de organelas e núcleo. Ao final de cada técnica é apresentado quais reagentes usar e como prepará-los.

Palavras-chave: Microscopia Óptica. Microscopia de Imunofluorescência. Microscopia Confotal. Citometria de Flux. Imunocitoquímica. Anticorpos.

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RESUMEN
El presente capítulo tuvo como objetivo presentar las dos principales técnicas para el estudio celular: la microscopía óptica, de inmunofluorescencia y confocal, y la citometría de flujo. Ambas poseen técnicas específicas para la preparación de muestras, pero todas se basan en métodos inmunológicos o inmunocitoquímicos. El capítulo aborda los principios de funcionamiento de estos dos equipos y sus variaciones, así como el principio de las técnicas inmunológicas y el principio de la fluorescencia. También se presentan conceptos y técnicas de preparación de muestras de inmunocitoquímica, además de todos los procedimientos previos sobre cómo se producen los diferentes tipos de anticuerpos y cuáles deben elegirse para cada objetivo, ya sea para marcación de la superficie celular, intracelular o dentro de orgánulos y núcleo. Al final de cada técnica se indica qué reactivos deben utilizarse y cómo prepararlos.

Palabras clave: Microscopía Óptica. Microscopía de Inmunofluorescencia. Microscopía Confocal. Citometría de Flujo. Inmunocitoquímica. Anticuerpos.

1 INTRODUCTION

Cells are microscopic living beings that cannot be observed with the naked eye. All the knowledge that is known about cells was obtained by indirect means of amplification (such as the increase in the sample size by optical lenses) or transduction of cellular information (detection and transformation of analog information into electronic and digital information) in a way that the observer can have contact with such a tiny dimension. There is a whole technological development for equipment that detects cell structures and takes them to the observer, as well as a whole technology for sample preparation. Electronic, chemical and biological processes have to be combined to obtain the most accurate information about the cell and its contents. These techniques have become more sophisticated to the point that it is not only possible to study cell structure, but also the population, the identification of cell types and even metabolism. Two main pieces of equipment are mandatory for the study of cells: microscopy, which can be brightfield or fluorescence, and flow cytometry. Both approaches necessarily rely on sample preparation techniques; The essential ones are immunocytochemistry, immunohistochemistry and fluorescence technology. This chapter aims to show the principles of operation of these two pieces of equipment as well as the techniques for preparing samples. The language is technical and accessible, with the aim of serving the reader, especially students, who are starting their laboratory functions in undergraduate and graduate courses on the ways of cell characterization.

2 IMMUNOFLUORESCENCE: TECHNIQUES FOR FLUORESCENCE MICROSCOPY AND FLOW CYTOMETRY

2.1 DETECTION OF SPECIFIC PROTEINS

When we talk about protein detection, we immediately refer to immunochemistry, a technique that allows the localized study of specific cellular proteins using the antigen-antibody reaction.

Suppose we need to study a certain protein, however, we do not know in which cell or cell location this protein is found. Through appropriate techniques, this protein is isolated and purified. By injecting this protein (antigen) into a rabbit, for example, it will form a gamma globulin (antibody) with the ability to combine exclusively with the protein of interest, to the exclusion of any other protein. The antibody (Ab) or immunoglobulin (Ig) is produced due to the immune reaction induced in the rabbit by the "foreign" protein that is of mouse origin, for example. Thus, it is possible to obtain from the rabbit's blood a specific Ab for that protein.

As will be seen in this book, there are several techniques for the characterization of stem cells. When we talk about immunochemistry, other derived terms come to mind such as immunocytochemistry (labeling for cultured cells), immunohistochemistry (isolated tissue), immunoblotting (detection of proteins in gel race), immunoprecipitation (recovery of proteins), among others. However, the most widely used in the study of stem cells is immunofluorescence, where cells and tissues are marked for analysis in fluorescence microscopy and flow cytometry. These two techniques are used because they indirectly identify very specific proteins that indicate the differentiation potential of stem cells. Therefore, it is necessary to have prior knowledge of immunochemistry, which identifies through the specific antibody-antigen binding the protein linked to the characteristic, function or state of a cell.

The term widely used for when we want to find a cellular protein using immunochemistry is labeling. For example, when we want to know if stem cells express Oct-4, a protein found in embryonic cells, it is said that it will be labeled for Oct-4 in a particular cell line. The immunochemistry technique is divided into immunocytochemistry, for cell marking, and immunohistochemistry, for tissue marking, both of which are widely used for observing markings in brightfield microscopy. The technique was developed after the discovery of fluorochromes, which are substances that emit fluorescent light at a certain wavelength when excited by a light source with a shorter wavelength. Fluorochromes make the technique more sensitive, due to the contrast of the light emitted by fluorochromes with the dark background. This innovation could be called immunofluorescence, used for the observation of cells in fluorescence microscopy and in quantification in flow cytometry.

Fluorochromes can be conjugated directly to primary Ab, when the labeling is direct, or conjugated to secondary Ab, as the indirect immunochemistry technique. Later, the protocols described will allow us to visualize the steps of cell protein labeling. Direct marking has the advantage of avoiding some washing steps essential for the application of secondary Ab; another advantage is the low cost, because it does not require secondary Ab (**Figure 1**). Indirect labeling is more versatile, as it allows the use of the primary with a set of available secondaries, of different fluorochrome colors, which can be applied to labels of several simultaneous proteins identified with different colors. Another difference is that indirect labeling produces more fluorescence and is more sensitive than direct labeling, since secondary Ab can bind in quantity to a single primary Ab, and primary Ab, in turn, binds to a single epitope of the antigen, especially when it is monoclonal (**Figure 2**).

In the specific chance of immunofluorescence, Ab is conjugated with a fluorescent compound (fluorochrome).

Figure 1

Direct immunocytochemistry, where a primary Ab (which has a covalently bonded fluorochrome that emits a specific color) binds specifically to an antigen. The fluorescent labeling will show the location of the protein of interest in the cell under study

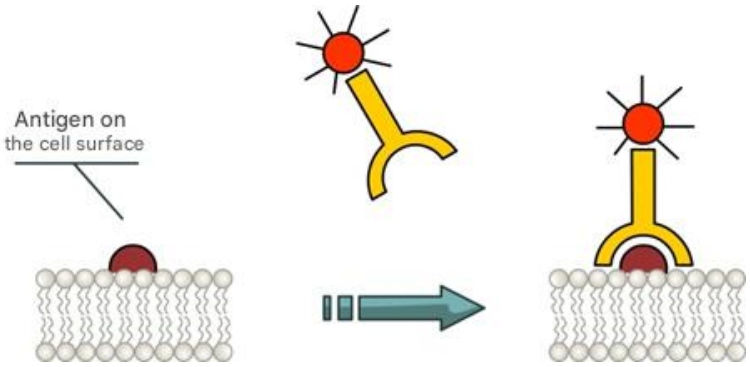
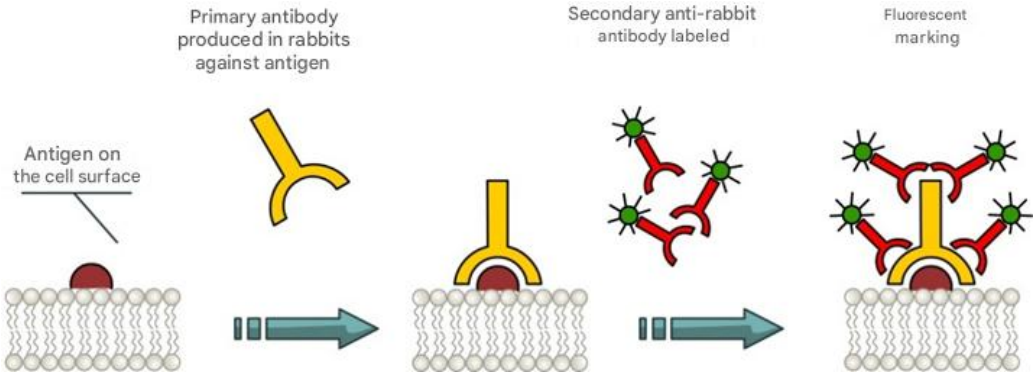


Figure 2

Indirect immunocytochemistry where a specific primary Ab binds to an antigen and, in a second moment, the secondary Ab (covalently bound to a fluorochrome), specific to the primary Ab, binds in quantity to the primary Ab molecule, increasing the final fluorescence



2.1.1 Principle of Immunocytochemistry

The principle of Immunocytochemistry is very simple, however care must be taken in selecting primary and secondary Abs, and pay close attention to the labeling procedures so as not to lose cells, obtain weak markings, or on the contrary, non-specific (**see protocol below**).

