USE OF ANTIBODIES IN IMMUNOFLUORESCENCE

LABORATORY MANUAL

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FOREWORD

The scope of this laboratory manual is to familiarize scientists who have not previously worked with antibodies with the topic, giving them tools to appropriately use antibodies in their research. This text might as well be useful to more experienced scientists who would like to review techniques regarding antibodies apart from those they regularly use. The target population is, in all cases, the researchers using the microscopy services offered by the *Centro de Microscopía de la Universidad de los Andes* and other related laboratories.

The main purpose of this text is, rather than providing the answers, posing the relevant question of what could be done better. This is so because antibodies differ greatly one from the other, therefore making generalizations a risky task. The text also intends to provide a deeper understanding of how antibodies and immunofluorescence techniques work, which is crucial for troubleshooting and experimental design. So instead of telling the researcher what to do it tells how things work.

This text is organized as follows: first we address the questions of what antibodies are and which properties they have, we then discuss several aspects of immunofluorescence techniques, later we address the matter of experimental design to finally present general and particular guidelines for the use of antibodies as well as some useful protocols.

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CONTENTS

INTRODUCTION

Immunofluorescence is a powerful technique for enhancing microscopic studies of biological samples. It makes use of the ability of antibodies to bind to specific proteins to exclusively stain those target proteins in the sample. The result is the possibility to observe and quantify protein and gene expression, with a high contrast and signal-to-noise ratio.

Antibodies were discovered at the end of the XIX century. Research on antibodies first focused on the immune system, but scientists rapidly realized the potential they had in other domains [1], being fluorescence microscopy on of them. Today, antibodies are industrially produced and can be readily bought like other laboratory materials. Handling and making good use of them is a more delicate issue, which I expect to address in this text, given their high cost.

Antibodies revolutionized the field of microscopy, mainly because they allowed for quantification. The feature of antibodies of making microscopy quantifiable broadens the possibilities for a technique that decades ago was merely qualitative.

More recently, with the development of advanced optical microscopy techniques such as single-molecule microscopy and super-resolution microscopy, the potential of antibodies in microscopy has once again boosted.

Immunofluorescence has thus become a mandatory technique for the study of biological samples.

BASICS ON ANTIBODIES

1. Antibody structure and function

Antibodies are proteins produced by the B cells of the immune system as a response to a certain antigen. There are several types of antibodies, but they all follow a similar structure to that of the paradigmatic IgG. These are composed by two heavy and two light chains linked by several disulfide bonds. The light and heavy chains have a highly variable region called the Fab fragment (Fragment having the Antibody Binding site) and a highly conserved Fc fragment (Fragment that Crystalizes). The structure resembles a Y, with the Fab fragments forming the two arms and the Fc fragments forming the tail. The Fc fragment is common to all antibodies produced by a certain animal species, enabling other species to recognize exogenous antibodies [2], [3].

The typical molecular weight of an IgG antibody is about 150kDa [1] while its typical dimensions are 14.5 nm x 8.5 nm x 4.0 nm, with the two antigen binding sites are separated by 13.7 nm [4], [5].

