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REVIEW

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Histoblot: A sensitive method to quantify the expression of proteins in normal and pathological conditions

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Summary. The histoblot (*in situ* immunoblotting) technique is a simple, reproducible, and sensitive method for protein detection that allows both protein quantitation and analysis of tissue distribution. This easy and fast method allows the direct transfer of native proteins from unfixed frozen tissue sections by mechanical pressure to an immobilizing matrix. Proteins are directly blotted onto nitrocellulose membranes that are then immunolabelled similar to a western blot, but the result is an immunohistochemical imprint of the section retaining all proteins. The histoblot combines advantages of western blot and immunohistochemical methods and yields optimal accessibility of proteins blotted on membranes whilst also preserving anatomical resolution. In addition, it avoids chemical modifications, crosslinking, or semi-denaturation of proteins, which can alter the access of antibody to epitopes, as introduced by conventional immunohistochemistry. Therefore, the histoblot often enables the use of antibodies that do not recognise the target protein in fixed tissue samples. This method has become a trusted alternative to reveal and compare the regional distribution and expression profile of different proteins in the brain in physiological and pathological conditions. In addition, the technique exhibits a high subregional resolution, although is not suitable to unravel protein distribution at the cellular and subcellular levels. In this review, we introduce the histoblot procedure used in our laboratory on brain sections for the identification of quantitative changes of neurotransmitter receptors, ion channels and other signalling molecules in the brain. We also discuss the potentialities, limitations, and fundamental principles of this technique.

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Introduction

Detection and quantification of specific proteins or enzyme activity are central to understand their function in different cell populations of tissues and organs. In the brain, the expression profile of neurotransmitter receptors, ion channels, effector enzymes or any other signalling proteins provide essential information to unravel their roles in specific neuron populations and within a given neuronal network. Although many techniques are widely used to study the expression patterns of many molecules in different brain regions (Luján, 2010), protein detection is mostly carried out using western blot and immunohistochemistry. Each technique comes with its own set of advantages and disadvantages. For example, whilst native proteins can be studied with western blots, tissue samples must be homogenized, resulting in the loss of cellular distribution. Whilst the resolution using immunohistochemistry is higher, the sensitivity of protein detection can be lost for some antigens due to fixation methods that modify antigenic sites. These technical limitations have led to the development of more sensitive methods such as the histoblot.

The histoblot is a simple technique, highly reproducible and sensitive, which allows for protein quantification and tissue distribution. Basically, brain

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sections are placed on a nitrocellulose membrane to transfer their proteins. After, the membrane is incubated with specific antibodies against the protein of interest. The result is an immunohistochemical imprint of the target protein, present in the tissue section (Fig. 1). Due to the number of primary antibodies commercially available and to the simplicity of its protocol, histoblot has become an efficient analytical technique that allows a better understanding of cellular biology.

In the present review we discuss the histoblot procedure which combines advantages of biochemical, histo- and immunocytochemical methods to yield optimal accessibility of molecules blotted on membranes, whilst retaining the preservation of anatomical resolution. We will discuss further the potentialities, restrictions and fundamental principles of the histoblot.

Histoblot: a technique combining the advantages of western blot and immunohistochemistry

The histoblot, also called *in situ* immunoblot or immunohistoblot, is a simple yet powerful technique to study the relative abundance and regional mapping of virtually any protein in research, diagnostic and therapeutic testing. This technique was first described in the laboratory of Stanley B. Prusiner (Taraboulos et al., 1992) to map the distribution of the cellular isoform of the prion protein (PrPC) in infected and non-infected brains. Furthermore, Okabe et al. (1993) developed a similar histoblot technique for the specific detection of proteins and enzymes in thymus and brain tissue. Soon afterwards, it was then applied to screen large areas of human tissues from patients with parkinsonism for the presence of pathological prion protein (Jendroska et al., 1994).

The histoblot relies on the specific interaction of antibodies with antigens present in the sample. This technique combines the advantages of western blot, as primary antibodies are used to detect the protein of interest, and immunohistochemistry as it allows one to visualize the brain area in which the protein of interest is expressed. Western blot, also called protein blotting or immunoblotting, is one of the most widely used detection techniques in the life science laboratory to study quantitative changes in protein levels from different samples (Towbin et al., 1979; Burnette, 1981). Shortly after this biochemical method was first described, it started to be employed to study the expression of proteins contributing to Alzheimer´s disease (Wolozin et al., 1986), drug resistance (Kartner et al., 1983) or proteins causing AIDS (Schüpbach et al., 1984). With this technical approach, molecules are differentially immobilized on the surface of blotting membranes, thus achieving optimal accessibility for detection and quantification of protein expression. Since target molecules are extracted from native tissue samples, western blot is highly sensitive. However, there are some limitations to the technique. The procedural steps are lengthy, and the homogenization of the tissue sample causes the loss of the anatomical and cellular distribution of all the proteins (Aguado and Luján, 2019; Molnár, 2021). Furthermore, the dissection of some brain regions can be difficult due to their location and irregular anatomical shape and the softness of fresh unfixed tissue samples. Moreover, if the brain area to be studied is small, a greater number of animals will need to be used to increase the amount of tissue. Finally, delays in brain extraction and processing of samples gives rise to proteolytic degradation resulting in inaccurate results, one of the main problems when working with *post-mortem* human tissue.

Immunohistochemistry (IHC) is an efficient laboratory method used to visualise the distribution and localization of a variety of proteins in different parts of a biological tissue, using antigen-specific antibodies (Konno and Watanabe, 2021). For the last forty years, IHC has revolutionised the practice of cytology, histology, and pathology, becoming an essential experimental and diagnostic method (Swanson, 2016). When applied to the brain, IHC can reveal the anatomical and cellular distribution of receptors, ion channels and many other signalling molecules. It is based on specific antigen-antibody reactions. However, immunohistochemical techniques also haves some limitations. For example, they have lower sensitivity than western blots due to the requirement of tissue fixation for adequate preservation of antigenicity and morphology (Luján, 2021). Most fixatives introduce covalent modifications and cross-linking of amino groups in proteins and/or denature proteins, giving rise to their conformational changes which decreases the binding of the primary antibodies in the tissue (Molnár, 2013a; Luján, 2021). In addition, all immunohistochemical techniques have other inherent limitations impacting on the efficiency of immunolabelling. One example is the differences in the quality of fixation during transcardial perfusion or by immersion, together with the possibility of protein loss from the tissue during processing steps (Molnár, 2013a). This is the main reason why IHC is not suitable to establish quantitative comparisons of protein expression levels in different brain samples.