2.1.1.1 Primary antibody

The entire immunological technique for labeling cellular proteins begins with the objective of the molecule of interest. Usually this molecule is a protein and, in immunological aspects, it is our antigen. The labeling of cell structures or molecules is based on the specific affinity between antigen and Ab. These molecules of interest are associated with a cell structure, region, function, or cellular state. Taking the Oct-4 molecule as an example once again, it is a transcription factor that promotes the transcription of genes linked to cell renewal and the embryonic state of cell undifferentiation. Its marking indicates that the cell is undifferentiated at an embryonic stage.

Once the antigen of interest has been identified, the next step is the choice of the primary Ab. The choice of primary Ab will depend on how the immunocytochemistry process will be performed, whether a single labeling, double or multiple labeling, direct or indirect labeling, the specificity of the secondary (or the secondary is chosen by the characteristic of the primary), intracellular or extracellular labeling. These criteria are commented below and in the protocols at the end of the microscopy and flow cytometry chapters, since each immunocytochemistry technique is specific to each type of analysis to be performed.

Ab cells are produced by the B cells of animals immunized by the antigen of interest. There are monoclonal and polyclonal Abs. Monoclonals are generated by a single clone of B cells from a single animal (host) that results in a homogeneous set of Ab specific to a single epitope (region of the antigen recognized by an Ab; an antigen can have multiple epitopes). Monoclonal Abs have the advantage of low probability of cross-reaction, contributing less to nonspecific markings (in the general sense, when markings of molecules other than those of interest occur), and low background marking. Polyclonal Abs, on the other hand, are generated from different clones of B cells of an animal and recognize various epitopes of the antigen of interest; have the advantage of being more tolerant to the influences of fixation and labeling methods and processes that can hinder the interaction between Ab and antigen.

In addition to the types of Ab we have the animals where they are produced and the isotypes or classes generated. Among the animals used for the production of Ab we have mice, rats, hamsters, rabbits, goats, horses, monkeys, among others. Ab have two types of chains, two light and two heavy and both chains have constant and variable regions (antigen recognition). The isotypes or classes are associated with the structure of the constant regions of the heavy chain and are classified into IgA (*alpha*), IgB (*beta*), IgD (*delta*), IgE (*epsilon*), IgG (*gamma*) and IgM (*mu*). The Ab classes can be subdivided into subclasses, as occurs in

humans, where the IgA and IgG classes are subdivided into IgA1 and IgA2, and IgG1, IgG2, IgG3 and IgG4. Other animals have other subclasses. As many Ab are produced in mice, it has subclasses that we should know: IgG1, IgG2a, IgGb and IgG3. We still have the isotypes related to the light chain: *kappa* (κ) and *lambda* (λ) are present in all the isotypes of the heavy chains, but always alone, never together – for example, the heavy chain isotype IgM can have the light chain κ isotype, or the λ, but never both together. There are differences in the amino acid sequence between the κ and γ chains, but all κ chains of the different heavy chain isotypes (IgA, IgB, IgD, IgE, IgG and IgM) are the same, as are the λ chain.

2.1.1.2 Secondary antibody

Before we start choosing the most appropriate secondary Ab for a given marking, let's try to understand which formats of secondary Ab are marketed, their structure and use.

In contrast to primary Ab, most commercialized secondary Abs are polyclonal, purified directly from animal serum that has been immunized according to the type of a region of the primary Ab that we wish to bind, i.e., the fragment or class or subclass of a specific Ab of a specific species In addition to the type of Ab, the purification method used will also influence the final characteristic of the secondary Ab.

As stated above, Abs have light and heavy chains and their constant and variable regions, so that, depending on the type of immunization done and the enzymatic treatment, we can isolate different formats of secondary Abs. Thus, when we talk about secondary Abs, some will be prepared to bind in all parts of a complete IgG (including both the light and heavy region, L+H), or only in the Fab or Fc region, or only in the γ region of the IgG. There are other Ab specific to the μ region of IgM, or to the light chain type such as κ or λ.

3 WHAT ARE THE ADVANTAGES AND DISADVANTAGES OF USING A SPECIFIC SECONDARY AB FOR A COMPLETE ANTIBODY FRAGMENT OR MOLECULE?

A complete IgG molecule (Anti-IgG (H+L)), having a Y shape, has two heavy chains (larger, approximately 50kDa each) and two light chains (smaller, approximately 25kDa each) and are joined together by disulfide bonds and hydrophobic interactions. This type of secondary Ab is produced by injecting an animal (goat, for example) with a complete primary Ab IgG from another species (mouse, for example). The secondary Ab produced is a polyclonal Ab that recognizes the heavy and light chain of the primary Ab. The downside is that light chain is common for all classes of Ab or immunoglobulin (Ig), which can lead to

cross-reactions. In labeling for cells in culture this problem is not as critical, but for tissue labeling, as there are many cell types, this problem becomes more common. When there is no concern with the type of Ig class (independent of the subclass) or to give a strong signal or when the type of Ig of the primary is not known, this secondary Ab format is very advantageous. Let's look at other types of Ab.

Heavy chain specific anti-Ig (α, γ, μ): taking an anti-IgG Ab as an example, its production is carried out by injecting a complete IgG into a host. Then, the Ab produced in this host go through an absorption process, where they are incubated with Ab IgG from other species attached to a column (i.e., there is no Ab in the column of the species of interest). The anti-IgG Abs that are not of interest have bound to the Abs of the column and the anti-IgG of the species of interest is recovered. This approach avoids cross-linking with other species and reduces the background due to its better specificity. This secondary Ab recognizes all fragments of an IgG: complete IgG and the fragments Fab, F(ab')2 and Fc. As light chains are present in all Ab classes, there is absorption of anti-IgG that are specific to the K and L chains, which prevents cross-reactions by this type of bonding. So, in the same way as the anti-IgG Ab, other types of Ab specific to other classes can be obtained, such as IgA, IgG, IgM. Its use is interesting when it is intended to perform double labeling where one primary Ab IgG and another IgM can be used, using the secondary anti-IgG conjugated with a fluorochrome and the anti-IgM conjugated with another fluorochrome.

Fc-specific anti-IgG: This type of Ab is specific only to an IgG class like the heavy-chain-specific anti-Igs described earlier. The difference is its specificity only by the Fc portion of the IgG molecule. Its production is done by injecting only the Fc portion of IgG, without the light chains and without the upper portion of the heavy chains, removed by enzymatic digestion by pepsin. This type of Ab further diminishes background and unspecific undesirable bonds.

Fragment F(ab')2: consists of a complete IgG without the Fc region, by digestion with pepsin. Fragment F(ab')2 is the two arms of Ab's Y-structure. With a molecular weight of approximately 110 kDa (less than the full 150 kDa IgG molecule) it is very useful for having better penetration and facilitating antigen recognition. These fragments, because they do not have the Fc portion, have the advantage of not binding to the Fc receptors of the immune system's defense cells. The fragment F(ab')2 is used to produce anti-F(ab')2 that recognizes only the light chains and the upper portion of the heavy chain of a primary Ab, not recognizing the Fc portion.

Fab fragment: this fragment is produced by digestion with papain removing the Fc portion and the bond between the bivalent molecule F(ab')2 generating two Fab fragments. With a molecular weight of approximately 50kDa, this fragment has greater penetrating power. This fragment is not recognized by Fc receptors. Its main use is to block nonspecific bonds with internal immunoglobulins in the sample, especially in double-labeling procedures.

3.1 CHOOSING THE MOST APPROPRIATE ANTIBODY

As previously stated, the choice of primary Ab depends on the molecule of interest to be labeled. Necessarily the primary Ab has to be reactive to the molecule of a given species. For example, a human cell that will be labeled for CD34, a surface molecule found in hematopoietic precursors, the primary Ab will have to be anti-human CD34 produced in an animal of a different species than the one that will be labeled, for example mouse. As Ab are mostly produced abroad, its commercial classification will be written in English, therefore, the primary Ab in question will be marketed as *Monoclonal Mouse IgG1 Anti-Human CD34*, that is, it is a primary, monoclonal Ab, produced in mice, it is of class IgG1, specific for the Cd34 molecule of humans. In addition to this classification, every commercialized Ab comes with manufacturer's specifications that will have to contain information on cross-reactivity with proteins from other species, whether it is a reabsorbed Ab and for which species it was reabsorbed. Another important piece of information is how Ab should be stored, as most are valid for six to twelve months, among other specifications.