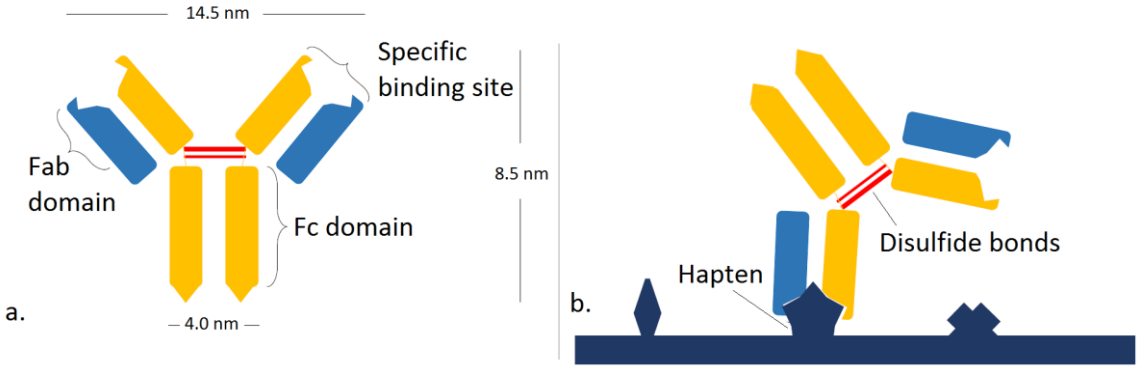

Figure 1: Structure of IgG antibodies. IgG antibodies are the paradigm of antibody structure. **a.** Main components of the IgG antibodies: Two heavy chains (orange) and two light chains (blue) linked by a disulfide bonds (red) form a Y shape. The Fab domain, which is highly variable, makes up the binding site**.** The Fc domain, which is highly conserved among the antibodies produced by a single species is characteristic and allows for inter-species recognition. **b.** Antibodies only bind to specific haptens (target proteins) as the variable Fab domain matches them but no other proteins.

2. Specificity, affinity, and avidity

The *specificity* of an antibody is its ability to recognize a specific target in the presence of other targets (epitopes). The specificity of an antibody is strongly dependent on its conformational determinants. The denatured protein may, for instance, not bind to the specific epitope. This is particularly relevant for monoclonal antibodies, which target a single epitope. The conformation of antibodies could vary due to changes in pH, temperature, salt concentration, association with other proteins, and fixation. The *affinity* of an antibody is a measure of its binding strength with a monovalent epitope. Antibodies with high affinity bind larger amounts of antigen with a greater stability in a shorter time than those with low affinity and are preferable for immunochemical techniques. The *avidity* of an antibody is a measure of the overall binding intensity of the antibody with a multivalent antigen presenting multiple epitopes

3. Polyclonal and monoclonal antibodies

Complex antigens are large molecules, thus antibodies can only bind to a section of them. Such a section is called an epitope. Polyclonal antibodies recognize several epitopes on a single antigen. Monoclonal antibodies only recognize one epitope[6]. This fact makes monoclonal antibodies more specific, while polyclonal antibodies may be more promiscuous and have a higher chance of binding to proteins different from their target. However, avidity is greater for polyclonal antibodies as they can bind to different parts of the same antigen [7].

4. pH, temperature, light, and contamination sensitivity

Antibodies are extremely sensitive molecules. In order to preserve the specificity of their binding site (the core essence of an antibody), their structure may not be altered. Changes in pH and temperature as well as exposure to light may damage antibodies.

pH Sensitivity

Antibodies are very sensitive to changes in the pH of the medium in which they are. Media with basic pH strongly and irreversibly damage the structure of antibodies, even altering the molecular size or causing decomposition; while media with acidic pH, although less harmful, can cause the antibodies to precipitate. The charge of antibodies can also change in certain pH ranges, which may inhibit adhesion to its target protein [1], [8]. Antibodies are therefore buffer-selective.

Temperature sensitivity: conformation and kinematics

Antibodies are also very sensitive to changes in temperature. High temperatures will, of course, denature them. Most antibodies are more stable at low temperature, so storage temperatures are usually below 4°C. However, thawing may increase the tendency of some antibodies to aggregate (especially for IgG3 and for enzyme-conjugated antibodies), therefore temperature below the freezing point should be avoided for these antibodies [9].

Temperature not only affects the strength of the antigen-antibody bond by changes in their structure but also by changes in their kinematics. The reaction

$$antigen + antiboy \rightleftarrows antigen - antibody\ complex$$

has a thermal optimum that depends on the chemical properties of the antigen and the antibody, but in most cases is expected to stabilize at low temperature [8]. This is so because when temperature decreases so does the kinetic energy of antibodies and antigens. The potential binding energy between an antibody and an antigen as a function of separation has a valley-like region (if they are too close they repel each other, whereas specific attraction increases energy as they separate until the bond is finally broken), as is shown in figure 2. If an antibody with energy $E > H_{bound}$ approaches an antigen, it may overcome the potential barrier of height H_{bound}. If $E < H_{unbound}$ the binding will become permanent. When temperature decreases H_{bound} and $H_{unbound}$ change slightly, but E usually changes more significantly, allowing for more bound antigen-antibody complexes. For some antibodies this is of no practical use as the resulting change in the binding probability is unaffected[8]. But if E decreases, so does the probability of an antibody approaching an antigen; this unwanted effect may be resolved by giving the antibodies and the antigens more time to

