**Immunofluorescence Protocol**

First, the sample is prepared by immersing it in a 30% sucrose solution for cryoprotection. Then, it undergoes rapid freezing with dry ice and ethanol. The frozen sample is stored at -70ºC until the day of sectioning. Using a cryostat, sections of the desired thickness (in this instance, 40 microns) are cut from the sample.

1. Allow the samples to reach room temperature for 1 hour.
2. Wash the samples three times for 10 minutes each in PBS with gentle shaking.
3. Block the samples by adding blocking solution and incubate for 2 hours at room temperature with gentle shaking.
4. Incubate the samples with primary antibodies in the incubation solution for 16-18 hours (overnight) at 4ºC with gentle shaking.
5. Wash the samples three times for 10 minutes each in PBS with gentle shaking.
6. Incubate the samples with secondary antibodies for 2 hours at room temperature in darkness.
7. Perform three washes for 10 minutes each in PBS with gentle shaking (in darkness).
8. Mount the samples using 10-15 µl of Vectashield mounting medium (with or without DAPI).
9. Cover the samples with a coverslip and seal them with varnish.
10. Store the samples at 4ºC, protected from light, until analysis.

|  |  |  |  |
| --- | --- | --- | --- |
| **Antibody** | **Dilution** | **Catalog** | **Notes** |
| Rat anti-Iba1 | 1:200 (1:1000) | ab283346 | For 40-micron slices, we could extend the titration to 1:1000 on the 3 primary antibodies (Iba1, GFAP, NeuN). The host is different for each antibody (rat, mouse, rabbit) to perform immunofluorescence of the three cells at the same time. When choosing secondary antibodies, note that the Alexa is specific to each host and that the excitation wavelength does not interfere with each other. |
| Mouse anti-GFAP | 1:400 (1;1000) | MAB360 |
| Rabbit anti-NeuN | 1:300 (1:1000) | ABN78 |
| Goat anti-rat A-555 | 1:1000 | ab150158 |
| Goat anti-mouse A-488 | 1:1000 | ab150113 |
| Goat anti-rabbit A-647 | 1:1000 | ab150079 |

**Phosphate Buffer Solution (PBS):**

|  |  |
| --- | --- |
| **Reagent** | **Amount** |
| Sodium Chloride (NaCL) | 8.06g |
| Potassium Chloride (KCl) | 0.22g |
| Sodium Phosphate (Na2HPO4) | 5g |
| Potassium Phosphate (KH2PO4) | 0.2g |
| Distilled water | Top up to 1L |

Adjust pH to 7.4. Store in refrigeration.

**Blocking Solution:**

|  |  |
| --- | --- |
| **Reactivo** | **Cantidad** |
| Goat serum o Bovine Serum Albumin | 5% |
| Triton X-100 | 0.1% |
| PBS | 94.9% |

Prepare at the moment. Use 300-400 microliters per well.

**Primary Antibody Incubation Solution:**

|  |  |
| --- | --- |
| **Reactivo** | **Cantidad** |
| Goat serum o Bovine Serum Albumin | 5% |
| Triton X-100 | 0.1% |
| Anticuerpos | If the titration is 1:1000, then 0.1% for each antibody but this could vary depending on the brand of the antibody, the thickness of the tissue, and the type of tissue. |
| PBS | Top up (approximately 94%) |

Prepare at the moment. Use 300-400 microliters per well.

**Secondary Antibody Incubation Solution:**

|  |  |
| --- | --- |
| **Reactivo** | **Cantidad** |
| Goat serum o Bovine Serum Albumin | 5% |
| Triton X-100 | 0.1% |
| Antibodies | If the titration is 1:1000, then 0.1% for each antibody but this could vary depending on the brand of the antibody, the thickness of the tissue, and the type of tissue. |
| PBS | Top up (approximately 94%) |

Prepare at the moment. Use 300-400 microliters per well.

**Theoretical Framework**

**Antigen Retrieval**

Most tissues fixed with aldehydes (cross-linking method) require antigen retrieval. Aldehyde fixation methods form methylene bridges between proteins, which can obscure some antigenic sites. Therefore, antigen retrieval allows for breaking these methylene bridges, exposing antigen sites, and enabling antibody binding.

Two commonly used methods are Heat-Induced Epitope Retrieval (HIER) and Proteolysis-Induced Epitope Retrieval (PIER). We will focus on the latter.

Detergents are amphipathic molecules containing nonpolar tails and polar heads. They are generally used for cell lysis (membrane disruption). Being amphipathic, they can break protein-protein, protein-lipid, and lipid-lipid bonds, denature proteins and other macromolecules, and prevent nonspecific binding in immunohistochemical tests.

Detergents have nonpolar (hydrophobic) tails and polar (hydrophilic) heads. This molecular structure is very similar to that of phospholipids in cell membranes. When dissolved in water at the appropriate concentration and temperature, amphipathic molecules assemble into structures with their heads facing outward and tails inward (away from water). Due to their molecular differences, detergents form spherical micelles, while phospholipids form bilayers. Thus, because of their similar structures, detergent can penetrate the phospholipid bilayer and subsequently alter the cell membrane.

Another way they alter cell structure is through the hydrophobic core (tails) of micelles, which can bind to hydrophobic regions of proteins. A parameter that defines membrane solubility is the aggregation number of micelles, which is the number of detergent molecules they contain. The length of the hydrophobic region is proportional to its hydrophobic capacity, which is usually constant in detergents, while the hydrophilic region (heads) is variable, thus giving each detergent its characteristics. Therefore, detergents are classified into three groups: ionic, nonionic, and zwitterionic.

We will only mention nonionic detergents since they are the ones we will use in our protocol. Nonionic detergents have noncharged hydrophilic heads. They are considered mild surfactants, as they break protein-lipid and lipid-lipid bonds, but not protein-protein bonds, nor do they denature proteins. This allows the membrane to be solubilized while maintaining its structure. Two important families of nonionic detergents are the Triton and Tween families.

The Triton family: all its members are similar with slight variations in their aggregation number per micelle. Triton X-100 is commonly used for the isolation of complex membrane proteins and for immunoprecipitation procedures.

The Tween family: Commercially known as Tween, polysorbates are surfactants with a polar head and a long polyethylene chain. They are mild surfactants as they do not affect protein activity and are effective solubilizers. They are commonly used as washing agents in immunoblots and ELISA tests to minimize nonspecific antibody binding and remove unbound portions.

**Blocking**

Before using antibodies to detect proteins, remaining binding sites must be blocked to prevent nonspecific binding with secondary antibodies and to avoid false positive results. Otherwise, antibodies would bind to the initially used binding sites that immobilized the proteins of interest. In theory, any protein, even if it does not have affinity for the binding site, can be used for blocking. However, in practice, certain proteins block better than others because they bind to membranes or other immobilization surfaces more effectively.

Blocking buffer solutions vary in content and may be made of milk, serum, or highly purified proteins to block binding sites on membranes. Serum from the same species as the secondary antibody is commonly used. Blocking can be performed at room temperature for 10-30 minutes.

Insufficient blocking results in false positives, with antibody binding to undesired sites. Conversely, excessive blocker concentrations can obscure antigen-antibody interactions, reducing signal.

Goat serum is widely used to block nonspecific binding sites with antibodies. Additionally, it blocks Fc receptors of primary and secondary antibodies in the sample.