The choice of primary Ab also depends on the specificity of the secondary Ab, as well as the choice of secondary depends on the type and class of the primary Ab. In the example above, since the primary is an IgG1 immunoglobulin produced in mice, the secondary has to be specific anti-IgG1 against mice. As we saw earlier, there are several formats marketed for primary and secondary Ab. In the present example, we can use a mouse IgG1-specific anti-Fab or anti-F(ab')2 secondary that will bind to the Fab chain of this molecule. The same happens for the anti-Fcγ specific to the gamma heavy chain (which characterizes the IgG molecule) and anti-IgG resorbed to other classes of molecules of the same species of interest (and from other species that may have cross-reactions), all of them bind in the Fc portion of the IgG1 primary given as an example. Abs that are reabsorbed for a given species are advised when there is nonspecific labeling due to cross-reaction with molecules of the cells of the species to be labeled. In our example above, if there is cross-reactivity, the use of a resorbed Ab for human is indicated. However, this procedure is more used when it is known

that there are human immunoglobulins (which cause cross-reactions) and when the epitope is not very reduced in the sample to be labeled. It will often be necessary to mark proteins that are inside some intracellular organelle, some researchers resort to Fab or F(ab')2 chains which are smaller and, therefore, easier to penetrate. Sometimes it is not known what the class of a primary Ab is, so one can turn to an anti-IgG secondary (H+L) that reacts with immunoglobulins of other classes, since they share the same light chain class κ and λ.

This is the simple procedure for choosing the specificity of Ab. On the other hand, the choice of structure will depend on whether it is necessary to be more specific, more permeable or if it needs to reduce the background, so you should consult above which configuration fits best, if specific for Fc, Fab or complete molecule. This type of concern must be taken into account, for example, when we make multiple appointments.

The dilution of the use of both the primary and secondary is suggested by the manufacturer, however, it will always be necessary to standardize the marking, titrating an optimal concentration that minimizes nonspecific markings and maximizes specific ones. Many factors influence the standardization of Ab concentration, such as fixation, permeabilization, incubation time, temperature, whether the protein is abundant or scarce, whether internal or membrane, the cell type and the technique to be used as immunocytochemistry or immunohistochemistry, and the analysis used as microscopy or cytometry, among other techniques.

Table 1

Various types of primary Abs and their abbreviations

Hu = human	*Rb = rabbit*	*Chaos Knight = chiken*
Ms = mouse	*Shp = sheep*	*Bov = bovine*
Gt = goat	*Hrs = horse*	*Sw = Swine*
GP = guinea pig	*Sy Hms = Syrian Hamster*	

4 ANTIBODY-CONJUGATED MOLECULES

Fluorochrome is used in fluorescence microscopy for visualization of individual structures and molecules and in cytometry for cell classification, both by the immunofluorescence technique. Fluorochrome is a molecule that has the property of absorbing light energy at a certain wavelength (excitation) and emits fluorescent light at a wavelength longer than excitation. This occurs due to the loss of energy between absorption

and emission (**Figure 3**). The physicochemical characteristics of fluorochrome are what define which wavelength of light it is excited and which wavelength of light it emits.

Figure 3

In the process called fluorescence, when a beam of light, of a certain wavelength, for example, in the blue range, falls on a fluorochrome, it absorbs photons, and the electrons of the molecule that were in a current state gain energy and are raised to a higher energy layer (1). Due to energy losses by a process called vibrational relaxation, which generates heat for the environment, the excited electrons fall into a less energetic electron shell (2). As this electronic state is unstable, the electrons return to their original position releasing energy in the form of emitted light (3). In this case, the emitted light has a shorter wavelength (green, for example) than the excitation light due to the loss of energy to the environment

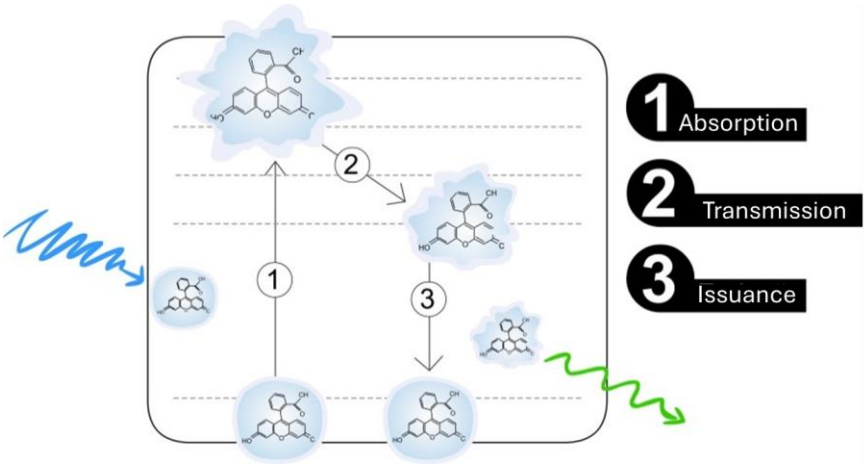
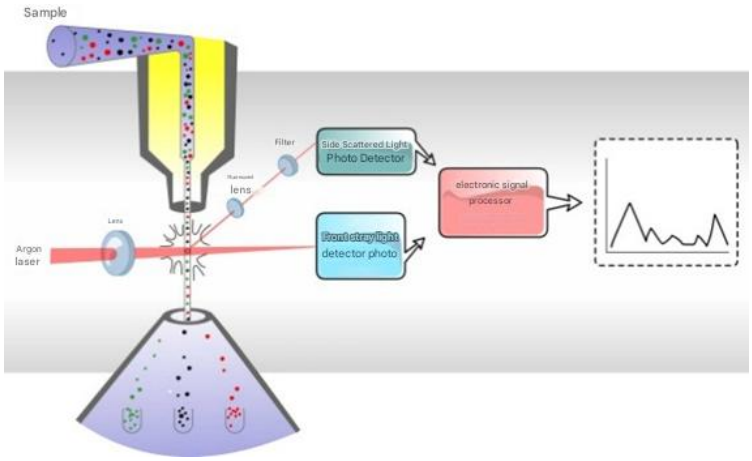


Table 2

Description of the characteristics of the most commonly used fluorochromes



In addition to fluorescence microscopy working with the property of antibody-antigen specificity, there are molecules that have affinity for certain cellular structures, such as DAPI and Propidium Iodide (PI), which are DNA intercalators and are routinely used to mark nuclei and perform cell viability testes, as is the case with PI.

It is also possible to visualize fluorescence in live cells. GFP, a green fluorescent protein found in jellyfish, can be used, along with the recombinant DNA technique. The gene for this protein can be introduced into cells in culture. The cells will produce the GFP protein and emit a green fluorescence when excited.

Stem cells transfected with the GFP gene can be injected into animals intravenously or intraperitoneally (for example) and, subsequently, the location of these cells in different organs and tissues of the body is verified, showing the capacity to graft a certain type of stem cell.

Another example of the usefulness of fluorescence microscopy is the detection of pH or Ca2+ concentration in the cell interior. *Acridine Orange* (AO) in an acidic environment, such as that of lysosomes, has an emission wavelength in the red range, while in a more neutral environment, such as the cytosol, it emits in an emission wavelength in the green range. AO is used to assess lysosomal loss. The fura-2 molecule also changes the emission wavelength when bound with Ca2+, used to check the intracellular concentration of Ca2+.

5 FIXATION AND PERMEABILIZATION

Before moving on to immunofluorescence protocols, it is necessary to understand a little about fixation and permeabilization methods. The first step for cell labeling, after culture, is to fix the cells to prevent degradation and preserve the quality of the cellular components by immobilizing the antigens while retaining cellular and subcellular structures through cross-linking of methylene bonds and Schiff bases between the bases of the amino acid residues of proteins. The main factors that influence the quality of the marking are the time, until the fixation occurs, the concentration of the fixative and the temperature at which the process occurs. A very long fixation time can mask the epitopes of the molecule of interest and prevent Ab binding; Each fastener has its ideal minimum and maximum time.

In the fixation process, what is mandatory is to fix the cells as soon as possible, to avoid degradation. Throughout the fixation process, the cells die, but the structures are preserved. In fluorescence microscopy, there are analyses with live cells (or non-fixed cells) where, during the process, the cells are kept in a small chamber that keeps them at a

concentration of 5% CO2, in a sterile environment, on a heating stage that maintains the temperature at 37°C. But here, we will only address the methods that use fixed cells, which are important for cell characterization.

Good fasteners have to have some desirable characteristics: good penetration capacity, enabling the fixation of the material completely; the coagulation of proteins has to be total and non-violent, but promote immediate cell death; it must not alter cell morphology and structures, maintain soluble elements in cells and tissues; and preventing the appearance of artificial structures (artifacts that can generate false analyses).

The choice of fixator will depend on the structure to be studied. There is no ideal fastener and often mixtures of different fasteners are used where the characteristic of each one will meet the needs of each type of structure to be observed. In the study of some nuclear structures such as chromatin, nucleolus and spindle filaments (during cell division), acid fixatives are used; and for the study of nucleoplasm, mitochondria and vacuoles, basic fixatives are used.