Unique advantages of histoblot to map protein expression

Histoblot has several advantages over other methods to study the qualitative and quantitative expression profile of proteins: (a) Histoblot is a very convenient and highly reliable approach to map and quantify the expression levels of virtually any plasma membrane and cytoplasmic protein in the brain (Ciruela et al., 2010; Fernández-Alacid et al., 2011; Ferrándiz-Huertas et al., 2012; Aguado et al., 2016b; Alfaro-Ruiz et al., 2019, 2021; Martín-Belmonte et al., 2020a,b, 2021), with a spatial resolution similar to *in situ* hybridisation histochemistry, and an enhanced spatial resolution

compared to the western blot; (b) transferred proteins are readily accessible for immunochemical analysis on the surface of nitrocellulose membranes, thus enabling the use of antibodies which do not recognize the target protein in fixed tissue samples (Aguado and Luján, 2019; Molnár, 2021); (c) Histoblot provides high sensitivity and improved consistency in immuno-labelling; two essential features to perform reliable quantitative comparisons of overall expression levels of proteins in different brain regions (Fernández-Alacid et al., 2011; Ferrándiz-Huertas et al., 2012); (d) Histoblot exhibits subregional resolution and affords quantitative analyses in different nuclei or in the different layers/subfields of a given brain region (Fernández-Alacid et al., 2011); (e) The amenability of the histoblot system to experimental manipulation and its high signal-to-noise ratio suggest that it may help in localizing molecules that are poorly detected by standard techniques, as in the case of fixation-sensitive antigens (Fernández-Alacid et al., 2011); (f) only a small amount of reagents are required for studying transferred proteins (Okabe et al., 1993); (g) The basic steps of histoblot techniques are relatively simple, cheap and easily implemented in most laboratories without a major investment in specialized equipment (Aguado and Luján, 2019; Molnár, 2021) (Fig. 1); (h) At the time of tissue sectioning, adjacent sections to the histoblot samples can be used for different proteins (Fernández-Alacid et al., 2011) (Fig. 2); and (i) since the proteins are blotted in their native configuration, they are in an optimal condition for further biochemical or physiological characterization (Aguado and Luján, 2019; Molnár, 2021).

Limitations of histoblot

Similar to other techniques, histoblotting does have some limitations: (a) Histoblot can only be used if a primary antibody against the protein of interest is available (Aguado and Luján, 2019); (b) Histoblot is not suitable to unravel protein distribution at the cellular and subcellular levels (Aguado and Luján, 2019; Molnár, 2021); (c) At the time of tissue sectioning, adjacent sections to the histoblot samples cannot be used for routine immunohistochemistry, and thus a direct comparison between tissue morphology and antigenic expression is not possible; (d) The same protein transfer cannot be used for numerous sequential analyses; (e) Each antibody must be independently optimized; and (f) primary antibodies that produce specific labelling on histoblots often fail to recognise the same protein targets in fixed tissue samples using conventional immunohistochemistry (Aguado and Luján, 2019; Molnár, 2021).

Preparing for a reliable histoblot

The use of quality reagents and correct methodology are key aspects for the detection of protein expression with the histoblot technique. There is very little information available to overcome the technical limitations of histoblot. The technique is based on seven

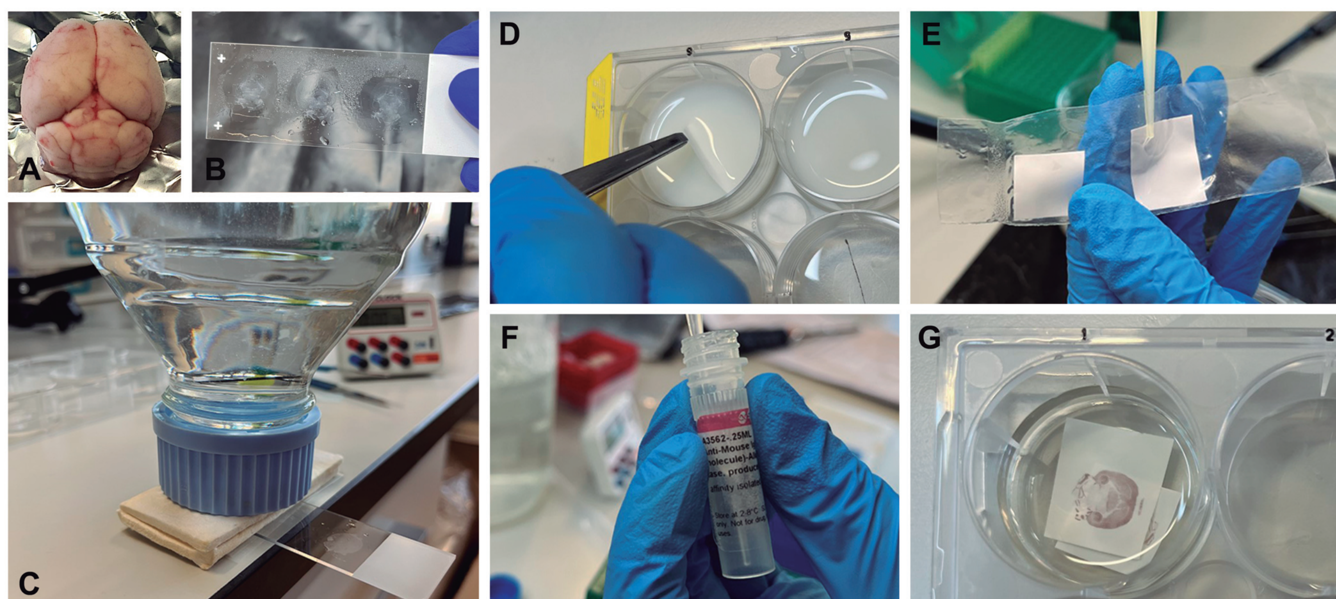

Fig. 1. Main procedural steps of the histoblot method from brain sample to immunolabelling. **A, B.** Sample preparation by extracting the brain and freezing at -80ºC until sectioning in a cryostat to obtain 10 μm-thick sections, which are collected on slides. **B.** Sectioning of brains in a cryostat. **C.** Mechanical pressure for direct transfer of proteins from brain tissue to an immobilizing blotting matrix. **D.** Blocking the membrane in non-fat dry milk to reduce non-specific binding. **E.** Antigen detection by incubating membranes with primary antibodies specific for the target protein. **F.** Incubation of membranes with a secondary antibody linked to alkaline phosphatase. **G.** Development and detection of the signal on the membrane.

steps: 1) preparation and sectioning of the samples; 2) transfer of proteins in the section to a membrane; 3) membrane blocking for reducing non-specific protein binding; 4) use of specific primary antibodies to detect the protein of interest; 5) incubation with conjugated secondary antibodies (e.g. the enzyme alkaline phosphatase); 6) development and detection of the signal; and, 7) quantification of the resulting immunohistochemical imprint using densitometry software (Fig. 1). Deficiencies in any of these steps can significantly compromise the results. For a detailed protocol of our histoblot technique we refer the reader to the step-by-step description in Aguado and Luján (2019). Below, we review some of the critical steps to establish a reliable and reproducible protocol from day to day.