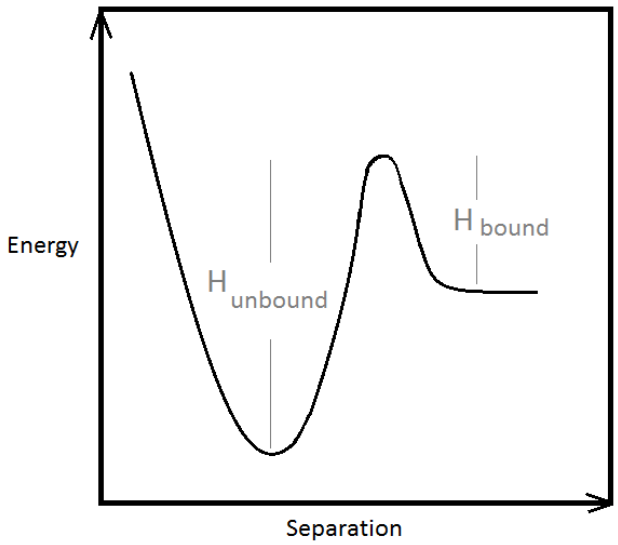

Figure 2: Potential energy of antibody-antigen binding as a function of their separation. If an antibody with energy $E > H_{bound}$ approaches an antigen, it may overcome the potential barrier of height H_{bound}. If $E < H_{unbound}$ the binding will become permanent. When temperature decreases H_{bound} and $H_{unbound}$ change slightly, but E usually changes more significantly, allowing for more bound antigen-antibody complexes.

bind[1]. This is of special importance when the performed stains are of low specificity or brightness, which we will discuss in greater detail in the experimental design section.

Light sensitivity

Antibodies are not as sensitive to light as fluorochromes (conjugated fluorescent molecules) are. Therefore antibodies that are conjugated to a fluorophore should not be exposed to light. Sometimes they come in dark, opaque aliquots, but that is not always the case. If the conjugated antibody comes in a transparent recipient, it must be stored covered in aluminum foil, or inside an opaque container or bag. When working with them, antibodies conjugated with a fluorochrome should only be exposed to light as much as it is necessary and then kept aside in the dark.

Contamination sensitivity

Antibodies are extremely sensitive to contamination, especially if it is of biological nature. They should only be handled using clean gloves, with sterile micropipette tips, and inside a laminar flow cabinet (this is particularly important if the lab in which you work handles bacteria, viruses or fungi). It is not advised to use a burner to keep the environment sterile as the heat may affect the antibodies.

5. Primary and secondary antibodies

Primary antibodies target specific proteins that are present in your samples. It is best if they are highly specific to avoid background. Secondary antibodies target (primary) antibodies produced in a certain species, as it is explained in the *Antibody Structure and Functions* section. So if your primary antibody is produced in mouse, for instance, your secondary antibody **must** an anti-mouse produced in any other species (goat anti-mouse). In direct immunofluorescence only primary antibodies –with a conjugated fluorophore- are used. In indirect staining both primary and secondary antibodies are used, but it is the secondary antibody which has the fluorophore conjugated.

[1] For some antibodies, such as anti-D in red cells from blood, the time needed to reach equilibrium at 4°C is 20 times longer than at 37°C [8].

BASICS ON IMMUNOFLUORESCENCE

1. Immunofluorescence techniques

Immunofluorescence is just one group of techniques among all fluorescence microscopy techniques. In immunofluorescence the samples do not exhibit autofluorescence, which is why one must stain them. In our case of study this is done by applying antibodies linked to fluorescent molecules that bind to the target proteins. There are several ways in which the fluorophores are linked to the antibodies; allowing us to speak of direct and indirect fluorescence.

Direct fluorescence uses primary antibodies which target the desired protein and which are conjugated with the fluorescent molecule. Indirect fluorescence uses primary antibodies which target the desired proteins but which do not have a conjugated fluorophore; instead, it also uses secondary antibodies which target the primary antibodies –but not the protein- and which do have a fluorophore conjugated. This is shown in figure 3.