The best known and most used fixative is formaldehyde. In immunohistochemistry it is commonly used at a concentration of 10% (for histological specimens) and in cytochemistry at a concentration of 1 to 4% (for cells adhered in a monolayer on coverslips, petri dishes, or culture bottles); In flow cytometry, it is used at a concentration of 1 to 2% (for cells in suspension). Formaldehyde is marketed in a 37% solution, however, we consider it as 100% and from there the dilution is made, so a 10% solution prepared for fixation will actually have a concentration of 3.7% formaldehyde. Fixation occurs by the covalent cross-linking between tissue proteins (primarily by the amino acid lysine), conferring rigidity to the cytoskeleton. It is an excellent fixative, as it preserves the morphology of the cells, especially the cytoplasmic environment, and the cells can be stored for a long period, a few months, at 4°C (it is recommended to change the fixative weekly, never letting it dry and keeping the cells always immersed). Paraformaldehyde, which is also widely used for the preparation of fixing solutions, is a polymerized form of formaldehyde that is depolymerized after heating.

As many factors influence fixation, different fixatives can be tested to optimize antigen preservation, distribution, and morphology of other cellular constituents. For example, some epitopes are lost on aldehyde fixation but conserved on alcohol fixation, and vice versa. Fixatives and alternative methods should be employed to improve the quality of the fixed sample, such as the use of methanol at −20°C (for 5 minutes) or formaldehyde fixation followed by a brief (1 minute) exposure to methanol at 0°C. Fixation with methanol is most

effective for localizing elements of the cytoskeleton. Alcohols work by extracting lipids and precipitating remaining protein, while aldehydes react by promoting cross-links that better preserve membranous structures.

In practice, we often realize that the material, even if properly fixed, the epitope is not accessible to Ab. In this case, the material undergoes a permeabilization process that permeabilizes the cell membrane, allowing Ab to penetrate the cell, reaching its interior and the interior of cytoplasmic organelles. Permeabilization reagents are basically detergents that make the membrane porous and denature the fixed protein, exposing the epitope. Among the permeabilizers are saponins, Triton X-100 from 0.2 to 0.5% or SDS that are always used after the fixation process. Alcohols such as methanol and ethanol are widely used as permeabilizers, while they are used as fixatives, having a double role. Often a short treatment with these reagents prior to Ab incubation is sufficient to expose the epitope. Special attention should be directed towards ensuring that access to the epitope is the same if the protein has undergone a displacement: for example, the protein that moves between the cytoplasm and the nucleus is not always equally accessible to the Ab.

Fixation using organic solvents such as alcohols removes lipids (a property that allows you to skip the permeabilization step) and dehydrates the cells; precipitation and denaturation also occur, in which the solubility of protein molecules is reduced and the hydrophobic interactions that give many proteins their tertiary structure are disrupted. The most common precipitation fixative is ethanol and methanol, mainly for the preservation of frozen materials and smears. Acetone is also widely used. Protein denaturants, methanol, ethanol and acetone, are rarely used alone for cell and tissue fixation, except when studying only nucleic acids. Acetic acid is a denaturant that is sometimes used in combination with the other precipitation fixers. Alcohol, by itself, is known to cause considerable shrinkage and hardening of tissues during fixation, while acetic acid is the only one associated with tissue swelling, combining the two can result in better preservation of tissue morphology.

Immunofluorescence used in conjunction with microscopy is and remains the best way to locate and determine the distribution of proteins within cells. The preparation of the cells for labeling with the primary Ab follows two important steps: 1) fixation with formaldehyde or paraformaldehyde, which aims to stabilize soluble and insoluble proteins in their native locations, followed by washing with detergents for permeabilization, but which can remove some soluble proteins that have not been well fixed; and 2) permeabilization and fixation with

organic solvents such as ethanol and methanol, which allows the precipitation of soluble and insoluble proteins in their original locations.

Fixation with formaldehyde or paraformaldehyde prior to permeabilization should preserve protein distribution, however, soluble proteins may undergo cell redistribution during the process of this technique. Many proteins that have a uniform cytoplasmic distribution have a coarse, punctual distribution and in some cases reticular appearance. It is common to observe a redistribution where proteins have a punctual and concentrated form around the nucleus, and often inside the nucleus. The cell type influences a lot, where in some cases the redistribution of proteins does not occur and in other cases it occurs.

The use of alcohols to fix and permeabilize tends to precipitate structural and soluble proteins, avoiding loss. However, this method also leads to redistribution of proteins, presenting a characteristic of reticular and fibrous texture, and redistribution into or around the nucleus, as well as to the nucleolus. A decrease in fluorescence is also observed when compared with labeled proteins in living cells.

The study of the genetic arrangement in the cell nucleus requires preservation of native nuclear structure and visualization of genetic regions using chemically modified DNA probes for FISH. The use of confocal microscopy provides the most accurate information about the nuclear structure. However, the penetration of DNA probes into cell nuclei and the preservation of native infrastructure is sometimes limited by the bulky cytoplasm and plasma membrane. These difficulties can be avoided if the relative position of the regions of interest of the chromosomes is not changed in nuclei prepared by the usual method in cytology, which consists of fixation by methanol and acetic acid. This attachment damages the plasma membrane and eliminates the cytoplasm, allowing easier penetration of DNA probes into the nucleus. However, at the same time, it induces dehydration of the nucleus, which can result in the breakdown of the chromosomal structure.

On the other hand, the fixation of nuclei with methanol and acetic acid causes substantial increase in the nuclear diameter. This probably occurs by widening the contact between the core and the blade, widening and flattening the core. Chromosomes from the regions that are closest to the nuclear membrane will be displaced, away from the nuclear center in these nuclei. On the other hand, the regions that are closest to the nuclear center are not much influenced by this flattening.

In flow cytometry, unfortunately, there is still no consensus regarding an optimal cell fixation for the multiparameters to be analyzed. Some strategies are employed to improve

the quality of a sample marking. The use of paraformaldehyde prior to fixation with alcohols follows the premise that the cross-links formed prevent the exit of proteins from the cell after permeabilization with methanol. The same was observed with the use of ethanol. It was observed that the use of methanol as a permeabilizer in place of Triton X-100 helped both in membrane permeabilization and in morphological preservation by measuring the light *scatter* parameter in a cytometer. This is very important since in cytometry it is interesting to select cell populations by morphology, as when we study circulating blood cells.

Both the paraformaldehyde concentration and the fixation temperature affect the immunofluorescence of intracellular proteins, the properties of light scattering cell, and cell DNA fluorescence in measurements using propidium iodide. As observed, the increase in intracellular protein immunofluorescence occurs at high concentrations of paraformaldehyde (2%), at a fixation temperature of 37°C. However, these are recommendations for single-parameter analysis for the measurement of intracellular proteins using immunofluorescent Abs. In multiparameter studies, such as light scattering and DNA content analyses, concentrations of 0.25% paraformaldehyde and fixation temperatures below 37°C are recommended, which improves the quality of the labeling in relation to the appearance of false aneuploidy, probably caused by a differential chromatin condensation and different degrees of nuclear protein cross-linking and/or DNA denaturation induced by paraformaldehyde. To better situate the difficulty of labeling DNA content, aneuploidy is the alteration in the amount of DNA that can be caused by the increase of chromosomes in transformed cells, as occurs in tumor cells. Changing the amount of genetic material also changes the intensity of the immunofluorescence of the marker used to mark DNA. If the interest is to study only the cell content, it is recommended to fix it using only ethanol, which avoids the appearance of false aneuploidies.

Formaldehyde is more used when we want to visualize cytoplasmic structures and preserve cell morphology, while alcohols are more requested in sample preparation when the compartment of interest is the nuclear content. However, one should always test both techniques or both together to define which method best represents the nature of the living cell. One must always take into account the molecule to be observed, its location and the cell type studied; and to use common sense due to the empiricism of fixation techniques.

5.1 SOME EXAMPLES OF FIXATION AND PERMEABILIZATION

Acetone

Fix cells in acetone at -20°C for 5-10 minutes. No permeabilization steps required after fixing acetone.

Methanol

Fix cells in methanol at -20°C for 5-10 minutes. No permeabilization step required to fix methanol next.

Ethanol

Fix cells in 95% ice-cold ethanol and 5% glacial acetic acid for 5-10 minutes

Methanol and acetone.

Methanol and acetone 1:1.Make the mixture at the time of use and fix the cells at -20ºC for 5-10 minutes.

Methanol and ethanol

Methanol and ethanol 1:1.Make the mixture at the time of use and fix the cells at -20ºC for 5-10 minutes.

Paraformaldehyde and Triton permeabilization

Fix at 2-4% paraformaldehyde for 10-20 minutes. Wash 2X with PBS for 5 min. Permeabilize with 0.5% Triton X-100 for 10 minutes. Paraformaldehyde and Methanol

Fix at 4% paraformaldehyde for 10-20 minutes. Wash 2X with PBS for 5 min. Permeabilize with methanol at -20 °C for 5-10 minutes.