Sample preparation

The first step in an histoblot protocol is tissue preparation. In our laboratory we routinely work with brain tissue (Fig. 1A,B). Histoblot only requires that the whole brain is frozen immediately after separation from the skull. Frozen samples allow the sectioning of the brain in the direction selected (sagittal, coronal, or horizontal), and also limit proteolytic degradation that starts when samples are not processed immediately after brain extraction.

Any organ or tissue can be used for histoblotting. When using brain tissue, the collection step should be done in a cold temperature regardless of the sample type employed (i.e., brain tissue of experimental animals extracted from the skull, brain tissue of experimental animals after the terminal procedure of an *in vivo* experiment, brain tissue by biopsy or brain samples dissected *post-mortem*) to prevent rapid protein denaturation and degradation. In addition, to obtain reproducible data, brain samples should remain frozen at -80º until sectioning and undergo as little manipulation as possible to avoid periods at higher temperatures and freeze/thaw cycles, which have an adverse effect on the quality of sectioning and protein conservation.

Transfer and blocking

The next important step is the blotting procedure, where the membrane plays an essential part of this process (Fig. 1C). Two types of membrane, nitrocellulose and polyvinylidene difluoride (PVDF), are used for histoblot. Nitrocellulose membranes have been more widely used (Ciruela et al., 2010; Fernández-Alacid et al., 2011; Ferrándiz-Huertas et al., 2012; Aguado et al., 2016b; Alfaro-Ruiz et al., 2019, 2021; Martín-Belmonte et al., 2020a,b, 2021) due to their high affinity for protein and retention abilities. Other laboratories have used PVDF membranes (Okabe et al., 1993), which provide better mechanical support and can be used for reprobing. This however produces higher background staining. It has been reported that nitrocellulose membranes can be applied for most soluble proteins, while PVDF membranes are recommended when proteins are membrane-bound (Okabe et al., 1993).

The transfer of tissue proteins onto the membrane matrix can be achieved in a very simple and quick way by mechanical pressure of the sections of unfixed frozen tissue against nitrocellulose membranes previously moistened with transfer buffer (Fig. 1C). Previous

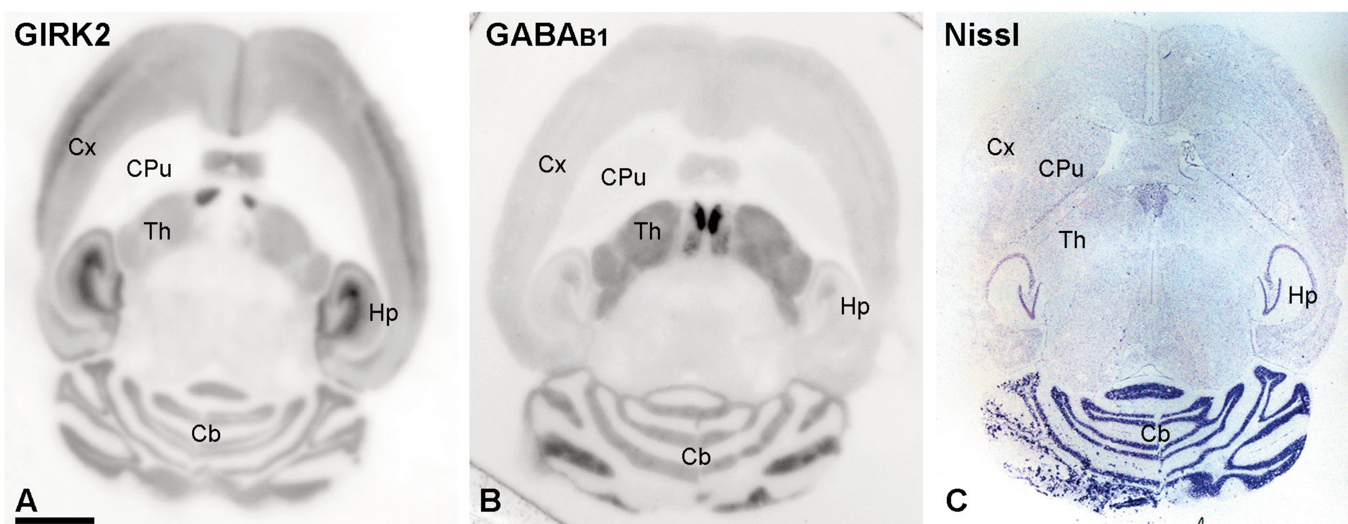

Fig. 2. Expression of proteins in serial sections and identification of brain regions in adjacent cryostat sections stained with cresyl violet. **A, B.** Distribution of the GIRK2 subunit of GIRK channels and the $GABA_{B1}$ subunit of GABAB receptors in serial sections of the adult mouse brain. **C.** Nissl-stained serial sections can be used to identify the different brain regions after histoblotting. Abbreviations: cortex (Cx), cerebellum (CB), hippocampus (Hp), dorsal thalamus (Th) and caudate putamen (CPu). Scale bar: 2 mm.

studies demonstrated that the time necessary to transfer proteins from the tissue to the membrane is very short for all proteins irrespective of their molecular weights (Okabe et al., 1993). Most laboratories use 30 seconds (Molnár, 2021) or 1 min (Aguado and Luján, 2019). Furthermore, the amount of protein bound to the membrane is proportional to the section thickness up to 10 µm, without any significant change for section thickness over 10 µm (Okabe et al., 1993).

A common problem observed during protein transfer to a membrane is the appearance of air bubbles and fuzzy images. Air bubbles arise due to the incorrect assembly of the transfer stack. The use of a plastic rod or a thick pencil is effective at reducing air bubbles. Fuzzy images arise when the mechanical pressure on the transfer stack moves laterally instead of totally vertical.