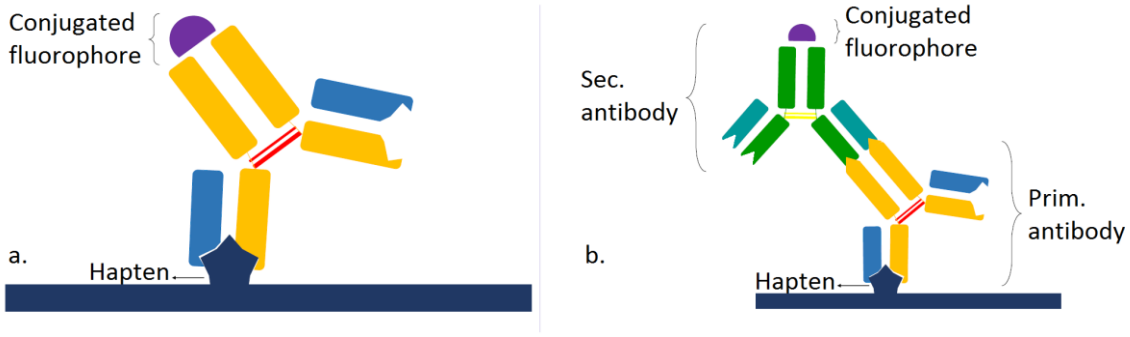

Figure 3: Direct vs. indirect immunostaining. **a.** In direct staining a primary antibody that targets the desired hapten is applied. This antibody has a conjugated fluorophore. **b.** In indirect staining a primary antibody that targets the desired hapten is applied. Next, a secondary antibody that targets the primary antibody and that has a conjugated fluorophore is applied.

Simplicity may lead us to think that direct fluorescence should be preferred, but this is seldom the case. Historically, this was the most common technique to be used, but that is no longer true as indirect fluorescence is more flexible. For example, the researcher could have access to only one microscope with few filters but have the need to stain different proteins in different samples. Thus it would be practical to buy several primary antibodies

that target the desired proteins and buy the few secondary antibodies that the filters allow, not needing to worry about buying the specific primary antibody with the specific conjugated fluorophore. Antibodies for direct fluorescence are also more expensive and less common than antibodies for indirect fluorescence, and that is a crucial point.

2. Microscope requirements

Color and filters

To work with immunofluorescence we need a fluorescence microscope. Such a microscope can illuminate the sample with light of one color and detect exclusively the fluorescence that is emitted by the sample, which is light of another color. The microscope must then have a fluorescence lamp (different from the lamp that produces white light for transmitted light illumination) or a set of lasers, and a set of filters.

Fluorescence lamps for microscopes are generally mercury or xenon lamps that emit a white continuous spectrum. The light of such lamps must be filtered by color, so that light of the appropriate color reaches the fluorophores and excites them. White or color LEDs can also be used. If the microscope uses color LEDs or laser sets, there is no need for filters in the illumination path. Once the fluorophore is excited it will emit light of greater wavelength (which is equivalent to light of less energy), i.e. light which is farther from violet and closer to red in the spectrum. The microscope must have a detection filter that allows light which is emitted from the sample to pass but blocks illumination light.

Microscopes with white fluorescence lights have a limited set of filters, so one must check if the available filters are appropriate for the desired fluorophore[2]. To do this one can compare the excitation and emission spectra of fluorophores with different filters sets. Some filters' and fluorophores' manufacturers have a tool for this in their websites[3]. On the other hand, microscopes with laser sets for fluorescence can illuminate with many more specific wavelengths, but do still need filter sets for detection, which again imposes a limit on which fluorophores can be imaged.

[2] The available resources for optical microscopy at the Centro de Microscopía can be found at https://investigaciones.uniandes.edu.co/index.php/es/centro-de-microscopia/microscopio-confocal/equipo.
[3] Common examples are: Thermofischer SpectraViewer (https://www.thermofisher.com/co/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html), Chroma SpectraViewer (https://www.chroma.com/spectra-viewer), Olympus FluoView Resource Center (http://www.olympusconfocal.com/java/dualprobes/), Zeiss Interactive Fluorescence Dye and Filter Database (https://www.micro-shop.zeiss.com/index.php?s=218486695447501&l=en&p=de&f=f&a=i)

Temporal Resolution

In many cases, the use of antibodies requires cell fixation and excludes the possibility to study cell dynamics –under these circumstances the study of cell structure or simply the study of protein expression is more valuable. However, sometimes the researcher is interested in having not only spatial but also temporal resolution. For such cases, the scientist will have to decide which microscope to use.

Confocal microscopes have excellent contrast and signal-to-noise ratio, but achieve this at the cost of time resolution. Acquiring a single frame in a confocal microscope can take up times in the order of minutes. For the study of cell dynamics, such times are usually too long. Regular fluorescence microscopes can acquire images at a much faster rate. This is so because they do not illuminate point by point, as confocal microscopes do. The limitation for these microscopes comes from the camera specifications. Most cameras can acquire images at rates up to the order of 10Hz, but that depends on the size of the image. For acquiring fast images with good contrast and good signal-to-noise ratios TIRF (Total Internal Reflection Fluorescence) is the ideal technique.