6 FLUORESCENCE MICROSCOPY

Fluorescence microscopy is a great technique for localizing cellular components, either in living cells or in fixed cells. This technique relies on labeling with a fluorescent compound (fluorochrome) following immunohistochemical or immunocytochemical procedures for fluorescence analysis, as mentioned earlier. A fluorescent compound absorbs light at a certain wavelength (excitation) and emits fluorescent light at a longer, more specific wavelength. Fluorescence microscopes have a set of filters that allow only light to pass through with the desired wavelength.

Fluorescence optical microscopes are typically configured for both the bright light field (transmitted light) and fluorescence microscopy. Both methods use the same principle of optical treatment: the transmitted light is produced by a tungsten lamp, focused on the sample by the capacitor, following the trajectory to the observer; The light for fluorescence is produced by a mercury lamp that is impeded, transmitted, or reflected by a set of filters and

mirrors. The focus of the sample always begins with brightfield microscopy, in which clear light (white) allows the visualization of the structure of the cells that have or are not marked by immunological techniques or staining. After the sample is focused, the microscope configuration can be changed for fluorescence analysis.

As seen earlier, the researcher chooses the molecule, protein, enzyme or structure to be observed and the mark with a specific Ab, which is associated with a fluorochrome and which corresponds to a color to be visualized. The technique for fluorescence microscopy allows the visualization of more than one cell marking, it is up to the researcher to make the best combination of markers that emit colors at wavelengths that can be separated by the filters available in the microscope where the observation will be made.

The most commonly used light source in fluorescence microscopes is the mercury lamp or xenon lamp, which emit light at wavelengths (e.g., 365, 400, 440, 546 and 580 nm) that cover most of the absorption characteristics of the most commonly used fluorochromes. The light emitted by the light source passes through an excitation filter that has the ability to let a certain wavelength pass through (bound to the chosen fluorochrome and which specifically absorbs this wavelength). Then the beam of light is reflected by a filter or dichroic mirror towards the sample. The light emitted by the sample (with a length longer than the emission light, due to the loss of energy to the environment) will be isolated by the emission filter to the observer or captured by a camera. Following an example, fluorescein (FITC) and rhodamine are widely used fluorochromes, and as a result fluorescein returns to the observer the color green and red respectively, so we can distinguish two markings in the same sample.

Fluorescein has maximum absorption at a wavelength of 490 nm and emits most efficiently at a length of 525 nm; rhodamine absorbs at 550 nm and emits at 580 nm. A double tagging with these two fluorochromes requires that a set of filters be combined for each tag. In the case of fluorescein, the excitation filter (BP 490/30 nm) prepares the beam of light emitted by the lamp with a wavelength of 490 nm, which is the absorption range of this fluorochrome; a dichroic mirror (MD 510 nm) will reflect the emitted light towards the sample; due to the energy decay the sample will emit light at the longer wavelength of 525 nm, a low-pass emission filter (LP 520 nm) will ensure it will eliminate other light components that are not desired; The same dichroic mirror that reflected the light emitted from 490 nm to the sample will now let through the light with a wavelength of 525 nm that will be viewed by the observer or captured by a camera attached to the microscope. The same will occur for

rhodamine, which will have a set of filters suitable for its range of light absorption and emission.

The disadvantage is that we have the limitation of the image not being seen with both markers simultaneously, as it is only possible to use one filter configuration at a time – the overlapping of both colors (or more, in other cases) is only possible after the acquisition of images of the same field for each fluorochrome, which is done by an image manipulation program.

The objective in fluorescence microscopy has the function of condensing, augmenting and collecting the fluorescence emitted by the fluorochrome of a sample. These and other points, such as the numerical aperture, the wavelength it can transmit, and the immersion medium used, must be taken into account before observing a sample. All of these features work together to determine a good image of the observed sample. The numerical aperture (NA) is the characteristic of the objective collecting light: the higher its value, the greater the amount of light collected by the lens; and it is associated with the proximity of the objective to the sample.

As the light is refracted by the medium between the sample and the objective, depending on the distance, when very close to each other (which occurs in the use of high-magnification objectives, with 100X) it is necessary to use immersion oil to correct the refraction of the emitted light and, consequently, improve the efficiency with which the light is collected by the objective, increasing the NA and the amount of light collected. Resolution, which by definition is the shortest distance between two objects that can still be distinguished as two separate objects, is linked to NA and wavelength: the larger the NA and the shorter the wavelength, the smaller the distinguishable distance between two objects, the higher the resolution.

Enlargement or enlargement is the ability to make the image larger than it is. Usually, the maximum magnification is 1000X (100X the magnification of the objective times 10X the magnification of the eyepiece). The magnification and the NA are related, because the more the image is amplified, the more it loses brightness, however, this can be compensated for the higher the NA

7 CONFOCAL MICROSCOPY

The confocal microscope is a more sophisticated fluorescence microscope, it has the same optical principle as the fluorescence microscope, however, the main difference is the

use of laser as a light source. The confocal microscope turns out to be more sensitive due to the laser that produces a more directed beam of light and waves at the length of light dedicated to the excitation of certain flurochromes, increasing its efficiency. Thus, we have lasers in the range of: Ultraviolet (excites in the ultraviolet range 405 nm), Argon (excites the range of violet and blue – 488 nm), Helium-Neon (excites in the green range – 543 nm), for example. The use of laser for excitation gives greater focusing power to the microscope, which makes it possible to explore the cell in depth. With the help of software and the electromechanical system of the confocal microscope, it is possible to perform "cuts" of images in different layers of the cell or tissue.

8 PROTOCOL FOR IMMUNOFLUORESCENCE UNDER MICROSCOPY.

8.1 MATERIALS

- Cells growing in culture with approximately 70 to 80% confluency.
- 2 to 4% formaldehyde (the more concentrated the better the fixation, however, it increases background autofluorescence).
- Buffer Solution: PBS (*Phosphate Buffer Saline*), pH7.4.
- Blocking solution: PBS/BSA, pH7.4 with 5% bovine albumin (BSA).
- 0.1% Triton 100X in buffer solution.
- Culture medium appropriate to the cell of interest.
-Trypsin
- Primary ab .
- Secondary Ab (specific to the type of Ig of the species from which the primary Ab was obtained) bound to a fluorochrome.
- Mounting medium.
- 35mm Petri dishes.
- Blade and coverslip.
- Becker (two)
-Distilled water
-Tissue
-Tongs.
-Enamel

8.2 PROCEDURE (DIRECT AND INDIRECT DIALING)

The difference between **direct and** indirect **dialing** is described in step 11 of this procedure. All equipment, material and reagent that will come into contact with living cells must be previously sterilized.

1. Disaggregate the cells in culture using trypsin.
2. On 35 mm plates, place the coverslips (previously treated with poly-L-lysine).
3. Seed the cells on the coverslip and complete with cell type-specific culture medium up to 2 mL. Leave adhering for 24 hours.

The cells adhere within 4 hours, but leaving it overnight ensures a morphology more suitable for appreciation after fixation. The confluency of 70 to 80% helps with morphology in most cell cultures.

4. Vacuum medium and wash 2 X with PBS.

Washes include gentle cell resuspension and spin for 5 min at 300g (approximately 1000 rpm). It is interesting to calculate the speed in rpm of each centrifuge.

5. After washing, add 1 mL of 2% formaldehyde to each well. Leave the cells to fix for 15 min at room temperature (TA). Fixation can also be done by solvents such as acetone absolute and 90% methanol or 70% ethanol at -20°C for at least 5 min.

In the case of using acetone or alcohols, there is no need for permeabilization since these solvents fix and permeabilize at the same time. That way, go directly to item 8.

If you use formaldehyde and want to stop the procedure here, the fixation can last up to a month at 4°C as long as you change the fixative every week.

Formaldehyde is interesting for membrane and protein labeling of cytoplasmic compartments, in addition to preserving cell morphology well. When you want to mark chromatin, the ideal fixatives are alcohols.

6. Vacuum the fixative and wash 2 X with PBS, for 5 min, each wash.
7. Permeabilization can be done with detergent using 0.1% of Triton X-100 for 10 min. Solvents such as absolute acetone or 90% methanol or 70% ethanol can be used at -20°C for 5 min, if the fixation has been done with formaldehyde.

If you want to label proteins within membrane-enveloped compartments such as vesicles, endoplasmic reticulum, or Golgi, you can increase the permeabilization time to up to 20 min. In this case, it is necessary to standardize to find the ideal time. When you want to mark chromatin, the ideal fixatives are alcohols.

8. Vacuum the fixative and wash 2 X with PBS, for 5 min, each wash.

9. After washing, add 1 mL BW/BSA and leave to block for 10 to 30 min at RT.

Blocking is done to reduce non-specific bonds with the primary Ab, avoiding non-specific markings. During the blockage do not let the cells dry out, add more blocker if necessary.