Blocking is an important step for histoblot (Fig. 1D), to prevent interactions between the membrane and the antibody against the target protein (Okabe et al., 1993). Moreover, it helps to reduce background staining and to avoid non-specific binding. It is carried out by placing the membrane with the transferred proteins in a dilute solution of non-fat dry milk for 1 hour (Aguado and Luján, 2019). Non-fat dry milk is the first choice because it is inexpensive and widely available. However, milk proteins are not compatible with all antibodies. In such cases, blocking solutions consisting of bovine serum albumin, goat serum albumin or fish skin gelatin (3-5% w/v) are also recommended. When background staining is high, fish gelatine can be used to reduce it significantly, due to the lack of IgG that can cross-react with primary antibodies (Tönnes et al., 1999). In addition, the casein that is present in the milk can be recognized by phospho-specific antibodies resulting in nonspecific binding and high background staining. As a rule, the use of inefficient blocking solutions or insufficient blocking incubations result in increased background staining. However, an excess of blocking incubations can also result in poor protein signal intensity. Therefore, we recommend that before the initiation of any histoblot experiment, researchers should first determine the best blocking reagent for their primary antibodies and type of membrane and the duration of blocking incubation. This will help to obtain reproducible results.

Primary antibodies and their validation

The quality of the primary antibody is the most critical factor that we have observed. This is because the histoblot is based both on the specificity and high affinity of antibodies in recognizing the protein of interest. The histoblot technique is based on the specificity and high affinity of antibodies to recognize and bind to the target antigen. Although primary antibodies for many different proteins are commercially available, they are not cheap. In addition, these antibodies and those in house-made are not equally good in efficiency and consistency. It is frequent to find that many primary antibodies do not detect the target protein when tested in the laboratory by histoblot. Moreover, they simply may not be available for a particular target protein. Many primary antibodies work for both histoblot and immunoblot due to their technical similarities. However, not all antibodies that work for histoblot or immunoblot provide immunolabelling for IHC due to the need for fixation.

Both polyclonal and monoclonal antibodies are widely used for histoblot. The theoretical advantage of polyclonal antibodies is that they can recognise different epitopes on the same protein, while monoclonal antibodies recognise single epitopes (Konno and

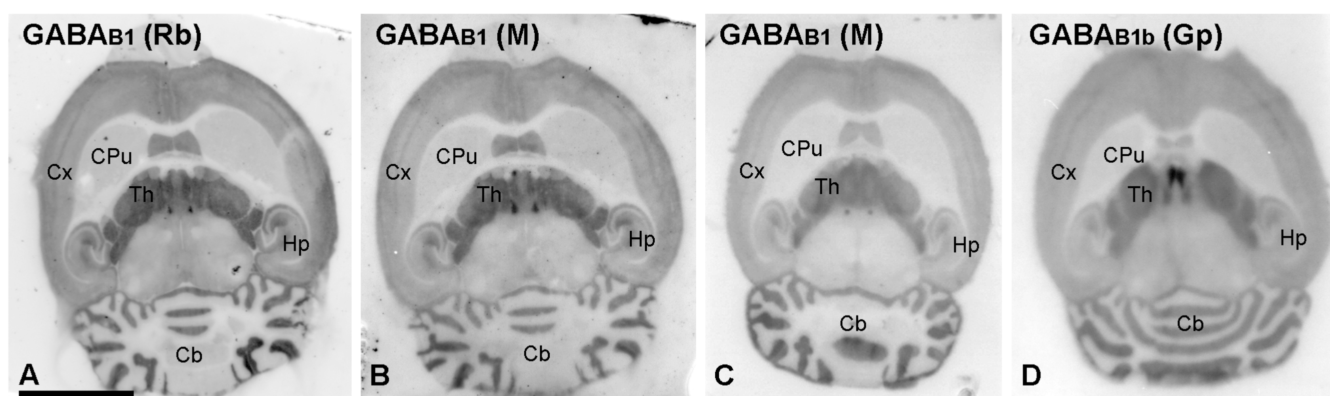

Fig. 3. Evidence favouring the specificity of a given antibody. Histoblots of mouse brain horizontal sections using four different antibodies against $GABA_B$ receptors. **A.** Antibody anti-$GABA_{B1}$ raised in rabbit (B17, aa. 525-539 of mouse $GABA_{B1}$). **B.** Monoclonal antibody anti-$GABA_{B1}$ (aa. 873-977 of rat C-terminus of $GABA_{B1}$, Neuromab, UC Davis/NIH, CA, USA). **C.** Monoclonal antibody anti-$GABA_{B1}$ (sc-166408; D-2, aa 929-958 of rat C-terminus of $GABA_{B1}$, Santa Cruz, CA, USA). **D.** Antibody anti-$GABA_{B1}$ raised in guinea pig (aa. 1-18 of isoform $GABA_{B1b}$). The working efficiency of these antibodies vary slightly, but the four of them produce the same labelling pattern. The specificity of these antibodies has been previously tested in $GABA_B$ KO mice, but when such information is absent for a target protein, the similar staining patterns are good circumstantial evidence favouring their specificity. Scale bar: 0.4 mm.

Watanabe, 2021). In our experience, quality and intensity of labelling is antibody-dependent, regardless of whether they are polyclonal or monoclonal (Fig. 3). However, the major disadvantage of polyclonal antibodies, especially those from a commercial source, is that their supply is limited by each batch available, thus compromising reproducibility of histoblot experiments. Nevertheless, optimum dilution should be established for each primary antibody, although in most cases concentration should be around 1 µg/ml following overnight incubation at 4°C. Lowering the concentration of the primary antibody and the incubation time can help to reduce nonspecific labelling.

The specificity of immunolabelling obtained with histoblots can readily be determined using positive and negative controls (Molnár, 2013a). Specificity refers to the ability of the primary antibody to recognize and bind to the target antigen. The best negative control is the use of tissue obtained from an animal lacking the target antigen (Fernández-Alacid et al., 2011; Martín-Belmonte et al., 2020b) or cells reduced in a knock down situation (e.g., using si or sh RNA). This is the most informative control, when available. Important supporting information about the primary antibody can be obtained comparing histoblot expression pattern with distribution patterns provided using immunohistochemistry and in situ hybridization techniques (Rhodes and Trimmer, 2006).

A good control when tissue from knockout animals is unavailable is using two or more different antibodies raised against non-overlapping epitopes of the same target protein (Fig. 3). If these antibodies produce the same labelling pattern, we can have good circumstantial evidence favouring their specificity (Kimura et al., 2002; Rhodes and Trimmer, 2006; Molnár, 2013a). However, it is also possible that two or more antibodies recognize the same antigen, but the same unrelated cross-reactive molecule too (Holmseth et al., 2012). Other frequently used negative controls include the omission of the primary antibody, or changing the primary antibody for pre-immune serum, obtained from immunized animals before the first injection of the antigen (Molnár, 2013a,b). A very popular negative control used in the past has been the pre-absorption of primary antibodies with excess of the corresponding antigenic peptide or protein (Molnár, 2013a,b), thus blocking the labelling of endogenous proteins by the saturation of antibodies with the antigens. However, the researcher should be aware that this control will only show that the primary antibody binds to the antigen and should not exclude the possibility of cross-reactions with other proteins.