Image Background

Image background is a major issue in traditional microscopes because they illuminate the sample longitudinally while only the sample section that is in focus is imaged. Confocal microscopes do not have this problem because they illuminate the image through a pinhole and therefore can focus the light only around the desired plane. For imaging samples such as tissues confocal microscopy is the ideal technique, as it dramatically reduces the image background. For imaging samples such as cells on a slide, this is not critical, thus they can be imaged in a traditional fluorescence microscope.

EXPERIMENTAL DESIGN

Many variables must first be optimized to obtain images of the desired quality when working with antibodies. These include the concentrations of the antibodies, the incubation times and temperatures, the fixation method, the blocking solution, the time exposure of the photographs, and the illumination intensity for example. In order not to lose track of the changes made, one must play with these variable one at the time and keep proper records of each experiment.

Where to begin then? For most of these there are good starting points. For instance, the cell fixation/permeabilization method must be one that preserves the structure that one wants to study and that does not compete with the primary antibody for binding sites. Antibodies manufacturers have recommended working concentrations for their antibodies depending on the application.

GUIDELINES

Immunostaining requires attention and concentration. It is very much like cooking: you can ruin everything in just a second, but if you follow the recipe carefully you'll achieve great results. Before you begin cooking, all the ingredients must be ready, the timer set, and, most importantly, the mind focused on what you're doing. [4]

General outlines:

When working with antibodies:

i. Everything must be clean and sterile.
ii. Time matters: don't rush the protocol, neither leave samples sitting.
iii. Keep focused: distraction may lead to forgetting to put antibody on a sample, or putting it twice.
iv. Have your implements ready.
v. Once the staining process begins and before it ends antibodies may never dry (we will discuss that in greater detail in the *Work with a humid chamber* subsection).
vi. Only make one change per experiment: multiple variables will make you lose track.
vii. Take note of everything you do.

Specific outlines:

Now I will present some more specific guidelines for several parts of working with antibodies. Later I will give some handy protocols.

1. Handling of antibodies

Before working with antibodies you must understand that they are very delicate supplies and that they all work differently. Next, I will present some general guidelines for handling antibodies, but you should always read and follow the handling instructions given by the manufacturer before working with any antibody.

[4]These words –although not exact- I owe to Joel Klahr, a kind friend of mine who taught me almost everything I know about antibodies. They are pure, brief wisdom. They have helped me, so I hope they get fixed in your memory as they did in mine.

Temperature

The researcher must avoid frequent freezing and thawing. If the antibody is to be used continuously, it is usually preferable to store it at 4°C instead of freezing it. Thawing and dethawing can also be avoided by aliquoting the stock of antibody. **Warning: Antibodies should always be stored according to the instructions given by the manufacturer.** The manufacturer will note the ideal storage temperature for continuous and sporadic use. Follow this instructions closely as you may damage costly laboratory supplies if you fail to do so.

Aliquoting

The manufacturer will indicate if the antibody may be aliquoted (**not all antibodies can be aliquoted** as they are buffer or concentration sensitive, for instance), and if that is the case they will also indicate in which buffer and at what concentration it should be diluted.

pH

If aliquoting is recommended by the manufacturer, do it following their instructions carefully. Measure the pH of your buffer before diluting the antibodies in it. For staining PBS is the most common dilution buffer as it is the most gentle for most cells. Only dilute antibodies in PBS (or PBS-based blocking solution) just some minutes before staining.

Contamination[9]

When aliquoting antibodies it often useful to add sodium azide to a final concentration of 0.02% (w/v) to prevent microbial contamination. Some antibodies already contain this preservative. Verify that by checking the composition of the storage buffer in their data sheet.

Do not use sodium azide if staining live cells with antibodies (it is toxic for most other organisms as well). Sodium azide will also interfere with any conjugation that involves an amine group. You may remove sodium azide from your antibodies by dialysis or gel filtration.