10. While the cells are blocked, dilute the primary Ab (at the dilution recommended by the manufacturer or at the concentration found after titration of the Ab in the labeling standardization process) in 25 µL of 5% PBS/BSA.
11. Aspirate the blocker and add the 25 µL of diluted Ab over the coverslip area (use a 200 µL tip, letting a drop form at the tip and touching the drop on the surface of the coverslip, with contact the drop spreads). Cut out a piece of the parafilm the size of the coverslip and place it lightly over the coverslip – this will prevent the solution from evaporating. Incubate the cells for 1h to TA, protected from light. The control group should take only PBS/BSA and accompany the experiment group throughout the process.

If the primary Ab is already conjugated with a fluorochrome the procedure performed is called **direct labeling**, so go straight to step 14. If there is a need to use a secondary Ab conjugated with a fluorochrome, the procedure performed is called **indirect labeling**, so follow the next item.

In some cases, a long incubation period will be necessary, even for convenience. In these cases, to avoid evaporation and maintain a humid environment, a container with buffer solution should be used, closed, and left at 4°C, where incubation can extend for one night.

12. With the aid of tweezers, carefully remove the parafilm, aspirate the solution with the primary Ab and wash 3 X with PBS or TBS for 5 min.

TBS can be used in place of PBS, it has Tween 20 which is a detergent that facilitates the permeabilization and washing of cells.

13. Dilute the secondary Ab in 25 µL of PBS or TBS according to the manufacturer's recommended dilution or at the concentration found after Ab titration in the labeling standardization process.
14. After washing, repeat in the same way as step 11, apply 25 µL of Ab dilution and place a piece of parafilm on the coverslip and incubate for 1 h at TA, protected from light.
15. Wash the coverslips as in step 12, finishing with the addition of 1 mL PBS.

16. Setting a 20 or 200 µL pipette to approximately 15 µL is enough to mount the blade with the mounting medium. Add the mounting medium on a clean blade so that the drop formed on the ferrule is just deposited on the blade and leave it on the bench.
17. With the help of tweezers, hold the coverslip and immerse it in distilled water (use two Beckers to make two washes). This step should be done very gently so that the breakdown of the coverslip and the loss of cells does not occur.

The removal of the coverslip from the bottom of the well, with the help of the forceps, is easier if the well has PBS. The fact that the coverslip is submerged in the PBS prevents the cells from drying out.

18. Vertically, touch the ends of the coverslip on an absorbent paper so that all the water is absorbed.
19. Carefully touching one end of the coverslip over and next to the edge of the droplet of the mounting medium, the medium will contact the entire end of the coverslip. Lay the coverslip, with the side where the adhered cells are, towards the slide. Notice that the middle fills the space under the coverslip so that it does not form bubbles and spreads the excesses.
20. Carefully place an absorbent paper on the blade to remove excess from the mounting medium.

This procedure prevents the mounting medium from preventing the application and drying of the enamel.

21. Apply the enamel around the coverslip in order to seal the cells that are between the coverslip and the blade. The slides can be stored for a few months at 4°C, but no more than a year.

This procedure prevents oxidation and drying of the cells. Commercial mounting media allow for better conservation of fluorescence intensity (check with manufacturer). If using a medium formed with glycerol and water, it is suggested that the cells should be analyzed within one to two weeks.

22. Take the slides for analysis under a fluorescence microscope.

Tip: Prolonged exposure of the labeled cells under the microscope's fluorescence excitation light depletes fluorochrome's ability to produce fluorescence.

Often at the end of the protocol the marking is nonspecific or very weak, one of the causes is the concentration too high or below the ideal. In this case, the concentration of primary and secondary Abs should be standardized through several dilutions until the ideal

one is found. Other causes for a weak marking may be insufficient permeabilization that did not allow Ab to enter intracellular compartments – in many cases smaller fragments of Ab are used to facilitate reaching the antigen; for nonspecific labeling perhaps the error is in the blocking step, the fixative may also cause increased autofluorescence, or perhaps the use of Ab more specific can solve the problem. Molecule redistribution can often occur, that is, soluble molecules are displaced from their natural regions to other different regions, in which case different types of fixatives or the use of double fixation should be tried.

8.3 REAGENTS & SOLUTIONS

Coverslips treated with Poly-L-lysine

Apply 25 µL of 1 mg/mL of poly-L-lysine on each coverslip, leave on at AT for 10 min. Gently wash the coverslips 3 X with water and let them dry.

PBS (Phosphate-buffered saline)

0.144g H2PO4

8.0 g NaCl

0.2 g KCl1.44 g Na2HPO4

Dissolve everything in 800 mL of H2Od

Adjust pH to 7.4 with HCl

Complete with H2Od for 1 L

Store at room temperature.

Formaldehyde, 2%

As commercial formaldehyde comes in a 37% solution, its dilution is done considering this solution as 100%. Thus, dilute 2 mL of the commercial formaldehyde solution in 98 mL of PBS, pH 7.4. If you wish to use paraformaldehyde, dissolve 4 g of paraformaldehyde powder in 10 mL of distilled water and heat to 60°C. Dilute in a 1:1 ratio with 2x concentrated PBS, pH 7.4.

Always prepare before use, it is not interesting to stockpile.

Methanol 90%

Just dilute in the proportion of 90% methanol and 10% distilled water.

Always prepare before use, it is not interesting to stockpile.

Ethanol 70%

Only dilute in the proportion of 70% ethanol and 30% distilled water.

Always prepare before use, it is not interesting to stockpile.

TBST (Tris-buffered saline with Tween-20*)*

Initially prepare Tris· Cl, 1 M

Dissolve 121 g Tris Base in 800 ml H2Od

Adjust pH 7.5 with concentrated HCl

Adjust the volume to 1 L with H2Od

Filter if necessary

Store up to 6 months at 4°C or TA

100 mM Tris-Cl, pH 7.5

0.9% NaCl

0.1% Tween-20

Store for up to 2 months at 4°C

BSA 5% (Bovine serum albumin)

5g BSA

100mL PBS

Prepare on the spot, do not stock up.

Triton X-100, 0.1%

99.9 mL PBS

0.1 mL Triton X-100

Store for up to 6 months at TA and protected from light.

Mounting Medium

Prepare the ratio of 50% glycerol to 50% PBS. Since glycerol is very viscous, check if the pipette has pulled in all the desired amount of glycerol and homogenize several times so that all the glycerol comes out of the pipette and is diluted in the PBS. Store at 4°C.

9 FLOW CYTOMETRY

Flow cytometry is a technique used to count, examine, and classify microscopic particles suspended in a flowing liquid medium. Flow cytometry has the following concepts: cytometry (cells), metry (measure), and flow (movement). Its principle is primarily based on a fluid system that allows cellular individualization, an optical system capable of detecting the light scattered as it passes through a cell and the wavelength emitted by a fluorochrome, and an electronic system for (physical-virtual) conversion, processing, organization and manipulation of data.

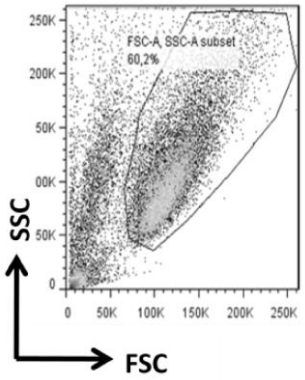
The flow cytometer device has optical-electronic detection as its principle, identifying different cells by detecting the light they scatter and the fluorescence they emit (if they are labeled with fluorochromes) when they pass through a laser beam (**Figure 4**). It allows the measurement of several parameters simultaneously, making it possible to analyze the physical and chemical characteristics of a single cell. This device is similar to a fluorescence microscope, however, instead of producing an image of the cell, it offers quantitative and qualitative data of a large number of cells in a short period of time, being possible to detect up to 10000 cells per second.

The cytometer basically has:

- A hydrodynamic flow system: which individualizes and directs cells to the point of incidence of the laser.
- A light radiation source: usually these are either a mercury lamp (different wavelengths) or Argon (488nm), Helium-Neon (HeNe, 543 nm) and Ultraviolet (UV, 405 nm) lasers.
- Filter system: intended for filtering the color spectrums emitted by fluorochrome (ultraviolet, blue, green and red, basically).
- Detectors (photodiodes or photomultipliers) for detection of scattered or emitted light.
- A unit for data processing and signal analysis, transforming the electrical signals emitted by the detectors into graphs that can be interpreted (machine-user interphase).