The best positive control is the *histological control*. This includes areas of the tissue where the presence of the target antigen in the sample is known and is not the purpose of the assay (Luján, 2010). For example, a histoblot experiment using an antibody specific for a cerebellar neuronal marker should include brain sections containing the cerebellum. The primary antibody should target only neurons or areas of the cerebellum and not cross-react with other brain areas. If the researcher does not see any labelling in the positive control one can surmise that something did not go well during the experimental procedure. However, correctly interpreting positive controls in histoblot is not easy, particularly when primary antibodies are manufactured against targets that are either expressed ubiquitously in the tissue or have low levels of expression. Another common problem when using antibodies which leads to incorrect interpretations, is when an antibody cross-reacts with other proteins and produces a Nissl-like staining in the brain of a given species. The same antibody applied to a mouse brain can produce a non-specific staining, while producing a specific pattern of labelling in rat brain (Fig. 4).

Secondary antibodies and detection system

Secondary antibodies are required for the indirect detection of a target protein recognised by a primary antibody (Fig. 1F). As several secondary antibodies can bind to a single primary antibody, their main role is the amplification of detectable signals. This is particularly useful to detect proteins with low expression levels. For histoblots, most primary antibodies produce good immunolabelling using alkaline phosphatase-conjugated secondary antibodies incubated for 90 min at room temperature or overnight at 4°C (Aguado and Luján, 2019). Longer incubation times may be sometimes required, but in our experience, it is not recommended. This is because non-specific binding and background labelling will increase. Similar to the conventional western blot, horseradish peroxidase-conjugated secondary antibodies in combination with enhanced chemiluminescence can also be used, but the resolution of the resulting blot is reduced (Molnár, 2021).

The speed of the alkaline phosphatase reaction depends on the antigen and concentration of immunoreagents, both the primary and secondary antibodies. The development of the colour reaction must be carefully monitored. In our laboratory, this normally takes between 5-30 minutes to obtain strong labelling. Longer reaction times normally increase background labelling. Immunolabelled areas should appear dark grey/black, whereas areas lacking the target protein and control sections should appear white.

A common problem observed during alkaline phosphatase reaction is low signal detection when the target protein cannot be detected after 20-30 min of exposure. This can happen due to an incomplete protein transfer, inappropriate dilution of primary/secondary antibodies or simply due to the poor quality of the primary antibody for this technique.

Reproducibility

The reproducibility of immunoassays is always an important concern in biomedical research (Gough, 2015). Histoblot analysis involves several inter-

dependent steps that can be influenced by subjective choices of the user, thus resulting in substantial sources of error and misinterpretation of data (Aguado and Luján, 2019; Molnár, 2021). A well characterized histoblot protocol is essential to avoid common pitfalls and determine which assay parameters are most critical for reproducible results. Reproducibility depends on accuracy and precision. Accuracy shows how closely the measured values represent the true value of the target protein in the sample. Precision shows the variation and error of measurements. Histoblot data should aim to be both precise and accurate, to help ensure that the reported observations convey meaningful information about the experimental samples.

Background subtraction

Images obtained with histoblots contain the specific signal generated by the immunodetection of the target protein and the non-specific signal generated by smears or by the membrane itself. As in the western blot technique, the aim of this step is to reduce/remove the confounding error introduced by background signal. The simplest method to subtract the background from the histoblot image (thus eliminating potential differences in optical densities across different sections) is by performing ~8 to 10 different background determina-tions near the brain protein-containing areas of the immunolabelled nitrocellulose membranes. The average of these