2. Working with a humid chamber

A humid chamber is necessary for immunostaining. It is simply a plastic box or a petri dish, for example, adequated to keep the inner environment humid and dark. The easiest way to make one is to take a petri dish or the box of a 96-well plate for Western blots and then line it with aluminum foil. For the process of staining a wet paper towel is placed in the box

with some support (like two wooden sticks) so that the sample (most usually a slide) does not lie directly on the humid paper.

3. Cell preparation

Cells for staining are usually prepared from three sources: adherent cells, suspension cells, and tissue. Cells from different sources are to be prepared differently. *Adherent cells* can be grown on culture dishes or directly on coverslips or slides. The latter is preferred when good image quality is needed. For more detailed information see protocols 2 and 3.

4. Troubleshooting

Background reduction

Strong background is mainly due to non-specific binding of either primary or secondary antibodies. Proteins, by nature, exhibit non-specific binding[10], i.e. they bind to haptens different from their specific antigens[11]. Non-specific binding can be reduced via blocking and rinsing. Blocking works in the following way: non-reactive proteins such as BSA, casein and those proteins present in sera from other animals block binding sites [12]. Rinsing works differently: when blocking has not been enough, one can remove the already bound antibodies to non-specific sites by performing several rinses with a washing buffer. This works with the antibody kinematics as the potential energy of non-specific binding is low, so they are easily pushed away from the binding site.

PROTOCOL 1

ATTACHING SUSPENSION CELLS TO SLIDES

In order to detect intracellular antigens in cells that grow in suspension it is best to fix the cells to a solid substrate before fixation. This can be done on coverslips or slides.

To achieve this one can deposit some volume with cells in suspension and let them dry in air; by then they will have precipitated and attached to the glass. Another method is to use a centrifuge. Centrifugation also spreads the cells slightly, allowing better resolution of internal structures.

We will now present the protocol for cell attaching using a centrifuge [13]:

Materials:

Cells growing in suspension
PBS 1X
Microscope slides
Cytocentrifuge

Method:

1. (Optional) Wash cells by centrifugation at 400g and suspend them in PBS at $1x10^6$ to $2x10^6$ cells/ml
2. Mount microscope slides in a Cytocentrifuge with hard covers. Add 0.1ml - 0.5ml of cell suspension to the reservoir.
3. Accelerate rapidly to 1200g and centrifuge for 5 -10 min.
4. Dry the cell monolayer in air for 15-20 min.
5. Either fix the cells or use them in a direct immunostaining procedure.

PROTOCOL 2

FIXING ATTACHED CELLS IN ORGANIC SOLVENTS[5]

Materials:

Cells attached to a coverslip, slide, or plate
PBS 1X
Organic solvent[6] such as methanol, acetone, or methanol-acetone (1:1) at -20°C

Method:

1. Rinse the coverslip, slide, or plate with some droplets of PBS with the help of a Pasteur pipette.
2. Drain well by suction, but to not let the specimen to dry.
3. Put some droplets of the organic solvent on the specimen.
4. Incubate for 2 minutes.
5. Drain by suction + (rinse with PBS + drain by suction)x5.
6. Let dry.

If cells are not to be used immediately, they can be stored at -20°C. Fixed cells at this temperature can be stored for as long as years. To store them, simply cover the coverslip, slide, or plate with aluminum foil, tag it properly, and put it in a sealed plastic bag in the freezer.

Troubleshooting [13]**:**

Problem: Fixed antigens do not yield a strong antibody-antigen reaction.
Solution: Instead of the solvents listed above, incubate the sample in 5% glacial acetic acid, 95% ethanol for 5 minutes at -20°C. Wash as usual. (This method may destroy the antigenicity of many antigens so it should not be used as a first choice).

[5] Organic solvents such as methanol and acetone not only fix structures but also permeabilize the cell membrane [14].
[6] Methanol is the most commonly used for fixing. For the fixation of certain structures the other solvents are preferred (microtubules are best fixed with methanol-acetone 1:1, for example).

PROTOCOL 3

FIXING ATTACHED CELLS IN PARAFORMALDEHYDE OR GLUTARALDEHYDE[13]

Caution:

Glutaraldehyde is toxic. Work in a fume hood.