Figure 4

Principle of flow cytometry



9.1 WORKING PRINCIPLE AND ANALYSIS

The cells, through a fluid system, are directed to a point (question mark) where they are intercepted by a beam of light, of a certain wavelength. Optical detectors, which

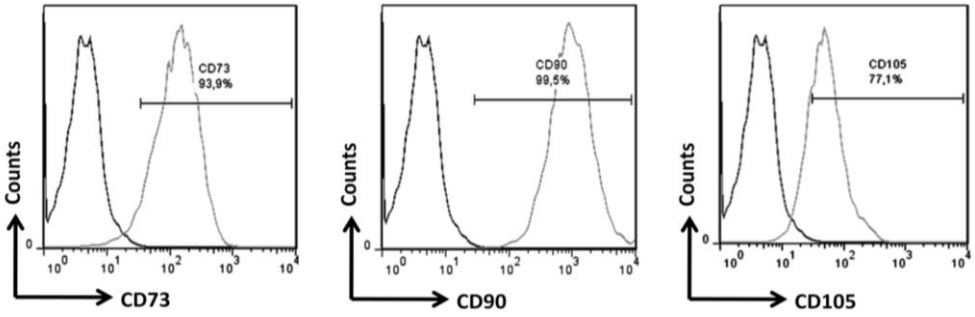
correspond to certain parameters, are pointed at the point of intersection. In flow cytometry, a parameter is understood as characteristics such as volume, cell complexity, and wavelength emitted by a fluorochrome. There are three types of detectors, one positioned in the line of the light beam (*Forward Scatter* or FSC) that detects the scattered light frontally; one positioned perpendicular to the direction of the light beam, responsible for detecting the light scattered laterally (*Slide Scatter* or SSC); and other types of detectors, each responsible for detecting a certain wavelength emitted by a fluorescent substance, and also positioned perpendicular to the light beam (**Figure 4**).

The cells of interest prepared for flow cytometry analysis should be suspended in a 5 mL round-bottomed polystyrene tube, suitable for flow cytometry. A system, which uses positive pressure, collects the cells from the tube and takes them to the flow chamber. The cells, upon entering the chamber, suffer an effect called hydrodynamic focus. The chamber fluid (*sheath fluid*) has a higher velocity in relation to the sample fluid, the difference in the velocity of the two fluids and the narrowing of the chamber allows the flow to behave in a laminar way, which leads to acceleration and concentration of cells in the center of the flow as the sample fluid gains speed and becomes narrower. As the cells are accelerated, they move away from each other, allowing them to pass through the question mark, where they are hit by a beam of light (**Figure 4**).

The cells are focused by the beam of light in the question mark. The beam of light when it hits the cell undergoes frontal and lateral dispersion. There is a detector for frontally scattered light that is in the line of the light beam; and a detector for sideways scattered light that is perpendicular to the light fixture; these detectors are represented by the FSC and SSC parameters, respectively. Thus, the FSC detector will provide characteristics on cell size and volume while the SSC detector will provide characteristics on intracellular complexity related to the amount of membranes such as nucleus, vesicles, Golgi complex, cytoplasmic reticulum, membrane roughness and cytoplasmic granules. Thus, we can obtain information in the form of graphs, on the computer screen, such as populations of cells classified in relation to their physical characteristics. For example, we can distinguish the different types of leukocytes by analyzing the population of cells found from the peripheral blood collected, or any other type of cells of interest, such as stem cells, always according to their size and the complexity of the membranes that constitute the cell body (**Figure 5**).

Figure 5

The combination of SSC (y-axis) and FSC (x-axis) allows a very good distinction between cell population and leukocyte residues or cellular remains. The stem cells shown have cell size and volume (FSC) and membrane complexity (SSC), such as organelles and nucleus. These cells are easy to notice, being shifted to the right. To the left, there are points that represent cell debris: small parts or remnants of membrane, small or large, but of low volume, therefore displaced closer to the y-axis



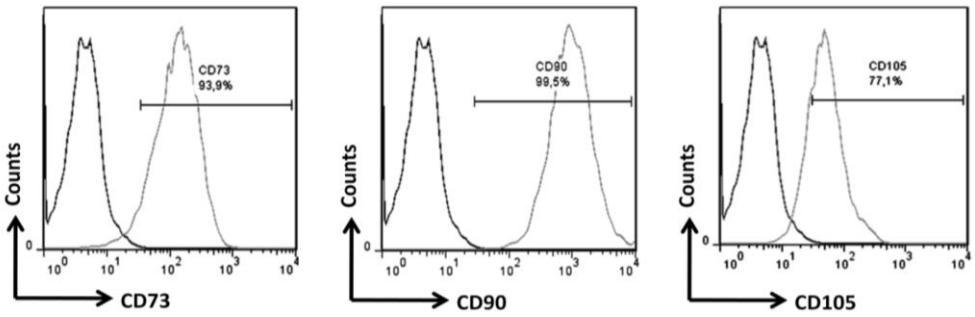
This type of approach to detecting cell populations uses a lot of graphical representation called dot *plot*. In this mode it is possible to check two parameters simultaneously. In the graph above (**Figure 5**), the x-axis represents the cell volume by the FSC parameter and the y-axis the SSC cell complexity parameter. In addition to the two defined parameters, the *dot plot* provides a third piece of information, which is the number of cells, where each point on the graph represents a single measured cell. Another much simpler mode of graphical representation is the histogram. In this mode it is possible to check the information of intensity and number of cells, as well as their proportion. In the example below (**Figure 6**), the intensity of the markers of cell molecules is represented on the x-axis and the number of cells is on the y-axis.

The flow cytometer also allows us to analyze cell populations that express certain types of molecules that can be labeled through the immunofluorescence technique with Ab linked to fluorochromes. The different types of fluorochromes, when excited by the light beam, emit certain wavelengths that are optically filtered and detected by detectors dedicated to a certain color range. More modern flow cytometers may have more fluorescence detectors, but at a minimum, they all come equipped with wavelength detectors in the green (FL1), orange (FL2), and red (FL3) range. For example, the FL1 detector detects the fluorochrome FITC, FL2 the fluorochrome PE and the FL3 fluorochrome APC. In stem cell research, this

approach becomes interesting because surface molecules are used to classify the state of differentiation and cell potency. Figure **6** shows a histogram of the number of cells by markers of cell potency.

Figure 6

Stem cells are shown to compare the expression of markers related to mesenchymal origin. The curve with the darkest stroke is the control, showing that this group of cells are not marked with Ab. The control serves to adjust the position of cells that do not have fluorochromes, thus presenting little fluorescence. The curve with a clearer line shows the cells that have undergone the immunofluorescence process: if the cells present the protein of interest, depending on the concentration of this protein, if in large quantities, the more Ab will bind to the cells, the stronger the intensity of the fluorescence and the more displaced to the right will be these cells. The y-axis shows the number of cells; The X-axis shows the intensity of the fluorescence emitted by the fluorochromes. In this case, the graphs show that the cells are positive for the molecules or markers for mesenchymal cells CD73, CD90 and CD105. The percentage shows the proportion of labeled cells for a given mesenchymal protein, with the unmarked control cells as the cutoff limit. Tip: even though the cells are not labeled, as is the case with the unlabeled negative control, they have their own fluorescence intensity detected by the flow cytometer. This should be calibrated so that the histogram of the control group is very close to zero on the x-axis, i.e., leaning against the y-axis



Another point to be observed is how the signal will be amplified, as there are linear and logarithmic amplifiers. The form of amplification is much more linked to the practical sense of observing the signs. The log form is commonly used for fluorescence, as it expands weak signals and compresses strong ones by visually eliminating extreme values, as it is used for marking analysis for stem cells. The Linear form is used when you want to analyze the signals as a whole – this type of amplification is widely used for cycle analysis.

9.2 APPLICATIONS OF FLOW CYTOMETRY

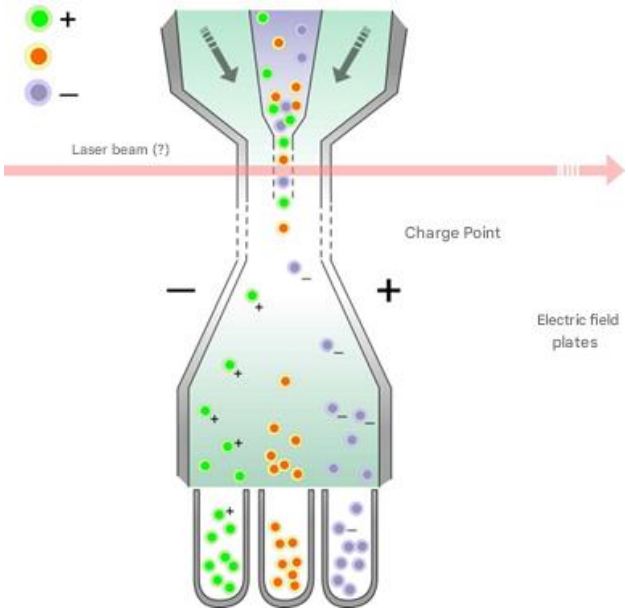
There are several possible applications using flow cytometry. The parameters that can be measured are: cell volume and morphological complexity, cell pigments such as chlorophyll, in cancerology (detection of a pathological cell; measurement of an abnormal DNA content), DNA and cell cycle (distribution of cells in the different phases of the cycle, presence of cells with abnormal DNA content, cell type analysis, cell kinetics, proliferation, etc.), RNA, analysis and classification of chromosomes, proteins, cell surface antigens (markers for identification of immune cell subtypes, for example), intracellular antigens (various cytokines, secondary mediators, etc.), nuclear antigens, enzyme activity, pH, intracellular ionized calcium, magnesium, membrane potential, membrane fluidity, apoptosis (quantification, measurements of DNA degradation, mitochondrial membrane potential, changes in permeability, caspase activity), cell viability, monitoring of cell electropermeabilization, pharmacology (characterization of multidrug resistance in tumor cells), glutathione, hematology (diagnosis, detection or identification of cell subtypes), various combinations (DNA/surface antigens, etc.). This list is very long and constantly expanding.