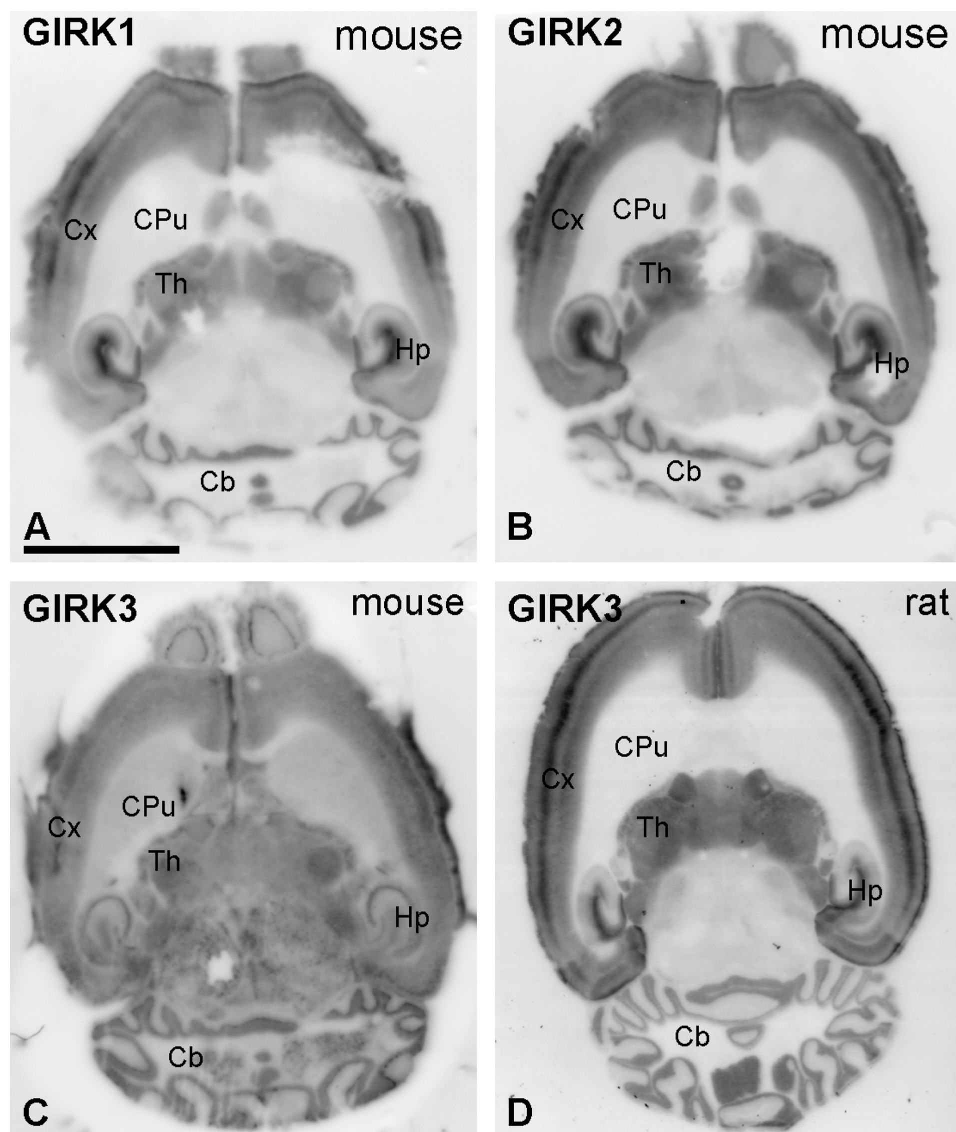

Fig. 4. Overlapping distribution of GIRK1 and GIRK2 subunits in the adult mouse brain. **A, B.** GIRK1 and GIRK2 exhibited broad and overlapping distributions in the adult brain, which help to predict putative formation of oligomeric complexes between GIRK1 and GIRK2 in different brain regions and subfields. Strong immunoreactivity for both channel subunits was detected in the neocortex (Cx), cerebellum (Cb), hippocampus (Hp), and thalamus (Th), with the lowest intensity in the caudate putamen (CPu). **C, D.** Interpretation of correct labelling patterns obtained with histoblots. Comparison of the regional distribution of the GIRK3 channel subunit in the mouse and rat brain. GIRK3 protein distribution is visualized on histoblots of brain horizontal sections using the same affinity purified anti-GIRK3 antibody. In mouse brain, GIRK3 exhibited a Nissl-like staining in all brain regions, whereas in rat brain it exhibited a specific pattern of labelling, similar to GIRK1 and GIRK2, and broad distribution in the neuropil of many brain regions. Scale bar: 4 mm.

background determinations is subtracted from the average pixel densities measured within the tissue or region of interest (Aguado and Luján, 2019; Molnár, 2021).

Quantitative detection and data analysis

Researchers frequently use the histoblot to assess quantitative differences, such as how protein abundance changes under different conditions or specific time points. Therefore, the data produced with the histoblot should be considered as semiquantitative because it provides a relative comparison of protein levels, but not an absolute measure (Fig. 5). The main reason for this is that the signal generated by detection is not linear across the concentration range of samples. For quantification, it is essential to ensure that the achieved image from the blot is sharp (Fig. 5). If samples are being compared for quantitative analyses, it is important to set accurately the thickness of the cryostat sections. This must occur in order to have the same amount of protein. Furthermore, samples must be processed in parallel in order to avoid differences in antibody concentration or alkaline phosphatase reaction. It has been established that the amount of protein bound to the membrane is proportional to the section thickness and the maximal binding quantity is reached in sections of 10 µm (Okabe et al., 1993).

For image acquisition and quantitative analysis, digital grayscale images of histoblots can be acquired by scanning the nitrocellulose membranes using a desktop scanner (Fig. 5). Different grayscale images must be captured and treated under the same conditions and parameters. This allows comparison of pixel densities (arbitrary units) of immunolabelling in different brain regions, different subfields of the same brain region, different time points or different treatments. Image analysis and processing can be performed using different softwares (Aguado and Luján, 2019; Molnár, 2021). To measure pixel density, circular cursors with a diameter of 0.1 mm should be placed on the region of interest. This region can be identified based on the use of adjacent Nissl-stained sections (Pickard et al., 2000; Kopniczky et al., 2005; Fernández-Alacid et al., 2011). To eliminate potential differences in optical densities across different sections, background correction must be performed, and the resulting average pixel density for the whole region from one animal must be counted as one 'n'. Pixel densities (arbitrary units, in grayscale images 0-255 range) can be plotted in a graph, and the appropriate statistical analysis can be performed on the samples. This will allow the detection of differences between brain regions of different animals, different time points or different experimental groups (Aguado and Luján, 2019; Molnár, 2021) (Fig. 5).

Presentation of representative histoblots

The accurate and representative presentation of histoblot data is an ethical concern when presenting our data to the scientific community. Data for histoblot are always presented in two forms: a representative image to demonstrate the quality of blot and the qualitative expression pattern of the target protein and a graph showing quantitative expression patterns (Fernández-Alacid et al., 2011; Ferrándiz-Huertas et al., 2012; Aguado et al., 2016a,b; Alfaro-Ruiz et al., 2019, 2021; Martín-Belmonte et al., 2020a,b, 2021). Due to the size of sections, it is not possible to run all experimental groups within the same blot to provide a representative image. Therefore, splicing of images from multiple blots

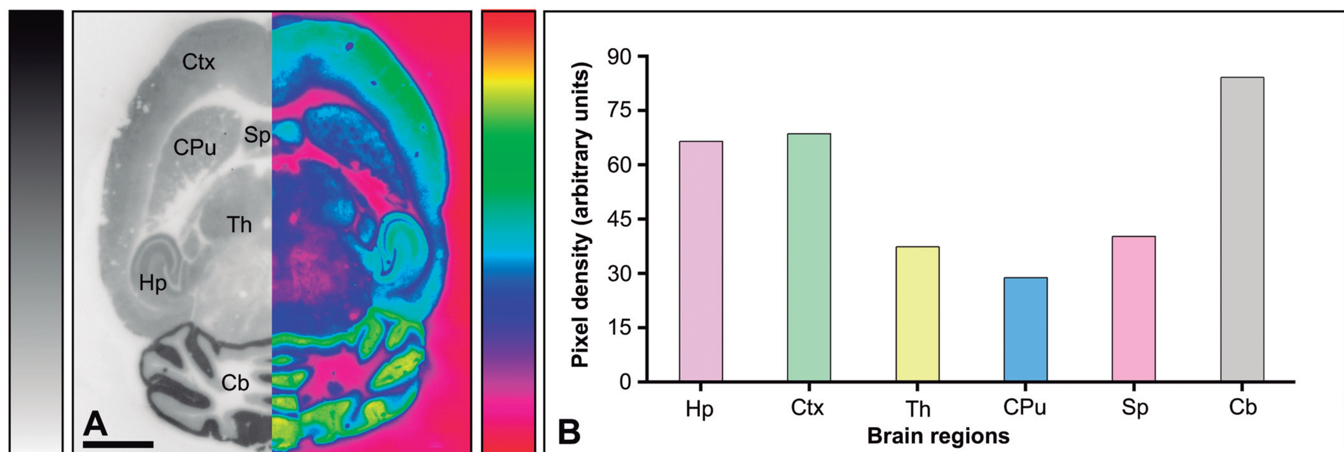

Fig. 5. Quantification of the resulting immunolabelling imprint. Sampling method used to compare immunoreactivities in different brain regions. **A.** Histoblots of coronal mouse brain sections were immunostained for the voltage-gated calcium (Cav2.1) channel. Grayscale histoblot images (left side) can be converted to colour gradients (right side) using gradient mapping with Image J software. Colour gradients illustrate the regional expression profiles of the Cav2.1 channel protein. **B.** Histogram showing densitometric measurements (arbitrary units) from the scanned histoblot to compare the protein densities in different brain regions. Cb, cerebellum; CPu, caudate putamen; Ctx, cortex; Hp, hippocampus; Th, thalamus; Sp, septum. Scale bar: 1 mm.