Materials:

Cells attached to a coverslip, slide, or plate
Ethanolamine (0.2M, pH 7.5) or sodium borohydride (0.5 mg/ml) in PBS (optional)
Paraformaldehyde (4%) <R> or glutaraldehyde (1%) (electron microscopic grade) in PBS
Permeabilization reagent, such as 0.2% Triton X-100, methanol, acetone, or methanol-acetone (1:1)
PBS 1X

Method:

1. Before the staining, prepare either the 4% paraformaldehyde solution or the 1% glutaraldehyde in PBS.
2. Wash the coverslip, slide, or plate gently with PBS.
3. For paraformaldehyde, incubate the sample in a 4% solution for 10 minutes at room temperature.
   For glutaraldehyde, incubate in a 1% solution for 1 hour at room temperature in a fume hood.
   Glutaraldehyde fixation is compatible with electron microscopic work, potentially allowing the specimen to be studied at the level of both light and electron microscopy. Paraformaldehyde fixation is not stable. If the paraformaldehyde fixation is followed by nonionic detergent lysis, long incubation in aqueous buffers will reverse the cross-linking and thus should be avoided. Therefore, the use of nonionic detergent permeabilization is not recommended for the detection of soluble antigens.
4. Wash the cells twice with PBS. At this stage, cells fixed with glutaraldehyde can be removed from the fume hood.
5. Permeabilize the fixed cells by incubating in one of the following reagents:
   - 0.2% Triton X-100 in PBS for 2 minutes at room temperature. Some antigens may need as long as 15 minutes; check this for each antigen.
   - Methanol for 2 minutes at -20°C.
   - Acetone for 30 seconds at -20°C.
   - For cells grown on tissue culture plates, methanol-acetone (1:1) for 30 seconds at -20°C.

6. (Optional) For glutaraldehyde, block free reactive aldehyde groups by incubating with 0.2M ethanolamine (pH 7.5) for 2 h at room temperature or by incubating with three changes of 0.5mg/ml sodium borohydride in PBS for 5 min each. In some cases, this may also help paraformaldehyde-fixed cells, but it is not necessary. This is recommended because free reactive aldehyde groups exhibit autofluorescence and may significantly increase background.
7. Rinse gently in PBS with four changes over 5 min.
8. Proceed to apply antibodies to the samples.

Reagent preparation:

To prepare paraformaldehyde (4%)

1. Add 8g of paraformaldehyde to 100ml of Mili-Q water.
2. Heat to 60°C in a fume hood. Add few drops of 1 N NaOH to help it dissolve.

   Caution*: Paraformaldehyde dissolves in water, releasing formaldehyde in the process; thus the preparation in a fume hood is essential.*

3. When the solid has completely dissolved, let the solution cool to room temperature, and add 100ml of PBS <u>2X.</u>

   This stock solution should be prepared fresh daily.

PROTOCOL 4

BINDING ANTIBODIES TO ATTACHED CELLS OR TISSUES

Materials:

Slides with attached cells (usually fixed as well)
Triton X-100
PBS 1X
BSA (Bovine Serum, Albumine)
Primary antibody
Secondary antibody, labeled (in case of indirect staining)
DAPI 300uM (Optional[7])
Gelvatol High Density
Cover slips
Humid chamber

Method:

Have ready:

I. Triton X-100 0.25% - 1%, BSA 1% in PBS (Cell membrane permeabilization solution)[8].
II. BSA 1% - 5% in PBS (Blocking solution)[9].
III. Calculations for desired volume of primary and secondary antibody, if necessary. This means that you should know what volume of diluted antibody you need, and given your working concentration, what volume of pure antibody and what volume of diluting buffer/solution that is.

Procedure:

Work in a humid chamber

[7] DAPI stains cellular nuclei. It is especially useful to recognize cells from deceiving cell-like structures.
[8] Triton X-100 is a strong cell permeabilizer. One should first use low concentrations as it may be too harsh on cells (see *Troubleshooting-Damaged cells).* Membrane permeabilization is important to allow effective rinsing inside the cell –crucial when working with intracellular antigens. Bear in mind that fixing may already have permeabilized the cell membrane if organic solvents were used.
[9] Fetal serum from the species in which the secondary antibody is produced can also be used as a blocking solution.
Buffer for Immunofluorescence (BIF) consisting of Bovine Fetal Serum 2% and BSA 1% in PBS may also be used when background is strong.

1. Add 100ul of TritonX-100 0.25% - 1%, BSA 1% in PBS to each slide.
2. Incubate in humid camber for 5min at 4°C.
3. Suction + (rinse with several droplets of PBS + suction)x3 times.
4. Have ready solution of primary antibody in blocking solution PBS-BSA 1% - 5%. It should not be ready more than 5min before application on slides.
5. Apply 200ul of primary antibody in PBS-BSA 1%.
6. Incubate in humid chamber at room temperature for at least 30 min.