9.3 FLUORESCENCE ACTIVATED CELL SORTER *(FACS*)

FACS is a technique based on flow cytometry with the objective of selecting one cell among thousands. A given cell that has an antigen of interest can be separated from others that do not have this antigen, if an Ab specific for this antigen is labeled with a fluorochrome. The FACS computer system associates the wavelength (color) of the fluorochrome as an event (in this case, a cell) that must be separated (**Figure 7**). In this way, a certain cell, with a specific marking, and that we want to separate, will receive an electric charge and, when passing through an electromagnetic field, this cell will be deflected towards one of the plates of the electromagnetic field, depending on the charge that this cell has received. The FACS technique is usually used to isolate different types of leukocytes. T cells, for example, have CD3 and Thy1.2 antigens; these molecules can be labeled with Ab conjugated with specific fluorochrome and associated electrical charge and the cells can be selected by this technique.

Figure 7

Fluorescence-activated cell separation (FACS). The cells are separated according to their marking and associated electrical charge. Thus, there are three possibilities: positive, negative or neutral charge



9.4 IMMUNOFLUORESCENCE PROTOCOL IN FLOW CYTOMETRY

For antigen labeling in flow cytometry, it is necessary that the procedure begins with the cells in suspension. We often work with cells that grow in suspension, but we also experiment with adhered cells. Regardless of the type of culture, after the experiment of interest, whether it is the addition of a substance, a drug, or the submission of the cell to some physical process such as irradiation, or simply reading the proliferative behavior or marking antigens for characterization, the cells must be resuspended and counted so that they can be read in the flow cytometer. The ideal number of cells for reading should be around 1x106 cells in total, but it is possible to read cells from 1x105 cells. The quantity is important because, during the cell antigen labeling procedure, the cells underwent several washes that include centrifugations and resuspensions in buffers to remove excess Ab and successive transfers to different tubes, procedures where a lot of cell loss occurs.

9.5 MARKING FOR SURFACE ANTIGEN DETECTION

The surface antigen labeling is performed with the live cells in suspension and at the end, if necessary, we fix it with 2% formaldehyde, in case the cytometer analysis cannot be performed immediately.

The procedure below is indicated for cell labeling in order to characterize the cells. Although the protocol below uses indirect labeling, in flow cytometry direct labeling is more attractive, as it avoids washing steps and incubation periods where more cell loss can occur.

Materials

Cells growing in culture with approximately 70 to 80% confluency.

Buffer Solution: PBS (*Phosphate Buffer Saline*), pH7.2, with 0.5% bovine albumin serum (BSA) and 2 mM Na2EDTA.

This buffer solution with EDTA is useful to prevent cell aggregation.

Culture medium appropriate to the cell of interest.

Formaldehyde at 2%.

Trypsin.

Primary ab .

Secondary Ab (specific to the type of Ig of the species from which the primary Ab was obtained) bound to a fluorochrome.

Pipettes.

15 mL conical tubes.

1.5 mL conical tubes.

5 mL round-bottom tubes (for flow cytometry).

***Procedure* (direct and indirect dialing)**

1. Disaggregate the cells in culture using trypsin.
2. Centrifuge the cells for 5 min at a speed of 300xg (approximately 1000 rpm) with the aid of the 15 mL conical tube.

It is interesting to calculate the speed in rpm of each centrifuge.

3. Resuspend the cells and count them. Subdivide them into 1.5 mL conical tubes in order to contain a quantity above 1x105 cells in total.
4. Resuspend the cells at 50 to 100 µL and add 10 µL of the diluted primary Ab at the concentration indicated by the manufacturer or at the concentration found after titration of the Ab in the labeling standardization process. Gently mix and incubate the cells at 4°C for 10 to 20 min or at the time indicated by the manufacturer.

5. Wash the cells by adding 1 to 2 mL of buffer solution.

Washing includes a gentle cell resuspension and centrifugation at 300xg for 8 min.

6. (Optional) Wash one more time.

If the marking is indirect, the procedure for adding the secondary Ab should be the same as it was done with the primary. If there is a need for multicolored marking, each Ab should be added independently, following the sequences of steps 4 through 6. Another option is to make a mixture of primary Abs and apply them to the cells simultaneously (as long as there is no interference between antigens; this is a procedure widely used when you have primary Abs already conjugated with fluorochromes).

7. Resuspend the cells in 300 µL, transfer them to a 5 mL round-bottomed tube, and keep them at 4°C if the reading is taken soon after. If you need to postpone the reading, you can fix the cells in 2% formaldehyde and keep them at 4°C – take the reading in less than a week.

When fixing the cells, first suspend in buffer solution and then add the formaldehyde so that the final concentration is 2%. This procedure prevents the cells from being aggregated if the cells were resuspended directly with the fixator.

9.6 LABELING FOR INTRACELLULAR ANTIGEN DETECTION – FIXATION AND PERMEABILIZATION

Many antigens of interest that we wish to label are inside the cell, in the cytoplasm, or in intracellular compartments such as endoplasmic vesicles and reticulum, even in the nucleus. In order for Ab to interact with the antigen that is inside the cell, it needs to enter the cell. To do this, the cell membrane must be permeabilized and the pores then formed must remain open. Therefore, initially, the cell must be fixed, which leads to cell death.

Materials

The materials required are the same as those used in the surface antigen labeling procedure described above, with the addition of the permeabilizer:

Permeabilization solution: 0.2% (v/v) Tween 20

***Procedure* (direct and indirect dialing)**

1. Disaggregate the cells in culture using trypsin.
2. Centrifuge the cells for 5 min at a speed of 300xg (approximately 1000 rpm) with the aid of the 15 mL conical tube.

It is interesting to calculate the speed in rpm of each centrifuge.

3. Resuspend the cells and count them. Subdivide them into 1.5 mL conical tubes in a total of 1x106 cells.

In this experiment, it is advisable to use a total of 1x106 cells per tube, since the process of fixation, permeabilization and subsequent washing will result in much greater cell loss than the procedure of cell surface antigen labeling.

4. Centrifuge the cells and remove the supernatant. Add 875 µL of iced buffer solution and mix gently. Add 125 µL of the fixing solution and mix again. Incubate for 30 min at 4°C.
5. Centrifuge and remove the supernatant. Add 1 mL of permeabilization solution to the cells. Incubate for 15 min at 37°C.
6. Add 1 mL buffer solution, centrifuge and remove the supernatant.
7. Add the primary Ab diluted appropriately in 100 µL of buffer solution to the cells. Mix gently and incubate for 30 min at 4°C.

A blockade step can be accomplished by incubating the cells for 1 min with 50 µL of human AB serum inactivated by heating. Lock before adding the primary Ab. This procedure reduces non-specific markings. If a membrane immunoglobulin test is performed, this step should not be performed.

8. Add 1 mL buffer solution and centrifuge for 5 min at 300xg.
9. Wash the cells once more by adding 1 mL of buffer solution.
10. If it is necessary to mark the primary Ab with a secondary conjugated with a fluorochrome (**indirect marking**), repeat steps 7 through 9. If Ab is already conjugated (**direct dialing**) go to the next step.

The Ab indicated as secondary is the F(ab')2 fragment format, because it is smaller, penetrates better into the cell, and eliminates the possibility of binding to Fc receptors.

11. Suppress the cells at 300 µL, transfer to a 5 mL round-bottomed tube, and keep them at 4°C if the reading is taken soon after. If you need to postpone the reading, you can fix the cells in 2% formaldehyde and keep them at 4°C – take the reading in less than a week.

Reagents

Formaldehyde, 2%

As commercial formaldehyde comes in a 37% solution, its dilution is made considering this solution as 100%. Therefore, dilute 2 mL of the commercial formaldehyde solution in 98 mL of PBS pH 7.4.

If you wish to use paraformaldehyde, dissolve 4 g of paraformaldehyde powder in 10 mL of distilled water and heat to 60°C. Dilute in a 1:1 ratio with 2x concentrated PBS, pH 7.4.

Always prepare before use, it is not interesting to stockpile.

Permeabilization solution: 0.2% (v/v) Tween 20

Mix 200 µl of Tween 20 with 100 ml PBS.

Store in amber vial for less than 1 month at 4 °C. Use it at room temperature.

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