is inevitable. However, in such cases a clear distinction between blots should be made (Fernández-Alacid et al., 2011; Ferrándiz-Huertas et al., 2012; Alfaro-Ruiz et al., 2019, 2021; Martín-Belmonte et al., 2020a,b, 2021). The inclusion of a representative positive and negative control is also recommended. If any alteration of an image is made, it will affect the entire image and hence this should be stated in the text or figure legend. Inappropriate manipulation of histoblot images is not ethical and can be considered as misconduct.

Alternative methodology to histoblot

The requirement of fresh or frozen tissue samples to perform histoblots (the main reason for its high sensitivity) precludes the possibility of using archived tissue samples for retrospective or prospective analysis (Dickson, 2002). A modification of the histoblot and IHC has been reported resulting in the paraffin-embedded tissue blot (PET blot), a technique combining the advantages of western blot, immunohistochemistry and histoblot (Schulz-Schaeffer et al., 2000). This method has been applied to study aggregate distribution in mouse and human tissue with amyotrophic lateral sclerosis (ALS) and follow the spread of synucleopathies in Alzheimer's disease (Kramer and Schulz-Schaeffer, 2007; Moh et al., 2010; Steinacker et al., 2014). It has also been used for the following: a) to discriminate between transmissible spongiform encephalopathies (TSE) strains inoculated into mice (Lezmi et al., 2006), b) to confirm the presence of the bovine spongiform encephalopathy (BSE) agents (Andréoletti et al., 2006), c) to detect PrP in the peripheral human tissues infected with CJD (Ritchie et al., 2004), and d) to detect atypical scrapie (Wemheuer et al., 2009).

The histoblot technique has also been adapted for the *in situ* hybridization technique, resulting in a simple and rapid method, known as *blot hybridization* (Gudkov et al., 1989). This approach is used to detect specific DNAs or RNAs in different tissues and organs (Gudkov et al., 1989). The method has been validated but its application has been very limited in the field.

Applications of the histoblot technique

Virtually any type of tissue can be analysed by histoblot, offering good analytical sensitivity. Regardless of the tissue and application, the data published so far show that many types of proteins with different molecular weights can be visualized using the histoblot technique. In this section we discuss the validated applications and the potential for new applications.

Expression of proteins in physiological conditions

The histoblot technique offers very high sensitivity and high signal to noise ratios, making it the method of choice for an analysis of the macroscale regional distribution of many proteins in the brain. For this reason, it has been successfully employed in several laboratories for; a) the mapping and quantitative comparisons in the expression of several subunits of ionotropic glutamate receptors (Benke et al., 1995; Wenzel et al., 1995; Tönnes et al., 1999; Pickard et al., 2000, 2001; Gallyas et al., 2003; Ball et al., 2010; Martín-Belmonte et al., 2020a), b) metabotropic glutamate receptors (Callaerts-Vegh et al., 2006; Jo et al., 2006; Morató et al., 2018; Martín-Belmonte et al., 2021), c) $GABA_B$ receptors (Martín-Belmonte et al., 2020b), d) adenosine receptors (Morató et al., 2017), e) nicotinic acetylcholine receptors (Court et al., 2000), f) Cav channel subunits (Ludwig et al., 1997; Ferrándiz-Huertas et al., 2012; Aguado et al., 2016a), g) K^+ channels (Fernández-Alacid et al., 2011; Alfaro-Ruiz et al., 2019, 2021), h) voltage-dependent Na^+ channels (Martínez-Hernández et al., 2013), i) G proteins and other signalling or trafficking proteins (Fajardo-Serrano et al., 2013; Aguado et al., 2016b; Morató et al., 2018; Roldán-Sastre et al., 2021) or j) enzymes such as PKC, NADPH diaphorase and alkaline phosphatase (Okabe et al., 1993). Importantly, the expression patterns described for all those proteins with histoblot entirely agree with the known expression and distribution pattern described using *in situ* hybridisation and immunohistochemistry. For example, the $GABA_{B1}$ subunit of the $GABA_B$ receptors showed a strong expression in the cerebellum and thalamus, moderate expression in the hippocampus, cortex and septum and weak expression in the caudate putamen and midbrain nuclei (Martín-Belmonte et al., 2020b), in agreement with the reported patterns of expression and distribution using in situ hybridisation and immunohistochemistry, respectively (Kaupmann et al., 1997; Margeta-Mitrovic et al., 1999). Another example, GIRK channel subunits showed rather similar expression patterns in adulthood, with strong labelling in the neocortex, cerebellum, hippocampus, and thalamus, moderate in midbrain and brainstem nuclei, and weak labelling in basal ganglia nuclei (Fernández-Alacid et al., 2011), consistent with previous studies using different approaches (Karschin et al., 1996).

Expression of proteins in pathological conditions

One of the most interesting applications of the histoblot is the detection of differences in the expression of different receptors and/or ion channels in pathological conditions using experimental animals. In mouse models of Alzheimer´s disease and tauopathy, histoblot has been a very valuable approach to unravel whether glutamate receptors, GABA receptors and potassium channels undergo changes in APP/PS1 mice and tau P301S mice (Martín-Belmonte et al., 2020a,b; Alfaro-Ruiz et al., 2021). We have recently determined the impact of amyloid-β (Aβ) pathology and tauopathy in the expression of GIRK channels (Alfaro-Ruiz et al., 2021). The histoblot technique established that the expression of GIRK channels is differentially affected in the two

transgenic mice. In APP/PS1 mice, a model of amyloid-β (Aβ) pathology, the expression of GIRK channels is not altered. In P301S mice, a model of tauopathy, the expression of GIRK1 and GIRK2 is significantly reduced in the hippocampus in a laminar-specific manner (Alfaro-Ruiz et al., 2021).