   For some reactions, incubating at 4°C, overnight (up to 24 h) or both may be useful.

7. Suction + (rinse with PBS + suction)x5.

   If one is working with direct immunofluorescence one should skip to step 15.

8. Have ready solution of secondary antibody in blocking solution PBS-BSA 1% - 5%. It should not be ready more than 5min before application on slides.
9. Apply 200ul of secondary antibody in PBS to each slide.
10. Incubate in humid chamber for 30 min[10] at room temperature (or 4°C).
11. (Optional) Apply 2ul of DAPI 300uM to each slide.
12. (Optional) Incubate in humid chamber for 5 min at 4°C.
13. Suction + (rinse with PBS + suction)x5. Leave almost dry
14. Let dry.
15. Apply gelvatol HD with a Pasteur pipette making sure there are no bubbles.
16. Put cover slip on top.
17. Let dry. Do not expose to light. Ready to be imaged.

[10] In case DAPI is used, incubate for only 25 min up to this point.

PROTOCOL 5

BINDING ANTIBODIES TO ATTACHED CELLS FOR SUPER-RESOLUTION MICROSCOPY

When immunostaining for a super-resolution microscopy that is based on stochastic blinking and bleaching of molecules it is mandatory to add a reducing agent. This protocol uses 2-Mercaptoetanol.

This protocol is based on protocol 4, with modifications after step 13.

Cells must be attached and fixed on cover slips, not slides, as the latter are usually too thick for the high magnification microscope objectives –which most commonly have short working distances- that are normally used.

Materials:
Same as those for protocol 4
2-Mercaptoethanol 14M
Microscope slides
Nail polish

Method:

1. Dilute 2-Mercaptoethanol to 14mM in PBS.

Follow protocol 4 up to step 13.

14. Apply 20ul 2-Mercaptoethanol 14mM on a microscope slide.
15. Drop cover slip with immunostained cells at an angle of 45° on top of the microscope slide making sure there are no bubbles.
16. Seal with nail polish.
17. Let dry. Do not expose to light. Ready to be imaged.

APPENDIX 1

PROTOCOL FOR EXTRACTION OF HUMAN LYMPHOCYTES

Human lymphocytes can be immunostained with many different antibodies, as they have numerous membrane markers. Lymphocytes are, therefore, a simple control for the effectiveness of antibodies. They can be used to test fixation or staining methods as well as antibody concentrations. One must bear in mind that the results obtained from experiments with human lymphocytes may not be exactly extrapolated to other type of cells.

This protocol is included as an appendix should it come in handy for the researcher.

***Caution:** This protocol includes extracting and handling human blood. All biosafety measures must be followed carefully. Extraction of blood may only be carried by trained personnel.*

Materials:

Human blood
PBS 1X
Ficoll

Method:

1. Extract blood. Transfer it to an empty, sterile falcon.
2. Double volume by adding PBS.
3. With the help of a Pasteur pipette, gently add 1ml ficoll for every 3ml of solution.
4. Centrifuge at 2500rpm for 15 min at room temperature.

   After centrifugation blood components will have separated. From top to bottom there will be the following 4 layers: plasma layer, white cloud of peripheral blood mono nuclear cells (lymphocytes) and basophils, ficoll, and erythrocytes.

5. With the help of a micropipette very gently extract the white cloud, paying close attention not to extract the other components as well (<u>especially ficoll or erythrocytes</u>) and deposit it in another falcon.
6. Complete volume with PBS to at least 5ml.
7. Centrifuge at 2500rpm for 5 min at room temperature.
8. Discard falcon's content (a white pellet will remain at the bottom. These are the cells).
9. Release pellet by hitting the bottom of the falcon.

10. Complete volume with PBS to at least 7ml.
11. Centrifuge at 2500rpm for 5min at room temperature.
12. Discard content.
13. Release pellet.
14. Raise to a certain volume with PBS.
15. Count cells in a Neubauer chamber.
16. Extract a volume and deposit in a new falcon as to obtain a desired cell concentration. Usual numbers are around 50 000 cells per 100ul.
17. Centrifuge at 2500rom for 5min at room temperature.
18. Attach and fix on slides/cover slips following protocols 1 and 2 or 3.

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