The histoblot technique was applied to detect differences in the expression of glutamate receptors following the induction of seizures in the hippocampus and cortex (Kopniczky et al., 2005; Világi et al., 2009; Borbély et al., 2015). It demonstrated that there was an increase of GluA1, GluA1(flop) and GluN2B labelling in most of the areas and layers of the hippocampus and a significant decrease of GluA2 staining intensity in the CA1 and dentate gyrus (Borbély et al., 2009). The altered receptors may increase the calcium permeability of hippocampal neurons, thus likely increasing the excitability of the hippocampus. In addition, following lateral entorhinal cortex lesion, the histoblot helped to identify an increase in GluN1 and GluN2B subunits of NMDA receptors and the GluK2 subunit of kainate receptors in deafferented layers of the hippocampus. This emphasises the importance of the lateral entorhinal area in the spread and regulation of hippocampal seizures (Kopniczky et al., 2005).

The use of the histoblot technique in human tissue was originally applied to small human brain samples for the almost exclusive detection of prion or amyloid proteins (Taraboulos et al., 1992; Jendroska et al., 1994; Ritchie et al., 2004). However, this method is also suitable for the rapid comparison of large, post-mortem clinical samples from the brain of patients with Alzheimer´s disease (Beliczai et al., 2008; Tousseyn et al., 2015) and has proven to be a useful screening method. Alzheimer's disease exhibits several neuropathological features, including neuritic plaques, neurofibrillary tangles, regionally variable neuronal loss and many different synaptic dysfunctions. Thus, many underlying mechanisms that may be involved in brain damage can be investigated using the histoblot method. Because of the significant quantity of information that the histoblot can provide, this method can be used for diagnostic purposes in the framework of human AD for characterizing and observing the progression of the disease.

Expression of proteins during development

Human brain development is a process that begins a few weeks after conception and is complete by early adulthood. During this period, the physiological and anatomical changes are driven by the orchestration of different events taking place in a temporo-spatial manner. This begins with neurulation and neuronal proliferation, followed by migration and differentiation, and ending with synaptogenesis, synapse pruning, circuit refinements and myelination. Current evidence points out that many of these brain developmental stages involve the appropriate expression and function of neurotransmitters, their receptors, and ion channels (Luján et al., 2005; Luján, 2010). Unravelling the expression pattern of those proteins during development is key to our understanding of how they contribute to developmental processes and function in the mature brain. The histoblot technique has helped to establish the expression of glutamate receptors, GABA receptors and ion channels during development (Benke et al., 1995; Wenzel et al., 1996, 1997; Tönnes et al., 1999; Jouhanneau et al., 2011; Fernández-Alacid et al., 2011; Ferrándiz-Huertas et al., 2012). For example, the GluN1 subunit, the obligatory structural and functional subunit of NMDA receptors, showed ubiquitous distribution during postnatal development (Benke et al., 1995). With regards to GluN2 subunits, the histoblot showed that NMDA receptors containing the GluN2A subunit are likely to be predominant throughout the brain, while those containing the GluN2B, GluN2C or GluN2D subunit represent more region-specific receptor subtypes (Wenzel et al., 1997). In particular, the GluN2D subunit was detected transiently expressed in certain brain regions, suggesting its role in such brain areas only during development (Wenzel et al., 1996). Kainate receptor subunits have also been analysed, showing their presence in layer IV barrel cortex during the first postnatal week, although likely lacking the high kainate affinity GluK4 and GluK5 subunits (Jouhanneau et al., 2011). Another example of important contributions of the histoblot technique has been shown for GIRK channels subunit during postnatal development. GIRK1, GIRK2 and GIRK3 subunits were expressed in the developing brain from the day of birth (P0), showing differences in a region- and subunit-specific manner (Fernández-Alacid et al., 2011).

Prediction in the formation of macromolecular complexes

The interaction of two or more proteins in different brain regions is detected using conventional co-immunoprecipitation performed on crude tissue extracts from dissected brain regions (Morató et al., 2021). However, this approach does not provide information on the specific neuron population and subcellular compartment where the molecular interaction is taking place. Based on the high sensitivity, anatomical and subregional resolution, the histoblot technique has established the overlapping expression profiles for GIRK channels and other proteins like RGS7, Gβ5 and $GABA_B$ receptors (Ciruela et al., 2010; Fernández-Alacid et al., 2011; Fajardo-Serrano et al., 2013; Aguado et al., 2016b). This regional expression match does not imply that the different proteins are co-expressed/co-assembled in the same neuron or subcellular compartment, as it is only circumstantial evidence for their association. However, the formation of macromolecular complexes was first predicted with histoblot, which was then confirmed with biochemical approaches (Ciruela et al., 2010; Fajardo-Serrano et al., 2013; Aguado et al., 2016b; Morató et al., 2017, 2018).

In the hippocampus, marked similarity in the expression pattern of the regulator of G-protein signalling protein RGS7, the type 5 G-protein β subunit, GIRK2 and $GABA_{B1}$ has been described, parallel to their functional and molecular association using electrophysiology and Co-IP respectively (Fajardo-Serrano et al., 2013). Moreover, metabotropic glutamate type 5 ($mGlu_5$) receptors are strongly expressed in CA1 pyramidal neurons and are co-distributed with contactin-associated protein 1 (Caspr1), a type I transmembrane protein member of the neurexin family; their association forming macromolecular complexes was confirmed using Co-IP techniques (Morató et al., 2018). In the cerebellum, the use of the histoblot technique showed the overlapping expression of GIRK1, GIRK3 and $GABA_{B1}$ (Ciruela et al., 2010) or RGS7, Gβ5 and RGS7-binding protein R7BP (Aguado et al., 2016b). In both cases, the formation of macromolecular complexes we demonstrated using Co-IP (Ciruela et al., 2010; Aguado et al., 2016b).

Conclusions

We have elucidated a simple, reliable, reproducible, and highly sensitive method for protein detection that allows both protein quantitation and analysis of tissue distribution. The histoblot is superior to conventional western blot as tissue integrity and anatomical resolution is maintained and has an advantage over routine immunohistochemistry in that unfixed samples can be examined. Moreover, our histoblot technique can be completed in timeframes shorter than western blots and immunohistochemical procedures. Without significant modifications, the general principles, methods, and basic protocol should be adaptable for use in many other signalling systems for almost all proteins in practically every organism and tissue. From this, we expect that histoblot can be applied to a variety of experimental conditions, including a wide spectrum of pathology.

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Consent for publication. All co-authors of this manuscript certify that it has not been submitted to more than one journal for simultaneous consideration and that the manuscript has not been published previously (partly or in full). The authors also can certify that our main study is not split up into several parts to increase the quantity of submissions, that none of the data presented here have been fabricated or manipulated and that we present our own data/text/theories/ideas. All authors and authorities have explicitly provided their consent to submit the present manuscript and in general we all agree with the ethical responsibilities of authors of the journal. Finally, all authors give consent for publication in Histology & Histopathology.

Competing interests. The authors of this manuscript declare that they have no competing interests.

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